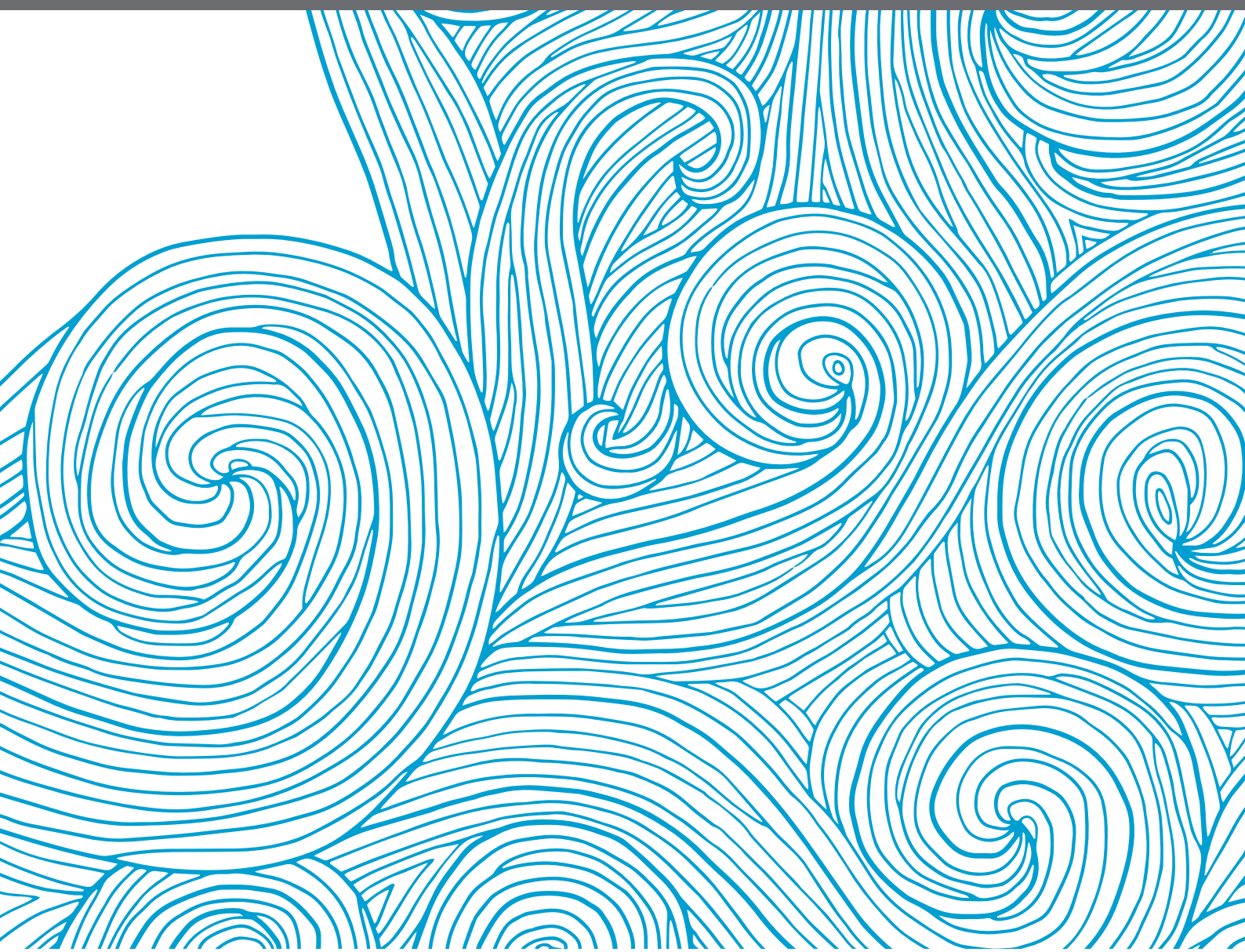
NEW CHALLENGES IN MARINE POLLUTION MONITORING

EDITED BY: Juan Bellas, Thierry Burgeot and Ketil Hylland
PUBLISHED IN: Frontiers in Marine Science and Frontiers in Environmental Science



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88963-518-4
DOI 10.3389/978-2-88963-518-4

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

NEW CHALLENGES IN MARINE POLLUTION MONITORING

Topic Editors:
Juan Bellas, Spanish Institute of Oceanography, Spain
Thierry Burgeot, Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), France
Ketil Hylland, University of Oslo, Norway

Citation: Bellas, J., Burgeot, T., Hylland, K., eds. (2020). New Challenges in Marine Pollution Monitoring. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-518-4

Table of Contents

frontiers in Marine Science

EDITORIAL
published: 22 January 2020
doi: 10.3389/fmars.2019.00820

Editorial: New Challenges in Marine Pollution Monitoring

Juan Bellas[1]*, *Ketil Hylland*[2] *and Thierry Burgeot*[3]

[1] Centro Oceanográfico de Vigo, Instituto Español de Oceanografía, IEO, Vigo, Spain, [2] Department of Biosciences, University of Oslo, Oslo, Norway, [3] Unit of Biogeochemistry and Ecotoxicology, Institut Français de Recherche pour l'Exploitation de la Mer, Nantes, France

Keywords: marine pollution, ecotoxicology, biomonitoring, water quality, bioaccumulation

Editorial on the Research Topic

New Challenges in Marine Pollution Monitoring

INTRODUCTION

There is abundant evidence that anthropogenic activities have polluted all compartments of the oceans, from the poles to the tropics, by different physical, chemical, and biological stressors. Chemical pollution is particularly tackled here with focus on legacy pollutants and newly emerging man-made compounds (xenobiotics) or anthropogenic forcing in the increase of natural chemical substances. It has been estimated that more than 100,000 chemicals are currently on the market [ECHA (European Chemicals Agency), 2017], and thousands of new substances are being introduced every year due to industrialization, intensive agriculture, and urban development. This has led to a continuous flow of chemical products to the oceans that have the potential to alter the structure of ecosystems by causing changes in the biotic communities that constitute them.

Traditionally, the assessment of marine chemical pollution would exclusively be based on chemical analysis of a limited set of potential pollutants in selected environmental matrices, and a comparison between their levels with those found in pristine areas not being subjected to direct human pressures. However, such chemical assessment of pollution only offers a partially suitable approach to the question of how marine organisms and ecosystem functioning are affected by pollutants. This can only be answered by means of an integrated assessment including both chemical analyses and biological tools that quantitatively link the levels of pollutants with their ecological effects, including new contaminants for which no analytical techniques have yet been developed. Ideally, we aim to detect disturbances caused by pollutants before ecosystems are affected. So we need sensitive indications for pollution effects that provide an *early warning* to allow taking measures to avoid ecological damage. As for this concern a unique effort based on a European consensus, has been developed by ICES/OSPAR (Davies and Vethaak, 2012).

A new challenge in marine pollution monitoring is also based on the harmonization of two European Union directives for the protection of the marine environment, the Water Framework Directive (WFD, 2000/60/CE) and the Marine Strategy Framework Directive (MSFD, 2008/56/CE). The latter established a legislative context demanding the use of effect-based tools for the assessment of pollution. These two directives were constructed according to two different strategies to assess the status of continental and coastal water ecosystems, following either a risk assessment approach (WFD) or an ecosystem approach (MSFD).

This Research Topic comprises 14 studies including original research articles, method developments, reviews, perspectives, and technology reports covering several aspects of marine pollution monitoring: integrated methodologies, distribution, and levels of legacy and emerging

OPEN ACCESS

Edited and reviewed by:
Hans Uwe Dahms,
Kaohsiung Medical University, Taiwan

***Correspondence:**
Juan Bellas
juan.bellas@ieo.es

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *13 November 2019*
Accepted: *18 December 2019*
Published: *22 January 2020*

Citation:
Bellas J, Hylland K and Burgeot T (2020) Editorial: New Challenges in Marine Pollution Monitoring. Front. Mar. Sci. 6:820. doi: 10.3389/fmars.2019.00820

pollutants and their impact on populations and communities of marine organisms, as well as new techniques for marine pollution monitoring.

INTEGRATED ASSESSMENT OF POLLUTION

An integrated approach for the assessment of marine pollution generates large and heterogeneous datasets including variables with very different metrics and distributions. All of these must be considered when selecting the method for data analysis. In this Research Topic, Regoli et al. applied a Weight of Evidence (WOE) model to results from the monitoring of offshore platforms, based on multiple lines of evidence including chemical analyses and biological responses. The assessment of environmental quality was summarized in terms of hazard quotients, providing a useful tool for the application of risk assessment in environmental management. Lehtonen et al. assessed pollution impacts in coastal areas of Finland with a WOE approach, using caged mussels. The integration of chemical and biological effect data revealed that the health of marine organisms in the area of study is being altered by pollution. Parmentier et al. assessed the effects of tributyltin (TBT) on the brown shrimp (*Crangon crangon*) and suggest that long-term endocrine disruption effects was the reason for previously unexplained declines in *C. crangon* and other crustacean populations in the German Bight. Förlin et al. studied perch (*Perca fluviatilis*) populations at reference sites from the Swedish National Monitoring Program where a decline in fish health had been previously observed. Alterations in several biological responses, confirmed by mRNA expression levels, were related to higher concentrations of natural, bioactive brominated compounds measured in the perch, attributed to algal blooms in the Baltic Sea. Yang et al. studied the distribution and levels of persistent organic pollutants in surface sediments of Qingduizi Bay. Polychlorinated biphenyls (PCBs) and hexachlorocyclohexane (HCH) levels were attributed to their extensive historical use in adjacent areas. However, the DDT profile showed recent usage and fresh inputs from aquaculture activities, with levels that could be potentially toxic to marine organisms.

POLLUTANTS OF EMERGING CONCERN

Pollutants of emerging concern are substances that have recently raised awareness due to their widespread use and presence in the environment and might represent a threat to ecosystems or to human health. These chemicals are not adequately regulated and urgent information is needed about their environmental distribution, bioaccumulation potential, and ecotoxicity. Among the substances of emerging concern, little attention has been paid to metals. Romero-Freire et al. reviewed the concentrations of Less-Studied Technology-Critical Elements (LSTCEs) (Nb, Ta, Ga, In, Ge, Te) in marine water and biota. Limited information of LSTCEs is available for estuarine and coastal waters and further efforts are needed to develop appropriate analytical procedures and certified reference materials. Among the pollutants of emerging concern, there has recently been increasing interest in microplastics (MPs). A number of studies have demonstrated that MPs pollution is widespread and ubiquitous in the marine environment, with potential to cause harm to biota. Tramoy et al. assessed plastic inputs from the Seine River basin to the sea using two methods, a statistical modeling approach and field results from floating booms. Both methods yielded similar estimates of plastic fluxes to the sea, ranging from 1,100 to 5,900 t/yr. Assumptions and uncertainties of both approaches were characterized. Schönlau et al. studied the levels of aryl hydrocarbon receptor (AhR) active compounds on field-deployed MPs. Bioassay-derived TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) equivalents were calculated with the DR CALUX® assay to evaluate the potential toxicity of hydrophobic organic compounds (HOCs) sorbed to MPs. The bioassay identified two bioactive fractions in the polymers and proved to be a valuable complement to chemical analysis in risk assessment of MP-sorbed compounds. Cormier et al. investigated the toxicity of organic pollutants sorbed to MPs using the zebrafish embryo test. No embryotoxicity was detected for virgin or spiked MPs, but oxybenzone- and benzo[a]pyrene-spiked MPs induced EROD activity. These results indicate that the standard acute embryo test would not be sufficiently sensitive to detect effects of MPs, and protocols including sublethal endpoints would be needed.

NEW TECHNIQUES AND TECHNOLOGY

The availability of suitable techniques and methods has typically hindered the study of environmental pollutants and their impact on marine ecosystems. The development of new techniques is crucial to overcome analytical limitations and other methodological difficulties to advance in the knowledge of their environmental behavior and effects. Fauvelle et al. developed an analytical method based on a single solid-phase extraction procedure followed by gas and liquid chromatography coupled with high resolution mass spectrometry (GC-MS and LC-HRMS) for the quantification of 40 organic compounds in natural seawater. The procedure, which allowed the quantification of five classes of legacy and emerging contaminants, was validated and applied to real seawater samples. Tato and Beiras compared the sensitivity of *Tisochrysis lutea* (T-iso), formerly *Isochrysis galbana*, with other microalgal species recommended for regulatory toxicity testing, using the 72-h growth-rate inhibition test. In general, T-iso showed higher sensitivity for most groups of toxicants than other species, meeting the acceptability criteria, therefore its use in marine toxicity testing is recommended. Paredes and Bellas present a perspective on the application of cryopreservation and cryobanking techniques to marine pollution monitoring. These techniques offer a potential solution to ensure the availability of biological material of stable quality throughout the year. A background of existing knowledge is presented and further development is discussed, including the need for extensive comparative testing with both fresh and cryopreserved biological material. Cyr et al. present an ocean glider-compatible fluorescence sensor capable

of continuously measuring polycyclic aromatic hydrocarbons (PAHs) concentrations in real time. The performance of the sensor was tested in laboratory with oil products and in three different deployments: an experimental flume tank, an urban harbor, and an off-shore oil and gas installation. Paris et al. studied the use of subsea dispersant injection (SSDI) in response to the *Deepwater Horizon* oil spill. Extensive BP Gulf Science Data were used to quantify petroleum dynamics throughout the spill, revealing no significant effect of the SSDI on the oil vertical distribution and concentration. These results question an uncritical SSDI application for deep-sea blowouts.

CONCLUSIONS

Marine pollution monitoring is a fundamental component in current environmental legislation which aims to preserve and protect marine ecosystems more effectively, and promoting their sustainable use. The articles of this Research Topic present some of the most important challenges to advance in this field. Integrated approach schemes need to improve data comparability between biological responses, which is frequently not satisfactorily done due to both confounding factors and methodological variability. A crucial knowledge gap is also the elaboration of internationally agreed assessment criteria for both environmental pollutants and biological responses. The knowledge about the environmental behavior and ecotoxicity of pollutants of emerging concern, including microplastics, is particularly relevant for integrative monitoring purposes, as well as the development of new approaches and technologies in marine pollution monitoring.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

We thank all authors, reviewers, and editors that have contributed to this Research Topic.

REFERENCES

Davies, I. M., and Vethaak, A. D. (2012). *Integrated Marine Environmental Monitoring of Chemicals and their Effects*. ICES Cooperative Research Report No. 315, 277.

ECHA (European Chemicals Agency) (2017). *Guidance for Identification and Naming of Substances Under REACH and CLP*. Version 2.1. ECHA-16-B-37.1-EN, Helsinki, 127.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

METHODS
published: 31 August 2018
doi: 10.3389/fmars.2018.00295

One-Single Extraction Procedure for the Simultaneous Determination of a Wide Range of Polar and Nonpolar Organic Contaminants in Seawater

Vincent Fauvelle, Javier Castro-Jiménez, Natascha Schmidt, Benoit Carlez, Christos Panagiotopoulos and Richard Sempéré**

Aix-Marseille Univ., Université de Toulon, CNRS, IRD, Mediterranean Institute of Oceanography (M I O), Marseille, France

OPEN ACCESS

Edited by:
Juan Bellas, Instituto Español de Oceanografía (IEO), Spain

Reviewed by:
Víctor M. León, Instituto Español de Oceanografía, Centro Oceanográfico de Murcia, Spain
Monica F. Costa, Universidade Federal de Pernambuco, Brazil

***Correspondence:**
Vincent Fauvelle
vincent.fauvelle@mio.osupytheas.fr
Richard Sempéré
richard.sempere@mio.osupytheas.fr

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *14 May 2018*
Accepted: *31 July 2018*
Published: *31 August 2018*

Citation:
Fauvelle V, Castro-Jiménez J, Schmidt N, Carlez B, Panagiotopoulos C and Sempéré R (2018) One-Single Extraction Procedure for the Simultaneous Determination of a Wide Range of Polar and Nonpolar Organic Contaminants in Seawater. Front. Mar. Sci. 5:295. doi: 10.3389/fmars.2018.00295

A rapid analytical method including one-single solid-phase extraction (SPE) procedure followed by gas and liquid chromatography coupled with high resolution mass spectrometry detection (GC–MS and LC–HRMS respectively) was developed for the quantification of 40 organic compounds ($1.6 < \log K_{ow} < 9.5$) in seawater including both legacy and emerging contaminants, with a focus on the most common plastic organic additives. This new method allowed for the analyses of nine organophosphate esters (OPEs), seven phthalates (PAEs), six bisphenols (BPs), five perfluorinated compounds (PFCs), and thirteen legacy organochlorinated compounds (OCs, including polychlorobiphenyles and pesticides) with recoveries in the ranges of 57–124% for OPEs, 52–163% for PAEs, 64–118% for BPs, 63–124% for PFCs, and 40–95% for OCs. As a result of (i) strict cleanup protocols, (ii) material, and solvent selection, and (iii) the use of an ISO 6 cleanroom for sample treatment, the procedural blank levels were always lower than 5 ng L^{-1}, even for the most abundant and ubiquitous compounds like tris-(2-chloro, 1-methylethyl) phosphate (TCPP) and diethylhexyl phthalate (DEHP). The quantification limits were in the ranges of 0.03–0.75 ng L^{-1} for OPEs, 0.03–0.25 ng L^{-1} for PAEs, 0.1–5 ng L^{-1} for BPs, 0.1–8 ng L^{-1} for PFCs, and 0.02–1.1 ng L^{-1} for OCs, matching seawater analysis requirements. Dissolved water phase samples collected in Marseille Bay (NW Mediterranean Sea) were analyzed using the developed method reveling the concentration of PAEs up to 140 ng L^{-1} (DEHP) and that of OCs up to 70 ng L^{-1} (α-endosulfan). For the first time, we also provided the concentrations of OPEs (TCPP up to 450 ng L^{-1}), BPs (bisphenol S up to 123 ng L^{-1}), and PFCs (PFOS up to 5 ng L^{-1}) in this area. A sampling station close to the municipal waste water treatment plant outfall exhibited the highest concentration levels for all compounds.

Keywords: plastic additive, seawater, organic contaminants, legacy contaminants, Phtalates, bisphenol, organophosphate esters

INTRODUCTION

Over the past decades, the dramatic increase in chemical diversity, production volume, uses and sources has led to the widespread occurrence of organic contaminants in all waterbodies including marine environments and numerous living organisms (Sousa et al., 2017). Organochlorine legacy contaminants [OCs, e.g., polychlorinated biphenyls (PCBs), organochlorine pesticides

(OCPs)] together with emerging substances (e.g., flame retardants, other organic plastic additives, pharmaceuticals) are increasingly monitored in relation with stricter national and European regulations (Allan et al., 2006). Moreover, the persistence of emerging contaminants, such as perfluorinated flame retardants (PFCs), together with their ubiquitous detection and high toxicity resulting in sub-ppt EU Environmental Quality Standard (EQS) prompted growing concern (Kaserzon et al., 2012). In addition to the direct release of chemicals in the environment, several organic compounds, such as plastic additives, can be indirectly introduced in aquatic environment following leaching of marine litter such as plastic debris (Net et al., 2015b; Murphy et al., 2016; Suhrhoff and Scholz-Böttcher, 2016; Paluselli et al., 2018b). The release of organic compounds initially included in plastic materials, e.g., phthalates (PAEs), organophosphate esters (OPEs), and bisphenols (BPs) is otherwise identified among the most critical hazards associated with plastic discharge in the environment (Hermabessiere et al., 2017; Hahladakis et al., 2018).

Substantially most of the above-mentioned chemical families exhibit endocrine-disrupting properties and are thus potentially associated with both health and environmental deleterious effects whose mechanisms are largely unknown (Messerlian et al., 2017; Barrios-Estrada et al., 2018), especially for mixtures of contaminants (Kim-Tiam et al., 2016). Most of them are found in marine environmental compartments, sometimes significantly exceeding $\mu g\ L^{-1}$ level in estuarine waters (Chau et al., 2015; Zhang et al., 2018) or being close to it in coastal waters (Pojana et al., 2007; Hu et al., 2014; Paluselli et al., 2018b), whereas offshore waters generally exhibit ng L^{-1} or lower levels (Brumovský et al., 2017; Lammel et al., 2017; Paluselli et al., 2018a). The various classes of contaminants cited above may induce their toxic effect via direct contact or by biomagnifying in the marine food web from phyto- and zooplankton, which are mainly affected by the dissolved water phase fraction of the contaminants (Kim-Tiam et al., 2016).

Although a number of previous reports on a reliable trace-level detection of different families of organic contaminants in the dissolved water phase have been published (Holadová et al., 2007; Pojana et al., 2007; Kaserzon et al., 2012; Assoumani et al., 2013; Hu et al., 2014; Chau et al., 2015; Net et al., 2015a; Brumovský et al., 2017; Lammel et al., 2017; Li et al., 2017; Paluselli et al., 2018a), they focused on one class of chemicals, making analyses of several classes of organic contaminants time-consuming. Thus, reliable detection of a large set of organic contaminants from different chemical families and polarities in natural water is only scarcely reported (Chau et al., 2015). Although solid-phase extraction (SPE), commonly followed by gas and liquid chromatography (i.e., GC and LC, respectively) coupled with mass spectrometry (MS) analyses, is now accepted as a method of choice for all the contaminant families mentioned above (Pojana et al., 2007; Kaserzon et al., 2012; Hu et al., 2014; Chau et al., 2015; Net et al., 2015a; Brumovský et al., 2017; Lammel et al., 2017; Li et al., 2017; Paluselli et al., 2018a), their fractionated elution from a one-single extraction step for simultaneous GC and LC analyses has been investigated only scarcely to the best of our knowledge.

The aims of this study are (i) to propose a systematic strategy for SPE fractionated elution step able to extract simultaneously a wide range of contaminants ($1.6 < \log K_{ow} < 9.5$) from seawater dissolved fraction for subsequent LC–MS and GC–MS analysis, (ii) to validate the analytical method at environmentally relevant concentrations, and (iii) to apply the method to real samples in order to verify its performance, in line with existing requirements for seawater monitoring.

MATERIALS AND METHODS

Chemicals and Reagents

Hexane, ethyl acetate (EtOAc), dichloromethane (DCM), acetone, and toluene were purchased from Promochem (Picograde, LGC standard). MeOH was provided by Biosolve (ULC-MS grade), whereas ultrapure water (MQ) was obtained from a Millipore (resistivity > 18.2 MΩ) Milli-Q system. All standards and key physical-chemical properties are listed in **Table 1**. PAEs were obtained from Supelco (USA), BPs and native PFCs from AccuStandard (USA), and isotope labeled PFCs from Wellington Laboratories (Canada). Individual native OPE and OC standards and labeled 2,4-DDT-*d8*, 4,4-DDT-*d8*, and α-endosulfan-*d4* were purchased from Dr. Ehrenstorfer GmbH (Germany), whereas labeled standards of tri-n-butyl-*d27*-phosphate, triphenyl-d15-phosphate, tri-n-propyl-*d21*-phosphate, malathion-*d7,* α-HCH-*d6*, and γ-HCH-*d6* were obtained from C/D/N Isotopes Inc. (Canada) and tris(2-chloroisopropyl)-*d18*-phosphate, tris(1,3-dichloro-2-propyl)-*d15*-phosphate, tris(2-chloroethyl)-*d12*-phosphate, $^{13}C_{12}$-PCB-28,−118, and−180 were from Cambridge Isotope Laboratories, Inc. (USA). Monomolecular stock solutions (250–100 ng μL^{-1}) were prepared in toluene except for PFCs and BPs (acetone). Working mix solutions were prepared in isooctane or acetone by dilution to 10 ng μL^{-1}.

Solid Phase Extraction Procedure

The SPE method developed here is based on a previous procedure described for PAEs by Paluselli et al. (2018a) and optimized with a fractionated elution step in order to recover different contaminant classes (OPEs, PAEs, BPs, PFCs, and OCs). A 1 L subsurface seawater sample was collected in Marseille Bay with a stainless-steel beaker, filtered through a precombusted (450°C, 6 h) 0.7 μm glass fiber filter the same day, and stored at −20°C if extractions were not performed the same day. The whole SPE protocol was conducted on a manifold under vacuum (Supelco Visiprep®). SPE cartridges were prepared in the laboratory [Waters Oasis hydrophilic-lipophilic balance (HLB)® bulk sorbent, 30 μm, 250 mg, glass cartridge, polytetrafluoroethylene (PTFE) frits] and conditioned with 3 cycles of acetone (5 mL), EtOAc (5 mL), DCM (5 mL), and finally 10 mL of MQ. The freshly collected seawater samples were spiked with the corresponding surrogate mix (**Table 1**, 100 ng $sample^{-1}$) before the sample loading on the cartridges (10 mL min^{-1}, ∼2 h). A subsequent washing step (5 mL MQ) allowed removing salts from the cartridges. The cartridges were then dried under vacuum for ∼2 h in order to remove any residual water that may affect subsequent GC–MS analysis. Special attention was

TABLE 1 | Identification and physico-chemical properties of target substances.

Name	Full name	CAS	Description	Monoisotopic mass (g mol^{-1})	log K_{ow}
ORGANOPHOSPHATE ESTERS (OPEs)					
TPP	Tripropyl phosphate	513-08-6	A	224.1177	1.9
TiBP	Tri-iso-buthyl phosphate	126-71-6	A	266.1647	3.5
TnBP	tri-n-butyl phosphate	126-73-8	A	266.1647	4
TCEP	tris-(2-chloroethyl) phosphate	115-96-8	A	283.9539	1.8
TCPPs	tris-(2-chloro, 1-methylethyl) phosphate	13674-84-5	A	326.0008	2.6
TDCP	tris-(2-chloro-, 1-chloromethylethyl) phosphate	13674-87-8	A	427.8839	3.7
TPhP	triphenyl phosphate	115-86-6	A	326.0708	4.6
EHDPP	2- ethylhexyl-diphenyl phosphate	1241-94-7	A	362.1647	5.7
TEHP	tris(2-ethylhexyl) phosphate	78-42-2	A	434.3525	9.5
TBP-*d27*	Tri-n-butyl phosphate-*d27*	61196-26-7	S	293.3341	
TPhP-*d15*	Triphenyl phosphate-*d15*	1173020-30-8	S	341.1649	
TCPP-*d18*	Tris (2-chloroisopropyl) phosphate-*d18*	-	S	344.1138	
TDCP-*d15*	Tris (1,3-dichloro-2-propyl) phosphate-*d15*	1447569-77-8	S	442.9781	
TPrP-*d21*	Tri-n-propyl phosphate-*d21*	1219794-92-9	IS	245.2495	
TCEP-*d12*	Tris (2-chloroethyl) phosphate-*d12*	1276500-47-0	IS	296.0292	
MAL-*d7*	malathion-*d7*	352438-94-9	IS	337.0800	
PHTHALATES (PAEs)					
DMP	dimethyl phthalate	131-11-3	A	194.0579	1.6
DEP	diethyl phthalate	84-66-2	A	222.0892	2.5
DBP	di-n-butyl phthalte	84-74-2	A	278.1518	4.5
DiBP	diisobutyl phthalate	84-69-5	A	278.1518	4.1
DEHP	diethylhexyl phthalate	117-81-7	A	390.277	7.6
BBP	benzylbutyl phthalate	85-68-7	A	312.1362	4.7
DnOP	di-n-octyl phthalate	117-84-0	A	390.277	8.1
DEHP-*d4*	diethylhexyl phthalate-*d4*	93951-87-2	IS	394.3021	
DEP-*d4*	diethyl phthalate-*d4*	93952-12-6	IS	226.1143	
DnBP-*d4*	di-n-butyl phthalte-*d4*	93952-11-5	S	282.1769	
BISPHENOLS (BPs)					
BPA	bisphenol A	80-05-7	A	228.115	3.3
BPAF	bisphenol AF	14878-61-1	A	336.0585	4.5
BPAP	bisphenol AP	1571-75-1	A	290.1307	4.4
BPF	bisphenol F	620-92-8	A	200.0837	2.9
BPP	bisphenol P	2167-51-3	A	346.1933	6.1
BPS	bisphenol S	80-09-1	A	250.03	1.9
BPZ	bisphenol Z	843-55-0	A	268.1463	5.4
4nOP-*d17*	4-n-octylphenol-*d17*	1219794-55-4	S	223.2738	
BPA-*d16*	bisphenol A-*d16*	96210-87-6	IS	244.2154	
PERFLUORINATED COMPOUNDS (PFCs)					
PFHA	perfluoro hexanoic acid	307-24-4	A	313.9801	3.5
PFHS	perfluorohexane sulfonic acid	3871-99-6	A	399.9439	3.2
PFOA	perfluorooctanoic acid	335-67-1	A	413.9737	4.8
PFOS	perfluorooctane sulfonic acid	1763-23-1	A	499.9375	4.5
PFOSF	perfluoro octane sulfonyl fluoride	307-35-7	A	501.9332	7.8
PFHA-*13C5*	perfluoro hexanoic acid-$^{13}C_5$	–	IS	318.9969	
PFHS-*13C3*	perfluoro hexanesulfonate-$^{13}C_3$	–	IS	402.9539	
PFOS-*13C8*	perfluoro octanesulfonate-$^{13}C_8$	–	IS	507.9643	
ORGANOCHLORINATED (OCs)					
PCB-28	polychlorobyphenyl-28	7012-37-5	A	255.9613	5.6
PCB-52	polychlorobyphenyl-52	35693-99-3	A	289.9224	6.1

(Continued)

TABLE 1 | Continued

Name	Full name	CAS	Description	Monoisotopic mass (g mol^{-1})	log K_{ow}
PCB-101	polychlorobyphenyl-101	37680-73-2	A	323.8834	6.5
PCB-118	polychlorobyphenyl-118	31508-00-6	A	323.8834	7.1
PCB-138	polychlorobyphenyl-138	35065-28-2	A	357.8444	7.2
PCB-153	polychlorobyphenyl-153	35065-27-1	A	357.8444	7.2
PCB-180	polychlorobyphenyl-180	35065-29-3	A	391.8054	7.9
4,4-DDT	4,4-dichlorodiphenyltrichloroethane	50-29-3	A	351.9147	6.9
HCB	hexachlorobenzene	118-74-1	A	281.8131	5.7
α -HCH	alpha-hexachlorocyclohexane	319-84-6	A	287.8601	3.7
β-HCH	beta-hexachlorocyclohexane	319-85-7	A	287.8601	3.7
γ-HCH	gamma-hexachlorocyclohexane	58-89-9	A	287.8601	3.7
α-Endosulfan	alpha-endosulfan	959-98-8	A	403.8169	
α -HCH-*d6*	alpha-hexachlorocyclohexane-*d6*	–	S	293.8977	
2,4-DDT-*d8*	2,4-dichlorodiphenyltrichloroethan-*d8*	–	S	359.9649	
PCB-180 *-13C12*	polychlorobyphenyl-180-$^{13}C_{12}$	–	S	403.8457	
γ-HCH-*d6*	gamma-hexachlorohexane-*d6*	60556-82-3	IS	293.8977	
PCB-28-*13C12*	polychlorobyphenyl-28-$^{13}C_{12}$	–	IS	268.0016	
PCB-118-*13C12*	polychlorobyphenyl-118-$^{13}C_{12}$	–	IS	335.9237	
α-Endosulfan-d4	alpha-endosulfan-d4	203645-57-2	IS	407.8420	
4,4-DDT-d8	4,4-dichlorodiphenyltrichloroethane-d8	93952-18-2	IS	359.9649	

A, S, and IS correspond to analyte, surrogate, and internal standard, respectively.

paid to ensure complete dryness since poly(divinylbenzene-co-*N*-vinylpyrrolidone) (Oasis HLB) is more wettable than conventional poly(styrene-co-divinylbenzene) SPE sorbents. Four eluting fractions covering a wide range of solvent polarity were studied separately by implementing a multi-elution step following the polarity gradient, in order to give a general scheme for potential further contaminant inclusion: 5 mL hexane (F1), 5 mL hexane/DCM 50:50 (v/v) (F2), 5 mL EtOAc (F3), and then 5 mL MeOH (F4). Depending on the chemical family one fraction might not be needed, but we chose to keep these four fractions that include both polar and nonpolar, as well as protic and non-protic solvents. The four fractions were collected separately in pre-combusted 22 mL glass flasks, evaporated to ∼1 mL under gentle N_2 flux, transferred into 2 mL pre-combusted conical glass vials, spiked with an appropriate internal standard (IS) mixture (**Table 1**, 100 ng sample^{-1}), and evaporated again to a final volume of approximately 50 μL for the GC–MS analysis or 1000 μL for the LC–high-resolution mass spectrometry (HRMS) analysis. The concentration factors were 20,000 for the GC-MS analysis and 1,000 for the LC–HRMS analysis. Considering the injection volumes on GC (2 μL) and LC (10 μL), 40 and 10 mL seawater volume equivalents were injected on each instrument. The whole procedure was conducted in an International Standards Organization (ISO) 6 cleanroom, and strict quality assurance and quality control (QA/QC) procedures were implemented (see the QA/QC section).

GC–MS Analysis

According to the literature, PAE, OPE, and OC analyses were conducted with Agilent 7820A Series GC coupled with Agilent 5977E MS, operating in selected ion monitoring and electron impact (70 eV) modes. The separation was achieved in a 30 m × 0.25 mm internal diameter × 0.25 μm HP-5MS capillary column (Agilent J&W). All target contaminants were quantified by the IS procedure. **Supplementary Table 1** shows selected ions for detection and quantification for each compound. The injection volume was 2 μL, and the helium carrier gas flow was 1 mL min^{-1}. The temperatures of the MS transfer line, ion source, and quadrupole were set at 300, 230, and 150°C, respectively. For OPEs and PAEs, the following conditions were applied: the injector temperature was 270°C (splitless), and the oven was programmed from 90 to 132°C at 3°C min^{-1}, to 166°C at 10°C min^{-1}, to 175 at 1°C min^{-1} (holding time: 2 min), to 232°C at 2°C min^{-1}, and then to 300°C at 25°C min^{-1} (holding time: 5 min). For OCs, the injector temperature was 250°C (splitless), and the oven was programmed from 90 to 140°C at 25°C min^{-1} (holding time: 10 min), to 158°C at 1°C min^{-1}, to 188 at 20°C min^{-1}, to 208°C at 1°C min^{-1}, and then to 300°C at 25°C min^{-1} (holding time: 5 min).

LC–HRMS Analysis

Simultaneous quantification of BPs and PFCs was achieved by analyzing 10 μL with LC–electrospray ionization quadrupole

time of flight mass spectrometry (LC-ESI-QTOF, Agilent 1290 Infinity LC system coupled with Agilent 6530 Accurate-Mass Q-TOF, Agilent Technologies, Les Ulis, France). LC was chosen for BPs and PFCs according to the literature. Syringe was washed externally with 1 mL MeOH for 20 s before injection to avoid contamination of the injection port or the following samples. Separation was achieved on an Agilent Zorbax Eclipse XDB reversed phase column (50 mm × 2.1 mm, 1.8 µm), with a temperature of 30°C. Elution was performed with MQ (A) and MeOH (B) under gradient conditions: 0 min 95:5 A/B, 1 min 95:5 A/B, 10 min 0:100 A/B, 15 min 0:100 A/B, 15.1 min 95:5 A/B, and 20 min 95:5 A/B. The ESI interface was operated in the negative mode (3.5 kV capillary voltage), and MS TOF mass acquisition was performed in the range of 50–600 m/z at a rate of 1 spectrum s^{-1}. The ESI parameters were set as follows: 300°C gas temperature, 11 L min^{-1} drying gas, 40 psig nebulizer, 350°C sheath gas temperature, and 11 L min^{-1} sheath gas flow. The TOF parameters were: 1,500 V nozzle voltage, 175 V fragmentor voltage, 65 V skimmer voltage, and 750 V octopole voltage. Ion chromatogram extraction was performed with 10 ppm mass tolerance, based on the exact monoisotopic mass (**Table 1**) and retention time (**Supplementary Table 2**). A typical chromatogram for selected compounds is available in **Supplementary Figure 1**.

Application to Seawater Samples

The method was applied to real seawater samples collected at l'Estaque, Frioul, and Cortiou sites in May 2017 in the Marseille city coastal area (**Figure 1**) from R/V Antedon II, and followed the protocol described in the "SPE procedure" section. L'Estaque is under the influence of the commercial harbor, Frioul is considered the least impacted site, whereas the Cortiou station is around 150 m from the municipal wastewater treatment plant outfall (WWTP, 2.1 10^6 population equivalent capacity, 1 10^6 effective population).

QA/QC

Most of the target chemicals may be found ubiquitously in technical laboratory items at each stage of sample processing (sampling, filtration, SPE, analysis), in particular the emerging contaminants. As a result, several systematic precautions were taken throughout the sample preparation processes: a pre-combusted (i.e., 450°C for 6 h) aluminum foil was used to prevent direct contact between seawater and plastic items (e.g., bottle caps); stainless steel, pre-combusted glassware and PTFE were used instead of polyethylene and polypropylene materials; all samples were processed in an ISO class 6 cleanroom (22°C, +15 Pa cleanroom pressure, 50 vol h^{-1} brewing rate); all lab hood was cleaned with MeOH; all materials were covered with a pre-combusted aluminum foil; and the cartridges were covered during drying. PTFE might be a source of PFCs. Therefore, its use was limited to SPE cartridge frits and SPE sample loading lines, and each PFTE item was cleaned drastically with MeOH in order to lower PFC contamination to acceptable levels. Corresponding procedural blanks were performed.

Other precautions were taken for the analytical part: a ultra-high pressure liquid chromatography grade solvent was chosen, the LC syringe was washed externally with MeOH for 20 s before each injection, and the column was flushed with both MeOH and MQ included in the LC gradient for 5 min. The GC injection syringe was washed 10 times with DCM and isooctane before and after each individual injection in order to minimize cross-contamination at the GC injection port. The QA/QC were based on (i) procedural blank quantification for each SPE batch, (ii) instrumental blanks every five samples, (iii) surrogates' recovery evaluation for each individual sample, (iv) the use of appropriate IS quantification, and (v) initial method validation for recovery and the limit of quantification for a target chemical in the matrix of interest. The average procedural blanks ($n = 3$) ranged from < the limits of detection to 3.4 ng L^{-1} (average: 0.35 ng L^{-1}), exhibiting generally very low amounts of target analytes (**Supplementary Table 3**). No particular instrumental blanks or cross-contamination was

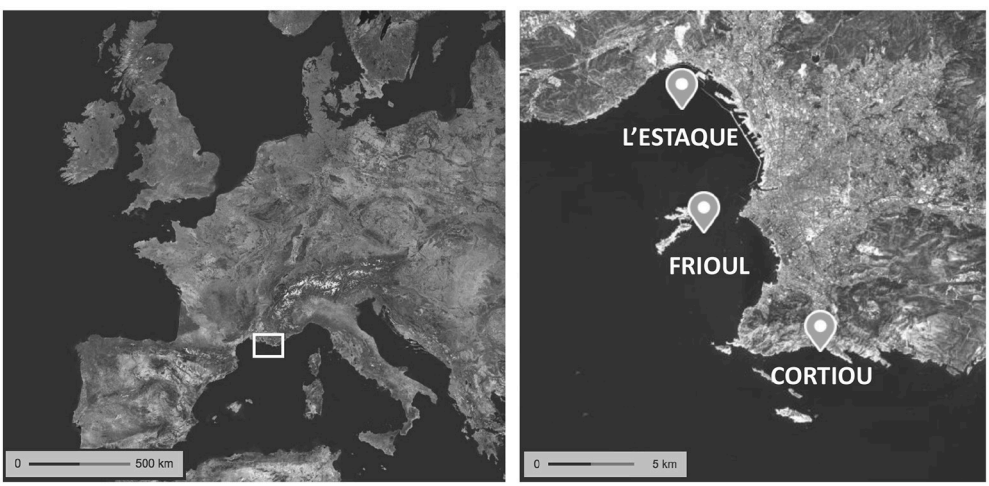


FIGURE 1 | Sampling locations in Marseille Bay. Map from the French National Institute of Geographic and Forestry Information.

TABLE 2 | Distribution of 40 target analytes in the four consecutive elution fractions.

	F_1: 5 mL Hexane	F_2: 5 mL Hexane/DCM 50:50 (v/v)	F_3: 5 mL EtOAc	F_4: 5 mL MeOH
ORGANOPHOSPHATE ESTERS (OPEs)				
TPP	10 ± 18	90 ± 18	0	0
TiBP	45 ± 40	55 ± 40	0	0
TnBP	19 ± 22	81 ± 22	0	0
TCEP	0	100	0	0
TCPPs	33 ± 58	67 ± 58	0	0
TDCP	0	100	0	0
TPhP	0	100	0	0
EHDPP	12 ± 17	88 ± 17	0	0
TEHP	47 ± 46	53 ± 46	0	0
PHTHALATES (PAEs)				
DMP	16 ± 27	84 ± 27	0	0
DEP	48 ± 35	52 ± 35	0	0
DBP	64 ± 35	35 ± 34	1 ± 1.1	0
DiBP	73 ± 26	26 ± 26	1 ± 0.8	0
DEHP	80 ± 19	20 ± 18	1 ± 0.5	0
BBP	10 ± 17	90 ± 17	0	0
DnOP	77 ± 21	22 ± 22	1 ± 0.8	0
BISPHENOLS (BPs)				
BPA	0	0	100	0
BPAF	0	0	34 ± 25	66 ± 25
BPAP	0	0	59 ± 19	41 ± 19
BPF	0	0	0	100
BPP	90 ± 17	10 ± 17	0	0
BPS	0	0	10 ± 10	90 ± 10
BPZ	0	0	0	100
PERFLUORINATED COMPOUNDS (PFCs)				
PFHA	0	0	9 ± 4	91 ± 4
PFHS	0	0	1 ± 1	99 ± 1
PFOA	0	0	0	100
PFOS	0	0	0	100
PFOSF	0	0	0	100
ORGANOCHLORINATED				
PCB-28	83 ± 11	12 ± 24	5 ± 11	0
PCB-52	91 ± 36	9 ± 27	0	0
PCB-101	92 ± 26	8 ± 35	0	0
PCB-118	93 ± 9	7 ± 22	0	0
PCB-138	89 ± 23	11 ± 12	0	0
PCB-153	92 ± 24	8 ± 26	0	0
PCB-180	91 ± 22	9 ± 30	0	0
4,4-DDT	77 ± 35	23 ± 25	0	0
HCB	100	0	0	0
α-HCH	85 ± 12	15 ± 26	0	0
β + γ-HCH	41 ± 28	58 ± 37	0	0
α-Endosulfan	100	0	0	0

The results are expressed as percent area of the sum of the areas found in the four fractions. The relative standard deviation is mentioned after each value (n = 3). Water Oasis HLB cartridges 250 mg, 1 L seawater sample.

observed during the GC–MS analyses. However, during the LC–HRMS analysis, we experienced instrumental blank issues, which were solved by a copious external syringe-washing step (MeOH flushing for 20 s) after each LC sampling. The nine isotope labeled surrogates (**Table 1**) added in the seawater samples before SPE showed acceptable recoveries in the range of 40–108% (**Table 3**). The LC matrix effects, i.e., ion suppression in the ESI source (Taylor, 2005), were always lower than 40% signal loss. These limited matrix effects were obtained by applying a lower SPE concentration factor than the one applied for the GC–MS analyses (1,000 vs. 20,000 see the "SPE procedure" section).

RESULTS AND DISCUSSION

SPE Optimization

The selection of an SPE sorbent that includes both polar and nonpolar moieties was the first requirement for retaining the whole range of target chemicals (1.6 < log Kow < 9.5). Among them, Oasis HLB is identified as the best candidate for extracting a wide range of contaminants (Kaserzon et al., 2012; Fauvelle et al., 2015; Net et al., 2015a). An important SPE step was the selection of an appropriate elution fraction. As our analytes cover a large polarity range, a preliminary fractionation experiment was

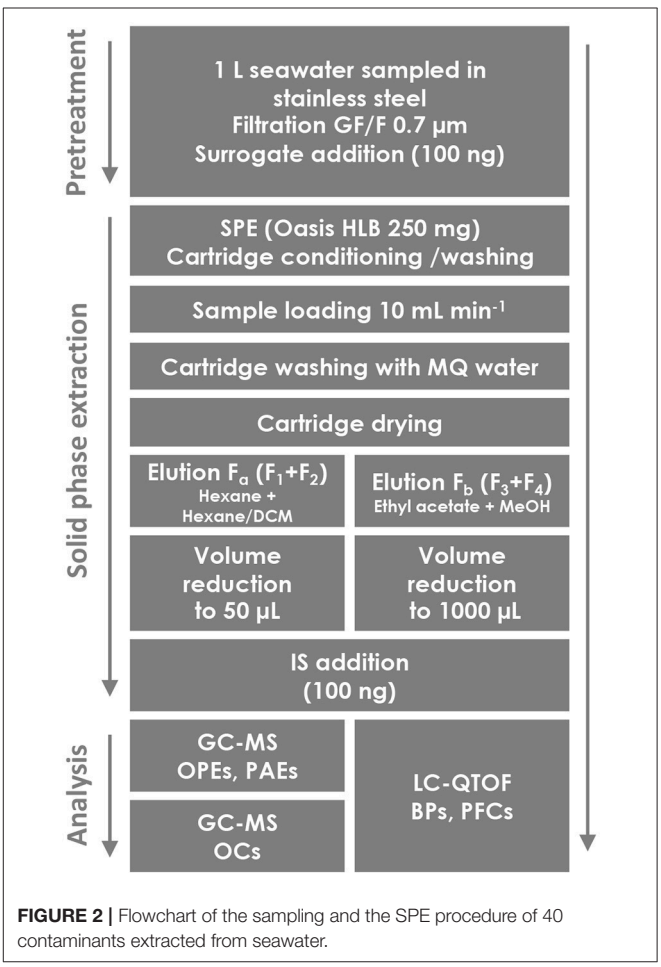


FIGURE 2 | Flowchart of the sampling and the SPE procedure of 40 contaminants extracted from seawater.

performed to better understand the HLB-to-solvent partitioning for each contaminant using a solvent polarity gradient: first nonpolar fraction (hexane), second slightly more polar fraction (hexane/DCM), third moderately polar fraction (EtOAc), and last polar fraction (MeOH). **Table 2** shows the distribution of all 40 target analytes in the four fractions defined in the "SPE procedure" section. OPEs were only found in F_1 and F_2 and not detected in F_3 and F_4. PAEs were mainly recovered in F_1 and F_2, whereas <1% was found in F_3, and they were not detected in F_4. OCs were also recovered predominantly in F_1 and F_2, while F_3 contained <5% of the total amount, and F_4 did not show detectable amounts. BPs were only found both in F_3 and F_4, except for BPP, which was detected only in F_1 and F_2. PFCs were otherwise only detected in F_3 and F_4. In summary, PAEs, OPEs, and OCs were recovered in the first two fractions, whereas BPs and PFCs were predominantly found in fractions F3 and F4. A limited number of PAEs and OCs were found in F3, with relative abundances always lower than 5%. One major exception for BPs was BPP, found mainly in F1, in line with its higher log K_{ow} value (i.e., 6.1, see **Table 1**).

Therefore, the final SPE protocol consisted in collecting F_1 with F_2 (called F_a thereafter) for the GC–MS analysis of OPEs, PAEs, and OCs and F_3 with F_4 (called F_b thereafter) for the LC–HRMS analysis of BPs and PFCs. As BPP was recovered in F_a, but analyzed by LC–HRMS, it was removed from the target list. A schematic view of the final protocol is available in **Figure 2**.

Method Validation

The final method was then validated at two environmentally relevant concentrations: 20 ng L^{-1} and 150 ng L^{-1}. Seawater samples (1 L) collected in the study area were spiked at the corresponding target concentrations with a mixture of native contaminants prepared in acetone (five replicates for each level, $n = 5$). Recoveries, the limits of quantification (LQs) are reported in **Table 3**. The LQs in pg injected were determined considering a signal/noise (S/N) ratio of >10 in the lowest calibration level, and the LQs in ng L^{-1} were derived from these values taking into account the corresponding SPE preconcentration factor. An acceptable sensitivity was reached working under the experimental conditions described above, with the LQs for the target contaminants ranging from 1 pg to 100 pg depending on the compound and the instrumental technique employed. The LQs in ng L^{-1} varied from 0.03 to 8 in seawater, with only five compounds having values ≥2 ng L^{-1}. The method recoveries for the native target contaminants (abbreviations are detailed in **Table 1**) and surrogates ranged from 57 ± 9 (TEHP) to 131 ± 16 (TCEP) for OPEs, from 52 ± 3 (DnOP) to 163 ± 131 (DEHP) for PAEs, from 88 ± 10 (BPAP) to 118 ± 26 (BPAF) for BPs, from 63 ± 9 (PFOSF) to 124 ± 5 (PFOA) for PFCs, and 48 ± 5 (PCB-180) to 133 ± 7 (α-endosulfan) for OCs. These values are in agreement with existing studies focusing on one chemical family: Hu et al. (2014) had recoveries between 67 and 118% for OPEs, Paluselli et al. (2018a) recovered PAEs in the range 95–110%, Pojana et al. (2007) found BPs in the range 50–98%, Kaserzon et al. (2012) determined recoveries between 36 and 129% for PFCs, and Lammel et al. (2017) found OCs in the range 75–103%. Similar recoveries were found for most compounds

TABLE 3 | Recoveries of the target contaminants ($n = 5$) with their associated standard deviation, LQs expressed as absolute amounts (pg) and ng L^{-1} in seawater.

Compound	Method recoveries in spiked seawater		LQ	
	20 ng L^{-1}	150 ng L^{-1}	(pg)	(ng L^{-1})
ORGANOPHOSPHATE ESTERS (OPEs)				
TPP	95 ± 9	108 ± 4	5	0.13
TiBP	119 ± 10	109 ± 5	1	0.03
TnBP	113 ± 16	107 ± 6	2	0.05
TCEP	124 ± 16	131 ± 5	10	0.19
TCPPs	122 ± 58	118 ± 4	10	0.25
TDCP	114 ± 9	120 ± 4	10	0.25
TPhP	111 ± 9	111 ± 4	5	0.10
EHDPP	74 ± 9	61 ± 6	5	0.13
TEHP	57 ± 9	61 ± 4	10	0.20
TBP-*d27*	88 ± 5	85 ± 4	2	0.05
TPhP-*d15*	91 ± 7	88 ± 4	5	0.13
TCPP-*d18*	109 ± 7	105 ± 10	30	0.75
TDCP-*d15*	111 ± 4	111 ± 15	11	0.28
PHTHALATES (PAEs)				
DMP	85 ± 5	80 ± 4	5	0.07
DEP	81 ± 8	82 ± 3	2	0.05
DiBP	137 ± 76	74 ± 15	1	0.03
DnBP	92 ± 7	82 ± 5	1	0.03
BBzP	82 ± 2	83 ± 4	5	0.09
DEHP	163 ± 131	73 ± 66	2	0.05
DnOP	57 ± 6	52 ± 3	10	0.25
DnBP-*d4*	92 ± 5	89 ± 1	1	0.03
BISPHENOLS (BPs)				
BPA	90 ± 22	88 ± 15	10	1.0
BPAF	112 ± 30	118 ± 26	1	0.1
BPAP	88 ± 13	92 ± 20	20	2.0
BPF	92 ± 19	90 ± 27	10	1.0
BPS	115 ± 9	112 ± 20	1	0.1
BPZ	96 ± 22	87 ± 17	50	5.0
4nOP-*d17*	64 ± 17	79 ± 4	30	3.0
PERFLUORINATED COMPOUNDS (PFCs)				
PFHA	69 ± 6	82 ± 2	80	8.0
PFHS	89 ± 10	73 ± 14	1	0.1
PFOA	113 ± 11	124 ± 5	20	2.0
PFOS	70 ± 9	85 ± 2	1	0.1
PFOSF	63 ± 9	76 ± 13	10	1.0
ORGANOCHLORINATED (OCs)				
PCB-28	70 ± 10	69 ± 9	10	0.18
PCB-52	75 ± 12	71 ± 3	12	0.31
PCB-101	63 ± 17	62 ± 4	15	0.38
PCB-118	63 ± 17	59 ± 4	10	0.25
PCB-138	60 ± 15	56 ± 4	20	0.42
PCB-153	70 ± 17	57 ± 3	20	0.46
PCB-180	53 ± 12	49 ± 3	20	0.50
HCB	48 ± 5	58 ± 3	12	0.29
α-HCH	76 ± 4	75 ± 1	20	0.49
β + γ-HCH	80 ± 3	81 ± 2	15	0.32
4,4-DDT	59 ± 15	55 ± 4	30	0.68
α-Endosulfan	110 ± 15	133 ± 7	45	1.07
α-HCH-*d6*	88 ± 7	95 ± 2	15	0.38
2,4-DDT-*d8*	69 ± 5	40 ± 2	1	0.02
^{13}C-PCB-180	65 ± 9	56 ± 1	20	0.45

at the two-environmental concentration tested. Therefore, most compounds showed acceptable recoveries. However, high relative standard deviations were found for DEHP and DiBP at the lower spiking level (i.e., 20 ng L^{-1}). The high recovery end observed could be due to a specific contamination of the spiked samples even if the blanks run in parallel did not show any significant contamination. Indeed, we measured a higher concentration of DEHP and DiBP in one single replicate, which explained the high relative standard deviations and recoveries for both compounds. Although many precautions were taken for minimizing contamination, some interference could still be attributed, for example, to random contamination of the SPE cartridges by residual plastic fibers present in the air. Therefore, we recommend analyzing duplicates of real samples to avoid false positive results, and thus we applied this strategy as described in the following section "Application to seawater samples." The recovery rates were not dependent on the compound polarity (e.g., nonpolar OCs showed a recovery range comparable to the one of more polar BPs), indicating that Oasis HLB is well adapted for our purpose.

Application to Seawater Samples

The method was applied to freshly collected marine samples from Marseille Bay at three sampling stations (**Figure 1**). The range of concentrations found at the three sites was between <LQ up to several hundreds of ng L^{-1}. Otherwise, concentrations close to LQ have been quantified, and accuracy was not affected. The duplicate samples were analyzed at each location (except for OCs, which were analyzed on a single replicate), and acceptable reproducibility was obtained at all sites, except for DEHP at the Cortiou station (79 and 132 ng L^{-1}). As DEHP LQ was substantially exceeded, the maximum analytical permissible error of 60% close to LQ (Lissalde et al., 2011) cannot explain fully the difference between both replicates. Therefore, this difference could also be attributed to laboratory contamination affecting one single sample from the duplicate. Among the 40 contaminants analyzed, 25 were found at least once (**Figure 3**). The three families of plastic organic additives exhibited the highest concentrations up to 462, 132, and 123 ng L^{-1} for the TCPP, DEHP, and BPS, respectively. Among the three sites, the highest concentrations of the targeted contaminants were found at Cortiou (150 m from Marseille WWTP outfall). The measured concentrations were generally in the range of those reported in previous studies in various marine environments (Pojana et al., 2007; Kaserzon et al., 2012; Hu et al., 2014; Chau et al., 2015; Net et al., 2015a; Brumovský et al., 2017; Lammel et al., 2017; Li et al., 2017; Paluselli et al., 2018a).

OPEs and PAEs represented from 78 to 85% of relative abundance of the five contaminant classes, whatever the site. OPEs were more abundant near the WWTP (∼60% at the Cortiou site), whereas PAEs were the most abundant at the most remote site (68% at Frioul). TCPP was always the most abundant OPE, with a relative abundance among OPEs between 77 and 84%. The sum of DEHP and DiBP was always higher than 75% of the total PAE concentration. BPs represented from 1% (Frioul) to 14% (Cortiou) of the total target contaminant concentration. BPS was the most abundant BP near the WWTP at Cortiou (∼90%), whereas BPA was the predominant BP in the other two sites (>80%). BPS was the most concentrated among all BPs (up to 123 ng L^{-1}, and 88% of all BPs), ahead of BPA, which points out the need for monitoring several compounds from each chemical family, for which less data is available. PFCs were quantified at very low levels in all samples (<1% of the total target contaminant concentration), with individual compound

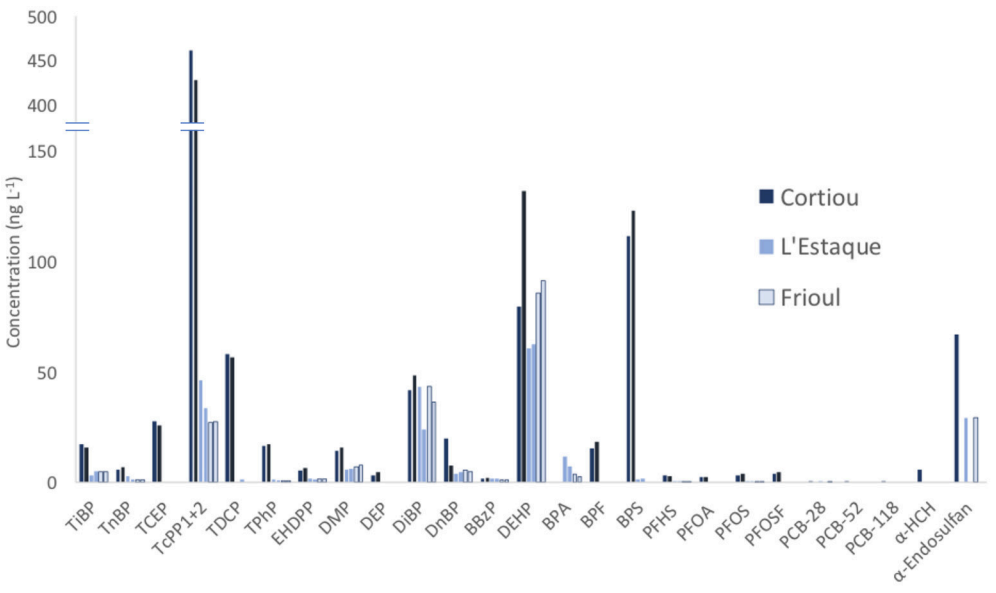


FIGURE 3 | Concentrations of contaminants found at the three sampling sites (Cortiou, L'Estaque, and Frioul) in the Bay of Marseille in May 2017. The duplicate samples are shown as two consecutive bars. The y-axis breaks between 150 and 400 ng L^{-1}. Not detected compounds are not shown. OCs were analyzed on one replicate at each site. The contaminant abbreviations are given in **Table 1**. Compounds are grouped by chemical family.

concentrations always lower than 5 ng L^{-1}, and only two OCs were found at concentrations clearly above the LQs (7–16% of the total target contaminant concentration depending on the site): α-endosulfan (30–70 ng L^{-1}, representing 90–100% of the 13 OCs concentration) and α -HCH (∼6 ng L^{-1}, representing <8% of OCs at all sites). PCBs-28,−52, and,−118 were found at very low concentration levels (0.4–0.6 ng L^{-1}) (**Supplementary Table 4**). The concentrations of the rest of OCs were below the LQs, most probably owing to the low amounts entering the bay and their preferential partitioning in the water particle phase (not considered in this study).

The EQSs from the European Union Water Framework Directive (WFD) in seawater are only available for DEHP (annual average: 1300 ng L^{-1}), PFOS/PFOA (annual average: 0.13 ng L^{-1}), and several OCs (annual average: from 4 to 300 ng L−1). Although an appropriate risk assessment cannot be conducted with the present data due to the limited number of samples, our results pointed PFOS as the most potentially harmful substance from our target list, exceeding the current EQS in seawater.

CONCLUSIONS

A reliable analytical method for a rapid measurement of five classes (OPEs, PAEs, BPs, PFCs, and OCs) of organic contaminants in natural seawater at trace levels was developed and validated. The method proposed in this study allowed the quantification of 40 organic contaminants presenting a wide range of physicochemical properties and sources in the environment, including both legacy and emerging contaminants. The implementation of a single SPE protocol using a sorbent, including polar and nonpolar moieties, allowed reaching acceptable performances both in terms of analyte recoveries and LQs. We proposed a systematic strategy for catching most of the organic contaminants, using the polarity gradient during the SPE elution step. Only ionizable organic substances could be missed because of the intrinsic properties of the selected sorbent, which could however been overcome by the use of mixed-mode ion exchange sorbents. This study focused mainly on plastic organic additives, which were found to be the most abundant contaminants in Marseille Bay, far ahead of conventional PCBs or OCPs. Therefore, our method represents a useful tool for screening and quantification of widely diffused plastic organic additives and indirect assessment of the impact of plastic waste on the dissolved seawater fraction, generally considered bioavailable.

AUTHORS CONTRIBUTIONS

VF, JC-J, and RS experimental design. NS and BC sampling. NS, BC, VF, and JC-J performed the experiments and analyses. VF, JC-J, RS, NS, and CP wrote the paper.

FUNDINGS

This study was financially supported by the JPI Oceans PLASTOX and Agence de l'Eau-CAREMED and Blue-POLUT projects. This research was also partially funded by the Labex OT-Med (no ANR-11-LABX-0061)—MEDPOP, funded by the French Government Investissements d'Avenir (ANR) through the AMIDEX project (no ANR-11-IDEX-0001-02). The project leading to this publication has also received funding from European FEDER Fund under project 1166-39417.

ACKNOWLEDGMENTS

The authors are grateful to Dorian Guillemain, Nagib Bhairy, Christian Grenz, Michel Lafont, and Deny Malengros from SAM-M I O platform and the crew and captain of the R/V Antedon II for operation at sea.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00295/full#supplementary-material

REFERENCES

Allan, I., Vrana, B., Greenwood, R., Mills, G., Knutsson, J., Holmberg, A., et al. (2006). Strategic monitoring for the European Water Framework Directive. *TrAC Trends Anal. Chem.* 25, 704–715. doi: 10.1016/j.trac.2006.05.009

Assoumani, A., Lissalde, S., Margoum, C., Mazzella, N., and Coquery, M. (2013). *In situ* application of stir bar sorptive extraction as a passive sampling technique for the monitoring of agricultural pesticides in surface waters. *Sci. Total Environ.* 463–464, 829–835. doi: 10.1016/j.scitotenv.2013.06.025

Barrios-Estrada, C., de Jesús Rostro-Alanis, M., Muñoz-Gutiérrez, B. D., Iqbal, H. M. N., Kannan, S., and Parra-Saldívar, R. (2018). Emergent contaminants: endocrine disruptors and their laccase-assisted degradation – a review. *Sci. Total Environ.* 612, 1516–1531. doi: 10.1016/j.scitotenv.2017.09.013

Brumovský, M., Bečanová, J., Kohoutek, J., Borghini, M., and Nizzetto, L. (2017). Contaminants of emerging concern in the open sea waters of the Western Mediterranean. *Environ. Pollut.* 229, 976–983. doi: 10.1016/j.envpol.2017.07.082

Chau, H. T. C., Kadokami, K., Duong, H. T., Kong, L., Nguyen, T. T., Nguyen, T. Q., et al. (2015). Occurrence of 1153 organic micropollutants in the aquatic environment of Vietnam. *Environ. Sci. Pollut. Res.* 25, 7147–7156. doi: 10.1007/s11356-015-5060-z

Fauvelle, V., Mazzella, N., Morin, S., Moreira, S., Delest, B., and Budzinski, H. (2015). Hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry for acidic herbicides and metabolites analysis in fresh water. *Environ. Sci. Pollut. Res.* 22, 3988−3996. doi: 10.1007/s11356-014-2876-x

Hahladakis, J. N., Velis, C. A., Weber, R., Iacovidou, E., and Purnell, P. (2018). An overview of chemical additives present in plastics: migration, release, fate and environmental impact during their use, disposal and recycling. *J. Hazard. Mater.* 344, 179–199. doi: 10.1016/j.jhazmat.2017.10.014

Hermabessiere, L., Dehaut, A., Paul-Pont, I., Lacroix, C., Jezequel, R., Soudant, P., et al. (2017). Occurrence and effects of plastic additives on marine environments and organisms: a review. *Chemosphere* 182, 781–793. doi: 10.1016/j.chemosphere.2017.05.096

Holadová, K., Prokupková, G., Hajslová, J., and Poustka, J. (2007). Headspace solid-phase microextraction of phthalic acid esters from vegetable oil employing solvent based matrix modification. *Anal. Chim. Acta* 582, 24–33. doi: 10.1016/j.aca.2006.09.005

Hu, M., Li, J., Zhang, B., Cui, Q., Wei, S., and Yu, H. (2014). Regional distribution of halogenated organophosphate flame retardants in seawater

samples from three coastal cities in China. *Mar. Pollut. Bull.* 86, 569–574. doi: 10.1016/j.marpolbul.2014.06.009

Kaserzon, S. L., Kennedy, K., Hawker, D. W., Thompson, J., Carter, S., Roach, A. C., et al. (2012). Development and calibration of a passive sampler for perfluorinated alkyl carboxylates and sulfonates in water. *Environ. Sci. Technol.* 46, 4985–4993. doi: 10.1021/es300593a

Kim-Tiam, S., Fauvelle, V., Morin, S., and Mazzella, N. (2016). Improving toxicity assessment of pesticide mixtures: the use of polar passive sampling devices extracts in microalgae toxicity tests. *Front. Microbiol.* 7:1388. doi: 10.3389/fmicb.2016.01388

Lammel, G., Spitzy, A., Audy, O., Beckmann, S., Codling, G. P., Kretzschmann, L., et al. (2017). Organochlorine pesticides and polychlorinated biphenyls along an east-to-west gradient in subtropical North Atlantic surface water. *Environ. Sci. Pollut. Res.* 24, 11045–11052. doi: 10.1007/s11356-016-7429-z

Li, J., Xie, Z., Mi, W., Lai, S., Tian, C., Emeis, K. C., et al. (2017). *Organophosphate esters* in air, snow, and seawater in the North Atlantic and the Arctic. *Environ. Sci. Technol.* 51, 6887–6896. doi: 10.1021/acs.est.7b01289

Lissalde, S., Mazzella, N., Fauvelle, V., Delmas, F., Mazellier, P., and Legube, B. (2011). Liquid chromatography coupled with tandem mass spectrometry method for thirty-three pesticides in natural water and comparison of performance between classical solid phase extraction and passive sampling approaches. *J. Chromatogr. A* 1218, 1492–1502. doi: 10.1016/j.chroma.2011.01.040

Messerlian, C., Martinez, R. M., Hauser, R., and Baccarelli, A. A. (2017). 'Omics' and endocrine-disrupting chemicals — new paths forward. *Nat. Rev. Endocrinol.* 13, 740–748. doi: 10.1038/nrendo.2017.81.

Murphy, F., Ewins, C., Carbonnier, F., and Quinn, B. (2016). Wastewater Treatment Works (WwTW) as a source of microplastics in the aquatic environment. *Environ. Sci. Technol.* 50, 5800–5808. doi: 10.1021/acs.est.5b05416

Net, S., Delmont, A., Sempéré, R., Paluselli, A., and Ouddane, B. (2015a). Reliable quantification of phthalates in environmental matrices (air, water, sludge, sediment and soil): a review. *Sci. Total Environ.* 515–516, 162–180. doi: 10.1016/j.scitotenv.2015.02.013

Net, S., Sempéré, R., Delmont, A., Paluselli, A., and Ouddane, B. (2015b). Occurrence, fate, behavior and ecotoxicological state of phthalates in different environmental matrices. *Environ. Sci. Technol.* 49, 4019–4035. doi: 10.1021/es505233b

Paluselli, A., Aminot, Y., Galgani, F., Net, S., and Sempéré, R. (2018a). Occurrence of phthalate acid esters (PAEs) in the northwestern Mediterranean Sea and the Rhone River. *Prog. Oceanogr.* 163, 221–231. doi: 10.1016/j.pocean.2017.06.002

Paluselli, A., Fauvelle, V., Schmidt, N., Galgani, F., Net, S., and Sempéré, R. (2018b). Distribution of phthalates in Marseille Bay (NW Mediterranean Sea). *Sci. Total Environ.* 621, 578–587. doi: 10.1016/j.scitotenv.2017.11.306

Pojana, G., Gomiero, A., Jonkers, N., and Marcomini, A. (2007). Natural and synthetic endocrine disrupting compounds (EDCs) in water, sediment and biota of a coastal lagoon. *Environ. Int.* 33, 929–936. doi: 10.1016/j.envint.2007.05.003

Sousa, J. C. G., Ribeiro, A. R., Barbosa, M. O., Pereira, M. F. R., and Silva, A. M. T. (2017). A review on environmental monitoring of water organic pollutants identified by EU guidelines. *J. Hazard. Mater.* 344, 146–162. doi: 10.1016/j.jhazmat.2017.09.058

Suhrhoff, T. J., and Scholz-Böttcher, B. M. (2016). Qualitative impact of salinity, UV radiation and turbulence on leaching of organic plastic additives from four common plastics - a lab experiment. *Mar. Pollut. Bull.* 102, 84–94. doi: 10.1016/j.marpolbul.2015.11.054

Taylor, P. J. (2005). Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. *Clin. Biochem.* 38, 328–334. doi: 10.1016/j.clinbiochem.2004.11.007

Zhang, Z. M., Zhang, H. H., Zhang, J., Wang, Q. W., and Yang, G. P. (2018). Occurrence, distribution, and ecological risks of phthalate esters in the seawater and sediment of Changjiang River Estuary and its adjacent area. *Sci. Total Environ.* 619–620, 93–102. doi: 10.1016/j.scitotenv.2017.11.070

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer VL and handling editor declared their shared affiliation at the time of the review.

frontiers in Marine Science

ORIGINAL RESEARCH
published: 30 October 2018
doi: 10.3389/fmars.2018.00389

BP Gulf Science Data Reveals Ineffectual Subsea Dispersant Injection for the Macondo Blowout

Claire B. Paris[1], Igal Berenshtein[1], Marcia L. Trillo[1], Robin Faillettaz[1], Maria J. Olascoaga[1], Zachary M. Aman[2], Micheal Schlüter[3] and Samantha B. Joye[4]*

[1] Department of Ocean Sciences, Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, FL, United States, [2] Fluid Science and Resources Division, Department of Chemical Engineering, The University of Western Australia, Perth, WA, Australia, [3] Institute of Multiphase Flows, Technische Universität Hamburg, Hamburg, Germany, [4] Department of Marine Sciences, University of Georgia, Athens, GA, United States

After the *Deepwater Horizon* oil platform explosion, an estimated 172.2 million gallons of gas-saturated oil was discharged uncontrollably into the Gulf of Mexico, causing the largest deep-sea blowout in history. In an attempt to keep the oil submerged, massive quantities of the chemical dispersant Corexit® 9500 were deployed 1522 m deep at the gushing riser pipe of the Macondo prospect's wellhead. Understanding the effectiveness of this unprecedented subsea dispersant injection (SSDI) is critical because deep-water drilling is increasing worldwide. Here we use the comprehensive BP Gulf Science Data (GSD) to quantify petroleum dynamics throughout the 87-day long blowout. The spatio-temporal distribution of petroleum hydrocarbons revealed consistent higher concentrations at the sea surface and in a deep intrusion below 1000 m. The relative importance of these two layers depended on the hydrocarbon mass fractions as expected from their partitioning along temperature and pressure changes. Further, analyses of water column polycyclic aromatic hydrocarbons (PAH) of GSD extensively sampled within a 10-km radius of the blowout source demonstrated that substantial amounts of oil continued to surface near the response site, with no significant effect of SSDI volume on PAH vertical distribution and concentration. The turbulent energy associated with the spewing of gas-saturated oil at the deep-sea blowout may have minimized the effectiveness of the SSDI response approach. Given the potential for toxic chemical dispersants to cause environmental damage by increasing oil bioavailability and toxicity while suppressing its biodegradation, unrestricted SSDI application in response to deep-sea blowout is highly questionable. More efforts are required to inform response plans in future oil spills.

Keywords: oil spill, deep-sea blowout, chemical dispersants, water column, Macondo, subsea dispersant injection, petroleum, Gulf Science Data

OPEN ACCESS

Edited by:
Juan Bellas, Instituto Español de Oceanografía (IEO), Spain

Reviewed by:
André Ricardo Araújo Lima, Universidade Federal de Pernambuco, Brazil
Markus Huettel, Florida State University, United States

****Correspondence:***
Claire B. Paris
cparis@rsmas.miami.edu

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *16 April 2018*
Accepted: *04 October 2018*
Published: *30 October 2018*

Citation:
Paris CB, Berenshtein I, Trillo ML, Faillettaz R, Olascoaga MJ, Aman ZM, Schlüter M and Joye SB (2018) BP Gulf Science Data Reveals Ineffectual Subsea Dispersant Injection for the Macondo Blowout. Front. Mar. Sci. 5:389. doi: 10.3389/fmars.2018.00389

INTRODUCTION

On April 20th, 2010, the Deepwater Horizon (DWH) blowout – one of the largest oil spill disasters in history – took place in the Gulf of Mexico at a depth of 1,522 m. A total amount of 4.1 million barrels of oil (McNutt et al., 2012) spilled over 87 days before the Macondo wellhead was capped on July 15th, 2010.

Since then, numerous studies have focused on this particular oil spill and revealed important characteristics of deep-sea blowouts. The dynamic physiochemical processes that transport petroleum hydrocarbons in the water column depend on the gas-to-oil ratio, the type and behavior of "live oil" (crude oil saturated with natural gas) under variable hydrostatic pressure, and on turbulent flows and ambient currents near the wellhead (Tolman, 1949; Aman et al., 2015). With a flow rate in the range of 50,000–70,000 bbl/d (Griffiths, 2012) and stratification-dominated currents, a live oil plume became trapped at levels of neutral buoyancy (Aman et al., 2015), leading to formation of intrusion layers (Camilli et al., 2010; Kessler et al., 2011). A noticeable dominant deep intrusion, the so-called "deep plume", was centered around 1,100 m, while secondary shallower intrusions were identified at about 800 and 300 m (Kessler et al., 2011; Spier et al., 2013). Further, the dissolution and biodegradation of the live oil as it rose in the water column lead to hydrocarbon partitioning between organic and aqueous phases. These key processes dictate the spatio-temporal dynamics of the oil spilled (Ryerson et al., 2011; Camilli et al., 2012). A detailed temporal analysis of the distribution of hydrocarbons in the water column is still lacking.

Recently, a comprehensive collection of more than 24,500 water samples from at least 67 Response and Natural Resource Damage Assessment (NRDA) studies were made available to the scientific community through the BP Gulf Science Data (GSD) (BP Gulf Science Data1, 2016; BP Gulf Science Data2, 2016). This dataset provides a unique opportunity to examine how the different petroleum hydrocarbons were transported and partitioned in the water column through time. What is more, massive amounts (*ca*. 771 thousand gallons) of chemical dispersants, namely Corexit® 9500 produced by Nalco-Champion (BP Gulf Science Data2, 2016; BP Gulf Science Data3, 2016), was injected directly at the wellhead as an unprecedented first response, to prevent rapid rise of oil to the surface.

Corexit 9500 is a powerful surfactant containing dioctyl sodium sulfosuccinate (DOSS) that lowers the interfacial tension between the oil and the water and enhances formation of neutrally buoyant micro-droplets (Wilson et al., 2015). This mechanism dictates that the enhanced dispersion increases the hydrocarbon content of the deep plume (Paris et al., 2012; Aman et al., 2015; Pesch et al., 2018), but DOSS could also increase the toxicity of the sequestered material (Kujawinski et al., 2011). Additionally, the amount of surfacing oil should slow, and sheens should be thinner, further downstream from the spill site (Socolofsky et al., 2015), which could help mitigate response efforts. Indeed, the droplet size distribution (DSD) generated during subsea blowouts has a strong impact on their outcome since they dictate the oil rising speed (Paris et al., 2012; Pesch et al., 2018). Yet, little is known about the impacts of subsea dispersant injection (SSDI) on the transport of petroleum hydrocarbons and its tradeoffs, beyond modulating the extent of environmental contamination by decreasing natural degradation and increasing water column toxicity. The impact of chemical dispersants on biodegradation is still debated (Kleindienst et al., 2015a,b).

The first objective of this study is to use the water column chemistry GSD to describe the monthly dynamics of light and heavy hydrocarbon mass fractions of the Macondo oil from May 5 to December 31, 2010. The second objective is to determine the relationship between the water column chemistry GSD and the variable dispersant injection volumes at the wellhead (BP Gulf Science Data2, 2016; BP Gulf Science Data3, 2016). This analysis provides the first quantitative insight as to whether SSDI is an effective control method during the turbulent release of live oil in the deep-sea.

MATERIALS AND METHODS

The GSD (BP Gulf Science Data1, 2016) Water Chemistry Data conforms to the Analytical Quality Assurance Plan and provides the concentrations on volatile hydrocarbons, polycyclic aromatic hydrocarbons (PAH), and saturated hydrocarbons (SHC) and benzene, toluene, ethylbenzene, xylenes (BTEX) in the water column from the sea surface to a maximum depth of 2,850 m. We filtered the BP GSD Water Chemistry Data by time from May 5, 2010 (first recorded BP GSD) to December 31, 2010 and by two radial distances from the blowout. We computed individual radial distances for each sample with the GIS WGS 1984 Geographic Coordinate System, and the World Behrmann Projected Coordinate System. Of the 13,218 water sample oil concentrations reported from offshore stations during the year 2010, 59% (7,741) and 26% (3.464) were collected within a radial distance of 100 and 10 km from Macondo, respectively (**Figure 1**). We used the water column samples within 100 km from the oil spill source for the spatio-temporal distribution of low (C5–C12) and high (C13+) hydrocarbon mass fractions, and within 10 km for the SSDI analysis. The spatial discretization of 10 km is specifically designed to test the assumption that SSDI would reduce the amount of oil rising directly at the response site (Socolofsky et al., 2015).

Spatio-Temporal Distribution of Hydrocarbon Mass Fractions

For deep-sea blowouts, the dissolution, biodegradation, and chemical composition of live oil influences partitioning of hydrocarbons throughout the water column (Jaggi et al., 2017). This partitioning behavior is based on the volatility and aqueous solubility of each hydrocarbon species (Ryerson et al., 2011; Reddy et al., 2012). To distinguish these processes, we classified the water chemistry results of PAH, SHC, and BTEX into two hydrocarbon fractions based on their molecular weight: a light fraction between five and twelve carbons (C5–C12) and a heavier fraction with more than thirteen carbons (C13+) (**Supplementary Table S1**). Concentrations within 100 km of the spill source ranged from 0.0008 to 58,730 μg/L and 0.0004 to 101,768 μg/L for the light and heavy fractions, respectively. Concentrations <0.05 μg/L are considered background concentrations derived from natural petroleum seeps (Wade et al., 2016). To obtain a unique concentration value per coordinate and sample date, values of replicates from different laboratories were averaged. The DWH

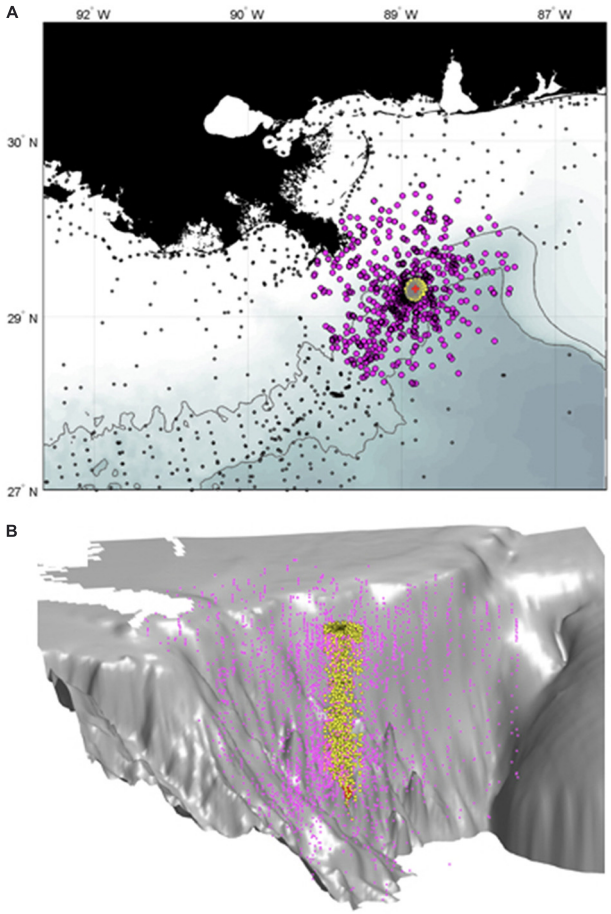


FIGURE 1 | BP Gulf Science Data (GSD) Water Chemistry Data stations collected during the *Deepwater Horizon* (DWH) blowout from May to December 2010. **(A)** Stations located within 100 km (magenta stations, n = 7,741) and 10 km (yellow stations, n = 3,464) perimeters from Macondo (red+) are used to analyze the daily vertical distribution of low (C5–C12) and high (C13+) molecular weight petroleum hydrocarbons, and to evaluate subsea dispersant injection (SSDI) volumes on the vertical distribution of polycyclic aromatic hydrocarbons (PAHs) around the response area, respectively. Green dots are GSD stations outside the study area (n = 5,477). The gray lines are at the 1,000 and 2,000 m isobaths. **(B)** Three-dimensional view of the selected stations relative to *Macondo* wellhead's depth (red circle, not to scale).

oil spill began with the drilling rig explosion on April 20, 2010, but the GSD collection was initiated on May 5, 2010. We report the monthly distribution of mean concentrations for the two mass fractions from May through December 2010 (**Figure 2**).

Subsea Dispersant Injection Analysis

To identify the potential effect of the SSDI on fresh live oil rising directly above the wellhead, we separate samples within a 10 km perimeter from Macondo (Ryerson et al., 2011; **Figure 1**). In addition to the PAH Water Chemistry Data (BP Gulf Science Data1, 2016; (**Supplementary Table S2**), we use the BP GSD records of surface and subsea dispersant application (BP Gulf Science Data2, 2016; BP Gulf Science Data3, 2016).

To assess the effect of the SSDI on Macondo oil dynamics, we use three complementary statistical approaches: (i) a regression tree analysis (RT) to explore the factors affecting oil concentrations, (ii) a generalized least squares (GLS) analysis to directly examine the hypothesis that increasing SSDI volume increases oil in the deep sea and reduces oil at the surface (Socolofsky et al., 2015), and (iii) linear regression models (LM) to detect if higher SSDI volume retains overall oil deeper in the water column. For each analyses, we analyzed the PAH samples and applied a logarithm transformation $\log(X + 1)$ on the PAH concentrations to stabilize variances. Hence, the response variables were (i) PAH concentration, (ii) the vertical ratio of the PAH concentrations between the deep (≥600 m) and shallow (<600 m) depth layers, and (iii) the depth center of mass of PAH concentration in the RT, GLS, and LM analyses, respectively. The different explanatory variables used are the volume of surface dispersant application [gal], the volume of SSDI [gal], the distance from the wellhead [km], the flow rate [bbl/day], and the time from initial blowout [days]. The hallmark effects of SSDI are an increase of oil entrainment in the deep plume and a decrease in the amount of oil surfacing directly above the response site (Socolofsky et al., 2015). The effectiveness of SSDI may therefore be observed quantitatively through changes in subsea oil concentration and/or the relative oil concentration between the sea surface and the subsea. All analyses were performed with R statistical software, using the R open-source software with the packages "rpart," "rpart.plot," "nlme," and "visreg" (R Core Team, 2018).

Regression Tree Analysis

The RT splits maximize the variation between the groups and minimizes the variation within groups using the sum of squares. The analysis initially generates a full tree, following by an optimal trimming of the tree, based on the 1SE rule, with a 10% cross-validation and a complexity-parameter of 0.039.

Generalized Least Squares Analysis

We examine the range of potential SSDI effects by computing the ratio between the geometric mean oil concentration of various depth layers where the nominator is always the upper layer, hereafter referred to as *oil vertical ratio*. SSDI effect is examined on its application day (not lagged), with the assumption that Corexit® 9500 would reach equilibrium adsorption to the water-oil interface on the timescale of seconds (Wade et al., 2016). We also examine the next day effect (1-day lagged). First, collinearity between explanatory variables is tested using Variance Inflation Factor (VIF; cutoff threshold = 2). Second, we checked for temporal dependence of the multiple regression model residuals using Ljung–Box test. We then used the GLS analysis to account for the autocorrelation and evaluated the association between the explanatory variables and the response variables. Finally, we used a Likelihood ratio test to find the best fitted model. The addition of the temporal auto-correlation term resulted in a significantly better GLS model (Likelihood ratio test, $X^2 = 11.46$, $p < 0.001$). A negative slope of the *oil vertical ratio* GLS regressions would indicate that the SSDI procedure was effective. Alternatively, no significant slope or a positive slope for the *oil*

vertical ratio would demonstrate no SSDI effect (**Supplementary Table S3**).

Linear Regressions Analysis

The application of SSDI should limit the rise of the oil in the water column (Socolofsky et al., 2015). Applying higher volume of SSDI should enable to treat a higher proportion of oil overall, and therefore retain it deeper in the water column. To detect for the presence of a relationship between the volume of SSDI applied and the overall vertical distribution of PAH concentrations, we split the SSDI volume into 500 L/d bins (ranging from 0 to 20,500 L/d, 22 bins in total) and computed the depth center of mass of PAH concentrations (Z_{cm}) for each bin. The Z_{cm}, which therefore summarizes the vertical distribution of PAH concentrations, were then tested against SSDI volume with linear regression.

RESULTS

Spatio-Temporal Analysis of Hydrocarbon Mass Fractions

Oil droplets rise to the surface depending on their buoyancy relative to the surrounding density stratification (Paris et al., 2012). Due to their varying size and biodegradation, they have different rise rates and follow different trajectories. The lighter, more volatile fraction (C5–C12) are generally consumed rapidly (Valentine et al., 2010; Crespo-Medina et al., 2014), while the heaviest category (C13+) resides longer in the water column before eventually reaching the surface.

Despite irregular sampling employed during collection of the BP GSD, a clear signal emerges through time (**Figure 2**). We find high concentrations of the lighter fraction (C5–C12) forming a deep intrusion between 1000 and 1300 m (Camilli et al., 2010) from May to July (red dots, **Figure 2A**). As reported in Crespo-Medina et al. (2014), the deep plume started to break down after July when the discharge stopped, with lower concentrations onward (green dots, **Figure 2B**). A clear gap in intermediate waters with background concentrations suggested acceleration of degassing droplets under decreasing pressure (Pesch et al., 2018) and/or biological consumption of this low molecular weight hydrocarbon category in the water column (Bagby et al., 2017).

We found elevated concentrations of heavy mass fraction (C13+) in the upper 200 m mostly near the DWH response site to about 80 km downstream from May through December 2010 (**Figure 2B** and **Supplementary Figure S1b**). This mass fraction was also trapped in the deep intrusion layer within 25 km from Macondo from May to July (**Figure 2B** and

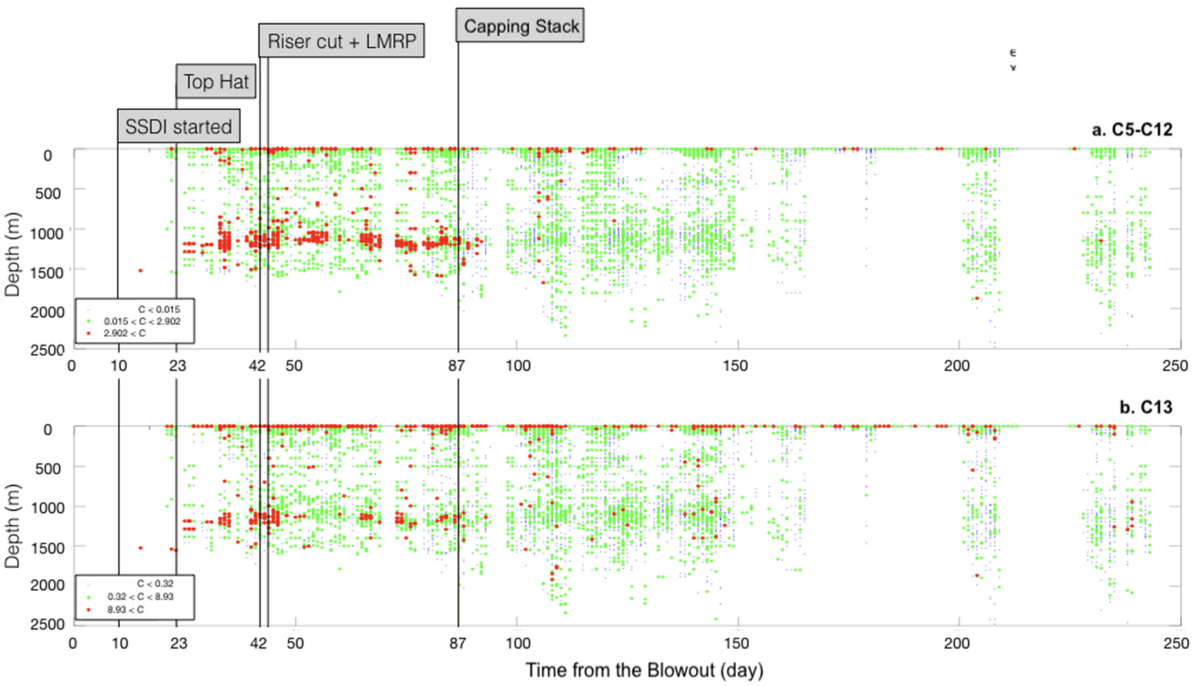


FIGURE 2 | Daily vertical distribution of petroleum hydrocarbon concentrations in the Gulf of Mexico (GoM) from the *Deepwater Horizon* blowout until December 31, 2010. **(A)** Concentrations of low (C5–C12) and **(B)** high (C13+) molecular weight hydrocarbon fractions. Values are computed into a grid of 10 m depth over the entire GoM. Oil-contaminated samples are represented by red dots for values above the 90 percentile (2.902 and 8.93 μg/L for C5–C12 and C13+, respectively) and by green dots between the median and the 90 percentile (0.015 μg/L < C5–C12 < 2.902 μg/L and 0.32 μg/L < C13+ < 8.93 μg/L); blue dots are samples that are below the median to uncontaminated samples (0–0.015 μg/L and 0–0.32 μg/L for C5–C12 and C13+, respectively); white area has no samples. The key dates of the various attempts to stop the oil spill are indicated: (1) SSDI started on April 30 (day 10 after the blowout); (2) top hat on May 13 (day 23); (3) riser cut followed by the Lower Marine Riser Package (LMRP) on June 4th (day 42); and (4) finally the Stacking Cap on June 15th (day 87). Note: no data were recorded in BP Gulf Science Data (GSD) before May 5, 2010.

Supplementary Figure S1b). Significant concentration values were also observed throughout the water column, suggesting additional sequestration in secondary intrusion layers. In contrast to the lighter mass fraction, we observed concentrations values higher than the 90 percentile after the well was capped, from August through December (red dots, **Figure 2B**). Indeed, these heavier oil compounds contained primarily PAHs, which are persistent pollutants that are effectively transported by currents (Camilli et al., 2010). We also observed concentrations above the median later in the year (green dots, **Figure 2B**). The unprecedented amount of chemical dispersants injected at depth may also have inhibited biodegradation (Kleindienst et al., 2015b), increasing the residence time of the oil in the Gulf of Mexico. Whether chemical dispersants inhibit biodegradation of different crude oil components is vigorously debated (Kleindienst et al., 2015a,b), but it is clear that oil degradation is not necessarily accelerated after dispersant addition (Rahsepar et al., 2016), potentially undermining the utility of SSDI.

Subsea Dispersant Injection

Variable volumes of the powerful ionic dispersant Corexit® 9500 (BP Gulf Science Data2, 2016) were deployed for the first time subsea, directly at the discharging wellhead (**Supplementary Figure S2**). Periods with no treatment alternated with periods of low, high, and peak SSDI volumes from April 20 to July 15, 2010. For example, a peak application in early June accounted for nearly 8% of the total SSDI volume of 771,272 gal (**Supplementary Figure S1**). Presumably, chemically dispersed oil should be sequestrated at depth via a shift in the spectrum of the oil DSD toward slow-rising micro-droplets (Aman et al., 2015); this should lead to a significant fraction of the spilled oil not reaching the sea surface – or at least not directly at the response site above the wellhead (Socolofsky et al., 2015). There is no empirical evidence of this effect. While water depth (RT; highest rank: 0.53) and time from the blowout (RT; rank: 0.23) are the two most important explanatory variables of the variance in PAH concentrations, the amount of chemical dispersants injected at the wellhead does not control the PAH distribution (RT; lowest rank: 0.04). Consequently, SSDI does not appear in any branches of the RT (**Figure 3**). The first split of the tree partitions early deep samples with high PAH concentrations (**Figure 3**) collected before May 13th, 2010 (day 23) —indicating the presence of a deep intrusion without chemical dispersants (**Figure 4** and **Supplementary Figures S2, S3**). Indeed, the intrusion occurs when a multiphase, buoyant jet entrains seawater. This date coincides with the Top-Hat operation, when methanol was injected into a dome placed on the discharging riser pipe to prevent hydrate formation (McNutt et al., 2012). The second split distinguishes surfaced oil (depth < 2 m) of intermediate concentration from the subsea oil (node b, **Figure 3**). Water Chemistry Data samples are further partitioned after day 42 (June 2, node f). This date corresponds to the partially successful leaking lower marine riser package (LMRP) cap operation (**Figure 2** and **Supplementary Figure S2**). This cap system was collecting 25,000 bpd of oil directly above blowout preventer (BOP) by two containment systems: a riser pipe connected to a surface vessel and a choke line connected to a submersible (McNutt et al., 2011, 2012; Griffiths, 2012). This explains why samples taken after June 2 were characterized by lower oil concentration (node f, **Figure 3**). At the same time, the SSDI volume was increased by threefold (**Supplementary Figure S2**).

Samples collected between May 13th and June 3rd are further partitioned by depth. Water sampled between 1099 m and 1291 m captures the deep intrusion (nodes c, **Figures 3**, **4**) with higher oil concentrations relative to samples collected deeper (node e, **Figure 3**) and shallower (node d, **Figure 3**). We find no evidence of SSDI effectiveness since deep and shallow samples are not characterized by particularly high and low oil concentrations when SSDI is applied (**Figure 4**). The absence of relationship between SSDI volume and oil concentrations is supported by the results of the GLS regression analysis (**Supplementary Figure S4**), indicating no change in the oil vertical ratio with SSDI volume (slope $= -10^{-7}$, $t = -0.1$, $p = 0.9$, (**Supplementary Figure S5a** and **Supplementary Table S2**) even when considering a lagged effect of SSDI. Similarly, the depth center of mass of oil concentrations was not affected by the volume of SSDI applied ($F = 0.93$, $R^2 = 0.04$, $p = 0.34$, **Supplementary Figure S5b**).

DISCUSSION

If the SSDI had the desired effect, we would see a negative effect, denoting higher oil concentration in the deep plume with increased SSDI. In theory, deep injection should have increased the petroleum hydrocarbon concentration values at depth by enhancing the natural formation of micro-droplets that intrude in the deep plume (Aman et al., 2015; Chan et al., 2015). Instead, the analysis captures the dynamics of the blowout containment attempts and demonstrates that SSDI volume does not explain the high PAH concentrations found between 1099 and 1291 m (**Figures 3**, **5** and **Supplementary Figure S1**). The finding of a dense petroleum hydrocarbon layer around the expected trap height without dispersant indicates the natural formation of a dominant intrusion, owing to the atomization of the live oil that was turbulently dispersed from the broken pipe. A large pressure-drop of approximately 9 MPa across the 16 m BOP stack and the high flow rates (*ca.* $Q = 0.08$ m^3 s^{-1}) (Griffiths, 2012) could provide sufficient energy dissipation rates for the atomization of live oil into micro-droplets (Li et al., 2008). If initial droplet were tiny (i.e., generally below <300 μm, Li et al., 2008), it is unclear that addition of dispersants would generate significantly smaller droplet sizes.

A combination of temperature- and pressure-dependent processes may also have played a large role on the behavior of the Macondo blowout, and consequently on the oil concentration distribution in the water column. The impact of these deep seafloor processes is presumably larger than the role of SSDI on the uncontrolled release of gas-saturated live-oil (McNutt et al., 2012; Oldenburg et al., 2012). These processes include: (1) a sudden pressure drop at the BOP upon exit of the

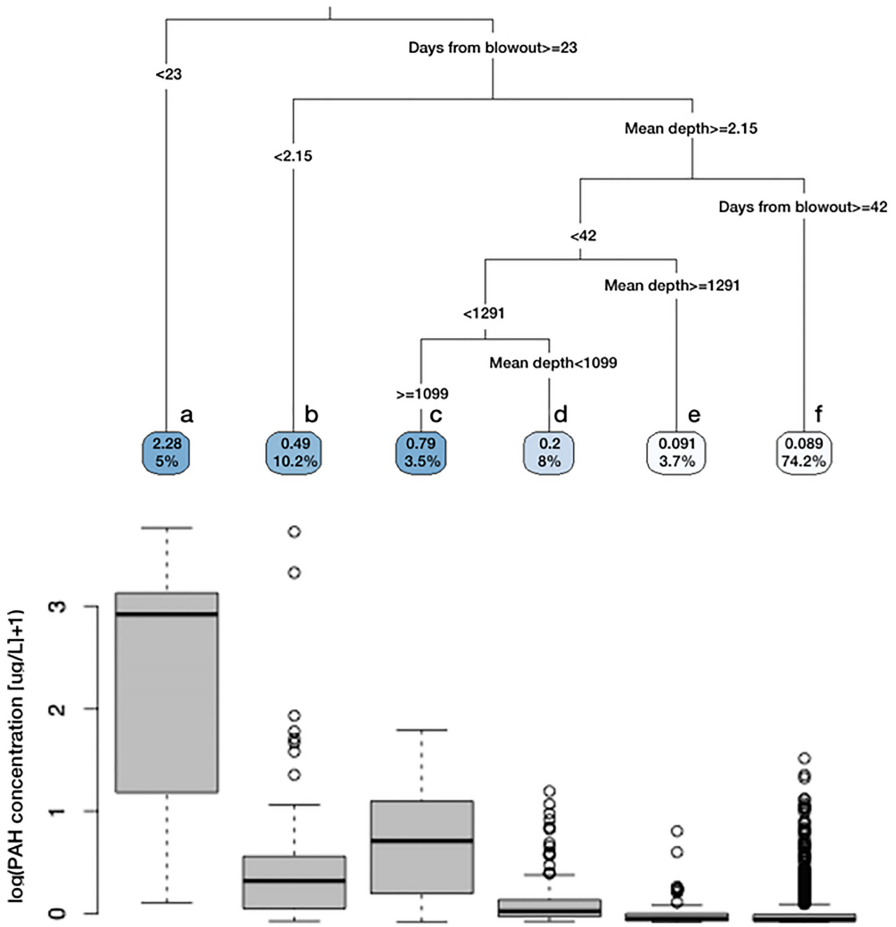


FIGURE 3 | Factors explaining oil distribution during the Macondo blowout. Regression tree of the log-transformed concentration [log(μg/l + 1)] of polynuclear aromatic hydrocarbon (PAH) as response variable against depth of the sample [m], time from the blowout [days], distance from the wellhead [km], subsea dispersant injection (SSDI, [gal]), and surface dispersant application [gal] as explanatory variables. The numbers in each terminal leaf (or node) are the mean concentration of PAH and the percentage of samples; the darker leaf shade, the higher the explanatory power of the branch. Box plots show the distribution of PAH concentration values in each node (*n* = sample size). The tree partitions 1428 samples collected from May 5th to July 12th within 10 km from Macondo and explains 39% of the variance in PAH concentrations. No effect of SSDI volume is detected within the six main nodes (a–f).

multiphase jet into the ambient seawater (Aliseda et al., 2010; Griffiths, 2012) leading to rapid expansion of the dissolved gas (Oldenburg et al., 2012), which would atomize the live oil into micro-droplets (Malone et al., 2018); (2) cold water and gas combined under high pressure (i.e., 5°C and 15.45 MPa at Macondo; Oldenburg et al., 2012) enhanced the formation of gas hydrates that may have encapsulated some crude oil, which may have decreased their buoyancy (Joye et al., 2011); or (3) as live oil rises in the water column and hydrostatic pressure decreases, degassing should increase the apparent droplet size. "Growing" droplets would then rise faster than expected from their initial size at the wellhead (Paris et al., 2012; Pesch et al., 2018). Such complex live oil processes may have shifted the initial DSD toward smaller droplets that remained suspended thousands of meters below the surface (Camilli et al., 2010) without the need of chemical dispersants (Aman et al., 2015), while more buoyant droplets at the tail of the size distribution may have expanded; degassing would have accelerated the droplet ascent in the water column. To date, these processes are unaccounted for in the response options for uncontrolled subsea oil spills. More work is necessary to measure the dispersion dependence on pressure released and the turbulent kinetic energy and dissipation rate at the blowout.

Moreover, efforts to control the Macondo blowout and repair the riser modulated both the pressure at the wellhead and the flow rate (McNutt et al., 2011; Griffiths, 2012) and influenced the DSD of the oil spewing from the wellhead independently of the oil treatment with Corexit. In particular, a drop of pressure in the BOP during early May (May 5–8, 2010) increased the turbulent energy (Griffiths, 2012) and the Riser Cut operation during early June (June 1–5, 2010) increased the flow rate by about 4%, both mechanically dispersing the oil into a plume of micro-droplets (Tolman, 1949; Griffiths, 2012; Aman et al., 2015). The timing of these operations coincided with increased SSDI volume and oil collection at

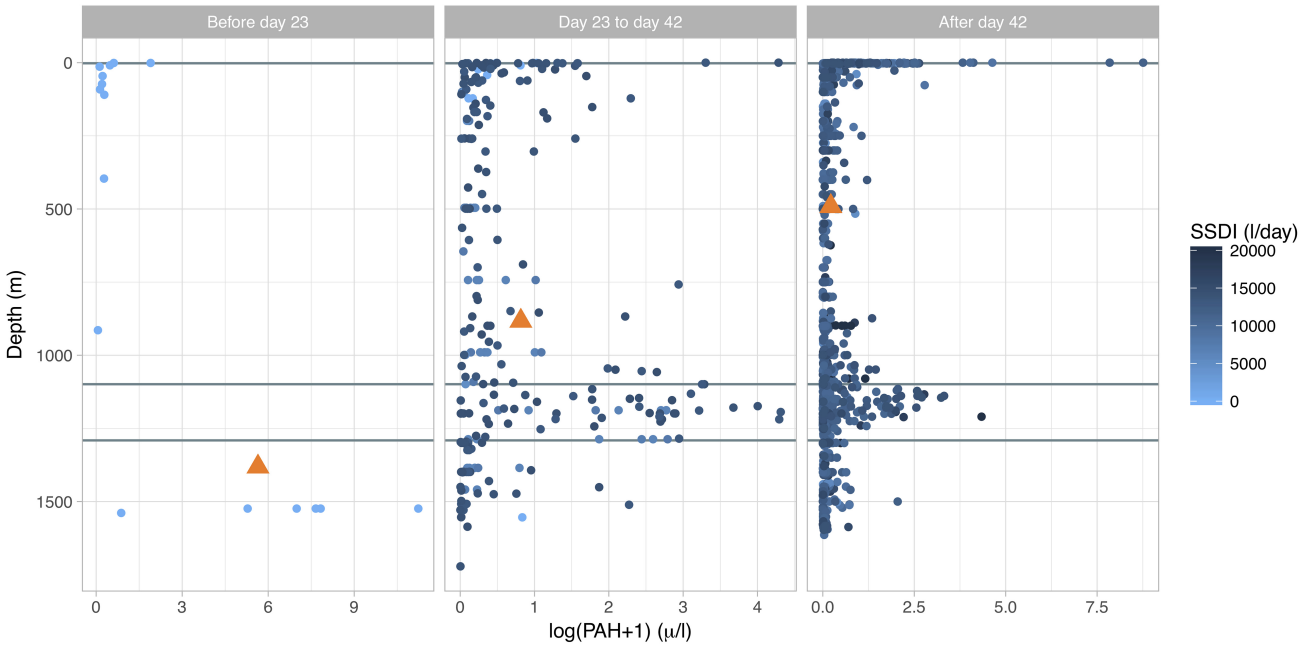


FIGURE 4 | Polycyclic aromatic hydrocarbon (PAH) concentration as a function of depth and Subsea Dispersant Injection (SSDI) volume (marker color). The panels correspond to the temporal splits (before and after day 23 and 42) from the hierarchical regression tree (RT) analysis of factors explaining the spatio-temporal variance in PAH concentrations (see **Figure 3**). The light blue lines correspond to the significant splits by depth (2, 1099, and 1291 m) in the RT analysis (see **Figure 3**). Orange triangles indicate the center of mass of the data points presented in each panel.

the wellhead (**Supplementary Figure S2**). The combination of these efforts would have prohibited objective visual evaluation of the effect of SSDI from surface responders at the DWH site.

The spatial-temporal distribution of oil constituents in the water column depends on each species' aqueous phase solubility and volatility. The lighter mass fraction (C5–C12) dissipates earlier from the environment and its chemical signature was weak after the capping of Macondo. Alternatively, the heaviest mass fractions (C13+) were still present in high concentration up to 5 months beyond the blowout. This petroleum hydrocarbon fraction contains most of the highly toxic PAHs. Yet, both fractions form the deep plume centered around 1100 m, even in the absence of, or low SSDI during May (**Figure 2** and **Supplementary Figures S2, S3**).

Here, several methods were applied to assess the effect of SSDI on the oil vertical distribution and PAH concentration throughout the Macondo blowout, but none detected significant results. This study therefore provides the first quantitative insight as to whether SSDI is an effective control method during the turbulent release of live oil in the deep-sea. Highly turbulent mixing at the wellhead may have generated natural dispersion (Aman et al., 2015; Boufadel et al., 2018). At the same time, the oil continued to surface near the DWH response site even under high SSDI volumes, presumably displacing the rising oil downstream (Socolofsky et al., 2015). This indicates that processes acting on DSD through time and space (Fingas, 2011) were not accounted for in the response strategy. The lack of effect of the SSDI volume variation on the vertical distribution of petroleum hydrocarbons does not mean that the oil droplet interfacial tension with water was not affected, but rather indicates that thermo-physical and chemical processes had a stronger effect on the uncontrolled release of gas-saturated oil and free gas. There was an extreme pressure drop at the Macondo wellhead (Aliseda et al., 2010; Wereley, 2011), leading to rapid outgassing and fractioning of the oil into fine droplets (Malone et al., 2018; Pesch et al., 2018). Extensive GSD revealed no significant SSDI effect on the oil vertical distribution throughout the DWH spill. These conclusions were previously suggested and supported both by numerical simulation and high-pressure experiments (Paris et al., 2012; Aman et al., 2015). Given the adverse toxic effects of Corexit® 9500, which can also suppress natural oil degradation (Kleindienst et al., 2015b), intense SSDI as response for deep-sea blowouts should be revised. Natural processes of mechanical dispersion may therefore have overcome SSDI as an effective response tool during the DWH oil spill. More work is necessary to better understand these fundamental mechanisms and measure the emulsification dependence on pressure released and the turbulent kinetic energy and dissipation rate at the blowout.

DATA AVAILABILITY

Data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC) at https://data.gulfresearchinitiative.org (doi: 10.7266/N7902251, 10.7266/N7VQ3139, 10.7266/N70G3HK6).

AUTHOR CONTRIBUTIONS

CP, MO, MT, ZA, IB, and RF designed the study. IB, CP, RF, and MT analyzed the data. CP, IB, RF, SJ, ZA, and MS wrote the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the Gulf of Mexico Research Initiative (GoMRI). CP, MT, ZA, and MS acknowledge support from the Center for the Integrated Modeling and Analysis of the Gulf Ecosystem (C-IMAGE II). MO and SJ acknowledge support from the GoMRI CARTHE and ECOGIG Consortia, respectively. IB and CP were funded by a National Academies of Sciences (NAS) Gulf Research Program. CP and IB are also funded by the National Academies of Sciences Gulf Research Program.

ACKNOWLEDGMENTS

We thank writer Dallas Murphy for his editorial help.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00389/full#supplementary-material

REFERENCES

Aliseda, A., Bommer, P., Espina, P., Flores, O., Lasheras, J. C., Lehr, B., et al. (2010). *Deepwater Horizon Release Estimate of Rate by PIV. Report to the Flow Rate Technical Group*. Available at: https://engineering.purdue.edu/~wereley/oilspill/Deepwater_Horizon_June_V.pdf [accessed November 29, 2011].

Aman, Z., Paris, C. B., May, E., and Johns, M. (2015). High-pressure visual experimental studies of oil-in-water dispersion droplet size. *Chem. Eng. Sci.* 127, 392–400. doi: 10.1016/j.ces.2015.01.058

Bagby, S., Reddy, C., and Aeppli, C. (2017). Persistence and biodegradation of oil at the ocean floor following Deepwater Horizon. *Proc. Nat. Acad. Sci. U.S.A.* 113, E9–E18. doi: 10.1073/pnas.1610110114

Boufadel, M. C., Gao, F., Zhao, L., Özgökmen, T., Miller, R., King, T., et al. (2018). Was the deepwater horizon well discharge churn flow? Implications on the estimation of the oil discharge and droplet size distribution. *Geophys. Res. Lett.* 45, 2396–2403. doi: 10.1002/2017GL076606

Bp Gulf Science Data1 (2016). *Chemical Analysis of Oil Samples from the Gulf of Mexico and Adjoining States from May 2010 to March 2014. Filename: WaterChemistry_W-01v02-01_xTab*. Available at: https://data.gulfresearchinitiative.org/data/BP.x750.000:0005

Bp Gulf Science Data2 (2016). *Subsea Dispersant Application Records Collected during the Deepwater Horizon (DWH) Accident Near the Mississippi Canyon block 252 Wellhead from April 30 to July 22, 2010 Filename: DispersantApplication_OTH-02v01-01*. Available at: https://data.gulfresearchinitiative.org/data/BP.x750.000:0018#

Bp Gulf Science Data3 (2016). *Application of Dispersants to Surface Oil Slicks by Aircraft and by Boat in Approved Areas of the Gulf of Mexico from April 22, 2010 to Final Application on July 19, 2010. Filename: DispersantApplication_OTH-01v01-01a*. Available at: https://data.gulfresearchinitiative.org/data/BP.x750.000:0017

Camilli, R., Iorio, D., Di, and Bowen, A. (2012). Acoustic measurement of the Deepwater Horizon Macondo well flow rate. *Proc. Nat. Acad. Sci. U.S.A.* 109, 20235–20239. doi: 10.1073/pnas.1100385108

Camilli, R., Reddy, C., and Yoerger, D. (2010). Tracking hydrocarbon plume transport and biodegradation at Deepwater Horizon. *Science* 330, 201–404. doi: 10.1126/science.1195223

Chan, G., Chow, A., and Adams, E. (2015). Effects of droplet size on intrusion of sub-surface oil spills. *Environ. Fluid Mech.* 15, 959–973. doi: 10.1007/s10652-014-9389-5

Crespo-Medina, M., Hunter, K., Meile, C., Diercks, A., Asper, V., Orphan, V., et al. (2014). The rise and fall of methanotrophy following a deepwater oil-well blowout. *Nat. Geosci.* 7, 423–427. doi: 10.1038/ngeo2156

Fingas, M. (2011). "Chapter 15 - oil spill dispersants: a technical summary," in *Oil Spill Science and Technology*, ed. M. Fingas (Boston, MA: Gulf Professional Publishing), 435–582.

Griffiths, S. (2012). Oil release from Macondo well MC252 following the Deepwater Horizon accident. *Environ. Sci. Technol.* 46, 5616–5622. doi: 10.1021/es204569t

Jaggi, A., Snowdon, R., Stopford, A., and Radović, J. (2017). Experimental simulation of crude oil-water partitioning behavior of BTEX compounds during a deep submarine oil spill. *Organic* 108, 1–8. doi: 10.1016/j.orggeochem.2017.03.006

Joye, S. B., MacDonald, I. R., Leifer, I., and Asper, V. (2011). Magnitude and oxidation potential of hydrocarbon gases released from the BP blowout. *Nat. Geosci.* 4, 160–164. doi: 10.1038/ngeo1067

Kessler, J., Valentine, D., Redmond, M., and Du, M. (2011). A persistent oxygen anomaly reveals the fate of spilled methane in the deep Gulf of Mexico. *Science* 311, 312–315. doi: 10.1126/science.1199697

Kleindienst, S., Paul, J. H., and Joye, S. B. (2015a). Assessing the impacts of chemical dispersants on microbial community composition and activity. *Nat. Rev. Microbiol.* 13, 388–396. doi: 10.1038/nrmicro3452

Kleindienst, S., Seidel, M., Ziervogel, M., Grim, K., Mailkin, S., Harrison, S., et al. (2015b). Chemical dispersants can suppress the activity of natural oil-degrading microorganisms. *Proc. Nat. Acad. Sci. U.S.A.* 48, 14900–14905. doi: 10.1073/pnas.1507380112

Kujawinski, E., Soule, M. K., Valentine, D. L., Boysen, A. K., Longnecker, K., and Redmond, M. C. (2011). Fate of dispersants associated with the Deepwater Horizon oil spill. *Sci. Technol.* 45, 1298–1306. doi: 10.1021/es103838p

Li, Z., Lee, K., King, T., and Boufadel, M. (2008). Oil droplet size distribution as a function of energy dissipation rate in an experimental wave tank. *Proc. Int. Oil Spill* 1, 621–626. doi: 10.7901/2169-3358-2008-1-621

Malone, K., Pesch, S., Schlueüter, M., and Krause, D. (2018). Oil droplet size distributions in deep-sea blowouts: influence of pressure and dissolved gases. *Environ. Sci. Technol.* 52, 6326–6333. doi: 10.1021/acs.est.8b00587

McNutt, M., Camilli, R., Crone, T. J., Guthrie, G. D., Hsieh, P. A., Ryerson, T. B., et al. (2011). Review of flow rate estimates of the Deepwater Horizon oil spill. *Proc. Nat. Acad. Sci. U.S.A.* 109, 20260–20267. doi: 10.1073/pnas.1112139108

McNutt, M., Chu, S., Lubchenco, J., Hunter, T., Dreyfus, G., Murawski, S. A., et al. (2012). Applications of science and engineering to quantify and control the Deepwater Horizon oil spill. *Proc. Nat. Acad. Sci. U.S.A.* 109, 20222–20228. doi: 10.1073/pnas.1214389109

Oldenburg, C., Freifeld, B., and Pruess, K. (2012). Numerical simulations of the Macondo well blowout reveal strong control of oil flow by reservoir permeability and exsolution of gas. *Proc. Nat. Acad. Sci. U.S.A.* 109:20255. doi: 10.1073/pnas.1105165108

Paris, C. B., Le Hénaff, M., Aman, Z. M., Subramanian, A., Helgers, J., Wang, D. P., et al. (2012). Evolution of the Macondo Well Blowout: simulating the Effects of the Circulation and Synthetic Dispersants on the Subsea Oil Transport. *Environ. Sci. Technol.* 46, 13293–13302. doi: 10.1021/es303197h

Pesch, S., Jaeger, P., Jaggi, A., Malone, K., Hoffman, M., Krause, D., et al. (2018). Rise velocity of live-oil droplets in deep-sea oil spills. *Environ. Eng. Sci.* 52:11. doi: 10.1089/ees.2017.0319

R Core Team (2018). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rahsepar, S., Smit, M. P. J., Murk, A. J., Rijnaart, H. H. M., and Langenhoff, A. A. M. (2016). Chemical dispersants: friend or Foe. *Mar. Poll. Bull.* 108, 113–119. doi: 10.1016/j.marpolbul.2016.04.044

Reddy, C., Arey, J., and Seewald, J. (2012). Composition and fate of gas and oil released to the water column during the Deepwater Horizon oil spill. *Proc. Nat. Acad. Sci. U.S.A.* 109, 20229–20234. doi: 10.1073/pnas.1101242108

Ryerson, T. B., Aikin, K. C., and Angevine, W. M. (2011). Atmospheric emissions from the Deepwater Horizon spill constrain air-water partitioning, hydrocarbon fate, and leak rate. *Geophys. Res. Lett.* 38:L07803. doi: 10.1029/2011GL046726

Socolofsky, S. A., Adams, E., Boufadel, M., and Aman, Z. (2015). Case study: intercomparison of oil spill prediction models for accidental blowout scenarios with and without Subsea Chemical Dispersant Injection. *Mar. Pollut. Bull.* 96, 1–2. doi: 10.1016/j.marpolbul.2015.05.039

Spier, C., Stringfellow, W., Hazen, T., and Conrad, M. (2013). Distribution of hydrocarbons released during the 2010 MC252 oil spill in deep offshore waters. *Environ. Pollut.* 173:224e230.

Tolman, R. (1949). The effect of droplet size on surface tension. *J. Chem. Phys.* 17:333. doi: 10.1063/1.1747247

Valentine, D. L., Kessler, J., Redmond, M. C., Mendes, S. D., Heintz, M. B., Farwell, C., et al. (2010). Propane respiration jump-starts microbial response to a deep oil spill. *Science* 330, 208–211. doi: 10.1126/science.1196830

Wade, T. L., Sericano, J. L., Sweet, S. T., Knap, A. H., and Guinasso, N. L. (2016). Spatial and temporal distribution of water column total polycyclic aromatic hydrocarbons (PAH) and total petroleum hydrocarbons (TPH) from the Deepwater Horizon (Macondo) incident. *Mar. Pollut. Bull.* 103, 286–293. doi: 10.1016/j.marpolbul.2015.12.002

Wereley, S. (2011). "Gulf oil spill particle image velocimetry (PIV) analysis, appendix 5, appendix D plume calculation team, PIV Report," in *Proceedings of the 10th Asian Control Conference (ASCC)*, Sabah.

Wilson, M., Graham, L., Hale, C., Maung-Douglass, E., Sempier, S., and Swann, L. (2015). "Oil spill science: persistence, fate, and effectiveness of dispersants used during the deepwater horizon oil spill," in *Oil Spill Science and Technology*, ed. M. Fingas (Houston, TX: Gulf Professional Publishing).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

TECHNOLOGY REPORT
published: 18 March 2019
doi: 10.3389/fmars.2019.00110

A Glider-Compatible Optical Sensor for the Detection of Polycyclic Aromatic Hydrocarbons in the Marine Environment

Frédéric Cyr[1]*, *Marc Tedetti*[2], *Florent Besson*[3], *Nagib Bhairy*[2] and *Madeleine Goutx*[2]

[1] Northwest Atlantic Fisheries Centre, Fisheries and Oceans Canada, St. John's, NL, Canada, [2] CNRS, IRD, Aix-Marseille Université, Université de Toulon, MIO UM 110, Marseille, France, [3] ALSEAMAR, Rousset, France

This study presents the *MiniFluo-UV*, an ocean glider-compatible fluorescence sensor that targets the detection of polycyclic aromatic hydrocarbons (PAHs) in the marine environment. Two MiniFluos can be installed on a glider, each equipped with two optical channels (one PAH is measured per channel). This setup allows the measurement of up to 4 different fluorescent PAHs: Naphthalene, Phenanthrene, Fluorene and Pyrene. Laboratory tests on oil products (Maya crude oil and Diesel fuel) as well as on marine samples near industrial areas (urban harbor and offshore installations) revealed that the concentration of the four PAHs targeted accounted for 62–97% of the total PAH concentration found in samples ($\sum$16 PAHs determined by standard international protocols). Laboratory tests also revealed that for marine applications, the calibration on Water Accommodated Fraction (WAF) of crude oil is more appropriate than the one on pure standards (STD). This is because PAH fluorescence is constituted in large part of alkylated compounds that are not considered with STD calibration. Results from three glider deployments with increasing levels of complexity (a laboratory trial, a field mission in non-autonomous mode and a fully autonomous mission) are also presented. During field deployments, the MiniFluo-glider package was able to detect concentration gradients from offshore marine waters toward the head of a Mediterranean harbor ($< 80\,ng\,L^{-1}$) as well as hydrocarbon patches at the surface waters of an oil and gas exploitation field in the North Sea ($< 200\,ng\,L^{-1}$, mainly Naphthalene). It is suggested that using only the WAF calibration, the concentration derived with the MiniFluo agrees within one order of magnitude with the concentration determined by Gas Chromatography coupled with Mass Spectrometry (overestimation by a factor 7 on average). These performances can be improved if the calibration is made with a WAF with PAH proportions similar to the one find in the environment. Finally, it is shown that the use of *in situ* calibration on water samples collected during the glider deployment, when possible, gives the best results.

Keywords: MiniFluo-UV, glider, SeaExplorer, fluorescence, hydrocarbons, PAH, oil spills, marine environment monitoring

OPEN ACCESS

Edited by:
Ketil Hylland,
University of Oslo, Norway

Reviewed by:
Antonio Olita,
Italian National Research Council (CNR), Italy
Laura Guimarães,
Universidade do Porto, Portugal

***Correspondence:**
Frédéric Cyr
Frederic.Cyr@dfo-mpo.gc.ca

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *15 October 2018*
Accepted: *21 February 2019*
Published: *18 March 2019*

Citation:
Cyr F, Tedetti M, Besson F, Bhairy N and Goutx M (2019) A Glider-Compatible Optical Sensor for the Detection of Polycyclic Aromatic Hydrocarbons in the Marine Environment. Front. Mar. Sci. 6:110. doi: 10.3389/fmars.2019.00110

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), a specific type of petroleum hydrocarbons, are lipophilic compounds consisting of two or more fused benzene rings. They are among the most widespread organic contaminants in the marine environment (Roose and Brinkman, 2005; González-Gaya et al., 2016). PAHs are persistent and mobile, can strongly bio-accumulate in food chains and are harmful to living organisms through carcinogenic and mutagenic effects (Kennish, 1991; Hylland, 2006). They are therefore recognized as high priority contaminants by the European Union and the US Environmental Protection Agency.

PAHs are introduced in the marine environment in mainly two different ways. The first type of contamination occurs as rare but catastrophic events related to well blowout, wrecked tankers, broken pipelines, etc. These situations require rapid assessment of the disaster in support of decision makers and environmental response. Such analyzes are however complex when not much is known about the extent of the spill apart from its surface signature visible from aerial or remote sensing surveys. The second type of contamination is more subtle and occurs as chronic releases in the environment, for example when small quantities of hydrocarbons are permanently found in water as a result of various industrial and urban activities. Such contamination requires a quasi-permanent monitoring of neighboring waters, specially in proximity of populated areas with economical activities based on beaches, water sports, fisheries, etc. In both cases, the workload associated with such measurements makes the temporal and spatial coverage of the area of interest difficult. This is because traditional measurement implies the collection of discrete water samples that need to be further analyzed in the laboratory.

In surface oceanic waters, total dissolved PAHs ($\sum$13-19 parent PAHs) display a wide range of concentrations: from 0.15 to 10 ng L^{-1} in open ocean (Witt and Matthäus, 2001; Stortini et al., 2009; Berrojalbiz et al., 2011), from 4 to 500 ng L^{-1} in coastal areas (Guitart et al., 2007; Valavanidis et al., 2008; Qiu et al., 2009) and from 100 to >1,000 ng L^{-1} in harbor waters (Zhou et al., 2000). It is worth noting that when taking into account alkylated derivatives (i.e., mono-, di-, tri- or tetra-methyl PAHs), the concentration of dissolved total PAHs ($\sum$ 32 parents + alkylated PAHs) may increase by a factor 1.5–3 in coastal and harbor waters (Guigue et al., 2011, 2014). In oceanic waters affected by intense oil spills, the concentrations of dissolved total (parents or parents + alkylated) PAHs may reach from 300 up to 100,000 ng L^{-1} (Reddy and Quinn, 2001; Zhou and Maskaoui, 2003; González et al., 2006). In these conditions of oil spill, the distribution of dissolved PAHs is generally dominated by low molecular weight petrogenic compounds, mainly naphthalene (Naph), phenanthrene (Phe), fluorene (Flu) and their alkylated derivatives (González et al., 2006; Zhou et al., 2013).

Due to their aromatic structure, PAHs are highly fluorescent in the ultraviolet spectral domain (UV: 200–400 nm). New techniques exploiting these fluorescence properties have been recently employed to overcome difficulties associated with laboratory measurements, and to increase the spatiotemporal coverage of the observations. Submersible UV fluorometers have been proposed to acquire real time, continuous and high frequency *in situ* measurements of dissolved PAHs/oil in natural or engineered waters (Zielinski et al., 2009; Conmy et al., 2014a,b). Many of these techniques were tested and validated during Deepwater Horizon spill, the largest spill in the recent history of oil and gas offshore industry. A good example of such new technique is the use of fluorescence properties of PAHs and autonomous underwater vehicles (AUV). These techniques were proven successful in tracking the hydrocarbon plume of Deep Water Horizon (Camilli et al., 2010). But while many fluorometers can detect the presence of oil in seawater (Conmy et al., 2014b), their performances usually depend on the correspondence between the choice of the sensor's spectral domain and the fluorescence signatures of the compounds found in the oil (Tedetti et al., 2012, 2013; Ferretto et al., 2014).

Unlike other AUVs, gliders have no propelling systems and their motion is driven by buoyancy changes (Davis et al., 2003; Rudnick et al., 2004). They cruise the ocean at relatively low speed (0.5–1 m s^{-1}) guided by satellite communications while acquiring variety of scientific parameters. Because of their relatively large autonomy and low utilization cost, they have received increased attention in ocean sciences research over the last decade, and are now increasingly used as standard pieces of ocean observing systems (Testor et al., 2010; Rudnick and Cole, 2011). When equipped with specific sensors, gliders could thus be used as powerful assessment tools to track dissolved PAHs in natural waters. One of these sensors is the *MiniFluo-UV*, hereinafter simply MiniFluo (Tedetti et al., 2013; Cyr et al., 2017), now fully operational on SeaExplorer gliders. Through laboratory work on the sensor alone and 3 scenarios of increasing complexity with the glider, it is shown below that glider-based optical measurements with the MiniFluo is a promising environmental assessment tool to monitor PAH concentrations in natural waters.

2. SENSOR DESCRIPTION AND METHOD

2.1. MiniFluo Sensor

The first version of the MiniFluo was developed between 2009 and 2011 as a field-portable fluorometer. In its original configuration, the MiniFluo was targeting the detection of Naphthalene-like (Naph-like) and Phenanthrene-like (Phe-like) fluorophores from discrete water samples (Tedetti et al., 2013). Here, and for the remaining of this study, *-like* suffix refers to the concentrations derived with the MiniFluo that target a certain PAH, although it is acknowledged that other compounds present in the environment may also fluoresce at close excitation/emission wavelengths ($\lambda_{Ex}/\lambda_{Em}$). Based on this first prototype, a second generation of the MiniFluo was developed during the period 2012–2015. This new submersible version is now compatible with underwater gliders SeaExplorer, and exhibits significant improvements in term of opto-electronic architecture (this version is first presented in Cyr et al., 2017). In addition to these improvements, the number of fluorophores to be detected has been extended with the addition of Fluorene-like (Flu-like) and Pyrene-like (Pyr-like) fluorophores. This

TABLE 1 | Excitation/emission wavelengths of the MiniFluo channels and their targeted fluorophores.

	λ_{Ex}(nm)	λ_{Em}(nm)
Phenanthrene	255	360
Fluorene	260	315
Pyrene	270	380
Naphthalene	275	340

Two channels among the following are possible for each MiniFluo (factory settings).

new version is now commercialized by ALSEAMAR, also the manufacturer of the SeaExplorer glider.

Two configurations are presented here: the "MiniFluo-1" for the detection of Naph-like and Phe-like, and the "MiniFluo-2" for the detection of Pyr-like and Flu-like. A first study using the actual version of the sensor was already published (Cyr et al., 2017), but the sensor was used to detect natural dissolved organic matter (DOM) fluorophores, and the study limited to MiniFluo-1 configuration without the emphasis on PAHs detection. The present study focuses on PAHs detection and the utilization of the MiniFluo/glider combination in the case of industrial contamination and/or risk-assessment applications.

Basic working principles relevant for this new application are recalled here, but we refer to previous studies for a more complete description of the sensor architecture and functioning (Tedetti et al., 2013; Cyr et al., 2017). The MiniFluo has two optical channels, enabling the simultaneous detection/quantification of 2 fluorescent compounds of interest (1 compound per optical channel). These channels may be chosen among a list of four $\lambda_{Ex}/\lambda_{Em}$ couples to be set up by the manufacturer (see list in **Table 1**). Because of firmware limitations, the installation of two MiniFluos with different configurations was not possible at the time the glider tests were performed (feature implemented since). While both MiniFluo-1 and MiniFluo-2 (Phe/Naph and Flu/Pyr) were used during laboratory measurements and for glider deployment in the experimental basin (polludrome), glider deployments in natural waters (Saumaty Harbor and North Sea) were carried out using only MiniFluo-1 (Phe/Naph). The glider deployments are described in section 2.3

2.2. MiniFluo Calibration and GC–MS Analyses

As reported in an earlier study (see Cyr et al., 2017, for a complete description), the conversion from relative unit (RU) signal of the MiniFluo ($C_{\mathrm{RU}} = \frac{C_c - N_D}{C_m - N_D}$) to mass concentration (C in ng L^{-1}) is done through the equation:

$$C = \frac{C_{\mathrm{RU}} - B}{SF}. \quad (1)$$

Here C_c is the measured count value of the detection photodiode, C_m the measured count value of the monitoring photodiode, $N_D = 4096$ the electronic noise of the circuit (*dark offset*). Parameters *SF* (*scale factor*, in RU L ng^{-1}) and *B* (*blank noise*, in RU) are obtained, respectively, as the slope and intercept of the calibration linear regression between the measured concentration and relative unit signal returned by the MiniFluo.

For each fluorometer (MiniFluo-1 and MiniFluo-2), the response of each optical channel (respectively Naph/Phe and Flu/Pyr) was calibrated using two different approaches (see also Cyr et al., 2017). The first calibration is performed on pure standards (STD), i.e., on the individual parent PAH compound. Each solution was prepared by solubilizing pure standards (Sigma-Aldrich $\geq$ 98%) in methanol, before realizing successive dilution in both ultra-pure (milli-Q) water and synthetic seawater (SSW) in order to obtain concentration ranging from 50 to 5,000 ng L^{-1}.

A second set of calibrations was conducted with water accommodated fraction (WAF) of oil in seawater in order to calibrate the sensors with solutions whose PAH composition is more representative of that found in the marine environment. These were realized using Maya Crude oil that naturally contains the targeted (parents) PAHs and their alkylated derivatives. The WAF was prepared by introducing 2 mL of crude oil ("Maya" type) at the surface of 1 L of filtered seawater (filter pore size 0.2 μm). The underlying water was then stirred with a magnetic stirrer for a minimum of 36 h. The water under the crude oil micro-layer is considered the WAF (Cyr et al., 2017).

The PAH composition of the WAF was determined in laboratory by Gas Chromatography coupled with Mass Spectrometry (GC–MS). This followed the CEDRE (Center of Documentation, Research and Experimentation on Accidental Water Pollution located in Brest, France) standard protocol that is part of the Bonn Agreement oil spill identification network OSInet certified ISO 9001:2015 (SGS-ICS). Compared to calibrations on STD, the calibration using WAF better captures the fluorescence of the entire PAH family of interest. The compound family, consisting of the parent (e.g., Naph, Phe, Pyr and Flu) and their alkylated derivatives, will be further referred as "Naphs," "Phes," "Pyrs," and "Flus," respectively. The WAF was diluted in SSW in order to obtain concentrations of 1.5, 3, 6, 12.5, and 25% of the initial WAF. For each of these WAF solutions, concentrations in Naphs, Phes, Pyrs et Flus were determined by GC–MS.

STD and WAF calibrations are carried with the MiniFluo connected to a computer via a communication box (see Tedetti et al., 2013, for picture and detailed description). The measurements are performed using quartz cuvettes placed on a cuvette holder containing prisms similar to the one found in the MiniFluo optical cap. In addition to measurements of each prepared solution mentioned above, a measurement in the absence of PAH (only milli-Q water or SSW) is also taken. The "detection limit" (*DL*) of the sensor is determined as the following: $DL = \frac{3 \times \sigma_{C_0}}{SF}$, where σ_{C_0} is the standard deviation of the blank measurement (i.e., only SSW or milli-Q water).

2.3. Glider Deployments

Three glider deployments were realized as part of this study. They are presented here in order of increasing level of complexity in terms of glider mission design. The first deployment is a validation of the sensor's response in a controlled environment at CEDRE in July 2017. The CEDRE

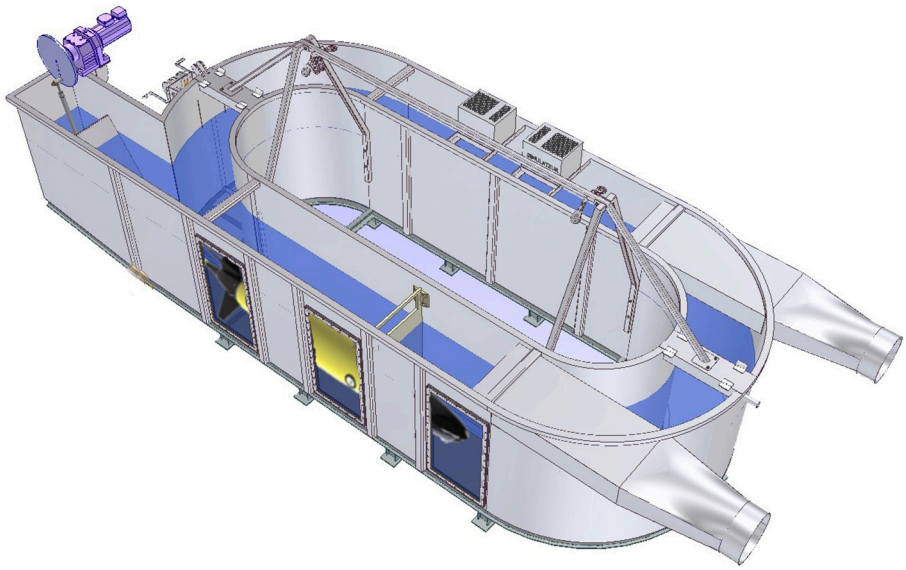


FIGURE 1 | Sketch of the "polludrome" at the Center of Documentation, Research and Experimentation on Accidental Water Pollution (CEDRE). The glider equipped with MiniFluo-1 and MiniFluo-2 was placed as illustrated here. Source of original image: G2B / CEDRE (modified with permission).

experimental basin (*polludrome*) is a controlled petroleum-impacted marine environment (flume tank) that reproduces a flow passing through the sensor's cap (see **Figure 1**). This aims to reproduce *in situ* conditions as if the glider was gliding in the ocean. The standard CEDRE procedure was applied. The day before the experiment, 50 mL petroleum fuel (Total S.A. Diesel) was gently poured into the 7 m^3 experimental basin and mixed (without surfactant addition) under a 1 $m\,s^{-1}$ current overnight to establish the WAF. The next day (∼14 h later), the glider was introduced in the polludrome, still under the same current. Then, the concentration of hydrocarbons was decreased by several steps via successive dilutions ($\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$) by removing each time about half of the polludrome volume before replacing it by seawater pumped from near-shore waters. Before being introduced in the polludrome, the water is filtered using a standard aquarium-grade *sand filtration* treatment that removes most large size particles and part of the dissolved organic material. At each concentration, 500 mL of water was sampled from the polludrome and the hydrocarbon fraction was extracted using a method of Stir Bar Sorptive Extraction (SBSE) and analyzed by GC−MS. While the MiniFluo was constantly sampling, only the average over about 2 min is taken as the measured fluorescence value for this concentration. This measurement was made after an approximate 10-min stabilization pause, a period determined by visual inspection of the MiniFluo signal after each dilution.

The second deployment was realized in natural waters. For this trial, the glider was deployed in Saumaty, a highly anthropized harbor located in Marseille metropolitan area (France) on October 11th 2016. The choice of this location was motivated by a known offshore-inshore gradient in PAHs concentration (Tedetti et al., 2013). For this 1-day mission, the glider was not performing normal dives, but was rather towed with a small inflatable embarkation approximately 1 m below the surface (see inset in **Figure 2**). The track of the deployment is a return transect from outside harbor (**Figure 2**). On the return transect, subsurface water samples were also collected at waypoints *S*01 to *S*05 for GC−MS analysis in the laboratory.

The third glider mission is a standard glider deployment that took place between November 18th and December 3rd 2016, near Troll oil and gas field in the North Sea (see map **Figure 3**). The mission was performed in partnership with Statoil and Havila Shipping and served as a demonstration scenario assessing the feasibility of using gliders to patrol in proximity of offshore installations. During this mission, the glider SeaExplorer SEA003 was deployed at waypoint *T*1 where 4 water samples were collected (at 1, 10, 25, and 40 m depths) for further GC−MS analysis in laboratory. The glider completed a return transect across the channel along the *T*1-*L*2-*L*1 section, before surveying the triangle delimited by waypoints *T*1-*T*2-*T*3-*T*1 near 6 offshore oil and gas installations (blue stars on **Figure 3** map).

For this last mission, the glider data were processed using the same procedure as in Cyr et al. (2017) and briefly recalled here. The glider was equipped with a pumped conductivity-temperature-depth sensor (Seabird's GPCTD) from which the conservative temperature (Θ), the absolute salinity (S_A) and the density anomaly referenced to the surface (σ_0) are derived using TEOS-10 toolbox (McDougall and Barker, 2011). This GPCTD is also equipped with a dissolved oxygen (O_2) sensor (Seabird's SBE-43F) from which concentrations are derived. A WetLabs ECO FLBBCD was also mounted on the glider for measurements of Chlorophyll-*a* (Chl-*a*) fluorescence ($\lambda_{Ex}/\lambda_{Em}$: 470/695 nm), backscattering at 700 nm (BB700) and humic-like fluorophore fluorescence ($\lambda_{Ex}/\lambda_{Em}$: 370/460 nm) expressed in $\mu g\,L^{-1}$ equivalent quinine sulfate units ($\mu g\,L^{-1}$ QSU). For this sensor, the manufacturer's calibration

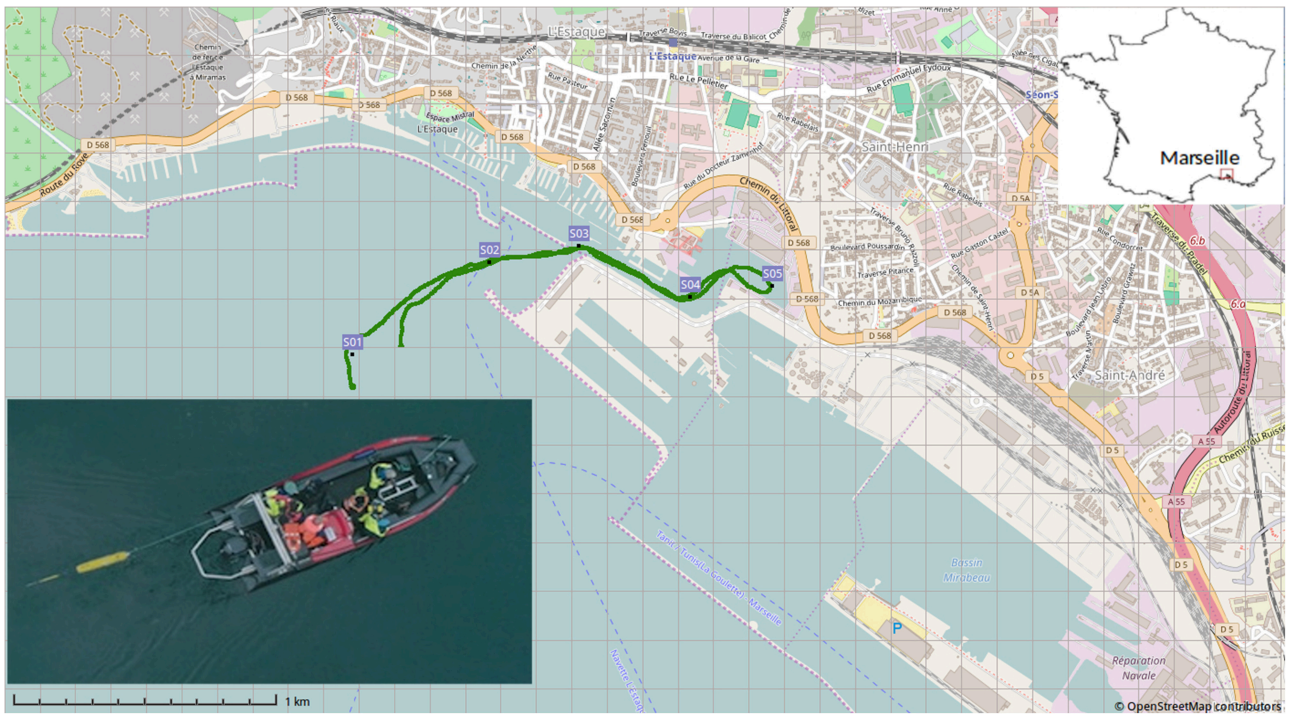


FIGURE 2 | Glider sampling track in Saumaty Harbor (main figure) near the city of Marseille, France (see upper inset for location). The glider was towed behind a small boat (see inset for an example from another similar mission). The photography in the lower inset was reproduced with the permission of Dr. J. Pearlman.

was used. Glider observations were processed with the Socib glider toolbox (Troupin et al., 2015) for cast identification and georeferencing. For the first deployment in the polludrome, the glider was equipped with MiniFluo-1 and MiniFluo-2, while for the two campaigns at sea the glider was only equipped with MiniFluo-1.

3. RESULTS AND DISCUSSION

3.1. Relative Proportions of PAHs Determined by GC–MS Analyzes

For the two WAFs prepared (laboratory calibration and polludrome experiment), **Table 2** (left half) shows the relative proportions of the 4 PAHs targeted among the total number of PAHs measured from GC–MS. Naphs (defined as the sum of parent and alkylated compounds) are the dominant PAH in both preparation, reaching 49.3% and 78.0% of the total amount PAH measured from the WAF, respectively, for the Maya used for laboratory calibration and for the Diesel used during the polludrome experiment. Among the 4 PAHs reported, Phes are the second dominant family (6.0% and 10.9%), followed by Flus (5.5% and 5.8%) and the Pyrs (1.3% for both preparations). The fact that the cumulative fraction of these 4 PAHs composes a large part of the total PAHs of both WAF (respectively 62.0% and 96.0%) is an indication that the choice of the compounds targeted by the MiniFluo is justified.

For the water samples collected in marine environment (**Table 2**, right half), the dominant PAHs are again Naphs, with a slightly higher proportion among total PAHs compared to the prepared WAFs (81.8% and 85.3%, respectively, for Saumaty and the North Sea glider campaigns). For the other compounds (Phes, Flus and Pyrs), the proportion are remarkably similar to the WAF prepared. Overall, the 4 PAHs analyzed corresponded to 94.7% and 97.0% of the total dissolved PAHs concentration measured by GC–MS.

Table 2 also highlights that the contribution of the parent compound is never dominant over the rest of the family. The alkylated compounds that are instead generally dominant, specially for Naphs in the marine environment, where they represent roughly 80% or more of the total PAH fraction. These results are in agreement with many works that have reported the dominance of low molecular weight PAHs and their alkylated homologs (particularly Naphthalenes) within the pool of total dissolved PAHs in seawater (González et al., 2006; Guigue et al., 2014; Adhikari et al., 2015; Fourati et al., 2018).

These observations provide the scientific background to prefer the use of the WAF calibration over STD to detect oil in the marine environment. A WAF with relative proportions of each parent/alkylated compounds close to those found in the environment is preferable, and would likely improve the measurement with the MiniFluo or other optical sensors. The ability of collecting water samples to assess the hydrocarbon composition of environment where fluorescence measurements are planned is thus an important aspect to consider. This aspect will be discussed in the next sections.

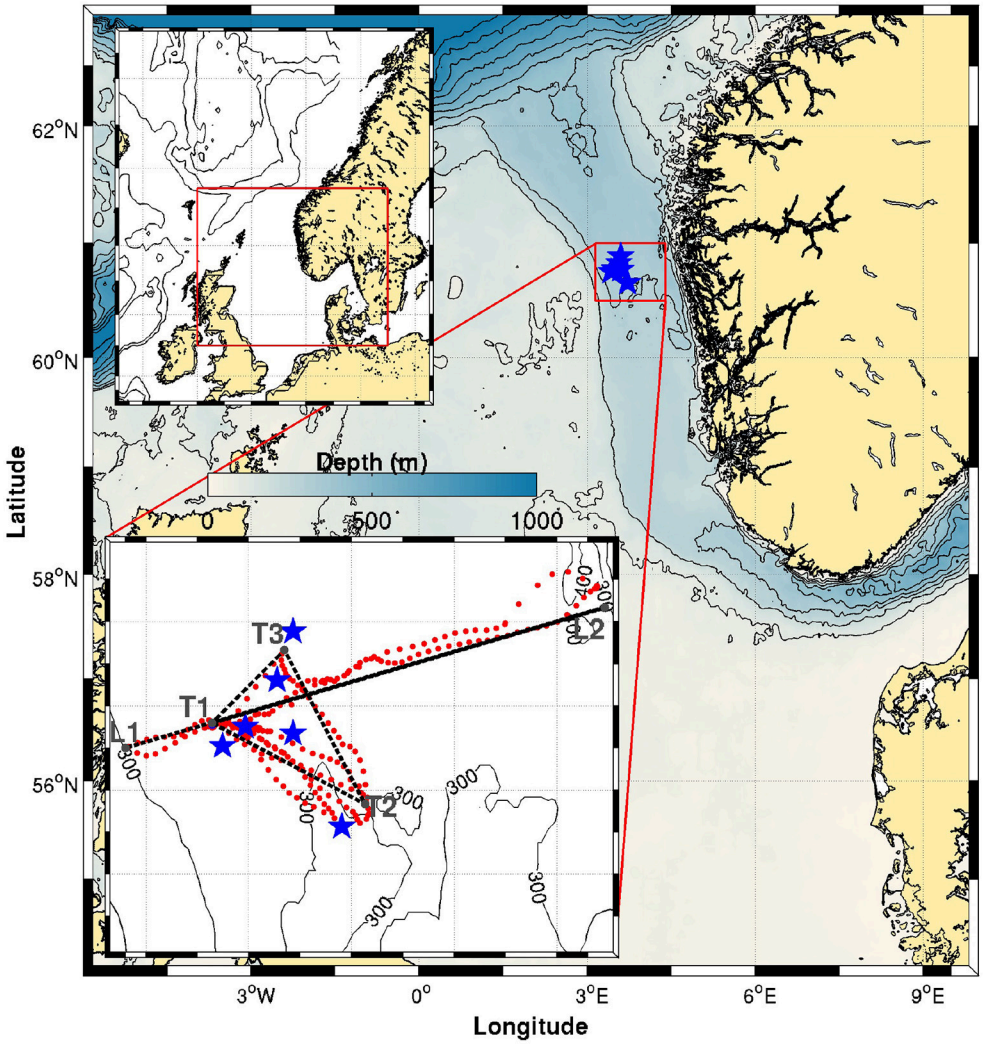


FIGURE 3 | Glider track completed during the deployment in the North Sea. Each red dot correspond to a glider yo (downcast+upcast). Offshore installations are shown with blue stars. Dark gray dots and reference letters are waypoints used for piloting and to delimit the transects referred to in the text.

3.2. Laboratory Calibrations

Calibration curves for each MiniFluo are presented in **Figures 4**, **5**, from which the scale factor (*SF*) and the blank noise *B* (respectively slope and intercept of the calibration curves) are determined for both STD and WAF. As mentioned above, the large proportion of alkylated compounds explains the higher *SF* for the WAF calibration compared to the STD curve. This explains why using a calibration on STD (lower *SF*) would overestimate both the parent PAH concentration and total (parent + alkylated) PAH concentration.

It is also observed that calibration with SSW leads to higher *SF* and *B* values compared to calibration with milli-Q water (**Figures 4A–D**). This discrepancy in calibration factors between ultra-pure water and seawater has been already pointed out by Tedetti et al. (2010) with the *EnviroFlu-HC* fluorometer (*TriOS Optical Sensors*) and attributed to matrix effect, mainly related to differences in salt content or pH. Moreover, the higher slopes for Phe compared to Naph in both milli-Q water and SSW may reflect a higher fluorescence capacity of Phe, which may be explained by the more elevated fluorescence quantum yield and/or molar absorption coefficient of Phe (relative to Naph) due to its higher number of conjugated π-electrons and its higher resonance energy (Valeur and Berberan-Santos, 2012).

The calibration of MiniFluo-1 with WAF shows R^2 coefficients of 0.995 and 0.970, respectively, for Naphs and Phes, that is slightly lower than for STD ($R^2 = 0.999$). This diminution may be attributed to the fact that the WAF dilution is difficult to achieve, specially at the highest concentrations. One solution would be to enrich the SSW with increasing concentration of crude oil rather than conducting dilutions.

Regarding calibrations made on STD in ultra-pure water, Flu presented much higher *SF* and *B* values compared to the three other PAHs, while Pyr displayed *SF* value close to that of Phe (**Figures 4A,B**, **5A,B**). The trend observed here for the four

TABLE 2 | Proportions (in %) of Naphthalene, Phenanthrene, Fluorene, and Pyrene in laboratory prepared WAF (Maya crude oil used in the laboratory and TOTAL Diesel fuel used in the polludrome; left two columns) and in the marine environment (Saumaty and North Sea; right two columns) as determined by GC–MS analysis.

	Laboratory WAF		Marine Environment	
	Maya crude oil	**Total diesel**	**Saumaty harbor**	**North sea**
Naphs total	**49.3** (7.2)	**78.0** (2.6)	**81.8** (9.0)	**85.3** (12.2)
Naphthalene	11.2 (3.4)	1.3 (0.5)	2.6 (0.3)	2.3 (0.7)
Methylnaphthalene	19.3 (4.0)	9.3 (2.1)	19.4 (4.7)	17.9 (2.1)
Dimethylnaphthalene	12.0 (1.0)	28.5 (2.9)	33.2 (4.1)	31.1 (2.3)
Trimethylnaphthalene	6.8 (1.1)	38.8 (4.3)	26.6 (2.0)	34.0 (9.0)
Phes total	**6.0** (2.3)	**10.9** (2.0)	**6.1** (5.6)	**5.3** (5.4)
Phenanthrene	1.7 (0.3)	2.4 (0.3)	2.3 (0.9)	1.4 (0.3)
Methylphenanthrene	1.9 (0.6)	4.7 (0.9)	1.7 (1.8)	1.0 (1.4)
Dimethylphenanthrene	1.4 (0.8)	3.2 (0.7)	1.7 (2.2)	1.6 (2.1)
Trimethylphenanthrene	1.0 (0.8)	0.6 (0.2)	0.5 (1.1)	1.4 (1.8)
Flus total	**5.5** (1.2)	**5.8** (0.9)	**5.1** (3.4)	**6.1** (6.2)
Fluorene	1.2 (0.1)	0.8 (0.1)	1.7 (0.3)	1.0 (0.4)
Methylfluorene	1.3 (0.2)	1.6 (0.2)	3.4 (3.1)	1.6 (2.1)
Dimethylfluorene	2.9 (1.0)	2.2 (0.4)	n.s.	3.4 (4.0)
Trimethylfluorene	n.s.	1.2 (0.2)	n.s.	n.s.
Pyrs total	**1.3** (0.9)	**1.3** (0.3)	**1.7** (1.4)	**0.3** (0.1)
Pyrene	0.1 (0.1)	0.4 (0.1)	0.9 (0.4)	0.3 (0.1)
Methylpyrene	0.3 (0.3)	0.5 (0.1)	0.8 (1.1)	n.s.
Dimethylpyrene	0.7 (0.5)	0.3 (0.1)	n.s.	n.s.
Trimethylpyrene	0.2 (0.4)	0.1 (0.0)	n.s.	n.s.
Total 4 main PAH	62.0	96.0	94.7	97.0

The total fraction of the family (parent + alkylated) relative to all PAH compounds present in the sample is highlighted in bold. The values reported are the average over the number of samples collected (N = 6 for Maya oil in laboratory, N = 11 for Diesel at Polludrome, N = 5 for Saumaty and N = 4 for the North Sea). Number in parenthesis are standard deviations for these same sampling. ns, non-significant concentrations.

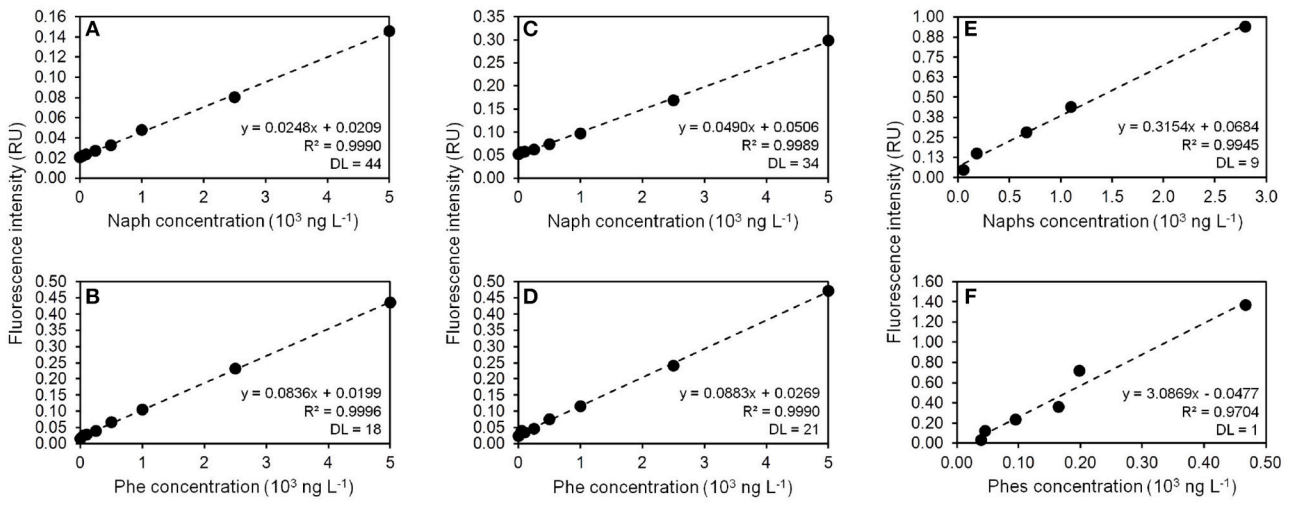


FIGURE 4 | Calibration of the MiniFluo-1 (MFL S/N #14) sensor (Naph and Phe) for pure standards in ultra-pure (milli-Q) water **(A,B)**, pure standards in synthetic seawater (SSW) **(C,D)** and with crude oil (Maya type) water accommodated fraction (WAF) containing parents and alkylated fractions of both PAH **(E,F)**. Concentrations of Naphs and Phes (for WAF only) were obtained by GC–MS analyses. *DL* is the detection limit in ng L^{-1}. Scale factor (*SF*) and Blank (*B*) values in Equation (1) are, respectively, the slope and y-intercept of the linear regression curve.

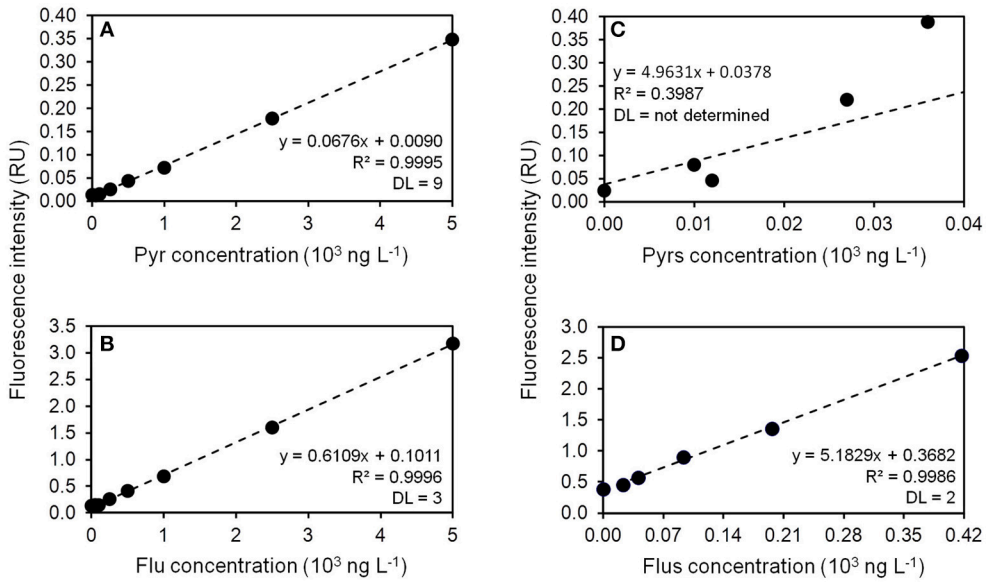


FIGURE 5 | Calibration of the MiniFluo-2 (MFL S/N #11) sensor (Flu and Pyr) for pure standards in ultra-pure (milli-Q) water **(A,B)** and with crude oil (Maya type) water accommodated fraction (WAF) containing parents and alkylated fractions of both PAH **(C,D)**. Concentrations of Flus and Pyrs were obtained by GC−MS analyses. *DL* is the detection limit in ng L^{-1}. Scale factor (*SF*) and Blank (*B*) values in Equation (1) are, respectively, the slope and y-intercept of the linear regression curve. Calibration curves with pure standards in synthetic seawater (SSW) are not available.

PAHs concerning the *SF* value (Flu >Phe >Pyr >Naph) has been already reported for the same kind of calibrations on STD in ultra-pure water from 3D fluorescence measurements performed using a laboratory spectrofluorometer (Ferretto et al., 2014). This trend in *SF* values reflects the intrinsic fluorescence capacity of the four PAHs (highest capacities for Flu and Phe, lowest for Naph) in accordance with their resonance energy (positive linear relationship between *SF* value and energy resonance for the four PAHs; Figure not shown). For the case of Pyrs and Flus, their proportions in the WAF (as analyzed by GC−MS) are less important than those of Naphs and Phes (**Table 2**). The concentrations range tested for those two compounds are thus more restricted than those for Naphs and Phes, especially for Pyrs (**Figures 5C,D**). In all cases, *SF* values (ratio of fluorescence intensity over PAH concentration) obtained when calibrating on WAF were higher than those obtained with STD calibration.

3.3. Validation in a Polludrome

PAH concentrations measured by GC−MS before the introduction of fuel in the basin (initial concentration) were already relatively high, with $C_{Naphs} = 887\,ng\,L^{-1} > C_{Phes} = 242\,ng\,L^{-1} > C_{Flus} = 98\,ng\,L^{-1} > C_{Pyrs} = 24\,ng\,L^{-1}$. This made the quantification of the concentrations at high dilution relatively difficult. This problem is probably related to an insufficient cleaning of the basin, and/or to non-negligible concentrations of PAHs in the water used for dilution (pumped from Brest Harbor). No measurements were performed in the water collection area, but concentrations measured in the European coastal marine environment generally vary in the range $10 - 500\,ng\,L^{-1}$ (Guigue et al., 2014), and are therefore not negligible. However, they remain below the initial concentration reported above (e.g., $1377\,ng\,L^{-1}$ for the $\sum$ 32 parents + alkylated PAHs). These relatively high values are likely due to a residual fraction of hydrocarbons that remains in the system after dilution (e.g., on the walls of the basin or in the pumping and filtering systems). In addition, the dilution system of the basin works in such a way that starting from the initial concentration (100% WAF), the total volume is reduced to its half before being completed by the filtered seawater (to reach 50% WAF), and so on until the minimum concentration is reached (6.5% WAF). Given the large volume considered here ($7m^3$) and the possible inertia of the drainage system, it is also probable that the dilution was less efficient than expected. Nevertheless, this system validated the calibration method and helped verifying that the sensor works as expected on the glider.

Table 3 shows the concentration of the 4 PAHs targeted here as determined by GC−MS in the experimental basin and measured by MiniFluo-1 (Naph, Phe) and MiniFluo-2 (Flu, Pyr) using the 2 calibrations previously discussed (STD and WAF). Because of technical issues, MiniFluo-2 was only deployed in the three dilutions with lowest concentrations. Due to the very low proportion of pyrenes in the fuel used to prepare the solutions, Pyrs concentration measured in the basin are very close to the detection limit and, although presented in **Table 3**, will therefore not be considered for further analysis.

Results derived from the MiniFluos using STD calibration overestimate the concentrations determined by GC−MS by a factor 3–15. This is expected because, as mentioned above, crude oil / fuel contains alkylated PAHs that fluoresce in the same spectral domain than their parent compound. The

TABLE 3 | Performances of the MiniFluo in the polludrome experimental basin.

	Proportions of diesel in the experiment basin (%)						Err ± std
	0	**6.5**	**12.5**	**25**	**50**	**100***	**(%)**
Naphs (ng L^{-1})							
GC−MS	887	2,735	2,927	4,523	7,960	12,890	
STD calib.	6,828	16,196	15,825	16,254	23,239	37,775	374 ± 176
WAF calib.	1,143	3,693	3,592	3,709	5,610	9,567	27 ± 5
Phes (ng L^{-1})							
GC−MS	242	365	563	693	828	1,361	
STD calib.	3,291	4,059	4,498	5,673	8,300	13,900	919 ± 189
WAF calib.	103	125	136	171	247	407	69 ± 6
Flus (ng L^{-1})							
GC−MS	98	200	197	351			
STD calib.	354	2,101	2,366	3,361			793 ± 319
WAF calib.	-10	196	227	345			32 ± 45
Pyrs (ng L^{-1})							
GC−MS	24	69	56	78			
STD calib.	6,643	16,196	15,825	16,254			$\sim 10^4$
WAF calib.	4,241	9,950	9,724	9,986			$\sim 10^4$

For each proportion of Diesel in the basin, GC−MS concentrations of Naphs, Phes, Pyrs and Flus (parent and alkylated compounds) are given (first line under each compounds in the table). These are compared to concentrations returned by the two MiniFluos (MiniFluo-1 for Naph/Phe and MiniFluo-2 for Flu/ Pyr) using calibration on pure standards (STD) solutions and with water accommodated fraction (WAF) of Maya crude oil. The mean relative error (err = $\frac{C_{MFL}-C_{GC-MS}}{C_{GC-MS}} \times 100\%$) and its standard deviation (std) for STD and WAF calibration are given in the right-most column of the table.

** 100% represent 50 mL of Diesel in the 7 m^3 experimental basin.*

calibration using Maya-based WAF however gives results that are in relatively good accordance with GC−MS measurements. Naphs and Flus concentrations are very close to those measured by GC−MS (by a factor 0.98 to 1.15), while Phes were slightly underestimated (factor 2–4). One assumption to explain this difference may be the different proportions of the parent and alkylated compounds between the Maya WAF used for calibration and the WAF (Total Diesel) from the experimental basin (**Table 2**).

While calibration with laboratory-prepared standard solutions is highly reproducible and appeared necessary for characterizing the sensor in terms of detection limit, performance compared to other sensors and aging, it appears that the calibration using WAF is a better approach to quantify petroleum products in seawater. Whenever possible, using the oil expected to be found on the exploitation field for sensor calibration will result in an even more accurate results. While WAF calibration captures the bulk part of the signal, it is shown in the next section that, when available, a third calibration using *in situ* GC−MS measurements may further refine the MiniFluo measurements.

TABLE 4 | Comparison between MiniFluo and GS−MS measurements during the Saumaty glider mission.

	Naphs (ng L^{-1})		Phes (ng L^{-1})	
Station	**GC−MS**	**MiniFluo/Naphs**	**GC−MS**	**MiniFluo/Phes**
S01	37.3	144.1	0.7*	28.1*
S02	42.0	171.8	0.8*	29.1*
S03	54.6	343.9	2.1	38.0
S04	51.3	693.9	6.6	55.1
S05	76.2	826.6	15.5	60.6
err ± std	672 ± 429%		912 ± 727%	

*Total Phenanthrenes and Naphthalenes concentration (parent and alkylated) determined by GS−MS and derived from the MiniFluo-1 using laboratory calibration on WAF (i.e., **Figures 4**, **5**) are reported. MiniFluo concentrations were determined from the average measurement over a 2-min period encompassing the time the water sample was taken. Location of stations are show on **Figure 2**. The mean relative error (err = $\frac{C_{MFL}-C_{GC-MS}}{C_{GC-MS}} \times 100\%$) and its standard deviation (std) between GC−MS and MiniFluo-derived concentrations are given in the bottom line of the table. Very low Phes concentrations near the GC−MS detection limit have been ignored during the error calculation.*

** Ignored for error calculation.*

3.4. Coastal Application in Marseille Metropolitan Area

The MiniFluo was tested in natural environment in Saumaty Harbor, Marseille Metropolitan area (see section 2.3). For this campaign, the glider was only equipped with MiniFluo-1 (Naph/Phe). Concentrations measured by the MiniFluo using laboratory WAF calibration are reported in **Table 4** and in **Figure 6** for Naph-like (top) and Phe-like (bottom). Results show a rapid increase of fluorescence as the glider advance toward the head of the harbor. Naphs and Phes concentrations determined by GC−MS are reported in the figure panels as star symbols. Using the laboratory WAF calibration, the MiniFluo overestimates Naph-like concentration by a factor 8 on average

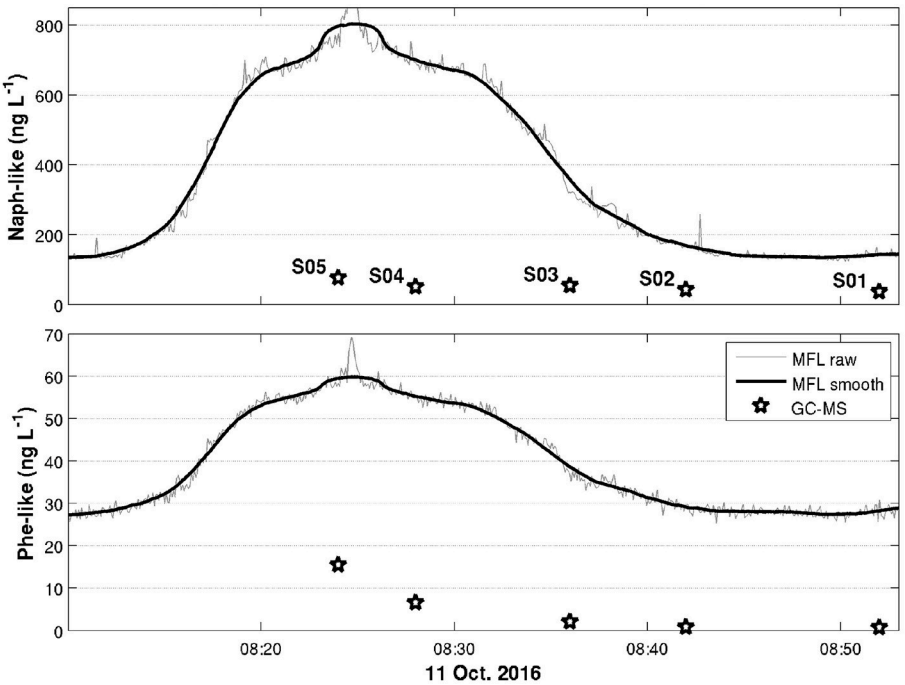


FIGURE 6 | Phenanthrenes **(Top)** and Naphthalenes **(Bottom)** concentration in Saumaty harbor. The thin gray line represent the full measurements from the the MiniFluo using laboratory WAF calibration. The thick black line is the smoothed timeseries using a 1-min running mean algorithm. Black stars are concentrations measured by GC−MS from collected water samples along the track.

(∼ 700% error) and Phe-like concentration by a factor 10 (∼ 900% error) on average over the water samples (the maximum over-estimation over a single measurement is a factor 18). These are reported in the last line of **Table 4** (note that for Phes, the two lowest concentrations near instrumental noise at S01 and S02 are ignored, see Table's footnote). While the MiniFluo was able to measure PAH concentrations very close to GC−MS concentrations during the polludrome experiment, an overestimation here is likely due to the fact that the waters in this urbanized area is a mixture of several organic pollutants or biological by-products (e.g., Tryptophan or Chl-*a*) that may fluoresce at wavelengths close to the targeted compounds. Other explanations includes the fact that we calibrated on a Maya crude oil WAF that have alkylated proportions not representative of those found in Saumaty (**Table 2**), or the presence of higher blank noise (background fluorescence levels) compared to the laboratory seawater used (SSW).

It is also possible to further improve the performances of the MiniFluo by performing site-specific (e.g., *in situ*) calibration. This type of *in situ* calibration is similar to what is routinely done on Chl-*a* or dissolved oxygen measurements during hydrographic surveys. **Figure 7** illustrates such calibration. Here panels *a* and *c* show the concentrations measured by the MiniFluo using *SF* and *B* calculated as the linear regression between the MiniFluo fluorescence and PAHs concentration determined by GC−MS at the 5 water collection stations (see panels *b* and *d* for the calibration curves). This figure illustrates how, with a certain number of discrete validation points, PAH concentrations can be adjusted for the entire timeseries to better represent the actual concentrations.

While the latter calibration method is always preferable, this extra step requires important means, such as the possibility of collecting sufficient water samples followed by PAH extraction and GC−MS analyzes. Such *in situ* calibration method should be considered, for example, when systematic monitoring surveys of the same region are required. When not possible, a WAF laboratory calibration seems sufficient to give an order of magnitude precision on the PAHs concentration (e.g., here an overestimation by a factor 8 and 10, respectively, for Naph-like and Phe-like), a precision sufficient to detect the presence of a major oil spill incident.

3.5. Offshore Application in the North Sea

As part of an offshore application scenario, the glider SEA003 equipped with MiniFluo-1 was deployed for 15 days in the North Sea in November-December 2016 (see section 2.3). Naph-like and Phe-like concentrations presented in this section are derived using laboratory WAF calibrations only (i.e., **Figures 4**, **5**). This is because while water samples were collected at deployment site (one station, 4 depths between 0-40 m), the concentration range encountered was relatively narrow and did not allow *in situ* calibrations of the MiniFluo channels. For this single station, **Table 5** however shows that PAH concentrations measured by the MiniFluo agree with the concentrations derived by GC−MS within 22% on average for Naphs and 873% (about factor 10) for Phes. In accordance with previous experiments (Polludrome

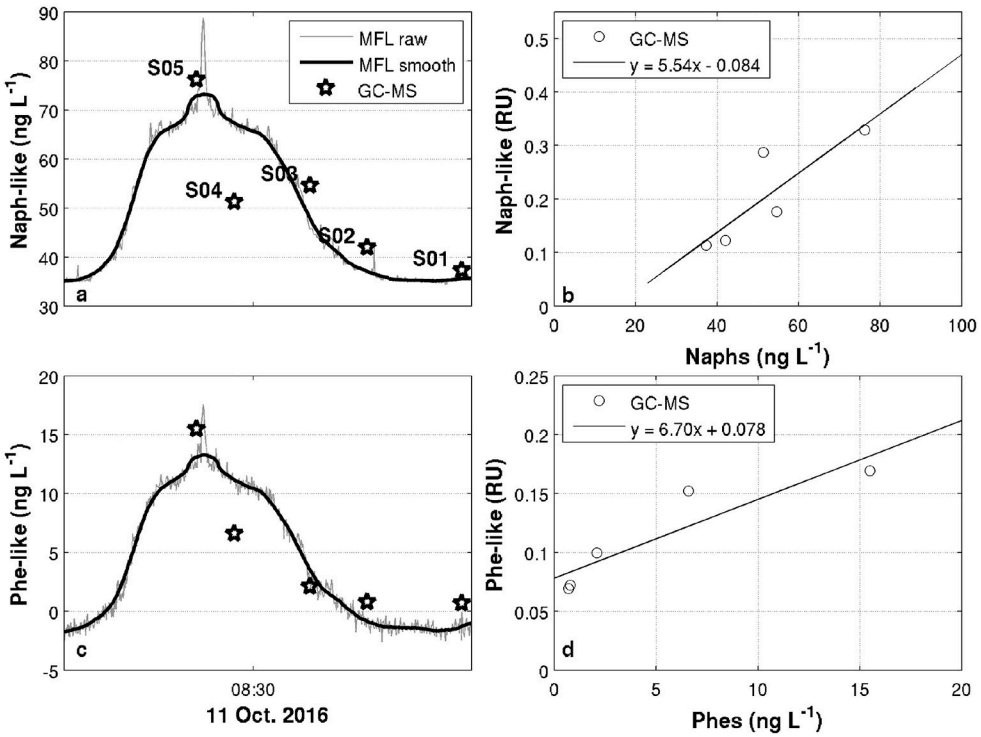


FIGURE 7 | Left column is similar to **Figure 6**, but using *in situ* calibration instead of laboratory calibration. The *in situ* calibration (right column) was calculated from the linear fit (solid line) between GC−MS data the mean relative-unit fluorescence returned by the MiniFluo over a 2-min period encompassing the water sample collection (black circles).

TABLE 5 | Comparison between MiniFluo and GS−MS measurements during the North Sea glider mission.

	Naphs (ng L^{-1})		Phes (ng L^{-1})	
Depth (m)	**GC−MS**	**MiniFluo/Naphs**	**GC−MS**	**MiniFluo/Phes**
0	152.4	126.4	12.6	24.4
10	186.6	123.4	1.9	24.1
25	137.0	123.8	24.0	23.9
40	81.1	102.5	1.0	23.3
err ± std	22 ± 9%		873 ± 908%	

Total Phenanthrenes and Naphthalenes concentration (parent and alkylated) determined by GS−MS and derived from the MiniFluo-1 using laboratory calibration on WAF (i.e., ***Figures 4***, ***5****) are reported. MiniFluo concentration were determined as the vertical average on 5-m bins encompassing the targeted GS−MS sample depth during the first glider dive. The mean relative error (err =* $\frac{C_{MFL}-C_{GC-MS}}{C_{GC-MS}} \times 100\%$*) and its standard deviation (std) between GC−MS and MiniFluo-derived concentrations are given in the bottom line of the table.*

and Saumaty), it is thus relatively conservative to expect that the concentrations measured here by the MiniFluo are within one order of magnitude to the true concentrations on average. Surface concentration of Naph-like and Phe-like concentrations averaged over the top 10 m of the water column are shown in **Figure 8**. Highest concentrations are found along the first transect line across the channel, east/southeast of *T*3 (approximately 20 November). This higher concentration patch has disappeared during the return transect. Another relatively high concentration patch was also detected at the northern edge of the channel (between 21–22 November). This location is near *Fensfjorden* entrance, a region with heavy marine traffic due to a large oil refinery and industrial area located at Mongstad. At the very end of the mission, while waiting for recovery at proximity of *T*1 waypoint, the MiniFluo also captured higher than average concentrations for a period of about 24 h (visible on the map only for Naph-like channel).

Figure 8 suggests a relative spatio-temporal heterogeneity of this region. This is not surprising given the tidal dynamics and the winter winds at play during the survey. However, the coherence in successive glider profiles with the MiniFluo, shows the ability of the sensor to detect hydrocarbon patches.

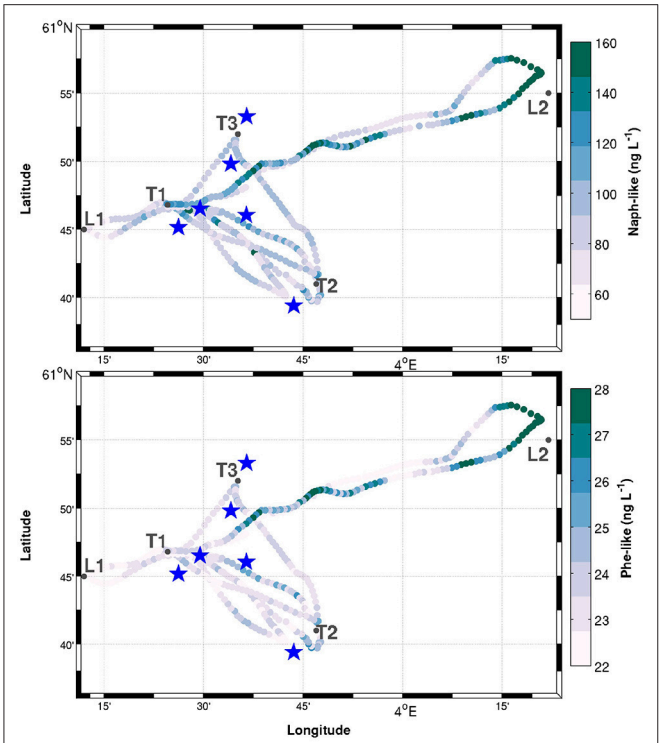


FIGURE 8 | Surface concentration of Naphs (upper) and Phes (lower) as measured with the MiniFluo along the glider track (averaged over the 0–10 m depth range). Offshore installations are highlighted with stars (see **Figure 3** for legend).

The maximum surface concentration encountered during the mission is about 200 ng L^{-1} for Naphs (Phes concentrations only reached about 30 ng L^{-1}). This is more than a factor 10 below the maximum admissible concentration of individual PAHs (2, 400 ng L^{-1}) set by the European Water Framework Directive (reported from Nasher et al., 2013).

Similar concentration levels are found deeper in the water column. Here only the first across-channel transect (projected along the *L*1-*L*2 line) is presented as it is the one with the most interesting features of this mission (**Figure 9**). As observed in **Figure 8**, patches of higher PAH concentrations (Phe-like and Naph-like) are found. This is specially clear in Phe-like (**Figure 9F**) in the top $\sim$ 100 m of the water column and roughly between 20–40 and 50–70 km. Interestingly, Phe-like signal is often contrasted with Chl-*a* signal, the former being maximum where the latter is minimum (specially clear in the 20–40 km range). Except very close to the surface, the Naph-like signal (**Figure 9E**) is different from Phe-like signal (note that some data near 30, 50, and 60 km were manually removed due to measurement problems). While Phe-like patterns are reproduced in Naph-like signal, the latter seems to also reproduce some of the Chl-*a* patterns (panel *E* exhibits features of both *D* and *F*). It is likely that the Naph-like channel also captures Tryptophan-like fluorescence that is associated with biological activities, and thus Chl-*a*. This is because Naphthalene $\lambda_{Ex}/\lambda_{Em}$ fluorescence couple is similar to the one of Tryptophan (Tedetti et al., 2010; Cyr et al., 2017).

Also worth noting is the relatively high Phe-like signal near the seafloor. This signal seems to reach surface concentration signal in plume-like patterns. These patterns are also reproduced in the humic-like fluorophore (higher concentration, **Figure 9G**), in the O_2 (lower concentrations, **Figure 9C**), and, to a lesser extent, in the Naph-like and Backscattering measurements (**Figures 9E,H**, respectively). These signatures may suggest hydrocarbon emanations from the seafloor entraining low oxygen concentration waters, or the sinking of organic matter containing hydrocarbon in degradation. These signatures also seem associated with a surface frontal zone located between 40 and 50 km (see isopycnals outcropping at the surface).

While analyzing in details the results of these campaign is outside the scope of this study, these results are one of the earliest demonstration of the large-scale interactions between hydrocarbon concentrations and physical-biochemical properties in an oceanic basin. Overall, this campaign successfully demonstrates that it is possible to use the glider-MiniFluo package as a powerful tool to monitor hydrocarbon concentrations in seawater. Such usage may include, the detection of natural seeps from the seafloor, the monitoring of a region for baseline studies or to monitor long-term effects, or the tracking of a spill in case of industrial accident.

4. CONCLUSION

This study summarizes the development of a glider-compatible fluorometer capable of detecting PAHs in the marine environment. Results suggest that a calibration of the MiniFluo on WAF is preferable over STD because the alkylated compounds often represent the largest part of a PAH family. It is thus important to consider not only the parent (such as a calibration on pure standard), but also the alkylated compounds when deriving concentration of a certain PAH in the marine environment. Results also show that when using such WAF calibration, it is relatively conservative to expect the concentrations derived from the MiniFluo and those determined by GC−MS to agree within one order of magnitude on average (e.g., overestimation by a factor 7 on average over the two *in situ* campaigns presented in this study). Because the relative proportion of the parent and alkylated compounds of a PAH family slightly differ from one type of hydrocarbon to the other, a calibration on a WAF with proportions close to the one in the targeted environment will give best results. In this sense, a calibration with different types of WAFs (i.e., different proportions of parent/alkylated hydrocarbons), but also on WAFs having undergone weathering/degradation processes, in order to better reflect the PAHs proportions found in the marine environment seems relevant for future improvement. This is because dissolved PAHs in the aquatic media are subjected to various transformation processes including, for example, biodegradation and photodegradation. These processes do not affect parent and alkylated compounds equally (Dachs et al., 1997; Diez et al., 2005), and thus likely lead to modifications of their

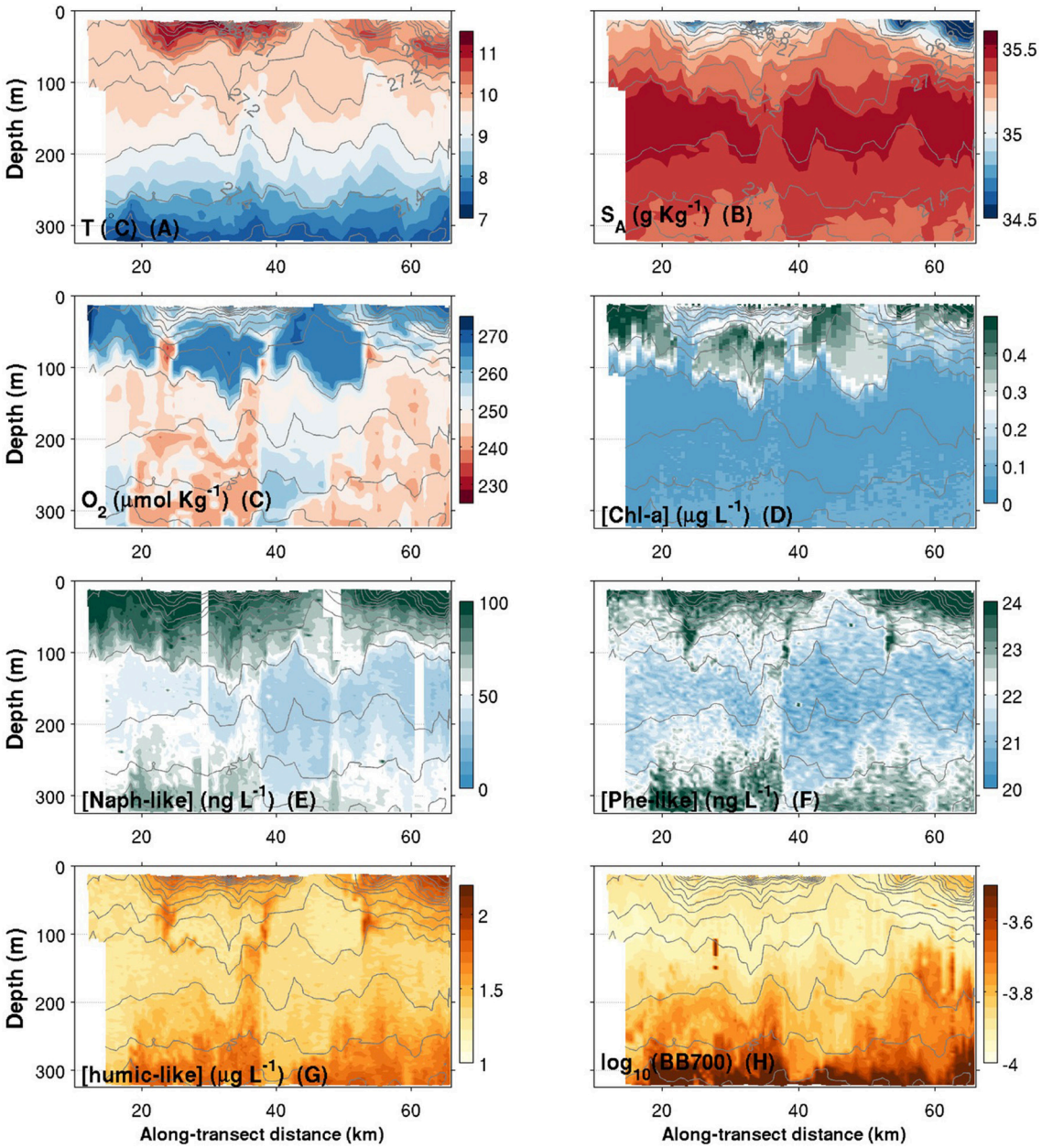


FIGURE 9 | Contours plots of various variables measured by the glider in function of depth and along-transect distance along the line *L1-L2* (see map **Figure 3**). **(A)** Conservative temperature; **(B)** Absolute salinity; **(C)** Dissolved oxygen concentration; **(D)** Chlorophyll-*a* concentration; **(E)** Naphthalene-like concentration; **(F)** Phenanthrene-like concentration; **(G)** Humic-like concentration; **(H)** Turbidity measured as the backscattering signal at 700 nm. Isopycnals are plotted in each panel with thin solid light gray lines (values identified in **A,B**).

relative abundances with time (in addition of possible spatial and seasonal effects). A series of laboratory controlled experiments dealing with the biodegradation and/or photodegradation of WAFs prior to their use for MiniFluo calibrations would thus be an interesting further improvement of the calibration procedure. As suggested earlier, however, a site-specific *in situ* calibration similar to what is performed with other biogeochemical sensors (e.g., dissolved O_2 or Chl-*a*), although more demanding in terms of logistics, is likely to give the best results. If performed during the glider mission, such calibration would also solve a large part of these biodegradation/photodegradation problems by providing an up-to-date response of the sensor in the natural environment. Overall, this study suggests new possibilities for research on industrial marine accidents,

while offering new tools to the industry to monitor PAHs in seawater.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

MG and MT initiated the development of the MiniFluo and its inclusion on the SeaExplorer glider, while FB is the R&D engineer in charge of the MiniFluo development at ALSEAMAR. MG led the laboratory calibration work and the polludrome experiment during which NB was responsible of the glider measurements. FC led the field work studies, including the data processing, and the writing of the manuscript. All authors contributed to the writing and the discussion around the manuscript.

FUNDING

This study is a contribution to the European project *NeXOS* - Next generation Low-Cost Multifunctional Web Enabled Ocean Sensor Systems Empowering Marine, Maritime and Fisheries Management, is funded by the European Commission's 7th Framework Programme - Grant Agreement N°614102.

ACKNOWLEDGMENTS

The authors would like to thank L. Beguery and ALSEAMAR, the SAM at MIO, J. Receveur and R. Jezequel at CEDRE, Statoil, Captains and crew of the R/V Antedon II and the Havila Troll, K. Kvalsund and N. Roar Hareide from the Runde Environmental Centre and personnel who participated to the development of the sensor: M. Abaker, C. Bachet, C. Guigue, C. Germain, P. Joffre and G. Wassouf. MG thanks IFREMER for hosting her during the writing. Two reviewers are greatly acknowledged for their comments.

REFERENCES

Adhikari, P. L., Maiti, K., and Overton, E. B. (2015). Vertical fluxes of polycyclic aromatic hydrocarbons in the northern Gulf of Mexico. *Mar. Chem.* 168, 60–68. doi: 10.1016/j.marchem.2014.11.001

Berrojalbiz, N., Dachs, J., Ojeda, M. J., Valle, M. C., Castro-Jiménez, J., Wollgast, J., et al. (2011). Biogeochemical and physical controls on concentrations of polycyclic aromatic hydrocarbons in water and plankton of the Mediterranean and Black Seas. *Glob. Biogeochem. Cycles* 25, 1–14. doi: 10.1029/2010GB003775

Camilli, R., Reddy, C. M., Yoerger, D. R., Van Mooy, B. A., Jakuba, M. V., Kinsey, J. C., et al. (2010). Tracking hydrocarbon plume transport and biodegradation at Deepwater Horizon. *Science* 330, 201–204. doi: 10.1126/science.1195223

Conmy, R., Del Castillo, C., Downing, B., and Chen, R. (2014a). "Experimental design and quality assurance: *in-situ* fluorescence instrumentation," in *Aquatic Organic Matter Fluorescence,* eds P. Coble, J. Lead, A. Baker, D. Reynolds, and R. Spencer (New York, NY: Cambridge University Press), 190–230.

Conmy, R. N., Coble, P. G., Farr, J., Wood, A. M., Lee, K., Pegau, W. S., et al. (2014b). Submersible optical sensors exposed to chemically dispersed crude oil: Wave tank simulations for improved oil spill monitoring. *Environ. Sci. Technol.* 48, 1803–1810. doi: 10.1021/es404206y

Cyr, F., Tedetti, M., Besson, F., Beguery, L., Doglioli, A. M., Petrenko, A. A., et al. (2017). A new glider-compatible optical sensor for dissolved organic matter measurements : test case from the NW Mediterranean Sea. *Front. Mar. Sci.* 4:89. doi: 10.3389/fmars.2017.00089

Dachs, J., Bayona, J. M., Raoux, C., and Albaigés, J. (1997). Spatial, vertical distribution and budget of polycyclic aromatic hydrocarbons in the Western mediterranean seawater. *Environ. Sci. Technol.* 31, 682–688. doi: 10.1021/es960233j

Davis, R., Eriksen, C., and Jones, C. (2003). "Autonomous buoyancy-driven underwater gliders," in *Technology and Applications of Autonomous Underwater Vehicles*, Chapter 3, ed G. Griffiths (London: Taylor and Francis), 37–58.

Díez, S., Sabaté, J., Viñas, M., Bayona, J. M., Solanas, A. M., and Albaigés, J. (2005). The Prestige oil spill. I. Biodegradation of a heavy fuel oil under simulated conditions. *Environ. Toxicol. Chem.* 24, 2203–2217. doi: 10.1897/04-604R.1

Ferretto, N., Tedetti, M., Guigue, C., Mounier, S., Redon, R., and Goutx, M. (2014). Identification and quantification of known polycyclic aromatic hydrocarbons and pesticides in complex mixtures using fluorescence excitation-emission matrices and parallel factor analysis. *Chemosphere* 107, 344–353. doi: 10.1016/j.chemosphere.2013.12.087

Fourati, R., Tedetti, M., Guigue, C., Goutx, M., Garcia, N., Zaghden, H., et al. (2018). Sources and spatial distribution of dissolved aliphatic and polycyclic aromatic hydrocarbons in surface coastal waters of the Gulf of Gabès (Tunisia, Southern Mediterranean Sea). *Prog. Oceanogr.* 163, 232–247. doi: 10.1016/j.pocean.2017.02.001

González, J. J., Viñas, L., Franco, M. A., Fumega, J., Soriano, J. A., Grueiro, G., et al. (2006). Spatial and temporal distribution of dissolved/dispersed aromatic hydrocarbons in seawater in the area affected by the Prestige oil spill. *Mar. Pollut. Bull.* 53, 250–259. doi: 10.1016/j.marpolbul.2005.09.039

González-Gaya, B., Morales, L., Méjanelle, L., Abad, E., Piña, B., Duarte, C. M., et al. (2016). High atmosphere - ocean exchange of semivolatile aromatic hydrocarbons. *Nat. Geosci.* 9, 438–442. doi: 10.1038/ngeo2714

Guigue, C., Tedetti, M., Ferretto, N., Garcia, N., Méjanelle, L., and Goutx, M. (2014). Spatial and seasonal variabilities of dissolved hydrocarbons in surface waters from the Northwestern Mediterranean Sea: Results from one year intensive sampling. *Sci. Total Environ.* 466–467, 650–662. doi: 10.1016/j.scitotenv.2013.07.082

Guigue, C., Tedetti, M., Giorgi, S., and Goutx, M. (2011). Occurrence and distribution of hydrocarbons in the surface microlayer and subsurface water from the urban coastal marine area off Marseilles, Northwestern Mediterranean Sea. *Mar. Pollut. Bull.* 62, 2741–2752. doi: 10.1016/j.marpolbul.2011.09.013

Guitart, C., García-Flor, N., Bayona, J. M., and Albaigés, J. (2007). Occurrence and fate of polycyclic aromatic hydrocarbons in the coastal surface microlayer. *Mar. Pollut. Bull.* 54, 186–194. doi: 10.1016/j.marpolbul.2006.10.008

Hylland, K. (2006). Polycyclic aromatic hydrocarbon (PAH) ecotoxicology in marine ecosystems. *J. Toxicol. Environ. Health A* 69, 109–123. doi: 10.1080/15287390500259327

Kennish, M. J. (1991). *Ecology of Estuaries: Anthropogenic Effects*, Vol. 1. Boca Raton, FL: CRC Press.

McDougall, T. J., and Barker, P. M. (2011). *Getting Started With TEOS-10 and the Gibbs Seawater (GSW) Oceanographic Toolbox*. Number May. SCOR/IAPSO WG127.

Nasher, E., Heng, L. Y., Zakaria, Z., and Surif, S. (2013). Concentrations and sources of polycyclic aromatic hydrocarbons in the seawater around Langkawi Island, Malaysia. *J. Chem.* 2013:975781. doi: 10.1155/2013/975781

Qiu, Y. W., Zhang, G., Liu, G. Q., Guo, L. L., Li, X. D., and Wai, O. (2009). Polycyclic aromatic hydrocarbons (PAHs) in the water column and sediment core of Deep Bay, South China. *Estuar. Coast. Shelf Sci.* 83, 60–66. doi: 10.1016/j.ecss.2009.03.018

Reddy, C. M., and Quinn, J. G. (2001). The North Cape oil spill: hydrocarbons in Rhode Island coastal waters and Point Judith Pond. *Mar. Environ. Res.* 52, 445–461. doi: 10.1016/S0141-1136(01)00100-3

Roose, P., and Brinkman, U. A. (2005). Monitoring organic microcontaminants in the marine environment: principles, programmes and progress. *Trends Anal. Chem.* 24, 897–926. doi: 10.1016/j.trac.2005.10.007

Rudnick, D. L., and Cole, S. T. (2011). On sampling the ocean using underwater gliders. *J. Geophys. Res. Oceans* 116, 1–12. doi: 10.1029/2010JC006849

Rudnick, D. L., Davis, R. E., Eriksen, C. C., Fratantoni, D. M., and Perry, M. J. (2004). Underwater gliders for ocean research. *Mar. Technol. Soc. J.* 38, 73–84. doi: 10.4031/002533204787522703

Stortini, A. M., Martellini, T., Del Bubba, M., Lepri, L., Capodaglio, G., and Cincinelli, A. (2009). n-Alkanes, PAHs and surfactants in the sea surface microlayer and sea water samples of the Gerlache Inlet sea (Antarctica). *Microchem. J.* 92, 37–43. doi: 10.1016/j.microc.2008.11.005

Tedetti, M., Guigue, C., and Goutx, M. (2010). Utilization of a submersible UV fluorometer for monitoring anthropogenic inputs in the Mediterranean coastal waters. *Mar. Pollut. Bull.* 60, 350–362. doi: 10.1016/j.marpolbul.2009.10.018

Tedetti, M., Joffre, P., and Goutx, M. (2013). Development of a field-portable fluorometer based on deep ultraviolet LEDs for the detection of phenanthrene- and tryptophan-like compounds in natural waters. *Sens. Actuat. B Chem.* 182, 416–423. doi: 10.1016/j.snb.2013.03.052

Tedetti, M., Longhitano, B. R., Garcia, A. N., Guigue, A. C., Ferretto, N., and Goutx, M. (2012). Fluorescence properties of dissolved organic matter in coastal Mediterranean waters influenced by a municipal sewage effluent (Bay of Marseilles, France). *Environ. Chem.* 9, 438–449. doi: 10.1071/EN12081

Testor, P., Meyers, G., Pattiaratchi, C., Bachmeyer, R., Hayes, D., Pouliquen, S., et al. (2010). "Gliders as a component of future observing systems," in *Proceedings of OceanObs'09: Sustained Ocean Observations and Information for Society, Vol. 1,* eds J. Hall, D. E. Harrison, and D. Stammer (Venice: ESA Publication WPP-306), 961–978.

Troupin, C., Beltran, J. P., Heslop, E., Torner, M., Garau, B., Allen, J., et al. (2015). A toolbox for glider data processing and management. *Methods Oceanogr.* 13–14, 13–23. doi: 10.1016/j.mio.2016.01.001

Valavanidis, A., Vlachogianni, T., Triantafillaki, S., Dassenakis, M., Androutsos, F., and Scoullos, M. (2008). Polycyclic aromatic hydrocarbons in surface seawater and in indigenous mussels (Mytilus galloprovincialis) from coastal areas of the Saronikos Gulf (Greece). *Estuar. Coast. Shelf Sci.* 79, 733–739. doi: 10.1016/j.ecss.2008.06.018

Valeur, B., and Berberan-Santos, M. N. (2012). *Molecular Fluorescence: Principles and Applications*. Weinheim: Wiley-VCH.

Witt, G., and Matthäus, W. (2001). The impact of salt water inflows on the distribution of polycyclic aromatic hydrocarbons in the deep water of the Baltic Sea. *Mar. Chem.* 74, 279–301. doi: 10.1016/S0304-4203(01)00020-2

Zhou, J. L., Hong, H., Zhang, Z., Maskaoui, K., and Chen, W. (2000). Multi-phase distribution of organic micropollutants in Xiamen Harbour, China. *Water Res.* 34, 2132–2150. doi: 10.1016/S0043-1354(99)00360-7

Zhou, J. L., and Maskaoui, K. (2003). Distribution of polycyclic aromatic hydrocarbons in water and surface sediments from Daya Bay, China. *Environ. Pollut. (Barking, Essex : 1987)* 121, 269–281. doi: 10.1016/S0269-7491(02)00215-4

Zhou, Z., Liu, Z., and Guo, L. (2013). Chemical evolution of Macondo crude oil during laboratory degradation as characterized by fluorescence EEMs and hydrocarbon composition. *Mar. Pollut. Bull.* 66, 164–175. doi: 10.1016/j.marpolbul.2012.09.028

Zielinski, O., Busch, J. A., Cembella, A. D., Daly, K. L., Engelbrektsson, J., Hannides, A. K., et al. (2009). Detecting marine hazardous substances and organisms: sensors for pollutants, toxins, and pathogens. *Ocean Sci.* 5, 329–349. doi: 10.5194/os-5-329-2009

Conflict of Interest Statement: The authors declare that the affiliation of FB, the manufacturer of the MiniFluo and the glider SeaExplorer, do not preclude to the scientific independence of this scientific work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

ORIGINAL RESEARCH
published: 10 April 2019
doi: 10.3389/fmars.2019.00151

Assessment of the Plastic Inputs From the Seine Basin to the Sea Using Statistical and Field Approaches

Romain Tramoy[1], Johnny Gasperi[1]*, Rachid Dris[1,2], Laurent Colasse[3], Cédric Fisson[4], Sarah Sananes[5], Vincent Rocher[6] and Bruno Tassin[1]*

[1] Université Paris-Est, Laboratoire Eau, Environnement, Systèmes Urbains (LEESU), UMR MA 102 – AgroParisTech, Créteil, France, [2] Animal Ecology I and BayCEER, University of Bayreuth, Bayreuth, Germany, [3] Association SOS Mal de Seine, La Bouille, France, [4] Groupement d'Intérêt Public (GIP) Seine-Aval, Espace des Marégraphes, Rouen, France, [5] Ministère de la Transition Ecologique et Solidaire, Courbevoie, France, [6] Syndicat Interdépartemental pour l'Assainissement de l'Agglomération Parisienne (SIAAP), Direction du Développement et de la Prospective, Colombes, France

OPEN ACCESS

Edited by:
Juan Bellas, Spanish Institute of Oceanography, Spain

Reviewed by:
Daniel González-Fernández, University of Cádiz, Spain
Giuseppe Suaria, Istituto di Scienze Marine (ISMAR), Italy

****Correspondence:***
Romain Tramoy
romain.tramoy@enpc.fr
Johnny Gasperi
gasperi@u-pec.fr

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *07 January 2019*
Accepted: *08 March 2019*
Published: *10 April 2019*

Citation:
Tramoy R, Gasperi J, Dris R, Colasse L, Fisson C, Sananes S, Rocher V and Tassin B (2019) Assessment of the Plastic Inputs From the Seine Basin to the Sea Using Statistical and Field Approaches. Front. Mar. Sci. 6:151. doi: 10.3389/fmars.2019.00151

Global estimations state that between 0.5 and 12.7 million metric tons of plastic enter the oceans each year. They are, however, associated with great uncertainties due to methodological difficulties to accurately quantify land-based plastic fluxes into the oceans. New studies at basin scale are thus needed for better model calibrations. Here, a modeling approach based on Jambeck's statistical method and a field approach are compared in order to (i) quantify plastic fluxes in the Seine River and (ii) characterize and constrain uncertainties of both approaches. Despite the simplicity of the statistical approach and rough extrapolations, both methods yield similar results, i.e., between 1,100 and 5,900 t/yr of plastic litter flowing into the Sea of which about 88–128 t/yr are removed by cleaning operations. According to the marine strategy framework directive (2008/56/EC), actions are required to quantify plastic fluxes entering the oceans. Among different methods, a better use of the data from the waste collection should be considered. The development of a national and homogenous platform listing all the collects would be a first step in that direction.

Keywords: litter, fluxes, catchment, waste collection, debris

INTRODUCTION

Since the 1950s, the production of plastic increased from 1.7 to 335 million of metric tons in 2016 (PlasticsEurope, 2017). Consequently, plastic litter has invaded marine and continental environments worldwide and became in the 2000s a global warning concern. According to global estimations, between 0.5 and 12.7 million metric tons of plastics are entering into the oceans each year, although known stocks are only about 300 thousand metric tons (Eriksen et al., 2014; Jambeck et al., 2015; van Sebille et al., 2015; Lebreton et al., 2017; Schmidt et al., 2017, 2018). Unexplored sea floors could then represent the final reservoir of plastic litter after sinking (Galgani et al., 2000; Kammann et al., 2018; Maes et al., 2018). Plastics constitute between 8 and 15% of waste mass generated by human activities (Hoornweg and Bhada-Tata, 2012). Nonetheless, and even if they are produced in urban areas, their physical and chemical properties make them the

predominant part of the litter in natural environments, even on isolated pacific islands, pristine sea ice or sea floors (Barnes et al., 2009). They also constitute an increasing threat for freshwater ecosystems (Eerkes-Medrano et al., 2015; Wilcox et al., 2015; Li et al., 2018). Plastic litter in marine ecosystems largely captured the attention of the scientific community, whereas most plastics would come from the continents via rivers (Schmidt et al., 2017). A better understanding of how plastics are transferred from continents to the oceans and in which amount is therefore of great interest, especially since it seems easier to remove plastic litter from almost unidimensional rivers than from tridimensional oceans.

According to the descriptor 10 of the Marine Framework Directive of 2008, actions are required to reach a good environmental status of ecosystems by 2020 with regard to marine litter[1]. This also accounts for assessing and monitoring riverine litter as a primary source of marine litter. Several statistical approaches have been developed in the last years to quantify the amount of plastics entering the oceans. They deal with visual counting as proposed by RIMMEL project (González-Fernández and Hanke, 2017), extrapolations from field data limited to punctual opportunistic measurements (Lechner et al., 2014; van der Wal et al., 2015) or statistical analysis at a global scale (Jambeck et al., 2015; Lebreton et al., 2017; Schmidt et al., 2017). Methods differ by their uncertainties and the size of plastic litter taken into account. For example, visual counting is focusing on macroplastics (>2.5 cm), field measurements on microplastics and extrapolation to larger plastic items (van der Wal et al., 2015), while statistics often disregard the size class of plastics. Fields measurements are most of the time focusing on buoyant plastics and ignore a potential plastic load under the surface water (Morritt et al., 2014) and statistical approaches, calibrated with the previous ones, are based on few key parameters poorly improved. To date, many methodological difficulties have prevented the development of a standard method to quantify plastic input entering into the oceans via rivers, because the plastic pollution is scattered, multi-origin and represents only a small fraction of the total waste production. Even in regions known for their inefficient solid waste management, particularly South-East Asia, leaks and losses of plastics to the environment are very low when compared to plastic production and consumption, which reaches several hundreds of millions metric tons (PlasticsEurope, 2017). All global estimations refer to the mismanaged plastic waste index (MPW) to quantify plastic input into the oceans (Jambeck et al., 2015; Lebreton et al., 2017; Schmidt et al., 2017). This index is based on the anthropogenic pressure considering (i) the amount of plastic waste that is not adequately disposed in landfills or incinerator plants according to the economic level of the considered territory (GDP), and (ii) a leakage rate set at 2% of the plastic waste generated, which accounts for accidental loss or littering. This leakage rate is used systematically even though it only relies on one United States report about littering behaviors and costs (MSWCONSULTANTS, 2009; see **Supplementary Data** of Jambeck et al., 2015). This rate thus constitutes one of the previously mentioned key parameters. Littering being intrinsically unquantifiable because it refers to unobservable loss, its rate of 2% is based on the ratio between the amount of litter generated in the United States in 2008 and the total national waste generation (**Supplementary Data** of Jambeck et al., 2015). Consequently, calculations at a global scale are linked to statistical weaknesses. These global estimates are nevertheless of main interest because they shed new light on the plastic pollution worldwide by giving orders of magnitude. Better constraining and identifying their limitations is essential to the development of a robust and standardized methodology for measuring plastic inputs entering the oceans.

Over the past 5 years, the microplastic occurrence and distribution of plastic litter in the Seine River were estimated and characterized (Gasperi et al., 2014; Dris, 2016; Dris et al., 2018), but no estimations of fluxes were performed yet. The present paper is a first step toward the construction of innovative methods to accurately estimate plastic fluxes in the Seine River. The Seine basin is relatively small, i.e., 78 600 km^2 and its rivers have low water flows, i.e., 300–350 m^3/s in average for the Seine river in Paris. They are under significant anthropogenic pressure with 16.7 Million inhabitants into the whole basin, of which ∼12 Million are gathered in Paris megacity. The Seine basin is the most dynamic territory at the national scale with 25% of the population, 50% of the fluvial traffic, 40% of the economic activity, and 30% of agricultural activities (Fisson et al., 2014; Lemoine and Verney, 2015). The seasonal contrast in the catchment is characterized by high water level periods during winter and very low water levels during summer that needs to be managed with dams and water reservoirs. The outlet of the catchment is a 170 km long meandering estuary in which the influence of tides and winds increase as the river mouth becomes closer. All these elements influence the plastic debris dynamics. Coupled with a strong anthropogenic pressure, plastic pollution is highly visible in the estuary – especially at low tide – with countless plastic debris stranded on the riverbanks. This makes the Seine basin a relevant experimental site to analyze plastic debris dynamics and assess the corresponding fluxes according to different approaches. Thanks to environmental associations and a general growing concern of the population, this initiative is supported by the French ministry in charge of the environment, public water agencies, local authorities and NGOs.

Two methods are successively discussed. The first approach is a conceptual modeling approach based on the statistical method developed by Jambeck et al. (2015). The use of regional and departmental data of waste generation per capita and plastic rate in domestic waste enables to better estimate the MPW generation than national data. Even though several key parameters (e.g., Leaking rate to the environment of 2%; Leaking rate to the Sea of 15–40%; GDP as a proxy for not adequately disposed waste) are still used in lack of better options. The second approach is based on field results, i.e., the percentage of plastic in debris captured by a network of floating booms deployed by SIAAP (public sanitation services of Ile de France) upstream and downstream Paris. The two methods are discussed in terms of robustness and sensitivity in order to estimate as much as possible their

[1]http://ec.europa.eu/environment/marine/good-environmental-status/descriptor-10/index_en.htm

uncertainties, and in particular those of the key parameters involved. In addition, the impact of clean-up procedures is discussed. Data collected during clean-up campaigns, especially from NGOs, are under-exploited and call for renewed interest in assessing pollution levels in rivers in general and in the Seine River in particular.

THE MODELING APPROACH

Data and Method

In the Seine catchment, the modeling approach was based on the following parameters: (i) the number of inhabitants, (ii) the annual amount of waste produced per capita, (iii) the associated plastic rate, and (iv) a 2% rate of leakage to the environment (Jambeck et al., 2015). The conceptual model is described in **Figure 1A**. According to Jambeck et al. (2015), the amount of MPW is also based on the GDP of countries to account for the municipal waste that are not well disposed. Since the GDP in France is high, this parameter is neglected because France is believed to manage municipal waste properly according to the world bank (Hoornweg and Bhada-Tata, 2012; **Supplementary Data** in Jambeck et al., 2015). Finally, only littering was considered, in which 15% to 40% are entering the Seine River to account for municipal street sweeping and other cleaning actions before it reaches the Seine River (Jambeck et al., 2015).

The annual waste production per capita was based on domestic residual waste – the fraction which is not sorted at home – and was extracted from the *Agence de l'Environnement et de la Maîtrise de l'Energie database* (ADEME[2]). The most recent and consolidated data from 2015 were used. The residual waste data are available at the department scale, which corresponds to the European NUTS-3 scale (NUTS: Nomenclature of Territorial Units for Statistics). Then, the average waste generation at the basin scale is weighted proportionally to the population of each department (see **Supplementary Data**). For departments partially included in the catchment of the Seine River, only the fraction actually included was considered using GIS layers containing population data for each municipality (GEOFLA® 2016 v2.2 Communes France Métropolitaine, IGN).

In this study, only domestic residual waste was considered, because it is the most important fraction in mass and also the better characterized in terms of amount, typology and plastic proportion (ORDIF, 2017a,b). It provides useful information about life styles of inhabitants living into the catchment and their use guarantees a good homogeneity among data like Jambeck et al. (2015) did at the national level using the world bank data (For France, these authors used the total municipal waste generation per capita and an associated plastic rate of 10%). Based on the domestic residual waste of more than 7.8 Million inhabitants in Paris megacity, the associated plastic rate is 16% (ORDIF, 2017a). This plastic rate was applied to the whole catchment, because there is no plastic rate associated to waste generation by department available. Then, the rate of 2% of littering used by Jambeck et al. (2015) was applied to estimate the amount of MPW since a more accurate rate is still not available. For the same reason, the 15 to 40% of MPW reaching the Seine River was also applied.

Results

Around one quarter of the French population, i.e., 16,7 Million people, is living into the Seine basin, of which ∼12 Million gathered in Paris megacity. The weighted average domestic residual waste generation is 276 kg/cap/yr for 2015, hence a total of plastic waste in the basin of 738,205 t/yr considering a plastic rate of 16% (**Figure 1B**). It can be noticed that results shows some discrepancies between departments. For example,

[2]http://carto.sinoe.org/carto/enquete_collecte/flash/#

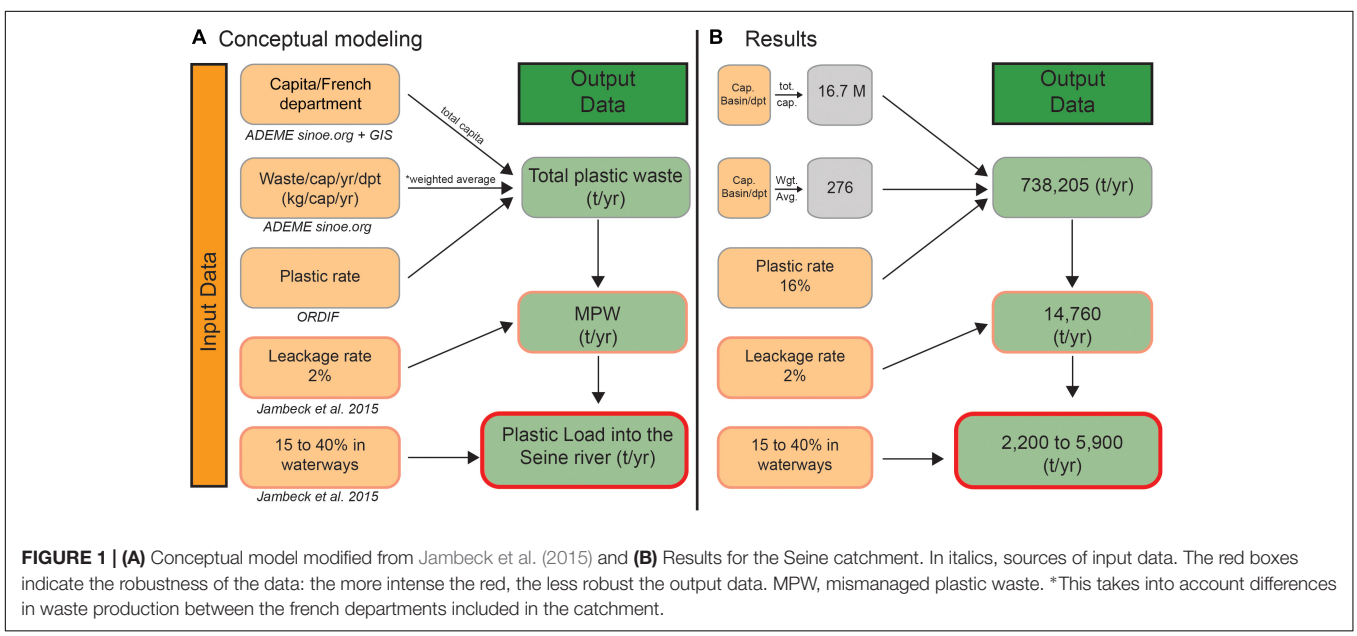


FIGURE 1 | (A) Conceptual model modified from Jambeck et al. (2015) and **(B)** Results for the Seine catchment. In italics, sources of input data. The red boxes indicate the robustness of the data: the more intense the red, the less robust the output data. MPW, mismanaged plastic waste. *This takes into account differences in waste production between the french departments included in the catchment.

the average in Paris megacity (288 kg/cap/yr) is 17% higher than the one in the rest of the basin (246 kg/cap/yr). It even goes up to 45% if only Paris city is considered when compared to the rest of the territory. This can be explained by the very high economic and touristic activity in Paris megacity, associated with the "on-the-go" consumption trend, generating much more waste than elsewhere (ORDIF, 2017a,b). The ORDIF reports shed new lights on discrepancies between urban and rural territories by comparing typologies of domestic residual waste between Paris megacity and the national averages. Results show a relatively low proportion of organics into the domestic residual waste from Paris megacity when compared to the national average. Whereas the proportion of plastic packaging (16%) – mainly films and PET bottles – and packaging board is higher than the national average (10% for plastic packaging). In addition, the densest areas suffer of a lack of space for selective collection, which contributes to increasing the amount of domestic residual waste in Paris megacity as well as reducing the recyclability potential.

Using those specific data from 2015 as inputs of the conceptual model, MPW is estimated at 14,760 t/yr, of which 15 to 40%, i.e., 2,200 to 5,900 t/yr entering into the Seine River (**Figure 1B**). This is less than the 20,000 t/yr of MPW estimated by Lebreton et al. (2017) in their study based on the total waste production for each world catchment using data from the World Bank. However, their calibrated model used to calculate plastic outputs yield only 9 to 45 t/yr of plastics entering the oceans from the Seine catchment. This discrepancy may be related to the lack of consideration for macroplastics and submerged debris due to rough calibrations of the predictive model. Calibrations were based on available and uncomplete local studies, which mainly report microplastic or mesoplastic concentrations measured at the surface. It illustrates the strong influence of input data into models, but also the great discrepancies between models and other methods of estimation coming from calibration issues.

The uncertainties related to the conceptual model approach is entirely based on the hypothesis that 15 to 40% of MPW reach the Seine River. However, those proportions are poorly documented and are linked to many environmental factors and local practices (street sweeping, hydrologic regimes, infrastructures, incivilities, associative cleaning, local policies, etc.). The same remarks are addressed to the 2% of littering, which relies on only one study from a US association of corporations: Keep America Beautiful, Inc. (MSWCONSULTANTS, 2009). This 2% rate is used in every global estimation as a baseline for littering estimates, whereas there is no reason to apply this rate in every country of the world including the Seine River in France. In conclusion, refining those parameters in each territory is of high interest to reduce uncertainties. Thus, another approach has been developped to be compared to the present results.

THE FIELD APPROACH

Data of the Floating Booms and Extrapolation Methods

The field approach is based on the amount of waste captured by the 26 floating booms of the SIAAP between 2007 and 2017, which are displayed on the Marne and the Seine River in Paris megacity (**Figure 2**). The covered period of data is larger than the previous method for several reasons. First, the localizations of the floating booms are not relative to the river morphology (e.g., meanders). They were designed and set up to collect macro-debris from combined sewer overflows during rainy events (Gasperi et al., 2012, 2014). Extending the covered period of data enables to account for inter-boom variability as well as inter-annual variability of the mean water flow. Second, we do not have the same resolution of data for the booms and the statistical approach as the oldest available data with the same precision are from 2013. Third, statistics of population and waste generation per capita did not drastically change between 2000 and 2015. The population increased of 10% in Paris megacity, while waste generation per capita decreased by 10% (ORDIF, 2017b). Thus, it still makes sense to compare the two methods, even though the resolution of data and the period considered are different.

Floating booms collect organics and plastic litter. The SIAAP records the monthly total masses collected in annual reports (see **Supplementary Data**). During the period 2007–2017, the mean annual water flow of the Seine River in Paris-Austerlitz Bridge was 308 m^3/s (see **Supplementary Data**). The plastic rate in those debris has been estimated by Gasperi et al. (2014) using debris collected in 10 booms corresponding to the red points in **Figure 2**. According to these authors, 1.4% in mass of the debris caught by the floating booms are plastics, with values ranging from 0.8 to 5.1%. The average of 1.4% was used to calculate plastic amounts captured by floating booms. However, the real efficiency of the booms for collecting floating debris is unknown. Then, hypotheses were assumed to estimate plastic fluxes. First, the plastic rate in the collected debris was considered constant for the whole year. Second, it is considered that plastics are uniformly distributed on the cross section of the Seine River. Following these hypotheses, the plastic amount captured by floating booms was extrapolated to a water flow ratio between the booms and the Seine River.

The water flow ratio R_Q corresponds to the ratio between water flows crossing the booms and the mean water flow of the Seine River, i.e., 308 m^3/s in Paris. Each boom is 7.5 m width for 0.3 m height covering 2.25 m^2 of the Seine River. In Paris megacity, the minimum water depth in the shipping line is 3.2 m according to navigation authorities[3]. With a mean water flow of 0.1 m/s, each boom is crossed by a water flow of 0.225 m^3/s, i.e., 5.85 m^3/s for the 26 booms. According to the water flow ratio method, booms capture a fraction $R_Q \sim 1/53$ of the mean water flow of the Seine River. Based on this ratio and data from the SIAAP, monthly mean concentrations of plastics in water $[Pl]_{monthly}$ (in mg/m^3 or in literature in $g/1000\ m^3$) were calculated using the following equation for the period 2007–2017:

$$[Pl]_{monthly} = \frac{M_{Pl}}{Q_{monthly} \times R_Q \times t_{(month)}} \quad (1)$$

[3]http://www.vnf.fr

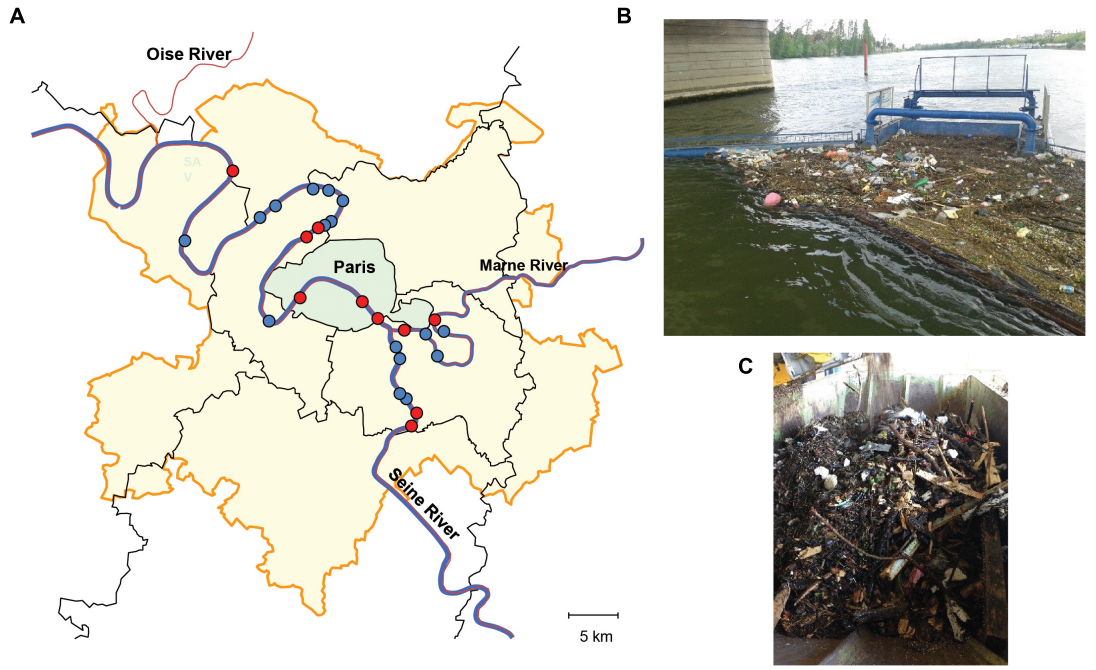


FIGURE 2 | (A) Geographical distribution of the floating boom network from SIAAP in Paris megacity with sampled booms by Gasperi et al. (2014) in red, **(B)** a picture from a floating boom and **(C)** an example of waste removal by boat. Modified from Gasperi et al. (2014).

With M_{Pl}, the mass of plastics in mg calculated by multiplying the monthly mass of debris in booms and the rate of plastics in debris (Gasperi et al., 2014), $Q_{monthly}$, the monthly mean water flow in m^3/s and $t_{(month)}$, the number of seconds in one month. Then, a log relation between plastic concentrations and water flow in the Seine River was constructed (**Figure 3**).

Results

Monthly plastic concentration values in the water filtered by booms were calculated using the equation (1). Between 2007 and 2017, they ranged from 50 to 390 mg/m^3, with an average at 173 ± 76 mg/m^3 and a median value at 165 mg/m^3. Those concentrations in the Seine River are in the range of concentrations compiled by Schmidt et al. (2017) who reported a mean value in worldwide rivers of 864.7 ± 5,461.3 mg/m^3, but they also reported a median concentration far smaller at ∼0.3 mg/m^3. At sea, mean values of plastic concentrations in accumulation zones in open oceans are between 300 and 600 g/km^2, similar to the average concentration (423 g/km^2) in surface waters of the Mediterranean Sea (Cozar et al., 2014; Cózar et al., 2015). Assuming a water layer of 30 cm, concentrations in the most polluted areas of the oceans reach 1 to 2 mg/m^3, which is two order of magnitude less than our estimations for the Seine River. Cleaning operations on land and in rivers would thus be more effective than in the open sea.

Monthly plastic concentrations in the Seine River are negatively correlated to monthly water flows (**Figure 3**). The range of plastic load flowing into the Seine River can be approximated using this relation with minimum and maximum annual water flow on the period 2007–2017 (**Figure 3**), i.e., x_1 = 190 m^3/s (in 2009) and x_2 = 521 m^3/s (in 2013), respectively. Assuming a uniform distribution of plastics on the cross section, the plastic load is then between 1,100 and 1,700 t/yr (see **Supplementary Data** for detailed calculations). This assumption of equal mass of plastic in the vertical profile is selected as best option for rough estimations, since non-floating litter loads remain unknown. Because floating booms cover barely 10% of the Seine river depth (3.2 m in Paris megacity), floating litter would represent between 110 and 170 t/yr.

Concentration-water flow relations are usually used to identify sources and mechanisms of diffusion of pollutants in rivers like for suspended sediments, particulate organic matter, nutrients or metals (e.g., Williams, 1989; Vink et al., 1999; Quilbé et al., 2006). By analogy, the significant relation ($p < 0{,}001$) between log concentration values of plastics trapped by booms and associated water flows depicts dilution processes of the plastic pollution with the increase of water flows (**Figure 3**). However, factors influencing the concentrations of plastics are numerous as suggested by the high inter- and intra-seasonality variability (summer/winter), with roughly 50% of the variance non-explained by this relation. This is probably the result of the combination between event-related plastic pollution, i.e., rain events, and a background pollution accumulation during dry weather periods from Paris megacity. Plastic leaks into the environment through incivilities or unintentional losses related to nomadic consumption would constitute relatively constant inputs, diluted by increasing water flows. For example, summer favors nomadic consumption close to the riverbanks. Then, storms promote the transfer of litters to the Seine River by runoff

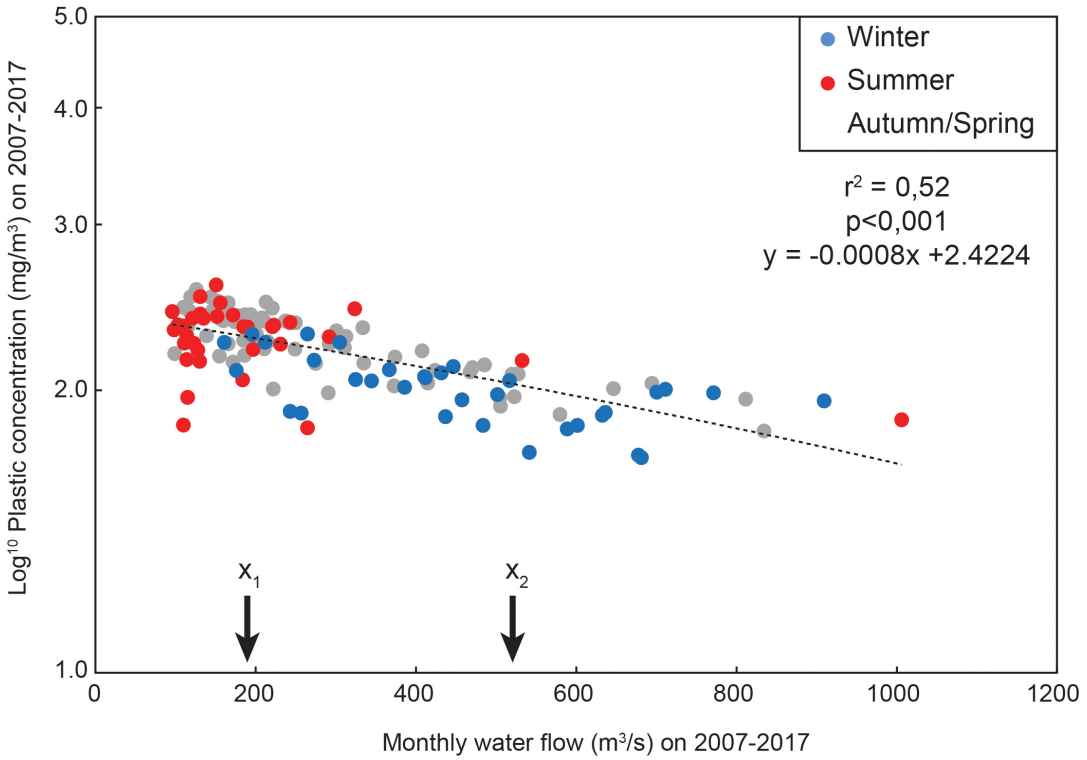


FIGURE 3 | Concentrations (log^{10}) of plastics in the Seine River relative to the monthly mean water flow filtered by 26 floating booms of the SIAAP between 2007 and 2017. Each point corresponds to a monthly mean water flow and associated log^{10} concentration of plastics during the same period. Minimum and maximum annual water flow for this period is, respectively, X_1 and X_2. Concentrations of plastics in mg/m^3 were calculated using (i) the monthly amount of debris collected by booms, (ii) an average plastic rate in debris of 1.4% (Gasperi et al., 2014), and (iii) a ratio between the mean water flow and water flow filtered by boom, i.e., 1/53. The highest mean water flow of 1,006 m^3/s corresponds to the flood of June 2016.

from their banks or from combined sewer overflows, resulting in event-related pollution. This could explain the wide range of plastic concentration values that is twice as large in summer as in winter. Nevertheless, while plastic amounts increase with water flows, it still remains insufficient to counteract dilution processes (**Supplementary Figure S1**).

According to internal SIAAP reports and **Supplementary Data**, no spatial trend (e.g., downstream or upstream Paris; **Figure 2**) can be highlighted regarding the amount of debris captured by floating booms. This suggests a constant input of debris in the whole area covered by the booms, which is in agreement with a strong and permanent anthropogenic pressure in Paris megacity. Other factors can influence the efficiency of floating booms and therefore the estimation of plastic fluxes. It must be noted that on one hand intense river traffic along the Seine and wind bring waste back to the banks, while on the other hand induced waves can push debris out from the booms. In addition, during high flood episodes, debris can escape from the booms. The flooding of the floodplain also decreases the chance for a boom to catch debris, while the potential amount of plastics increases as evidenced by the winter flood in 2018 (**Figure 4**). Municipalities downstream of Paris were particularly impacted by the presence of plastic litter on riverbanks or in sluices (e.g., Méricourt, Les Andelys). The real trapping efficiency of the booms is therefore unknown and might be evaluated using tracking waste as markers.

DISCUSSION

The various approaches lead to estimates ranging from 1,100 to 5,900 t/yr of plastics entering the Seine River. This is much higher than those estimated by Lebreton et al. (2017), i.e., 9–45 t/yr, mostly based on floating microplastic data and therefore lacking information on the macroplastic pool. However, when compared to estimations related to floating litter only, i.e., 110 to 170 t/yr, results are closer to each other suggesting a relatively good reliability of the Lebreton's model for floating litter flux. When compared to the population of the Seine River catchment, 1,100 to 5,900 t/yr of plastics yields between 66 and 353 g/cap/yr. Uncertainties leading to this wide range have been identified but could not be precisely assessed. However, both methods converge toward similar orders of magnitude, while their uncertainties refer to completely different parameters. In summary, the conceptual modeling approach has the advantage of being systematic, easy to apply, but therefore somewhat abstract and based on almost unverifiable assumptions (e.g., 2% leakage rate). In contrast, the field approach, based on

FIGURE 4 | Debris during the winter flood in 2018. Route de Muids, right riverbank, ∼120 km downstream Paris megacity, in February 2018, the 15th.

floating booms, is based on very concrete amount of plastic litter collected. However, it is limited in time and space, because floating booms are concentrated in Paris megacity and their efficiency can be very variable. In addition, only floating litter is being collected by booms. Thus, considering plastics as homogeneously distributed into the water column is a very strong hypothesis.

To date, few studies give orders of magnitude of plastic fluxes in rivers. When available, they are often difficult to compare with each other because of methodological issues. Most of the time, estimations are based on trawling devices or similar, with different mesh sizes. For example, estimates of plastic fluxes in the Danube are much lower than those estimated in the Seine with only 17 g/cap/yr (Lechner et al., 2014). But they are based on extrapolations of plastic amount trapped in fish larvae nets (500 μm mesh size) that were settled in Vienna without catching any floating macroplastics. Whereas macroplastics are the most dominant plastics in mass in open oceans (Eriksen et al., 2014). Despite devices used for similar methods are different, plastic load in rivers can often be easily converted in g/cap/yr. In low income countries like Vietnam, between 350 and 7,270 g/cap/yr of plastic debris (>2 cm) have been estimated for Nhieu Loc – Thi Nghe, a canal flowing into the Saigon River (Lahens et al., 2018). The authors estimate a median flow at 1,620 g/cap/year, which is five times higher than our largest estimation for the Seine River. Another study based on particle counting and trawling in the estuary of the Saigon River estimated between 7,500 and 13,700 t/yr of plastics entering the ocean, namely 938 to 1,713 g/cap/year (van Emmerik et al., 2018). At similar economic levels to France, estimated plastic fluxes into the great lakes in the United States are very similar to those estimated for the Seine River with 10,000 t/yr for ∼90 million inhabitants, meaning 111 g/hab/yr (Hoffman and Hittinger, 2017). Sources of plastics are mainly related to the huge lakeside population of Chicago, Detroit, Toronto, and Cleveland. However, the convergence of estimated plastic fluxes for the Great Lakes and the Seine River is not very surprising, as the authors also used the approach of Jambeck et al. (2015). This points to the need of diversity in methods to estimate plastic fluxes otherwise the risk is inter-validation between studies.

The RIMMEL protocol (González-Fernández and Hanke, 2017) proposes to count particles by unit of time and has been updated by van Emmerik et al. (2018) in Vietnam who converted particle counts in masses and coupled results with hydrological data to get annual plastic discharge. Following this method and using a mass per sampled plastic piece of 3.2 g (van Emmerik et al., 2018), 1,100 to 5,900 t/yr of plastics would be equivalent to 343.8 to 1,843.4 Million particles in 1 year. Considering that all plastic litter is buoyant, an observer should count between 11 and 58 particles per second. Even when only considering a floating litter flux, i.e., 110–170 t/yr, an observer should count 1.1 to 1.7 particle per second. Except during floods, such number of items is never reached with the real figure being closer to several items per minutes. This huge discrepancy might be related to the difference between what can be seen at the surface and what is really flowing under the surface water. According to Morritt et al. (2014), plastic flow below the surface water can be important as suggested in the Thames River, but the fraction of plastic remaining nearby to the surface in the Seine River remains unknown. This highlights the need to develop methods more closely connected to the field measurements with greater spatial and temporal representativeness.

One of the ways that should be explored for diversification of methodologies is the use of data collected by the numerous NGOs in charge of bank cleaning. The pollution of the riverbanks by macroplastics is extremely visible and contrast with low visual pollution in the river. This "environmental disorder" tends to raise awareness at economic, social and political levels. The influence of citizens results in the implementation of measures to collect plastic litter through different organizations: public (cleaning boats in the Hauts-de-Seine, SIAAP floating booms), private non-profit organization (Naturaulin in the Seine estuary) or NGO (La-Seine-en-Partage, OSE, SOS Mal de Seine, La Maison de l'Estuaire...). Other private actors, such as hydroelectric power plants, also catch part of the litter out of technical necessity, i.e., protecting the turbines from the presence of debris

TABLE 1 | Estimates of plastic amounts collected in the Seine river or on river banks.

Organization	Unit of data	Amount of plastics (t/yr)	Source	Reliability (1–5)
Naturaulin	Plastics removed are weighted since 2018	16	Data sheets from the Seine maritime authorities	5/5
La Maison de L'estuaire	Annual volumes	10	Report 2016–2018 of La Maison de l'Estuaire	3/5
SIAAP floating booms	Mass	27	SIAAP, Gasperi et al., 2014	4/5
La Seine en partage	Mass converted by the association from volumes (multi-source)	10	Pers. Com. La Seine en partage	2/5
Hydropower plants (Poses + Port−Mort)	Number of waste bins. Conversion to mass using a plastic rate from Gasperi et al., 2014	10–50	Pers. Com. hydrowatt	3/5
Hauts de Seine cleaning boats	Total mass (organics + plastics + others), conversion using the rate of valorized industrial waste	15	Pers. Com. hauts de Seine authorities	3/5
TOTAL		**88–128**	–	–

The reliability of the estimates is a subjective criterion which depends on the reference data and the operations required to convert them into a flux. The lowest reliability is rated 1, while the best is rated 5. Notice that Naturaulin displays now the exact mass of the collected plastics since 2018.

that could damage them. Although the list of clean-up operations is not exhaustive, the compilation and standardization of the data collected shows that these actors remove 88–128 t/yr of plastic throughout the river (**Table 1**). However, this estimate faces significant methodological difficulties because the data recovered differ in format or types of cleaning between actors. For example, run-of-river power plants outsource waste treatment, which is charged by the number of containers without any characterization being carried out on the collected litter. These problems of consistency in the data have been regularly pointed out since the macro-waste topic emerged some 20 years ago in France (Lerond, 1997; Poitou, 2003; Galgani et al., 2010).

Converting volumes of litter collected (with organics) into plastic mass or using already transformed data, without the associated methodology, does not facilitate the estimation of the impact of cleaning (**Table 1**). This figure of 88–128 t/yr should therefore be taken with caution. It is also almost negligible (1–12%) when compared to previous estimations of plastic fluxes. The compilation of a large amount of beach waste collection data from 130 countries shows that 250,000 metric tons of waste have been collected worldwide mostly since the 2000s, or around 12,500 t/yr (Schneider et al., 2018). Assuming that a similar cleaning effort is renewed each year and that all litter collected is plastic, the collection rate would be 0.1 to 2.5% of the estimated 0.5 to 12.7 million metric tons reaching the oceans (Jambeck et al., 2015; Lebreton et al., 2017; Schmidt et al., 2017). Accordingly, although useful from a societal and environmental point of view, these clean-up operations alone would not be able to solve the problem of plastics in the oceans (Schneider et al., 2018). In addition, most cleanings deal with stocks and not fluxes. In the case of the Seine River, regular cleanings in accumulation zones may prevent the development of a considerable litter stock along the river and reduce litter flux to the oceans by renewing the capacity of these zones to act as litter sinks. Consequently, the use of cleaning data could help to evaluate fluxes and even set up an indicator of the pollution rate of rivers by plastic litter. In the Seine Estuary, cleanings make already possible to qualitatively highlight the main impact of floods, rainfall events and great tides (Naturaulin and La Maison de l'Estuaire, pers. com.), in agreement with models that predict the maximum plastic inputs during winter in our latitudes (Lebreton et al., 2017). Such cleanings should be developed in other basins as programs of qualitative and quantitative monitoring measures of riverine litter according to the Descriptor 10 of the marine strategy framework directive (MSFD; see text footnote 1). Cleanings already proved their usefulness leading to restrictions at the European level of single use plastics for the coming years such as cotton bud sticks, cutlery, plates, stirrers, straws, sticks for balloons[4].

CONCLUSION

The combined use of a field approach and a conceptual modeling approach has led to estimates of plastic fluxes exported from the Seine to the Sea of the same order of magnitude, i.e., 1,100 to 5,900 t/yr of which 88–128 t/yr are removed by cleaning operations and dams. However, these estimates are based on strong assumptions and/or suffer from huge uncertainties that remain difficult to constrain in the current state of knowledge. This study also highlighted the role of the various actors involved in waste collection in the river. They can be an important source of data despite the lack of homogeneity and wide discrepancies in collection practices. Nevertheless, this study constitutes a first attempt at the national level. It calls for the development of new and innovative methods facing methodological difficulties discussed in this paper. For example, the transfer dynamics of plastics from the upstream to the downstream of the river should also be considered to accurately evaluate plastic inputs into the oceans but also the impact of mitigation policies.

Other actors in waste collection have yet to be identified. In the future, it would be interesting to set up a homogeneous database common to all actors at the national, European and international level. It could be used to develop an indicator of the pollution of the river by plastic litter and other anthropogenic items. Such an

[4]http://europa.eu/rapid/press-release_IP-18-6867_en.htm

indicator of river pollution by plastic litter would be able to meet the goals of the MSFD on the monitoring and assessment of litter entering the oceans through rivers.

AUTHOR CONTRIBUTIONS

RT, JG, and BT contributed to conception and design of the study. RT wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

Sources of founding are entirely supported by the ministry of the ecological and solidary transition.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2019.00151/full#supplementary-material

REFERENCES

Barnes, D. K. A., Galgani, F., Thompson, R. C., and Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *Philos. Trans. R. Soc. B Biol. Sci.* 364, 1985–1998. doi: 10.1098/rstb.2008.0205

Cozar, A., Echevarria, F., Gonzalez-Gordillo, J. I., Irigoien, X., Ubeda, B., Hernandez-Leon, S., et al. (2014). Plastic debris in the open ocean. *Proc. Natl. Acad. Sci. U.S.A.* 111, 10239–10244. doi: 10.1073/pnas.1314705111

Cózar, A., Sanz-Martín, M., Martí, E., González-Gordillo, J. I., Ubeda, B., Gálvez, J. Á, et al. (2015). Plastic accumulation in the mediterranean sea. *PLoS One* 10:e0121762. doi: 10.1371/journal.pone.0121762

Dris, R. (2016). *First Assessment of Sources and Fate of Macro- and Micro- Plastics in Urban Hydrosystems: Case of Paris Megacity*. Ph.D. thesis, LEESU, Paris.

Dris, R., Gasperi, J., and Tassin, B. (2018). "Sources and Fate of Microplastics in Urban Areas: A Focus on Paris Megacity," in *Freshwater Microplastics: Emerging Environmental Contaminants*, eds M. Wagner and S. Lambert (Cham: Springer International Publishing), 69–83. doi: 10.1007/978-3-319-61615-5_4

Eerkes-Medrano, D., Thompson, R. C., and Aldridge, D. C. (2015). Microplastics in freshwater systems: a review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. *Water Res.* 75, 63–82. doi: 10.1016/j.watres.2015.02.012

Eriksen, M., Lebreton, L. C. M., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., et al. (2014). Plastic pollution in the World's Oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. *PLoS One* 9:e111913. doi: 10.1371/journal.pone.0111913

Fisson, C., Leboulenger, F., Lecarpentier, T., Moussard, S., and Ranvier, G. (2014). *L'estuaire de la Seine: Etat de santé et évolution*. Rouen: GIP Seine-Aval.

Galgani, F., Fleet, D., Van Franeker, J., Katsanevakis, S., Maes, T., Mouat, J., et al. (2010). Marine Strategy Framework Directive Task Group 10 Report Marine Litter. JRC Scientific and Technical Report EUR 24340 EN - 2010.

Galgani, F., Leaute, J., Moguedet, P., Souplet, A., Verin, Y., Carpentier, A., et al. (2000). Litter on the Sea Floor Along European Coasts. *Mar. Pollut. Bull* 40, 516–527. doi: 10.1016/S0025-326X(99)00234-9

Gasperi, J., Dris, R., Bonin, T., Rocher, V., and Tassin, B. (2014). Assessment of floating plastic debris in surface water along the Seine River. *Environ. Pollut.* 195, 163–166. doi: 10.1016/j.envpol.2014.09.001

Gasperi, J., Zgheib, S., Cladière, M., Rocher, V., Moilleron, R., and Chebbo, G. (2012). Priority pollutants in urban stormwater: Part 2 – Case of combined sewers. *Water Res.* 46, 6693–6703. doi: 10.1016/j.watres.2011.09.041

González-Fernández, D., and Hanke, G. (2017). Toward a harmonized approach for monitoring of riverine floating macro litter inputs to the marine environment. *Front. Mar. Sci.* 4:86. doi: 10.3389/fmars.2017.00086

Hoffman, M. J., and Hittinger, E. (2017). Inventory and transport of plastic debris in the laurentian great lakes. *Mar. Pollut. Bull.* 115, 273–281. doi: 10.1016/j.marpolbul.2016.11.061

Hoornweg, D., and Bhada-Tata, P. (2012). *What a Waste: A Global Review of Solid Waste Managment*. Washington, D.C: The world bank.

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., et al. (2015). Plastic waste inputs from land into the ocean. *Science* 347, 768–771. doi: 10.1126/science.1260352

Kammann, U., Aust, M.-O., Bahl, H., and Lang, T. (2018). Marine litter at the seafloor – abundance and composition in the North Sea and the Baltic Sea. *Mar. Pollut. Bull.* 127, 774–780. doi: 10.1016/j.marpolbul.2017.09.051

Lahens, L., Strady, E., Kieu-Le, T.-C., Dris, R., Boukerma, K., Rinnert, E., et al. (2018). Macroplastic and microplastic contamination assessment of a tropical river (Saigon River, Vietnam) transversed by a developing megacity. *Environ. Pollut.* 236, 661–671. doi: 10.1016/j.envpol.2018.02.005

Lebreton, L. C. M., van der Zwet, J., Damsteeg, J.-W., Slat, B., Andrady, A., and Reisser, J. (2017). River plastic emissions to the world's oceans. *Nat. Commun.* 8:15611. doi: 10.1038/ncomms15611

Lechner, A., Keckeis, H., Lumesberger-Loisl, F., Zens, B., Krusch, R., Tritthart, M., et al. (2014). The Danube so colourful: a potpourri of plastic litter outnumbers fish larvae in Europe's second largest river. *Environ. Pollut.* 188, 177–181. doi: 10.1016/j.envpol.2014.02.006

Lemoine, J. P., and Verney, R. (2015). *Fonctionnement Hydro-sédimentaire de l'estuaire de la Seine*. Rouen: GIP Seine-Aval.

Lerond, M. (1997). Définition des conditions de mise en oeuvre du nettoyage de la Seine - Synthèse. *Parc Naturel Regional de Brotonne*

Li, J., Liu, H., and Paul Chen, J. (2018). Microplastics in freshwater systems: a review on occurrence, environmental effects, and methods for microplastics detection. *Water Res.* 137, 362–374. doi: 10.1016/j.watres.2017.12.056

Maes, T., Barry, J., Leslie, H. A., Vethaak, A. D., Nicolaus, E. E. M., Law, R. J., et al. (2018). Below the surface: twenty-five years of seafloor litter monitoring in coastal seas of North West Europe (1992–2017). *Sci. Total Environ.* 630, 790–798. doi: 10.1016/j.scitotenv.2018.02.245

Morritt, D., Stefanoudis, P. V., Pearce, D., Crimmen, O. A., and Clark, P. F. (2014). Plastic in the Thames: a river runs through it. *Mar. Pollut. Bull.* 78, 196–200. doi: 10.1016/j.marpolbul.2013.10.035

MSWCONSULTANTS (2009). *National Visible Litter Survey and Litter Cost Study*. Stamford, CT: Keep America Beautiful, Inc.

ORDIF (2017a). *Données de caractérisations locales des ordures ménagères résiduelles (OMR) en île de France*. Paris: ORDIF, Observatoire Régional des Déchets.

ORDIF (2017b). *LA GESTION DES DÉCHETS MÉNAGERS ET ASSIMILÉS EN ÎLE-DE-FRANCE*. Paris: ORDIF, Observatoire Régional des Déchets.

PlasticsEurope (2017). *Plastics - the Facts 2017, an analysis of European plastics production, demand and waste data*. Brussels: Association of Plastics Manufacturer.

Poitou, I. (2003). Les macrodéchets littoraux : une gestion publique empirique en région provence-alpes-Côte d'Azur. *Méditerranée* 100, 17–22. doi: 10.3406/medit.2003.3277

Quilbé, R., Rousseau, A. N., Duchemin, M., Poulin, A., Gangbazo, G., and Villeneuve, J.-P. (2006). Selecting a calculation method to estimate sediment and nutrient loads in streams: application to the beaurivage river (Québec, Canada). *J. Hydrol.* 326, 295–310. doi: 10.1016/j.jhydrol.2005.11.008

Schmidt, C., Krauth, T., and Wagner, S. (2017). Export of plastic debris by rivers into the sea. *Environ. Sci. Technol.* 51, 12246–12253. doi: 10.1021/acs.est.7b02368

Schmidt, C., Krauth, T., and Wagner, S. (2018). Correction to export of plastic debris by rivers into the sea. *Environ. Sci. Technol.* 52, 927–927. doi: 10.1021/acs.est.7b06377

Schneider, F., Parsons, S., Clift, S., Stolte, A., and McManus, M. C. (2018). Collected marine litter — a growing waste challenge. *Mar. Pollut. Bull.* 128, 162–174. doi: 10.1016/j.marpolbul.2018.01.011

van der Wal, M., van der Meulen, M., Tweehuijsen, G., Peterlin, M., Palatinus, A., Kovac Virsek, M., et al. (2015). *Final Report on Identification and Assessment of Riverine Input of (Marine) Litter*. Bristol: Eunomia Research & Consulting Ltd.

van Emmerik, T., Kieu-Le, T.-C., Loozen, M., van Oeveren, K., Strady, E., Bui, X.-T., et al. (2018). A methodology to characterize riverine macroplastic emission into the ocean. *Front. Mar. Sci.* 5:372. doi: 10.3389/fmars.2018.00372

van Sebille, E., Wilcox, C., Lebreton, L., Maximenko, N., Hardesty, B. D., van Franeker, J. A., et al. (2015). A global inventory of small floating plastic debris. *Environ. Res. Lett.* 10:124006. doi: 10.1088/1748-9326/10/12/124006

Vink, R., Behrendt, H., and Salomons, W. (1999). Development of the heavy metal pollution trends in several European rivers: an analysis of point and diffuse sources. *Water Sci. Technol.* 39, 215–223. doi: 10.2166/wst.1999.0549

Wilcox, C., Sebille, E. V., and Hardesty, B. D. (2015). Threat of plastic pollution to seabirds is global, pervasive, and increasing. *Proc. Natl. Acad. Sci. U.S.A.* 112, 11899–11904. doi: 10.1073/pnas.1502108112

Williams, G. P. W. (1989). Sediment concentration versus water discharge during single hydrologic events in rivers. *J. Hydrol.* 111, 89–106. doi: 10.1016/0022-1694(89)90254-0

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

ORIGINAL RESEARCH
published: 13 June 2019
doi: 10.3389/fmars.2019.00316

mRNA Expression and Biomarker Responses in Perch at a Biomonitoring Site in the Baltic Sea – Possible Influence of Natural Brominated Chemicals

Lars Förlin[1], Noomi Asker[1], Mats Töpel[2], Tobias Österlund[3], Erik Kristiansson[3], Jari Parkkonen[1], Peter Haglund[4], Suzanne Faxneld[5] and Joachim Sturve[1]*

[1] Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg, Sweden, [2] Department of Marine Sciences, University of Gothenburg, Gothenburg, Sweden, [3] Department of Mathematical Sciences, Chalmers University of Technology and University of Gothenburg, Gothenburg, Sweden, [4] Department of Chemistry, Umeå University, Umeå, Sweden, [5] Department of Environmental Research and Monitoring, Swedish Museum of Natural History, Stockholm, Sweden

OPEN ACCESS

Edited by:
Juan Bellas, Oceanographic Center of Vigo, Spanish Institute of Oceanography, Spain

Reviewed by:
Annalaura Mancia, University of Ferrara, Italy
Emilie Lacaze, Environment and Climate Change Canada, Canada

***Correspondence:**
Lars Förlin
lars.forlin@bioenv.gu.se

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *01 February 2019*
Accepted: *27 May 2019*
Published: *13 June 2019*

Citation:
Förlin L, Asker N, Töpel M, Österlund T, Kristiansson E, Parkkonen J, Haglund P, Faxneld S and Sturve J (2019) mRNA Expression and Biomarker Responses in Perch at a Biomonitoring Site in the Baltic Sea – Possible Influence of Natural Brominated Chemicals. Front. Mar. Sci. 6:316. doi: 10.3389/fmars.2019.00316

Perch (*Perca fluviatilis*) has been used in biological effect monitoring in a program for integrated coastal fish monitoring at the reference site Kvädöfjärden along the Swedish east coast, which is a site characterized by no or minor local anthropogenic influences. Using a set of physiological and biochemical endpoints (i.e., biomarkers), clear time trends for "early warning" signs of impaired health were noted in the perch from this site, possibly as a result of increased baseline pollution. The data sets also showed relatively large variations among years. To identify additional temporal variation in biological parameters, global mRNA expression studies using RNA sequencing was performed. Perch collected in 2010 and 2014 were selected, as they showed variations in several biomarkers, such as the activity of the detoxification enzyme CYP1A (EROD), the plasma levels of vitellogenin, markers for oxidative stress, white blood cells count and gonad sizes. The RNA sequencing study identified approximately 4800 genes with a significantly difference in mRNA expression levels. A gene ontology enrichment analysis showed that these differentially expressed genes were involved in biological processes such as complement activation, iron ion homeostasis and cholesterol biosynthetic process. In addition, differences in immune system parameters and responses to the exposure of toxic substances have now been verified in two different biological levels (mRNA and protein) in perch collected in 2010 and 2014. Markedly higher mRNA expression of the membrane transporter (MATE) and the detoxification enzyme COMT, together with higher concentrations of bioactive naturally produced brominated compounds, such as brominated indoles and carbazoles, seem to indicate that the perch collected in 2014 had been exposed to macro- and microalga blooming to a higher degree than did perch from 2010. These results and the differential mRNA expression between the 2 years in genes related to immune and oxidative stress parameters suggest that attention must be given to algae blooming when elucidating the well-being of the perch at Kvädöfjärden and other Baltic coastal sites.

Keywords: transcriptomics, biomonitoring, ecotoxicology, biomarkers, perch, brominated chemicals

INTRODUCTION

The aquatic environment is a final sink for most pollutants. Strategies for environmental monitoring and risk assessment are vital for maintaining the aquatic ecosystem. In particular, biochemical and physiological responses (biomarkers) are valuable tools to provide information and to assess the overall quality of the environment. Methods utilizing biomarkers have been used extensively to investigate exposure, effects and health status in fish (e.g., Larsson et al., 2003; van der Oost et al., 2003, Lehtonen et al., 2014; Asker et al., 2016; Hylland et al., 2017). For this purpose, biomarker measurement of the coastal perch (*Perca fluviatilis*) have been performed for 30 years in the Swedish National Monitoring Program. These field surveys are run in three reference sites, which started in Kvädöfjärden in 1988, in Holmöarna in 1995, and in Torhamn in 2001. The reason for selecting reference sites for these investigations was to provide information about possible temporal large-scale changes in the coastal ecosystems that may have been caused by changes over time due to different biotic and abiotic factors. In addition, the purpose was to build a database of background biological variables to be used as reference data in studies in polluted coastal areas (Sandström et al., 2005). Therefore, the coastal reference sites are located in areas without any known local or regional point sources and are away from large freshwater inflows.

A continuous decline in the health of fish from those reference areas has been demonstrated by using a combined biomarker and fish health parameter approach (Sandström et al., 2005; Hansson et al., 2006a; Hanson et al., 2009). The decline in fish health in the reference sites is obvious from the successive increase in EROD activity, elevated levels of white blood cell counts, disturbed plasma ion balances, and decreasing condition factors (CFs; a body mass index). Parameters related to fish reproduction also seem to be negatively affected, where the relative gonad size of perch from Kvädöfjärden has been continuously reduced since the start of the investigation in 1988. Based on the evaluation of the first 20 years of biomarker data from perch in Kvädöfjärden, it was suggested that the clear time trends that have been observed for EROD activity and the gonado somatic index (GSI) are related to increasing exposure to environmental contaminants (Hanson et al., 2009).

It is difficult to find a simple explanation for the indicated deterioration of fish health. The current knowledge about the actual chemical pollution at the reference sites and the resulting toxic body burden are only fragmented. At the Kvädöfjärden reference site, which is the focus of the present study, the fish body-burden of most "classic" pollutants, which are measured by the National Monitoring of Contaminants in Biota, such as DDT, HCHs, HCB, PCBs and heavy metals, have shown generally decreasing trends over time (Bignert et al., 2017). The occurrence of naturally occurring brominated compounds, such as brominated diphenylethers and dioxins, show temporal variations but no clear time trends in perch from Kvädöfjärden (Haglund et al., 2010). Other so-called "emerging pollutants," such as different PFASs, show generally increasing trends (Holmström et al., 2005; Faxneld et al., 2016; Bignert et al., 2017) in biota such as herring and guillemot eggs in the Baltic Sea. Many of these chemicals can cause adverse effects individually, but it is difficult to link to any individual chemical or part of a complex mixture of chemical compounds to the biomarker time trends. Nevertheless, chemical pollution is usually caused by a complex chemical cocktail that contains dozens of chemicals acting in concert. It has repeatedly been shown that the joint toxicity of chemical mixtures can be substantial, even if all individual compounds are present at only low, individually non-toxic concentrations (Kortenkamp et al., 2009).

In addition to that, the biomarker data from the perch in coastal sites in the Baltic Sea show clear time trends, some of the biomarkers show large variations between years. To investigate and compare variations in the perch biomarkers between two years, 2010 and 2014, which showed large variations, were selected for further studies. For example, the biomarker data from these 2 years showed that the EROD activities, plasma levels of vitellogenin and gonad sizes were higher in 2010 than in 2014 in female perch, whereas catalase and glutathione reductase activities and the plasma content of calcium were markedly lower in 2010 than in 2014. To broaden the biological toolbox for assessing the environmental impact of contaminants and to provide more information about the possible differences between years stored samples of perch liver from the 2010 and 2014 samplings were selected for RNA sequencing. The purpose was to identify differences in mRNA expression patterns that might explain the observed differences between years that might have been caused by different exposure scenarios. In addition, a non-targeted chemical analysis of muscle samples from perch collected in 2010 and 2014 was initiated. The purpose was to provide possible causation for the observed biological differences by analyzing possible differences in the content of brominated dioxins and dioxin-like compounds. Some of these compounds have been found in (and are likely produced by) primary production organisms, i.e., macro- and microalgae in the Baltic Sea (Haglund et al., 2007, 2010; Malmvärn et al., 2008; Unger et al., 2009).

MATERIALS AND METHODS

Sampling

Perch (*P. fluviatilis*) were caught with gill nets (mesh size: 30–33 mm bar length) by a local fishermen at the Kvädöfjärden site on the Swedish east coast (**Figure 1**). After being caught, fish were carefully released directly from the net and kept for 2 to 3 days to allow stress parameters to go down to base levels before sampling in corves situated at the sampling site (Hansson et al., 2006a and references therein). The sampling took place the last week in September every year between 1988 and 2017. All perch in this study were sexually mature females. At the sampling day, fish were killed by a sharp blow to the head, and blood was collected from the caudal vein with a heparin-prepared syringe. Fresh blood was used for measurement of the haematocrit, hemoglobin content, glucose levels and to produce blood smears for the blood cell count. Thereafter, blood was centrifuged for 90 s at 6,000 *g*, and the plasma was collected and stored at −80°C. After measuring the weight and length, the fish was cut open

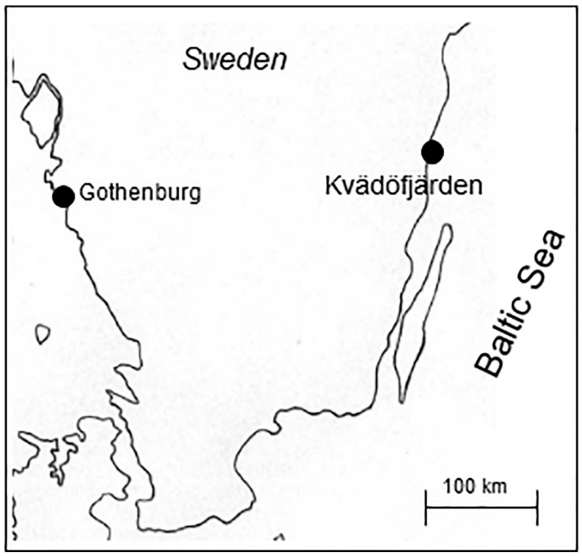


FIGURE 1 | Map indicating the sampling area, Kvädöfjärden, at the Swedish east coast.

and the bile collected with a syringe. The liver was excised and weighed, and one piece was shock-frozen in liquid nitrogen for measurement of enzyme activities. From year 2010 three extra pieces of liver were shock-frozen in liquid nitrogen in separate cryotubes for analyses of additional biomarkers or other analyses (e.g., RNA sequencing) and then were stored in the large liquid nitrogen containing tanks at the Department of Biological and Environmental Sciences, University of Gothenburg. Fish were weighed after dissection for the somatic weight (carcass weight). The fish carcass was frozen at −20°C and then sent for storage to Swedish Environmental Specimen Bank at the Natural History Museum in Stockholm. Ethical permission for the samplings was approved by the local animal committee in Gothenburg, Sweden.

Morphometric Indices and Age

The CF, liver somatic index (LSI) and gonad somatic index (GSI) were calculated as follows: CF = somatic weight (g) × 100)/length^3 (cm), LSI = liver weight (g) × 100/somatic weight (g), GSI = gonad weight (g) × 100/somatic weight (g). The age of the fish was determined by the otolith structures, as previously described (Svedäng et al., 1997).

Blood Parameters

Blood smears on glass slides were stained using May-Grunwald stain for 5 min, followed by Giemsa stain solution for 18 min. Slides were then rinsed in water and left to dry. Glass slides were analyzed microscopically; approximately 2000 cells were counted per glass slide under magnification (×400). The numbers of immature red blood cells, thrombocytes, lymphocytes, and granulocytes were calculated and presented as a percentage of the total blood cells counted. The total amount of white blood cells (WBC)was calculated as the sum of all thrombocytes, lymphocytes, and granulocytes and was presented as a percentage of the total blood cells. The erythrocyte volume fractions (haematocrit) were estimated using haematocrit capillary tubes followed by centrifugation of the blood using a haematocrit capillary centrifuge for 2 min and a microhaematocrit reader. The hemoglobin and glucose concentrations in blood were measured using a cuvette system from Hemocue, with assayed hemoglobin (HemoTrol; Eurotrol) and glucose (GlucoTrol-AQ; EuroTrol) as quality controls.

Plasma Electrolytes

Levels of the ions Na^+, K^+, Cl^-, and Ca^{2+} in the blood plasma were determined with Convergys ISE comfort Electrolyte Analyzer, Cölbe, Germany.

Preparation of Liver Samples

Liver samples were homogenized (glass/Teflon) in four volumes of 0.1 M Na^+/K^+–phosphate buffer (pH 7.4) containing 0.15 M KCl as previously described (Förlin, 1980). The homogenate was centrifuged at 10,000 *g* for 20 min, and the supernatant was re-centrifuged at 105,000 *g* for 60 min. The supernatant (cytosolic fraction) was aliquoted, and the pellet (containing the microsomal enzymes) was re-suspended in one volume of homogenization buffer containing 20% glycerol. All preparation steps were carried out on ice, and the samples were stored at −80°C until analyzed.

Biochemical Analysis

Ethoxyresorufin O deethylase (EROD) activity was measured in the liver microsomal fraction according to the method described by Förlin et al. (1994) using rhodamine as a standard. The reaction mixture contained a 0.1 M sodium phosphate buffer (pH 8.0), 0.5 mM ethoxyresorufin, and 25 to 50 ml of sample in a final volume of 2 ml. The reaction was started with the addition of 10 ml of 10 mM NADPH. The increase in fluorescence was monitored at 530 nm (excitation) and 585 nm (emission).

The activities of glutathione reductase (GR), glutathione S-transferase (GST) and catalase (Cat) activity was measured in the cytosolic fraction as previously described by Stephensen et al. (2002) and Sturve et al. (2005) and references therein. For GR the reaction mixture contained 0.1 mM DTNB (1-Chloro-2,4-dinitrobenzene) and 12 mM EDTA, the reaction was initiated by the addition of 4 mM GSSG (oxidized glutathione) and the absorbance was read at 415 nm. For GST the reaction mixture contained 2 mM CDNB (5,5′-Dithiobis (2-nitrobenzoic acid), 1 mM GSH (glutathione), 0.1 M Na phosphate buffer (pH 7.5). The absorbance was read at 350 nm. For the measurement of the Cat activity the samples were diluted in a 0.08 M KPO_4-buffer pH 6.5, and 0.08 M H_2O_2 was added to initiate the reaction. Reactions were measured at 240 nm.

Vitellogenin (Vtg) levels were measured with a competitive enzyme-linked immunosorbent assay according to Specker and Anderson (1994) as outlined previously (Parkkonen et al., 1999). Plasma samples were diluted (1:10,000 for samples from females) and incubated overnight at 4C with primary antibody against perch Vtg (diluted 1:20,000). The protein concentrations were measured with Folin Phenol reagent (Lowry et al., 1951).

Statistics

Biomarker data from female perch was analyzed with the Mann–Whitney *U*-test using IBM SPSS statistics 25. Time trends were tested with Spearman's correlation analysis.

RNA Sequencing

RNA Extraction

Liver samples from female perch (5 samples from 2010 and 5 samples from 2014) were homogenized in lysis buffer [RNeasy mini plus kit (Qiagen) using a TissueLyser (Qiagen)] at 25 Hz for 6 min. The total RNA was isolated according to the manufacture's instruction using 50% EtOH. The RNA quality was assessed using TapeStation (Agilent Technologies, United States), and the RIN quality values ranged between 9.4 and 10.0.

Library Preparation and Sequencing

The 10 individual samples were first barcoded, and a single Illumina TruSeq stranded library was then generated and sequenced two lanes of Illumina HiSeq2500 with a 2 × 126 bp setup. This resulted in 12.9–14.5 million reads generated per sample.

Filtering and Trimming of Data

The first ten bases of the reads were removed using fastx_trimmer v0.0.14 from the FASTX-toolkit[1]. The adapter and primer sequences were removed using cutadapt v1.3 (Martin, 2011). Bases below a Phred score quality threshold of 20 were then removed with fastq_quality_filter v.0.0.14, which was also from the FASTX-toolkit. An additional four bases in the 5′ end were then removed using fastx_trimmer after the result had been analyzed by fastqc v.0.11.4.

Transcript Assembly and Annotation

The reads from the 10 samples were then assembled independently using Trinity v.2.2.0 (Grabherr et al., 2011) with the digital normalization option and taking the strand specificity into account. The ten assemblies resulted in 53770–64140 transcripts, of which 40187–48272 had an assembly score greater than the optimized threshold determined by transrate v1.0.3 (Smith-Unna et al., 2016) (in **Supplementary Table S1**). To reduce transcript redundancy caused by sequence variability between different individuals, the transcripts with a good assembly score were clustered using CD-HIT (Fu et al., 2012) using a sequence identity cutoff of 97%. In addition, only transcripts represented in at least 2 individuals were kept for further analysis which resulted in 65658 sequences. A BUSCO v3.0.2 (Waterhouse et al., 2017) analysis of this dataset identified 58.8% complete, 11.0% fragmented and 30.2% missing transcripts. Transcripts were annotated using Annocript v.1.1.3[2] and the UniRef90 database (accessed in August 2016) using default settings.

Mapping and Statistical Analysis

The raw reads were mapped to the transcript assembly using bowtie2 v.2.2.2 (Langmead and Salzberg, 2012). Prior to mapping, the bases below a quality threshold of 24 were removed using fastq_quality_filter from the FASTX-toolkit. The number of reads mapped to each transcript was determined using samtools idxstats (Li et al., 2009).

The mRNA expression was analyzed using R version 3.4.2[3] and the package edgeR v. 3.20.9. Low-abundant transcripts with a total count lower than 4 reads across all samples were filtered out. The mRNA expression was then normalized using TMM normalization. Transcripts that were differentially expressed between the 2014 and 2010 populations were identified by fitting a GLM model implemented in edgeR, and the *p*-values were corrected by using the false discovery rate (FDR). Functional enrichment using gene ontology terms (GO-terms[4]) was carried out among all the significantly differentially expressed genes (FDR < 0.05) between 2010 and 2014, using the web service DAVID[5]. The UniProt ID of the best-scoring transcript annotation for each transcript was used as an input to the analysis.

Data Availability

The raw data files were deposited to the NCBI sequence read archive (SRA) with accession number PRJNA529638.

Chemical Analyses of Brominated Dioxin-Like Compounds

Fish muscle (without skin and subcutaneous fat) was used for the chemical analysis. Pooled samples were prepared from the 10 same individuals who are used for RNA sequencing, i.e., 5 samples from 2010 and 5 samples from 2014. In each pool, 5 fish with 20 gram muscle from each fish was used, i.e., 100 gram in total to each pool. The muscle was taken from the middle dorsal muscle layer (TemaNord, 1995). The muscle pieces were sampled from perch retrieved from the Swedish Environmental Specimen Bank, at the Swedish Museum of Natural History.

The fish samples were spiked with a suite of 13C-labeled chlorinated dibenzo-p-dioxin and dibenzofuran internal standards (1 ng each), homogenized with sodium sulfate, extracted with organic solvents, and fractionated according to planarity using activated carbon into three fractions containing, amongst others, (1) the bulk of PCBs, (2) the mono-ortho PCBs, and (3) the non-ortho PCBs and polybrominated and polychlorinated dibenzo-*p*-dioxins and dibenzofurans and dibenzofurans (Haglund et al., 2007). Fractions 2 and 3 contained the planar dioxin-like compounds. Those fractions were further fractionated according to the polarity on Florisil, and three fractions were collected (Norstrom et al., 1988). The two first contained non-polar contaminants, whilst the two latter contained fat and other semi-polar and polar compounds.

The non-polar fractions from Florisil were screened for brominated compounds using comprehensive two-dimensional gas chromatography (GC × GC; Zoex ZX2, Houston, TX, United States) electron-capture negative ion chemical ionization (ECNI) high-resolution mass spectrometry (HRMS; Agilent,

[1]http://hannonlab.cshl.edu/fastx_toolkit/

[2]https://github.com/frankMusacchia/Annocript

[3]www.r-project.org

[4]www.geneontology.org

[5]https://david.ncifcrf.gov/

7250, St. Clara, CA, United States). Extracted ion chromatograms (EICs) of the bromide ions were used to trace brominated compounds. The ECNI and electron ionization (EI) spectra were collected for all brominated compounds. Details on the GC × GC-HRMS analyses are given **Table 1**. An attempt was made to identify as many brominated compounds as possible using a combination of EI-MS library searches (NIST 17 libary; NIST, Gaithersburg, MA, United States) and manual spectra interpretation.

A semi-quantification was performed using the EI data and the most closely matched internal standard (same degree of halogenation) assuming the same molar response factor, and using the sum of all ions detected of each analyte and internal standard. The result of such a semi-quantification is assumed to be within a factor 2–3 of the correct value. The uncertainty in the ratios of the 2010 and 2014 data should, however, be much less. It should be similar to that of quantitative dioxin determinations, i.e., ca. 20% uncertainty (expanded uncertainty, $k = 2$).

RESULTS

Biomarker Time Trends

The time trend for liver EROD activity in perch at Kvädöfjärden has been reported earlier (Sandström et al., 2005; Hansson et al., 2006a; Hanson et al., 2009). In **Figure 2**, it can be seen that there is still a significant increasing time trend for the longer period from 1988 to 2017, despite a decreasing trend in EROD activity between the years 2009 and 2014. For GSI, the negative time trend reported before is also still significant for the longer time period, regardless of more stable GSI levels in the last 15 years (**Figure 3**). Other biomarker time trends in perch include an increase in the liver catalase activity since 2012 (**Figure 4**). In addition, it has also been indicated that plasma calcium ion content and blood glucose levels show clear increasing time trends in the perch at Kvädöfjärden (Larsson et al., 2016, in Swedish).

TABLE 1 | Settings used for the comprehensive analysis of complex samples using two-dimensional gas chromatography with high-resolution mass spectometry.

Instrument:	Agilent 7250 GC-QTOF
GC × GC modulator:	Zoex ZX2
1st GC column:	30 m, 0.25 mm, 0.25 μm film, Agilent DB5ms-UI
Modulation loop:	2 m, 0.25 mm, uncoated, non-polar deactivated
2nd GC column:	1.3 m, 0.25 mm, 0.10 μm film, Quadrex 70% phenyl-siloxane
GC carrier gas:	Helium 1.0 or 1.4 ml/min, constant flow mode
Inlet temperature:	300°C
Oven temperature:	90°C (1 min) – 5°C/min or 4°C/min, 300°C (2.5 min)
Transfer line temp:	300°C
Ion source temperature:	EI 250°C, CI 150°C
Modulator time/temp:	3 s, 0.35 s hot pulse, +100°C bias (cold jet kept at −90°C)
MS data range/rate:	m/z 45–430, 50 Hz
EI, electron energy:	70 eV, 1 mA filament current
CI reaction gas:	Methane (45% flow)

Comparison Between 2010 and 2014

Morphometric Indices

Fish that were collected in the years 2010 and 2014 were selected, since they displayed large differences in physiological parameters and biomarker levels. In total, 52 fish were included; 27 were sampled in 2010 and 25 were sampled in 2014. The sampled fish from the two different years were of the same age (3.6 and 3.5 years, respectively), but the fish sampled in 2014 were slightly longer and had significantly higher weight resulting in a significantly higher CF compared to the fish sampled in 2010. These fish also had significantly larger livers and liver somatic indices. However, fish sampled in 2014 had lower gonad weights, which led to significantly lower gonadal somatic indices. Morphometric indices are displayed in **Table 2**.

Blood Parameters

The amount of red blood cells, the haematocrit, was higher in fish sampled in 2014 compared to 2010, but this did not reflect in the hemoglobin content, which remained unchanged. Glucose levels were significantly lower in 2014, while blood lactate levels were higher, even though the difference was not statistically significant (**Table 3**).

The concentrations of all four plasma ions that were analyzed (Na^+, K^+, Ca^{2+}, and Cl^-) were higher in the fish sampled in 2014 compared to those in 2010, even though the difference was only significant for Ca^{2+} an Cl^- (**Table 3**).

The blood cell count revealed that the amount of all cells analyzed, except immature red blood cells (iRBC), were lower in fish sampled in 2014 compared to those sampled in 2010. The lower levels in the amount of the total WBC, lymphocytes, granulocytes and thrombocytes were significant for all cell types. However, the amount of iRBC was significantly higher in 2014, which corresponds to the increase in haematocrit (**Table 3**).

Biochemical Markers

When comparing fish sampled in 2014 with fish sampled in 2010, the results show lower phase 1 detoxification capacity and oestrogenic responses and higher oxidative stress-related responses. Both EROD activities and the vitellogenin levels were significantly lower in 2014 compared to those in 2010. However, the activities of the antioxidant enzymes that were analyzed (GR, GST, and CAT) were all higher in 2014 compared to 2010, with GR and CAT significantly higher (**Table 4**).

Transcriptome Analysis for 2010 and 2014

mRNA Expression Studies

Global mRNA expression studies using RNA sequencing were performed to identify additional temporal variations in biological parameters by using perch collected in 2010 and 2014. RNA sequencing analysis identified 65538 genes, of which 4803 had a significant difference in mRNA expression levels (FDR < 0.05). Of these, 2769 genes had a higher mRNA expression level and 2034 genes had a lower mRNA expression level in 2014 compared to 2010.

The genes coding for proteins with biological functions associated with the innate immune system, such as the

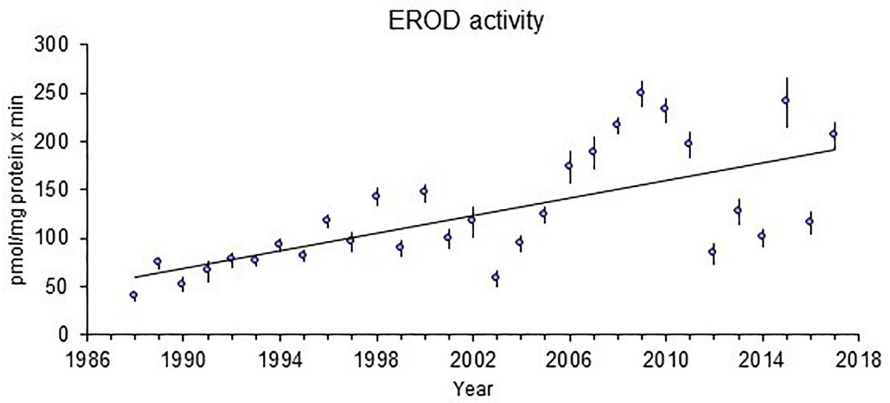


FIGURE 2 | EROD activity in the liver (pmol/mg protein x min) in female perch collected in the reference site Kvädöfjärden. The points indicate mean values ± standard error of 20–27 fish in each point, and the straight line represents a significant time trend ($P < 0.01$).

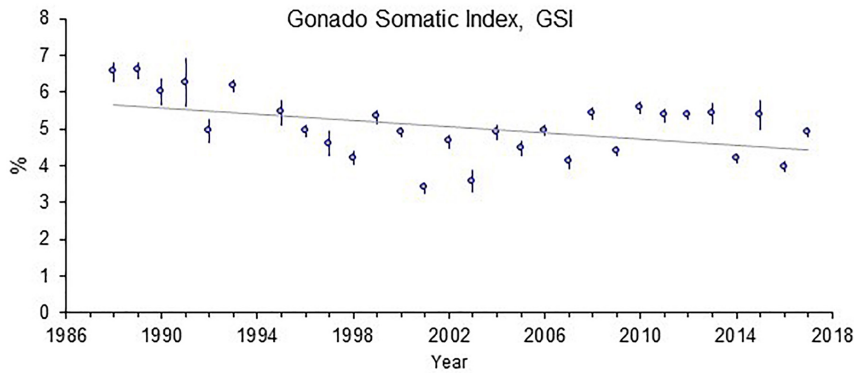


FIGURE 3 | Gonado somatic index, GSI (%) in female perch collected in the reference site Kvädöfjärden. The points indicate mean values ± standard error of 20–27 fish in each point, and the straight line represents a significant time trend ($P < 0.05$).

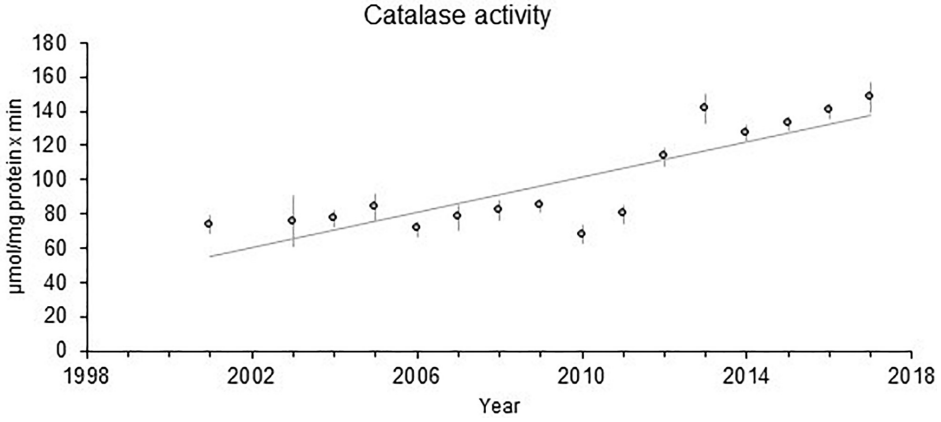


FIGURE 4 | Catalase activity (µmol/mg prot x min) in female perch collected in the reference site Kvädöfjärden. The points indicate mean values ± standard error of 20–27 fish in each point, and the straight line represents a significant time trend ($P < 0.01$).

toll-like receptor, complement components, serum amyloid A and lysozyme, had a higher mRNA expression level in fish collected in 2014 (**Supplementary Table S2**). Proteins encoded by genes that are involved in egg formation, such as vitellogenin and zona pellucida, had a higher mRNA expression level in 2010. In addition, several lectins had

TABLE 2 | Morphometric indices in female perch (*Perca fluviatilis*) sampled in Kvädöfjärden in 2010 and 2014[a].

Morphometric indices		
Year	**2010**	**2014**
Age (year)	3.6 ± 0.1	3.5 ± 0.2
Length (mm)	261.4 ± 3.7	269.7 ± 4.0
Weight (gram)	219.1 ± 10.4	264.2 ± 10.3*
Somatic weight (gram)	207.5 ± 9.9	253.5 ± 9.8*
Gonad weight (gram)	11.6 ± 0.6	10.5 ± 0.6
Liver weight (gram)	3.1 ± 0.2	4.3 ± 0.1*
Condition factor (CF)	1.20 ± 0.02	1.31 ± 0.02*
Gonad somatic index (GSI)	5.6 ± 0.2	4.2 ± 0.1*
Liver somatic index (LSI)	1.51 ± 0.02	1.75 ± 0.02*

[a]*Assessment performed on 27 (2010) and 25 (2014) individuals per site and shown as the mean ± standard error. * indicates significant differences between 2010 and 2014, $p < 0.05$.*

TABLE 3 | Blood parameters in female perch (*Perca fluviatilis*) sampled in Kvädöfjärden in 2010 and 2014[a].

Blood parameters		
	2010	**2014**
Haematocrit (%)	26.8 ± 0.4	28.6 ± 0.3*
Hemoglobin (g/L blood)	63.9 ± 1.2	62.8 ± 1.6
Glucose (nmol/L)	6.49 ± 0.26	5.24 ± 0.16*
Lactate (mg/100 ml plasma)	11.7 ± 1.5	15.8 ± 3.3
WBC (%)[b]	6.45 ± 0.22	4.84 ± 0.17*
Lymphocytes (%)[b]	3.09 ± 0.12	2.43 ± 0.09*
Granulocytes (%)[b]	1.04 ± 0.07	0.82 ± 0.04*
Thrombocytes (%)[b]	2.32 ± 0.11	1.60 ± 0.10*
iRBC (%)[b]	0.71 ± 0.04	0.84 ± 0.03*
K^+ (mmol/L blood)	3.49 ± 0.09	3.66 ± 0.08
Na^+ (mmol/L blood)	153.2 ± 0.8	155.4 ± 1.5
Ca^{2+} (mmol/L blood)	0.59 ± 0.03	0.88 ± 0.05*
Cl^- (mmol/L blood)	117.1 ± 1.1	122.7 ± 1.5*

[a]*Assessment performed on 27 (2010) and 25 (2014) individuals per site and shown as the mean ± standard error. * indicates significant differences between 2010 and 2014, $p < 0.05$.* [b]*Frequency (%) of WBC (white blood cells), lymphocytes, granulocytes, thrombocytes and iRBC (immature red blood cells) of the total number of blood cells counted.*

different mRNA expression levels in 2010 compared to 2014 (**Supplementary Table S2**).

The data seem to indicate differently expressed genes associated with oxidative stress, such as PPAR-alpha (peroxisome proliferator-activated receptor alpha) and G6PD (glucose-6phosphate dehydrogenase), and possibly indicate the antioxidant protein peroxiredoxin-6 with higher mRNA levels in 2014 than in 2010. The data also show markedly higher expression of mRNA levels for some membrane pumps, especially MATE1 (multidrug and toxin extrusion protein 1), in the 2014 collected fish (**Supplementary Table S2**).

The data show higher mRNA expression of genes coding for proteins involved in detoxification, especially of CYP 2, such as CYP 2K1, and phase II proteins, such as UPGT (glucuronosyltransferase), in the fish collected in 2010. In addition, the mRNA expression of genes coding for proteins that are involved in iron homeostasis, such as hepcidin, haptoglobin, and ferritin, showed different patterns in 2010 and 2014. For a ranked list of the top genes according to mRNA expression levels, see the **Supplementary Data** (**Supplementary Table S2**).

TABLE 4 | Activities and levels of biochemical markers in female perch (*Perca fluviatilis*) sampled in Kvädöfjärden in 2010 and 2014[a].

Biochemical markers		
	2010	**2014**
EROD [pmol/(mg protein × min)]	231.9 ± 12.7	100.8 ± 9.2*
VTG (mg/ml plasma)	1043 ± 86	477 ± 76*
GR [nmol/(mg protein × min)]	7.9 ± 0.2	10.6 ± 0.2*
GST [μmol/(mg protein × min)]	0.097 ± 0.003	0.100 ± 0.003
CAT [μmol/(mg protein × min)]	68.1 ± 10.2	127.5 ± 4.6*

[a]*Assessment performed on 27 and 25 individuals per site in 2010 and 2014, respectively, except for VTG, where 15 and 12 individuals were used per site in 2010 and 2014, respectively. Data shown as the mean ± standard error, and * indicates significant differences between 2010 and 2014, $p < 0.05$.*

Principal component analysis (PCA) using all of the sequenced transcripts identified differences in the mRNA expression during the separate time points, as the individual perch collected were clearly divided into two groups (**Figure 5**).

Gene Ontology Enrichment Analysis

Genes with differential mRNA expressions were analyzed for the overrepresentation of GO terms to identify differences in biological pathways and genes with similar biological functions (**Table 5**). When using the GO enrichment analysis tool (DAVID) against the human database, several groups of biological processes were found ($p < 0.001$). In total, 124 regulated biological processes were identified, such as the complement activity, the alternative pathway (GO:0006957), iron ion homeostasis (GO:0055072), and response to estradiol (GO:0032355) and cholesterol biosynthetic process (GO:0006695).

Analyses of Dioxin-Like Brominated Compounds

The non-targeted screening for brominated compounds in perch muscle revealed four compound classes, viz. brominated dibenzo-p-dioxins, carbazoles, indoles and methyl-indoles. The total concentrations (pg/g fresh weight) are given in **Table 6** and the individual concentrations (pg/g fresh weight) are given in **Supplementary Table S3**.

Five mono-, di-, and tri-brominated dioxins were identified in the chemical analyses. Three were found at higher concentrations in 2014, while two were higher in 2010. The total concentration of brominated dioxins was 1.8 times higher in 2010 (**Table 6**).

For the carbazoles, three dibrominated carbazoles were identified. One was only detected in 2010 and two were found at slightly higher concentrations in 2014. The total

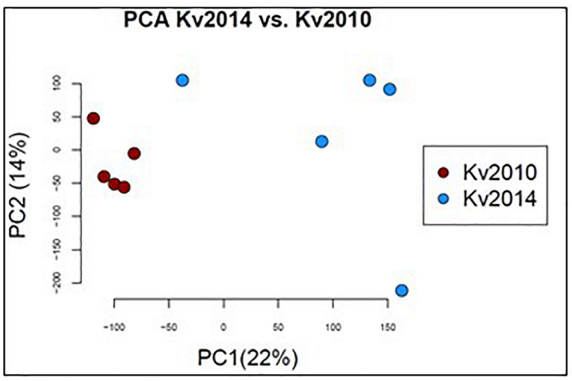


FIGURE 5 | Principal component analysis (PCA) performed on the 5 individuals from 2010 and the 5 individuals from 2014 using all 65538 transcripts. Individuals were separated between the different years.

TABLE 5 | Biological processes found in gene ontology (GO) terms enrichment analysis[a].

GO identifier	GO term: biological process	*p*-value
GO:0006957	complement activation, alternative pathway	1,70E-24
GO:0055072	iron ion homeostasis	6,88E-11
GO:0006954	inflammatory response	1,75E-08
GO:0032355	response to oestradiol	5,59E-08
GO:0032455	nerve growth factor processing	8,47E-08
GO:0035094	response to nicotine	1,11E-06
GO:0034379	very-low-density lipoprotein particle assembly	2,86E-06
GO:0006695	cholesterol biosynthetic process	7,89E-06

[a]*Functional annotation assessed by GO term enrichment analysis using all significantly differentially expressed genes (FDR < 0.05). The results are shown for the GO term biological process from the hierarchic level 4.*

TABLE 6 | Summation of the concentrations (pg/g fresh weight) of different chemical groups in female perch (*Perca fluviatilis*) sampled in Kvädöfjärden in 2014 and 2010 and the ratio between the two years.

	Sum 2014	Sum 2010	Ratio 2014/2010	Ratio 2010/2014
Brominated dioxins	2.8	4.9		1.8
Brominted carbazoles	3.2	47.4		14.8
Brominated indoles	752.7	126.2	6.0	
Brominated methylindoles	173.3	68.0	2.6	

concentration of the three dibrominated carbazoles was 14.8 times higher in 2010 perch muscle compared to that in 2014 (**Table 6**).

The chemical analyses also identified nine brominated indoles and four brominated methylindoles in the perch muscle. For the individual concentrations of indoles, all except one were higher in 2014 than in 2010, and the total concentration of brominated indoles was 6 times higher in 2014 than in 2010 (**Table 6**). For the four brominated methylindole concentrations, two were higher in 2014 and two were slightly higher in 2010. The total concentration of brominated methylindoles was 2.6 times higher in 2014 compared to that in 2010 (**Table 6**). Generally, the levels of both brominated indoles and methylindoles were higher in the 2014 perch muscle compared to 2010.

DISCUSSION

Biomarker Time Trends

It was published that studies of the coastal fish monitoring in the reference area of Kvädöfjärden show that the development of fish health status is not satisfactory (Balk et al., 1996; Sandström et al., 2005; Hansson et al., 2006a; Hanson et al., 2009; Larsson et al., 2011). The previously reported time trends for the GSI and EROD activity in the perch from Kvädöfjärden are still significant for the whole period from 1988 to 2017. For the EROD activity, the marked increase seen from year 2002 to 2009 was followed by a marked decrease between the year 2009 and 2014. After that, the EROD activity had varied between a relatively high to low level. For the GSI, the decreasing trend is still significant, mainly because of large relative gonad sizes during the first ten years of the time series, whereas the data for the last 15 years indicates more stable GSI levels.

The symptoms that were observed in the perch in the reference areas were similar to some of the known effects of certain organic pollutants and to the effects in fish that are exposed to complex mixtures of contaminants, e.g., fish living in areas close to industry effluent point sources or close to other large human activities (Andersson et al., 1988; Larsson et al., 2003; Sturve et al., 2005, 2014; Hansson et al., 2006b, 2014). However, the results of the monitoring of environmental pollutants in the reference areas are not in line with the apparent deterioration of health in coastal fish (Bignert et al., 2017). In Kvädöfjärden, the studies show that most of the organic pollutants and metals that were analyzed in perch were reduced or unchanged (Bignert et al., 2017). However, for some pollutants, e.g., mercury and DDE, in the perch's muscle, increases can be seen for a period of approximately 10 years from 2004 and onward. Increased or unchanged levels of a number of classic environmental pollutants indicate that there was a diffuse supply of these in Kvädöfjärden or a release from sediment. The latter could be supported by major changes in the bottom-dwelling community, especially with an increased occurrence of an invasive organism, the Polychaeta worm Marenzelleria (Michalek and Werner, 2012), whose behavior in sediments is suspected to cause the release of old chemicals from the sediment (Hanson et al., 2016, in Swedish; Hanson et al., manuscript). After 2011, the presence of Marenzelleria decreased again. The period of marked increase of the EROD activity seems to coincide with the occurrence of the digging invasive worm Marenzelleria (Hanson et al., 2016, in Swedish).

Studies show that a number of perfluorinated substances, hydroxylated brominated substances and siloxanes increased in Baltic Sea herring (Faxneld et al., 2014, 2016). Even in eggs from common guillemot from Stora Karlsö, most perfluorinated substances (PFAS) increased (Bignert et al., 2017). The number of chemicals is increasing substantially in our society, and most of

them are not monitored today. Exposure to a mixture of different known and unknown chemicals can, therefore, be an important part of the explanation for the observed health effects of the investigated coastal fish. In addition to the chemical pollution, various environmental factors, such as water temperature, food availability, salinity (Förlin et al., 2014, in Swedish; Hanson et al., 2016 in Swedish) and more, for example, algae blooming, can possibly affect fish biochemistry and physiology over time.

Comparison of 2010 and 2014

Detoxification Enzymes

Yearly monitoring since 1988 reveals, as expected, variations between years. Since it was of interest to compare the results from fish caught when the EROD activity was high with fish from years when the EROD was low, fish from 2010 to 2014 were selected for comparison and for further analyses.

The EROD activity showed significantly different levels in the female perch between the 2 years, with a 2.3 times higher activity in 2010. In our mRNA expression studies, different CYP1 (A, B, and C) genes could be identified, but no significant differential mRNA expression between the two time points could be seen. However, all identified CYP1 genes showed slightly higher mRNA expression levels in 2010 when compared to 2014, which could well be in line with the higher EROD activity level in 2010.

In contrast to the CYP1 genes, a few other phase I and phase II genes showed differential mRNA expressions between 2010 and 2014. The mRNA expression levels for genes coding for CYP2 proteins were 2.7 to 4.4 times higher in perch from the sampling in 2010 than in 2014. CYP2 proteins are important in the detoxification of a variety of endogenous and exogenous compounds. Only two of the expressed CYP 2 genes were identified to any certainty: CYP2K1 and CYP2J2. In fish species, CYP2K1 has been indicated to be involved in lipid metabolism and aflatoxin metabolism, and CYP2J2 is involved in the metabolism of arachidonic acid (Schlenk et al., 2008; Goldstein et al., 2010). For the phase II detoxification hepatic enzyme GST, the mRNA expression analysis showed three different GST genes that were significantly highly expressed in the 2010 fish. GST is a large family of proteins that catalyze the conjugation of reduced glutathione to a wide range of compounds that result in detoxification (Nebert and Vasiliou, 2004; Schlenk et al., 2008). The activity of this phase II enzyme did not show any significant difference between the 2 years. However, our catalytic GST assay is not specific to different GST proteins and may, therefore, not be able to detect the differences that are indicated for the individual GST mRNA levels. The RNA sequencing also identified genes for other detoxification proteins, including significantly higher mRNA expression of UDP glucuronosyltransferase (UDPGT) in 2010. UDPGT enzymes catalyze the conjugation of glucuronic acid to many hydrophobic compounds, thereby facilitating excretion of toxic compounds through bile and urine (Schlenk et al., 2008; Oda et al., 2015). The differences in mRNA expression of the phase I and II genes that are involved in the detoxification metabolism seem to indicate that fish from 2010 express slightly higher relative enzymatic detoxification capacity than fish from 2014. These differences may indicate different scenarios with respect to exposure to potentially toxic chemicals, but the differences are regarded to be relatively small.

Brominated Dioxin-Like Compounds

In the non-targeted chemical analysis of muscle samples from perch from 2010 and perch from 2014, the focus was to identify brominated dioxin-like compounds. No major differences in the levels of brominated dioxins were found between 2010 and 2014. In addition to brominated dioxins, brominated indoles, brominated methylindoles and brominated carbazoles could be found. Both halogenated carbazoles and indoles have been indicated to be aryl hydrocarbon receptor agonists and, thus, to have the potential to upregulate CYP1A and EROD activity (DeGroot et al., 2015; Mumbo et al., 2015). In the present study, the concentrations of the brominated indoles were generally higher in the 2014 collected fish than in the 2010 fish. This difference is not in line with the difference in EROD activity, with higher activities in 2010 than in 2014. This discrepancy may indicate that the brominated indoles are not causing, or are not the major causation for, the difference in EROD activities between 2010 and 2014.

Three different brominated carbazoles were detected. Two of them were slightly higher in 2014 than in 2010. Many halogenated carbazoles are persistent, express dioxin-like properties (Mumbo et al., 2015) and are possibly more potent inducers of the CYP1A/EROD system than are the indoles. The potency of the detected brominated carbazoles to induce the CYP1A/EROD system in the perch from Kvädöfjärden is not known, and it is not known whether the semi-quantified level found in the perch from Kvädöfjärden is high enough to cause effects in the CYP1A/EROD system. It can, therefore, not be ruled out whether brominated carbazoles play any role in causing differences in EROD activities between the 2 years.

Of the brominated compounds, the brominated dioxins, the brominated indole and methylindole groups are probably mainly of natural origin (Gribble, 1999; Haglund et al., 2010). The sources for these compounds can be macro- or micro-algae. Recently, brominated indoles were tentatively identified in red and brown algae from the Baltic Sea (Björklund, 2018). For the brominated carbazoles, the origins are more unclear with the anthropogenic origin, including sources such as the production of dyes, aluminum industry, rubber, coal combustion and more (Guo et al., 2014; Fromme et al., 2018). However, it has been suggested that carbazoles also have natural origins and seem to dominate in sediment from the great lakes (Barbosa et al., 2014; Guo et al., 2017). Since Kvädöfjärden is located away from large anthropogenic activities, it is likely that the major contribution of the carbazoles are natural sources in that area. In addition, halogenated carbazoles were recently tentatively identified in red and brown algae from the Baltic (Björklund, 2018).

It would be of great interest to perform quantitative measurements of the brominated carbazoles and indoles retrospectively in the perch from Kvädöfjärden. This would be a very important task for the future, as it is likely that historic release in the environment of these groups of halogenated compounds are buried in sediments (Guo et al., 2014, 2017). In addition, it is likely that there is an on-going release of these

compounds into the environment from current anthropogenic and/or natural activities (Guo et al., 2014, 2017; Fromme et al., 2018; Wu et al., 2018).

Membrane Pumps and Detoxification

The RNA sequencing also identified that genes coding for proteins in membrane pumps had a markedly higher mRNA expression in 2014 than in 2010. This was especially the case for the membrane pump that denoted multidrug and toxin extrusion protein (MATE), which was expressed more than one hundred times higher in the 2014 perch compared to the 2010 perch. Genes coding for MATE proteins have been characterized in zebrafish (Loncar et al., 2016). Genes for other drug membrane transporters also had a markedly higher mRNA expression in 2014 fish but not to the same extent as for the MATE gene. The membrane pumps/transporters mediate the excretion of drugs and other organic compounds into bile and urine and thus have an important function in the disposition of toxic compounds (Ferreira et al., 2014). The higher mRNA expression of these membrane transporters could indicate that the fish must handle the transportation of chemicals (endogenous or exogenous) out of the cells (and organism) to a larger degree in 2014 than 2010.

In this context, it is very interesting to the present study that the higher content of brominated compounds in 2014 may be reflected in a higher capacity to transport and excrete these brominated compounds in the 2014 fish than in the 2010 fish. We do not know if the indicated membrane pumps can handle the detected brominated compounds. However, it is of particular interest that the MATE transporter is known to be important as the flavonoid transporter (Lee et al., 2014). Plants are rich in flavonoids. This large group of compounds, many of which have probiotic properties, have been studied extensively for their role in food and health (Moon et al., 2006; Viskupciova et al., 2008). Recently, it has been shown that microalga, including cyanobacteria, contain flavonoids (Goiris et al., 2014; Singh et al., 2017). In the Baltic Sea, the blooming of microalga frequently occurs. Although we have not analyzed any polyphenolic flavonoids in our fish, we speculate that the high mRNA expression of the MATE gene may suggest exposure to flavonoids and thus more profound outbreaks of microalga/cynobacterial blooming close to Kvädöfjärden in 2014 than in 2010.

In addition, the mRNA expression level of the gene coding for the enzyme catechol O-methyltransferase domain-containing protein 1 (COMT-D1) may indicate higher exposure to flavonoids and thus greater algal blooming in 2014 than in 2010. This gene was indicated to have a ten times higher mRNA expression level in the 2014 fish compared to the 2010 fish. Catechol O-methyltransferase (COMT) is a family of enzymes that degrades catecholamines by methylation (such as dopamine and adrenalin) and catechol oestrogens, as well as various drugs and substances having catechol structure. It is known that flavonoids can also be methylated by COMT enzymes (Zhu et al., 1994; Lemanska et al., 2004). This finding strengthens the contention that the 2014 perch may well have been exposed to microalga blooms that produced flavonoids to a higher degree than did the perch that were sampled in 2010.

Algal blooming frequently occurs in the Baltic Sea. HELCOM provides yearly assessments of cyanobacterial blooms in the Baltic Sea in fact sheets (Öberg, 2017). The fact sheets provide an overall assessment of the blooming and a comparison between years in the entire Baltic Sea. They also provide an annual map to indicate the spatial distribution of the blooms. In the fact sheets for 2010, the overall assessment for 2010 blooms "can be considered to be normal" (Hansson and Öberg, 2010) and for 2014, the bloom "can be considered to be slightly above normal" (Öberg, 2014). In addition, the spatial distribution of the blooms show that in 2010, most of the blooms occurred in the southern part of the Baltic Proper, whereas in 2014, the bloom occurred more in the northern part of the Baltic Proper, which is much closer to the coastal reference site Kvädöfjärden. The information in the fact sheets, therefore, support the assertion that fish in 2014 were likely exposed to algal blooming to a higher degree than the fish in 2010 in the reference area of Kvädöfjärden.

Gonad Development and Vitellogenin

GSI was among the parameters that showed differences between the two time points. In the female perch of the age and size that were used in the present study, the gonads were in a state of rapid growth and development at the time when the fish were netted and sampled in the last week of September. In addition, plasma levels of vitellogenin showed differences between the 2 years. Vitellogenin is produced in the liver and is transported to the gonads, where they are incorporated into the eggs as yolk proteins. The GSI was larger and the plasma content of vitellogenin was higher in the 2010 fish. The same pattern with higher mRNA expression in 2010 was also seen for the genes coding for the protein vitellogenin and for the protein zona pellucida, which were also involved in the egg formation. These differences probably reflect that the 2010 fish were in a slightly more advanced stage in the development of eggs in the gonads (i.e., slightly ahead in growth and development of the gonads).

Whether the detected brominated chemicals may play a role in the difference in gonadal development between the 2 years is unknown. Whether these compounds and flavonoids, many of which have profound bioactive properties, affect the well-being of the perch in Kvädöfjärden would be a very important and interesting research topic in the future. Developmental toxicity by flavonoids in zebrafish, including, for example, the inhibition of aromatase and behavioral disturbances, has been indicated in earlier studies (Brugel et al., 2016). Aromatase converts androgens to oestrogens. Since oestrogens regulate vitellogenin synthesis, the lower plasma vitellogenin levels and lower mRNA expression of vitellogenin in 2014 fish compared to 2010 fish may be the result of aromatase inhibition by flavonoids. In the current state, this is only speculation but is an important hypothesis that is well worth pursuing to discover how or if naturally occurring algae blooms that produce more or less bioactive and toxic compounds may affect reproduction physiology in feral fish.

Oxidative Stress

Other differences between the 2 years seem to indicate that there is oxidative stress in fish in 2014 compared to fish that were sampled in 2010. The enzymatic activities of

catalase and glutathione reductase were both higher in 2014. Catalase metabolizes hydrogen peroxide to water and oxygen, while glutathione reductase maintains a correct intracellular ratio of reduced and oxidized glutathione. Glutathione is an antioxidant that is oxidized in antioxidant reactions (Halliwell and Gutteridge, 1999). However, these changes were not reflected in changes in the mRNA expression level; on the contrary, the gene coding for catalase in the perch was slightly higher in the 2010 fish. The gene for another important antioxidant enzyme, peroxiredoxin 6, was indicated to have a markedly higher expression level in 2014 than in 2010. Peroxiredoxin enzymes have the same function as catalase, which is to decompose hydrogen peroxide into water and oxygen. Possibly coupled to the indicated higher mRNA expression in of the peroxiredoxin is the higher mRNA expression of thioredoxin-like protein 1, which was also observed in the 2014 samples. This antioxidant can be related to the function of peroxiredoxin by reducing oxidized peroxiredoxin back to its reduced and active form in the reaction with hydrogen peroxide. A previous study by Sheader et al. (2006) show that both perodixiredoxins and thioredoxins are upregulated by cadmium generated oxidative stress. Taken together, these results suggest that the fish in 2014 had higher levels of intracellular hydrogen peroxide, which possibly derived from pro-oxidant-induced ROS generation. Previous studies show that cyanobacterial blooms lead to effects on the antioxidant defense system in fish and leads to oxidation of macromolecules such as lipids (reviewed in Paskerova et al., 2012).

A higher mRNA expression of the gene coding for the enzyme glucose-6-phosphate dehydrogenase (G6PDH) in the pentose phosphate pathway was also seen in the 2014 fish. G6PDH activity has previously been linked to metal exposure and oxidative stress (Grasset et al., 2016). This enzyme is important in the antioxidant system in organisms because the level of NADPH, which is required to maintain the level of intracellular antioxidant glutathione, is required. The higher mRNA expression of G6PDH and the other above-mentioned mRNA expressions indicate that perch from 2014 show a higher exposure of oxidants than those from 2010. The cause for the apparent higher mRNA expression of genes that are involved in oxidative stress is unknown, but the higher mRNA expression may have strengthened the contention of larger exposure to chemicals that originated from algal blooming in 2014 than 2010.

Several antioxidant genes are regulated by the nuclear transcription factor NRF2, which controls over 200 genes that are involved in the antioxidant system. The NRF2 protein is anchored to the cytoskeleton through KEAP1 and is released and translocated into the nucleus during oxidative stress. Our results show no differences in NRF2 mRNA expression between the 2 years, which suggests mild oxidative stress where the translocation of NRF2 present in the cytosol is enough for antioxidant gene control (Kobayashi and Yamamoto, 2005).

Immunology

In the present study, both the innate (non-specific) and the adaptive (specific) immune systems were differentially regulated. Differences in the white blood cell counts seem to indicate a more activated adaptive immune system in 2010. The higher levels of total WBC, lymphocytes, granulocytes and thrombocytes were significant for all cell types. The higher amount of lymphocytes in perch captured in 2010 also coincides with a higher level of mRNA coding for immunoglobulin chains (Ig heavy chain and Ig kappa chain V, V-I, V-III, and V-IV region, Ig mu chain), which possibly indicates a higher amount of antibody-producing B cells within the lymphocytes population.

A differential mRNA expression of genes coding for proteins that are known to be involved in the innate immune system were noted. For example, serum amyloid A, toll-like receptor 5 lysozyme and most of the complement system components (C1q-like protein, C3, C4-A, C5, C7 C8, C9 and factor H-related protein), had higher mRNA expression levels in fish collected in 2014. All of these proteins are involved in bacterial/viral recognition and elimination, thus taking part in the first line of defense against invading pathogens in vertebrates, including fish (Magnadóttir, 2006; Uribe et al., 2011).

Lectins are a group of proteins with the ability to identify and bind to a diverse set of carbohydrate structures and have multiple functions when expressed in animals, plants and microorganisms. In vertebrates and invertebrates, lectins are involved in cell adhesion and carbohydrate recognition in the circulatory system, as well as within cells. Lectins also have the ability to recognize carbohydrate residues on invading microorganisms, which leads to opsonization, agglutination and the activation of the complement system, thus taking part in the immune defense (Vasta et al., 2011). Several of the lectins that are identified in fish are known to be involved in the innate immune system (Russell and Lumsden, 2005; Vasta et al., 2011). Some fish lectins are also known to be induced after stimuli by different infectious agents (Bayne et al., 2001). Lectins such as collectin-12, the C-type lectin, fucolectin-5, galectin, ladderlectin, galactose-specific lectin nattectin and intelectin are found to be differently expressed in perch, some with a higher mRNA expression in 2010 and others with a higher mRNA expression in 2014.

Genes that are involved in iron homeostasis were differentially expressed in the data set, mostly with a higher mRNA expression in perch collected in 2014. Hepcidin is released upon iron overload (Hu et al., 2007), thereby initiating an increase in iron storage, which was expressed more than 500 times higher in 2014 (logFC at 9.1) than 2010. Other genes with a higher mRNA expression in perch collected in 2014 were haptoglobin and haemopexin binding to free hemoglobin and haeme, respectively (Wassell, 2000), in addition to serotransferrin, mitoferrin and the transferrin receptor protein, which were all involved in iron transport and uptake. Earlier studies in fish, such as turbot, channel catfish and sea bass (*Dicentrarchus labrax*), has shown that genes coding for proteins involved in iron transport and storage are expressed to a higher extent after bacterial infection (Rodrigues et al., 2006; Peatman et al., 2007; Millan et al., 2011). This would then indicate that iron homeostasis could have a suppressive effect on invading bacteria and hamper their growth and survival due to a low amount of available free iron.

The difference in the amount of WBC and the mRNA expression of genes that are involved in the innate immune system between 2010 and 2014 could be due to different types of bacterial or viral infections in the collected perch. However,

abiotic factors such as temperature, oxygen, salinity and pH can have stimulating or suppressive effects on the immune system (Bowden, 2008). Seasonal variation in the respiratory burst of phagocytes and the complement system activity has been noted in the common carp (*Cyprinus carpio*) (Buchtíková et al., 2011), as well as immunosuppressive effects of steroid hormones in male common carp (Watanuki et al., 2002). Feed additives may also modulate the immune system in fish (Akhter et al., 2015). Chemical pollutants that are found in the environment are also known to affect the immune system (Reynaud and Deschaux, 2006). In yellow perch (*Perca flavescens*) collected in areas that are polluted with heavy metals, PCBs and PAHs, different lysozyme and ceruloplasmin activity were found compared to that of the reference sites (Dautremepuits et al., 2008). In addition, naturally occurring toxins can have an effect on the immune system. Many secondary metabolites, such as the brominated indoles produced by marine organisms, have multiple and interesting bioactive properties, such as inhibiting inflammation, antibacterial and antiviral properties, and antioxidants (Gribble, 2015). In this context, it is interesting that the indole derivative can combat bacterial infection in marine organisms (Yang et al., 2017). Whether the brominated indoles may have this function in the perch from Kvädöfjärden requires further studies to elucidate. However, it is noted that in the fish from 2014, there was a lower number of WBC active when the levels of brominated indoles are higher than those of the fish from 2010, when the content of brominated indoles were lower.

CONCLUSION

In earlier studies, it was suggested that the time trends in certain biomarker responses in perch from the Kvädöfjärden reference site, studied for more than 30 years, can possibly be the result of changes in the baseline pollution. The RNA sequencing study identified approximately 4800 genes that had different mRNA expression levelswhen comparing perch collected from 2 years, 2010 and 2014, selected as they showed relatively large differences in biomarker levels. The results showed that these differentially expressed genes were involved in biological processes such as complement activation, iron ion homeostasis and cholesterol biosynthetic process. The differences in immune system parameters and responses to the exposure of toxic substances could be verified in two different biological levels (mRNA and protein) in perch collected in 2010 and 2014. The perch collected in 2014 showed higher concentrations of bioactive brominated compounds than did perch from 2010. Macro- and microalgae in the Baltic Sea where algal blooming frequently occurs, likely produce these chemicals. The transcriptomic analyses with the indicated higher mRNA expression of genes that are especially related to membrane transporters and methylation of catechols and the differential mRNA expression between the 2 years in genes related to immune and oxidative stress parameters suggest that attention must be given to algae blooming when elucidating the well-being of the perch at Kvädöfjärden and other Baltic coastal sites. The results from the present study therefore suggest that algal blooming and naturally produced chemicals may play a role in exposure to contaminants in the perch from this coastal site.

ETHICS STATEMENT

Ethical permission (Dnr 5.8. 18-02260/2018; Idnr 001380) for the samplings was approved by the ethical animal committee in Gothenburg, Sweden.

AUTHOR CONTRIBUTIONS

LF, JS, and NA planned and designed the experiments and drafted the manuscript. JP, LF, JS, NA, and SF collected the animals. MT, TÖ, and EK performed all computational and statistical analyses on the RNA sequences. JP conducted the biochemical analyses. PH conducted the chemical analysis. All authors contributed to and approved the final version of the manuscript.

FUNDING

This project was given financial support by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS) and by the Swedish Integrated Fish Monitoring Project, which was funded by the National Marine Monitoring Programme, Swedish Environmental Protection Agency.

ACKNOWLEDGMENTS

We would like to thank E. Nyberg and J. S. Öhlund of the Swedish Museum of Natural History for coordination and preparation of fish for the non-target analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2019.00316/full#supplementary-material

REFERENCES

Akhter, N., Wu, B., Memon, A. M., and Mohsin, M. (2015). Probiotics and prebiotics associated with aquaculture: a review. *Fish Shellfish Immunol.* 45, 733–741. doi: 10.1016/j.fsi.2015.05.038

Andersson, T., Förlin, L., Härdig, J., and Larsson, Å. (1988). Physiological disturbances in fish living in coastal water polluted with bleached kraft mill effluents. *Can. J. Fish Aq. Sci.* 45, 1525-1536.

Asker, N., Albertsson, E., Wijmark, E., Bergek, S., Parkkonen, J., Kammann, U., et al. (2016). Biomarker resöonses in eelpout from four coastal areas in

Sweden, Denmark and Germany. *Mar. Environ. Res.* 120, 32–43. doi: 10.1016/j.marenvres.2016.07.002

Balk, L., Larsson, Å., and Förlin, L. (1996). Baseline studies of biomarkers in the feral female perch (Perca fluviatilis) as tools in biological monitoring of anthropogenic substances. *Mar. Envinron. Res.* 42, 203–208. doi: 10.1016/0141-1136(95)00064-x

Barbosa, M., Vallentao, P., and Andrade, P. B. (2014). Bioactive compounds from macroalgae in the new millenium: implications for neurodegenerative diseases. *Mar. Drugs* 12, 4934–4972. doi: 10.3390/md12094934

Bayne, C. J., Gerwick, L., Fujiki, K., Nakao, M., and Yano, T. (2001). Immune-relevant (including acute phase) genes identified in the livers of rainbow trout, *Oncorhynchus mykiss*, by means of suppression subtractive hybridization. *Dev. Comp. Immunol.* 25, 205–217. doi: 10.1016/s0145-305x(00)00057-4

Bignert, A., Danielsson, S., Ek, C., Faxneld, S., and Nyberg, E. (2017). *Comments Concerning the National Swedish Contaminant Monitoring Programme in Marine Biota, 2017 (2016 years data), 10:2017*. Stockholm: Swedish Museum of Natural History.

Björklund, S. (2018). *Naturally Produced Organohalogens in Algae from the Baltic Sea and the Swedish West Coast*. Master thesis, Umeå University, Sweden.

Bowden, T. J. (2008). Modulation of the immune system of fish by their environment. *Fish Shellfish Immunol.* 25, 373–383. doi: 10.1016/j.fsi.2008.03.017

Brugel, S. M., Bonventre, J. A., and Tanguay, R. L. (2016). Comparative development toxicity of flavonoids using integrative zebrafish system. *Toxicol. Sci.* 154, 55–68. doi: 10.1093/toxsci/kfw139

Buchtíková, S., Simkova, A., Rohlenova, K., Flajshans, M., Lojek, A., Lilius, E.-M., et al. (2011). The seasonal changes in innate immunity of the common carp (*Cyprinus carpio*). *Aquaculture* 318, 169–175. doi: 10.1016/j.aquaculture.2011.05.013

Dautremepuits, C., Marcogliese, D. J., Gendron, A. D., and Fournier, M. (2008). Gill and head kidney antioxidant processes and innate immune system responses of yellow perch (*Perca flavescens*) exposed to different contaminants in the St. Lawrence River, Canada. *Sci. Total Environ.* 407, 1055–1064. doi: 10.1016/j.scitotenv.2008.10.004

DeGroot, D. E., Franks, D. G., Higa, T., Tanaka, J., Hahn, M. E., and Denson, M. S. (2015). Naturally-occuring marine brominated indoles are aryl hydrocarbon receptor Ligands/Agonists. *Chem. Res. Toxicol.* 28, 1176–1185. doi: 10.1021/acs.chemrestox.5b00003

Faxneld, S., Berger, U., Helander, B., Danielsson, S., Miller, A., Nyberg, E., et al. (2016). Temporal trends and geographical differences of perfluoroalkyl acids in baltic sea herring and white-tailed sea eagle eggs in Sweden. *Environ. Sci. Technol.* 50, 13070–13079. doi: 10.1021/acs.est.6b03230

Faxneld, S., Helander, B., Bäcklin, B.-M., Moraeus, C., Roos, A., Berger, U., et al. (2014). *Biological Effects and Environmental Contaminants in Herring and Baltic Sea top Predators*. Stockholm: Swedish Museum of Natural History.

Ferreira, M., Costa, J., and Reis-Henriques, M. (2014). ABC transporters in fish species: a review. *Front. Physiol.* 5:266. doi: 10.3389/fphys.2014.00266

Förlin, L. (1980). Effects of clophen A50, 3-methylcholanthrene, pregnenolone-16 α-carbonitrile and phenobarbital on the hepatic microsomal cytochrome P-450-dependent mono-oxygenase system in rainbow trout of different age and sex. *Toxicol. Appl. Pharmacol.* 54, 420–430. doi: 10.1016/0041-008x(80)90169-6

Förlin, L., Goksoyr, A., and Husoy, A.-M. (1994). "Cytochrome P450 monooxygenase as indicator of PCB/dioxin like compounds in fish," in *Biomonitoring of Coastal Waters and Estuaries*, ed. K. J. M. Kramer (Boca Raton, FL: CRC Press Inc.), 135–150.

Förlin, L., Larsson, Å., Hansson, N., Parkkonen, J., Nyberg, E., Faxneld, S., et al. (2014). *Fokus Kvädöfjärden: Varför mår kustfisken dåligt? (Title in English: Focus Kvädöfjärden: Why are Coastal Fish Not Healthy?) HAVET 2013/2014*. Available at: http://havsmiljoinstitutet.se/publikationer/havet/havet-2013-2014 (accessed April, 2014).

Fromme, H., Mi, W., Lahrz, T., Kraft, M., Aschenbrenner, B., Bruessow, B., et al. (2018). Occurrence of carbazoles in dust and air samples from different locations in Germany. *Sci. Total Environ.* 610-611, 412–418. doi: 10.1016/j.scitotenv.2017.08.070

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. Z. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28, 3150–3152. doi: 10.1093/bioinformatics/bts565

Goiris, K., Muylaert, K., Voorspoels, S., Noten, B., De Paepe, D., Bart, E. G. J., et al. (2014). Detection of flavonoids in microalgae from different evolutionary lineages. *J. Phycol.* 50, 483–492. doi: 10.1111/jpy.12180

Goldstein, J. V., McArthur, A. G., Kubota, A., Zanatte, J., Parente, T., Jönsson, M., et al. (2010). Identification and development expression of the full complement of cytochrome P450 genes in zebrafish. *BMC Genomics* 11:463. doi: 10.1186/1471-2164-11-643

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. *Nat. Biotechnol.* 29, 644–652. doi: 10.1186/1471-2105-12-S14-S2

Grasset, J., Ollivier, É., Bougas, B., Yannic, G., Campbell, P. G., Bernatchez, L., et al. (2016). Combined effects of temperature changes and metal contamination at different levels of biological organization in yellow perch. *Aquat. Toxicol.* 177, 324–332. doi: 10.1016/j.aquatox.2016.06.008

Gribble, G. W. (2015). Biological activity of recently discovered halogenated marine natural products. *Mar. Drugs* 13, 4044–4136. doi: 10.3390/md13074044

Gribble, W. G. (1999). The diversity of naturally occurring organobromine compounds. *Chem. Res. Rev.* 28, 335–346. doi: 10.1021/es100914r

Guo, J., Chen, D., Potter, D., Rockne, K. J., Sturchio, N. C., Giesy, J. P., et al. (2014). Polyhalogenated carbazoles in sedimens of lake michigan: a new discovery. *Environ. Sci. Technol.* 48, 12807–12815. doi: 10.1021/es503936u

Guo, J., Li, Z., Ranasinghe, P., Bonina, S., Hosseini, S., Corcoran, M. B., et al. (2017). Spatial and temporal trends of polyhalogenated carbazoles in sediments of upper great lakes: insight into their origin. *Environ. Sci. Technol.* 51, 89–97. doi: 10.1021/acs.est.6b06128

Haglund, P., Löfstrand, K., Malmvärn, A., Bignert, A., and Asplund, L. (2010). Temporal variations of polybrominated dibenzo-p-dioxin and methoxylated diphenyl ether concentrations in fish revealing large differences in exposure and metabolic stability. *Environ. Sci. Technol.* 44, 2466–2473. doi: 10.1021/es9038006

Haglund, P., Malmvärn, A., Bergek, S., Bignert, A., Kautsky, L., Nakano, T., et al. (2007). Brominated dibenzo-p-dioxins - a new class of marine biotoxins? *Environ. Sci. Technol.* 41, 3069–3074. doi: 10.1021/es0624725

Halliwell, B., and Gutteridge, J. M. C. (1999). *Free Radicals in Biology and Medicine*, Third Edn. Oxford: Oxford University Press.

Hanson, N., Förlin, L., and Larsson, Å. (2009). Evaluation of long-term biomarker data from prech (Perca fluviatilis) in the baltic sea suggests increasing exposure to environmental pollutants. *Environ. Toxicol. Chem.* 28, 364–373. doi: 10.1897/08-259.1

Hanson, N., Larsson, Å., Parkkonen, J., Förlin, L., Faxneld, S., Nyberg, E., et al. (2016). *Bottendjuren Påverkar Fiskens Hälsa (Title in English: Bottom Living Organisms Affect Fish Health.). HAVET 2015/2016*. Available at: http://havsmiljoinstitutet.se/publikationer/havet/havet2015-2016 (accessed May, 2016).

Hansson, T., Barsiene, J., Tjärnlund, U., Åkerman, G., Linderoth, M., Zebuhr, Y., et al. (2014). Cytological and biochemical biomarkers in adult female perch (Perca fluviatilis) in a chronically polluted gradient in the Stockholm recipient (Sweden). *Mar. Poll. Bull.* 81, 27–40. doi: 10.1016/j.marpolbul.2014.03.001

Hansson, T., Lindesjöö, E., Förlin, L. K., Balk, L., Bignert, A., and Larsson, Å. (2006a). Long-term monitoring of the the health status of female perch (Perca fluviatilis) in the Baltic Sea shows decreased gonad weight and increased hepatic EROD activity. *Aquat. Toxicol.* 79, 341–355. doi: 10.1016/j.aquatox.2006.07.001

Hansson, M., and Öberg, J. (2010). *Cyanobacteria Blooms in the Baltic Sea. HELCOM Baltic Sea Environment Fact Sheets*. Available at: http://www.helcom.fi/baltic-seatrends/environment-fact-sheets/

Hansson, T., Schiedek, D., Lehtonen, K., Vuorinen, P. J., Liewenborg, B., Noaksson, E., et al. (2006b). Biochemical biomarkers in adult female perch (Perca fluviatilis) in a chronically polluted gradient in the Stockholm recipient (Sweden). *Mar. Poll. Bull.* 53, 451–468. doi: 10.1016/j.marpolbul.2006.04.014

Holmström, K., Järnberg, U., and Bignert, A. (2005). Temporal trends of PFOS and PFOA in guillemot eggs from the Baltic Sea, 1968-2003. *Environ. Sci. Technol.* 39, 80–84. doi: 10.1021/es049257d

Hu, X., Camus, A. C., Aono, S., Morrison, E. E., Dennis, J., Nusbaum, K. E., et al. (2007). Channel catfish hepcidin expression in infection and anemia. *Comp. Immunol. Microbiol. Infect. Dis.* 30, 55–69. doi: 10.1016/j.cimid.2006.10.004

Hylland, K., Robinsson, C. D., Burgeot, T., Martinez-Gomez, C., Lang, T., Svavarsson, J., et al. (2017). Integrated chemical and biological assessment of

contaminant impact in selected European coastal and offshore marine areas. *Mar. Environ. Res.* 124, 130–138. doi: 10.1016/j.marenvres.2016.05.014

Kobayashi, M., and Yamamoto, M. (2005). Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid. Redox Signal.* 7, 385–394. doi: 10.1089/ars.2005.7.385

Kortenkamp, A., Backhaus, T., and Faust, M. (2009). *State of the Art Report on Mixture Toxicity. Report to the EU Commission, Directorate General for the Environment*. Available at: http://ec.europa.eu/environment/chemicals/effects/pdf/report_mixture_toxicity.pdf (accessed December, 2009).

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Larsson, Å., Hanson, N., Förlin, L., and Parkkonen, J. (2016). *Kustfisk – Hälsa. (Title in English: Coastal Fish – Health). HAVET 2015/2016*. Available at: http://havsmiljoinstitutet.se/publikationer/havet/havet2015-2016 (accessed May, 2016).

Larsson, Å., Förlin, L., Hanson, N., Parkkonen, J., Bignert, A., Nyberg, E., et al. (2011). *Kustfisken - Alltmer Påverkad av Miljögifter. (Title in English: Coastal Fish – Increasingly Affected by Contaminants.). HAVET 2011*. Available at: http://havsmiljoinstitutet.se/publikationer/havet/havet-2011 (accessed April, 2011).

Larsson, Å., Förlin, L., Lindesjöö, E., and Sandström, O. (2003). "Monitoring of individual organisms responses in fish populations exposed to pulp mill effluents," in *Environmental Impacts of Pulp and Paper Waste Streams*, eds T. R. Stuthridge, M. R. van den Heuvel, N. A. Marvin, A. H. Slade, and J. Gifford (Pensacola, FL: SETAC Press), 216–226.

Lee, J. H., Lee, J. E., Lee, H., Jun, H. J., and Lee, S. J. (2014). Multidrug and toxin compound extrusion proetin-1 (MATE1/SLC47A1) is a novel flavonoid transporter. *J. Agric. Food Chem.* 62, 9690–9698. doi: 10.1021/jf500916d

Lehtonen, K., Sundelin, B., Lang, T., and Strand, J. (2014). Development of tools for integrated monitoring and assessment of hazardous substances and their biological effects in the Baltic Sea. *AMBIO* 43, 69–81. doi: 10.1007/s13280-013-0478-3

Lemanska, K., van der Woude, H., Szymusiak, H., Boersma, M. G., Gliszczynska-Swiglo, A., Rietjens, I. M. C. M., et al. (2004). The effect of catechol O-demethylation on radical scavenging characteristics of quertin and Luteolin – A mechanistic Insight. *Free Radic. Res.* 38, 639–647. doi: 10.1080/10715760410001694062

Li, H., Handsaker, B., Wysoker, A., Fennel, T., Ruan, J., Homer, N., et al. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352

Loncar, J., Popovic, M., Krznar, P., Zaja, R., and Smital, T. (2016). The first characterization of multidrug and toxin extrusion (MATE/SLC47) proteins in zebrafish (Danio rerio). *Sci. Rep.* 6:28937. doi: 10.1038/srep28937

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish Shellfish Immunol.* 20, 137–151. doi: 10.1016/j.fsi.2004.09.006

Malmvärn, A., Zebühr, Y., Kautsky, L., Bergman, Å., and Asplund, L. (2008). Hydroxylated and methoxylated polybrominated diphenyl ethers and polybrominated dibenzo-p-dioxins in red alga and cyanobacteria living in the Baltic Sea. *Chemosphere* 72, 910–916. doi: 10.1016/j.chemosphere.2008.03.036

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17:3.

Michalek, M., and Werner, M. (2012). *Abundance and Distribution of Marenzelleria Species in the Baltic Sea. HELCOM Baltic Sea Environment Fact Sheets*. Available at: http://www.helcom.fi/baltic-sea-trends/environment-fact-sheets/

Millan, A., Gomez-Tato, A., Pardo, B. G., Fernandez, C., Bouza, C., Vera, M., et al. (2011). Gene expression profiles of the spleen, liver, and head kidney in turbot (Scophthalmus maximus) along the infection process with *Aeromonas salmonicida* using an immune-enriched oligo-microarray. *Mar. Biotechnol.* 13, 1099–1114. doi: 10.1007/s10126-011-9374-7

Moon, Y. J., Wang, X., and Morris, M. E. (2006). Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. *Toxicol. In Vitro* 20, 187–210. doi: 10.1016/j.tiv.2005.06.048

Mumbo, J., Henkelmann, B., Abdelaziz, A., Pfister, G., Nguyen, N., Schroll, R., et al. (2015). Persitance and dioxin-like toxicity of carbazole and chlorocarbazoles in soil. *Environ. Sci. Pollut. Res.* 22, 1344–1356. doi: 10.1007/s11356-014-3386-6

Nebert, D. W., and Vasiliou, V. (2004). Analyis of the glutathione gene family. *Hum. Genomics* 1, 460–464.

Norstrom, R. J., Simon, M., Muir, D. C. G., and Schweinsburg, R. E. (1988). Organochlorine contaminants in arctic marine food chains: identification, geographical distribution and temporal trends in polar bears. *Environ. Sci. Technol.* 22, 1063–1071. doi: 10.1021/es00174a011

Öberg, J. (2014). *Cyanobacteria Blooms in the Baltic Sea. HELCOM Baltic Sea Environment Fact Sheets*. Available at: http://www.helcom.fi/baltic-sea-trends/environment-fact-sheets/

Öberg, J. (2017). *Cyanobacteria Blooms in the Baltic Sea. HELCOM Baltic Sea Environment Fact Sheets 2017*. Available at: http://www.helcom.fi/baltic-sea-trends/environment-fact-sheets/ (accessed January, 2018).

Oda, S., Fukami, T., Yokoi, T., and Nakajima, M. (2015). A comprehensive review of UDP-glucuronosyltransferase and esterases for drug development. *Drug Metab. Pharm.* 30, 30–51. doi: 10.1016/j.dmpk.2014.12.001

Parkkonen, J., Larsson, D. G. J., Adolfsson-Erici, M., Petterson, M., Berg, A. H., Olsson, P.-E., et al. (1999). "Contraceptive pill residues in sewage effluent are estrogenic to fish," in *Proceedings, Sixth International Symposium, Reproductive Physiology of Fish*, Bergen, 362–364.

Paskerova, H., Hilscherova, K., and Blaha, L. (2012). Oxidative stress and detoxification biomarkers responses in aquatic freshwater vertebrates exposed to microcystins and cyanobacterial biomass. *Environ. Sci. Pollut. Res.* 19, 2024–2037. doi: 10.1007/s11356-012-0960-7

Peatman, E., Baoprasertkul, P., Terhune, J., Xu, P., Nandi, S., Kucuktas, H., et al. (2007). Expression analysis of the acute phase response in channel catfish (Ictalurus punctatus) after infection with a Gram-negative bacterium. *Dev. Comp. Immunol.* 31, 1183–1196. doi: 10.1016/j.dci.2007.03.003

Reynaud, S., and Deschaux, P. (2006). The effects of polycyclic aromatic hydrocarbons on the immune system of fish. *Aquat. Toxicol.* 77, 229–238. doi: 10.1016/j.aquatox.2005.10.018

Rodrigues, P. N., Vazquez-Dorado, S., Neves, J. V., and Wilson, J. M. (2006). Dual function of fish hepcidin: response to experimental iron overload and bacterial infection in sea bass (*Dicentrarchus labrax*). *Dev. Comp. Immunol.* 30, 1156–1167. doi: 10.1016/j.dci.2006.02.005

Russell, S., and Lumsden, J. S. (2005). Function and heterogeneity of fish lectins. *Vet. Immunol. Immunopathol.* 108, 111–120. doi: 10.1016/j.vetimm.2005.08.004

Sandström, O., Larsson, Å., Andersson, J., Appelberg, M., Bignert, A., Ek, H., et al. (2005). Three decades of Swedish experience demonstrates the need for integrated long-term monitoring of fish in marine coastal areas. *Wat. Qual. Res. J. Can.* 40, 233–250. doi: 10.2166/wqrj.2005.030

Schlenk, D., Celander, M. C., Gallagher, E. P., George, S., James, M., Kullman, S. W., et al. (2008). "Biotransformation in fishes," in *The Toxicology of Fishes*, eds R. T. Di Giulio and D. E. Hinton (Boca Raton, FL: CRC Press), 153–234. doi: 10.1201/9780203647295.ch4

Sheader, D. L., Williams, T. D., Lyons, B. P., and Chipman, J. K. (2006). Oxidative stress response of European flounder (Platichthys flesus) to cadmium determined by a custom cDNA microarray. *Mar. Environ. Res.* 62, 33–44. doi: 10.1016/j.marenvres.2006.03.001

Singh, D. P., Prabha, R., Verma, S., Meena, K. K., and Yandiger, M. (2017). Antioxidant properties and polyphenolic content in terrestrial cyanobacteria. *3Biotech* 7:134. doi: 10.1007/s13205-017-0786-6

Smith-Unna, R., Boursell, C., Patro, R., Hibber, J. M., and Kelly, S. (2016). TransRate: reference-free quality assessment of de novo transcriptomic assemblies. *Genome Res.* 26, 1134–1144. doi: 10.1101/gr.196469.115

Specker, J. L., and Anderson, T. R. (1994). Developing an ELISA for a model proetin – vitellogenin. *Biochem. Mol. Biol. Fishes* 3, 568–578.

Stephensen, E., Sturve, J., and Förlin, L. (2002). Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 133, 435–442. doi: 10.1016/s1532-0456(02)00129-1

Sturve, J., Balk, L., Liewenborg, B., Adolfsson-Erici, M., Förlin, L., and Carney Almroth, B. (2014). Effects of an oil spill in a harbor assessed using biomarkers of exposure in eelpout. *Environ. Sci. Pollut. Res.* 21, 13758–13768. doi: 10.1007/s11356-014-2890-z

Sturve, J., Berglund, Å., Balk, L., Broeg, K., Böhmert, B., Massey, S., et al. (2005). Effects of dredging in Göteborg harbour, Sweden, assessed by biomarkers in eelpout Zoarces viviparus). *Environ. Toxicol. Chem.* 24, 1951–1961.

Svedäng, H., Ojaveer, H., and Urtans, E. (1997). Interpretation of the otolith structures in viviparous blenny *Zoarces viviparus*. *J. Appl. Ichthyol.* 13, 137–142. doi: 10.1111/j.1439-0426.1997.tb00113.x

TemaNord (1995). *Nordic Environmental Specimen Banking: Methods in Use in ESB: Manual for Nordic Countries, 543*. TemaNord Scandinavia: Nordic Council of Ministers.

Uribe, C., Folch, H., Enriquez, R., and Moran, G. (2011). Innate and adaptive immunity in teleost fish: a review. *Vet. Med.* 56, 486–503. doi: 10.17221/3294-vetmed

Unger, M., Asplund, L., Haglund, P., Malmvärn, A., Arnoldsson, K., and Gustafsson, Ö. (2009). Polybrominated and mixed brominated/chlorinated dibenzo-p-dioxins in Sponge (Ephydatia fluviatilis) from the Baltic Sea. *Environ. Sci. Technol.* 43, 8245–8250. doi: 10.1021/es901705r

van der Oost, R., Beyer, J., and Vermeulen, N. P. E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Sci. Tech.* 42, 1058–1065.

Vasta, G. R., Nita-Lazar, M., Giomarelli, B., Ahmed, H., Du, S., Cammarata, M., et al. (2011). Structural and functional diversity of the lectin repertoire in teleost fish: relevance to innate and adaptive immunity. *Dev. Comp. Immunol.* 35, 1388–1399. doi: 10.1016/j.dci.2011.08.011

Viskupciova, J., Ondrejovic, M., and Sturdik, E. (2008). Bioavailability and metabolism of flavonoids. *J. Food Nutr. Res.* 47, 151–162.

Wassell, J. (2000). Haptoglobin: function and polymorphism. *Clin. Lab.* 46, 547–552.

Watanuki, H. T., Yamaguchi, T., and Sakai, M. (2002). Suppression in function of phagocytic cells in common carp *Cyprimus carpio* L. injected with estradiol, progesterone or 11-ketotestosterone. *Comp. Biochem. Physiol. C* 132, 407–413. doi: 10.1016/s1532-0456(02)00100-x

Waterhouse, R. M., Seppey, M., Simão, F. A., Manni, M., Ioannidis, P., Klioutchnikov, G., et al. (2017). BUSCO applications from quality assessments to gene prediction and phylogenomics. *Mol. Biol. Evol.* [Epub ahead of print].

Wu, Y., Tan, H., Zhou, C., Crimmins, S., Holsenb, M., and Chen, D. (2018). Bioacculmulation and spatial trends of polyhalogenated carbazoles in great lakes fish from 2004 to 20016. *Environ. Sci. Technol.* 52, 4536–4545. doi: 10.1021/acs.est.8b00427

Yang, Q., Pande, G. S. J., Wang, Z., Lin, B., Rubin, R. A., Vora, G. J., et al. (2017). Indole signaling and (micro)algal auxins decrease the virulence of *Vibrio cambelli*, a major pathogen of aquatic organisms. *Environ. Microbiol.* 19, 1978–2004.

Zhu, B. T., Ezell, E. L., and Liehr, J. G. (1994). Catechol-O-methyltransferase-catalyzed rapid O-methylation of mutagenic flavonoids. *J. Biol. Chem.* 269, 292–299.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past collaboration with one of the authors LF.

frontiers in Marine Science

ORIGINAL RESEARCH
published: 08 July 2019
doi: 10.3389/fmars.2019.00377

Application of a Weight of Evidence Approach for Monitoring Complex Environmental Scenarios: the Case-Study of Off-Shore Platforms

Francesco Regoli[1], Giuseppe d'Errico[1], Alessandro Nardi[1], Marica Mezzelani[1], Daniele Fattorini[1], Maura Benedetti[1], Marta Di Carlo[1], David Pellegrini[2] and Stefania Gorbi[1]*

[1] Department of Life and Environmental Sciences, Marche Polytechnic University, Ancona, Italy, [2] Institute for Environmental Protection and Research (ISPRA), Livorno, Italy

OPEN ACCESS

Edited by:
Thierry Burgeot, Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), France

Reviewed by:
Gabriela Verónica Aguirre-Martínez, Universidad Arturo Prat, Chile
Hans Uwe Dahms, Kaohsiung Medical University, Taiwan

***Correspondence:**
Francesco Regoli
f.regoli@univpm.it

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *22 December 2018*
Accepted: *17 June 2019*
Published: *08 July 2019*

Citation:
Regoli F, d'Errico G, Nardi A, Mezzelani M, Fattorini D, Benedetti M, Di Carlo M, Pellegrini D and Gorbi S (2019) Application of a Weight of Evidence Approach for Monitoring Complex Environmental Scenarios: the Case-Study of Off-Shore Platforms. Front. Mar. Sci. 6:377. doi: 10.3389/fmars.2019.00377

Multidisciplinary investigations based on integration of chemical and biological measurements, represent an added value to monitoring and management protocols, and their use is recommended by European Directives to evaluate the environmental status of aquatic ecosystems. However, assessing the overall significance of results obtained in different typologies of studies is often a difficult challenge. The aim of this work was to present a quantitative Weight Of Evidence (WOE) model (Sediqualsoft) to integrate huge amounts of heterogeneous data and to validate this approach in complex monitoring scenarios. Using the case-study of an off-shore platform field in the Adriatic Sea, procedures are presented to elaborate different typologies of data (lines of evidence, LOEs), including chemical characterization of sediments, bioavailability, biomarkers, ecotoxicological bioassays and benthic communities around three platforms. These data are initially evaluated by logical flowcharts and mathematical algorithms, which provide specific hazard indices for each considered LOE, before their different weighting and overall integration in an environmental risk index. The monitoring study selected for the WOE elaboration consisted on chemical analyses of trace metals, aliphatic hydrocarbons, polycyclic aromatic hydrocarbons carried out on 60 sediment samples; the same samples were also characterized for the status of benthic communities; bioavailability of metals from sediments was assessed in laboratory conditions on the polychaete *Hediste diversicolor*, while bioaccumulation of inorganic and organic chemicals and biomarker responses were measured in native and transplanted mussels; ecotoxicological properties of sediments were evaluated through a battery of bioassays determining algal growth of the diatom *Phaeodactylum tricornutum*, bioluminescence of the marine bacterium *Vibrio fischeri*, survival of the copepod *Acartia tonsa* and embryotoxicity of sea urchin *Paracentrotus lividus*. Overall, almost 7000 analytical results were elaborated and summarized in specific hazard

indices. The WOE integration of multiple typologies of data allowed more robust and weighted conclusions compared to the use of individual LOEs, highlighting the feasibility of this procedure for multidisciplinary monitoring and risk assessment approaches. On a practical side, the WOE evidences also suggested a revision of actual monitoring procedures. Overall, the proposed WOE model appeared as a useful tool to summarize large datasets of complex data in integrative indices, and to simplify the interpretation for stakeholders and decision makers, thus supporting a more comprehensive process of "site-oriented" management decisions.

Keywords: off-shore platforms, risk assessment, WOE integration, multidisciplinary approaches, monitoring

INTRODUCTION

Marine ecosystems are challenged by several anthropogenic stressors, including release of chemicals through a variety of sources (i.e., riverine effluents, urban sewers, direct discharge, accidents, and atmospheric deposition). The impact of pollutants is one of the most faceted issues to investigate since mixtures of organic and inorganic compounds can interact through complex mechanisms, modulate responsiveness to other stressors and thus affect several biological responses, from cellular and physiological processes, up to populations dynamics and ecosystem functioning (Newman and Clements, 2007): in this context, the development of accurate monitoring plans is crucial for environmental protection. Initial programs were typically aimed to quantify the presence and distribution of chemicals in the environment, but the importance of assessing biological effects of contaminants has gradually risen and actual monitoring practices recommend the integration of both chemical and biological approaches (Piva et al., 2011; Benedetti et al., 2014; Regoli et al., 2014; Bebianno et al., 2015; Vethaak et al., 2017). The use of multidisciplinary studies for the characterization of aquatic environment quality is highly encouraged also by European Directives such as the Water Framework Directive (WFD, Directive 2000/60/EC) and the Marine Strategy Framework Directive (MFSD, Directive 2008/56/EC), which require member states to evaluate and classify the ecological status of water bodies through the integration of different quality indicators. Strategies for integrated chemical and biological monitoring have been described and recently adopted by various international agencies or working groups such as OSPAR, HELCOM, MEDPOL, ICES (Broeg and Lehtonen, 2006; HELCOM, 2010; Davies and Vethaak, 2012; OSPAR, 2013; Hylland et al., 2017; Lyons et al., 2017; Vethaak et al., 2017): a recognized advantage of such approaches is the added interpretative value derived from the integration of multiple typologies of studies, thus improving our ability to describe and interpret variations of environmental conditions.

The Sediment Quality Triad was the first Weight of Evidence (WOE) approach, combining various lines of evidence (LOEs), such as chemical analysis, toxicity testing, and considering the in-situ benthic community structure (Chapman, 2007), to link contamination with their biological and ecological impacts. Quantification of chemicals is fundamental in environmental monitoring but, by itself, it does not provide information on transfer to biota and biological effects of contaminants (Benedetti et al., 2012). Laboratory bioassays are useful to evaluate the presence of acutely toxic compounds measuring specific biological endpoints in selected organisms: proper batteries integrating various classes of ecotoxicological bioassays have progressively been developed to cover different taxa across the main ecological and trophic positions. Also benthic studies have evolved in the last decade moving from qualitative descriptions, to long-term surveys up to the functioning of benthic communities and development of Ecological Quality Status Descriptors (Dauvin, 2015; Borja et al., 2016, 2017).

Compared to the original triad, additional LoEs have further enhanced our possibility to understand cause-effect relationships between chemical exposure and biological effects, providing a more robust basis for environmental control and management. Bioaccumulation of pollutants in resident or transplanted organisms is a sure proxy of bioavailability and transfer of chemicals from abiotic matrices to biota, being also recognized in some environmental quality standards. Sub-lethal alterations at molecular and cellular level (biomarkers) provide a sensitive indication of early changes which often represent the first warning signals before the onset of long-term toxicological effects or changes at higher levels of biological organization (Broeg and Lehtonen, 2006; Moore et al., 2006; Regoli and Giuliani, 2014; Benedetti et al., 2015). Various health indices have been described in the last years combining complex biomarker data from multiple cellular pathways, to summarize the severity of biological disturbance in a scientifically sound but also user-friendly format for environmental managers and decision makers (Beliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006; Dagnino et al., 2008; Piva et al., 2011; Benedetti et al., 2012; Marigómez et al., 2013).

The inclusion of multiple lines of evidence in a weight of evidence framework would thus represent a powerful approach within national and international strategies for monitoring environmental quality. However, the lack of standardized procedures for the integration of complex datasets of heterogeneous results, often prevents the adoption of such multidisciplinary approaches and their inclusion in decision-supporting procedures (Dagnino et al., 2008; Semenzin et al., 2008; Linkov et al., 2009; Piva et al., 2011; Benedetti et al., 2012). A multistep traffic-light system has been recently proposed for OSPAR, integrating data on contaminant and biological effects in background assessment concentrations (BACs), environmental

assessment concentrations (EACs) and their analogous criteria for biological responses. These values are generally derived for North Atlantic areas and species (Vethaak et al., 2017), and for each analyzed parameter a specific color is given if below BAC (blue), between BAC and EAC (green), or above EAC (red): the graphical combination of all the results allows to easily visualize the proportion of parameters assigned to each color, but the relative proportions of various colors in different parameters is not summarized in a quantitative evaluation to facilitate a comparison between samples.

Quantitative criteria would certainly improve the utility of multidisciplinary assessments increasing their capability to compare and discriminate between different environmental conditions. In a recent and quantitative WOE model (Sediqualsoft), logical flowcharts and mathematical algorithms were developed to elaborate data from various LOEs (e.g., sediment chemistry, bioaccumulation, biomarkers, ecotoxicological bioassays and benthic communities): synthetic and quantitative hazard indices are provided for each of the considered line of evidence, before their overall integration in the final WOE assessment (Piva et al., 2011). Independent elaborations for various LOEs are based on different criteria specific for various typologies of data, including the number, magnitude and typology of chemicals exceeding normative guidelines or natural concentrations measured in control organisms, while for biological responses the eco- toxicological relevance of measured endpoints and their variations compared to specific thresholds are considered (Piva et al., 2011). These criteria have been validated in numerous national and international case studies for environmental risk assessment associated with polluted sediments, harbor areas, or complex natural and anthropic impacts on the marine environment (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al., 2014; Bebianno et al., 2015; Mestre et al., 2017; Pittura et al., 2018).

Standardized procedures for integrating heterogeneous data are of utmost importance, particularly in multidisciplinary monitoring scenarios when the overall significance of completely different typologies of results need to be summarized to adequately communicate the risk in a simple way, without reducing the scientific soundness of the overall investigation. In this respect, this work was aimed to demonstrate the feasibility of the quantitative WOE approach as an innovative procedure for quantitative impact assessment of complex anthropogenic activities. The selected case-study was the monitoring of off-shore platforms, which are often regulated by normative requirements, precise sampling strategies and parameters to analyze for demonstrating the lack of negative interactions with several indicators of the marine environment. Despite the extensive analytical effort, a practical challenge remains the absence of effective and transparent approaches to synthesize the overall significance of such huge amounts of different results, ensuring that environmental impacts and hazards are properly quantified and compared on both a geographical and temporal scale. In this study, following prescriptions by the Italian Institute for Environmental Protection and Research (ISPRA), 3 Adriatic platforms were subjected to an extensive multidisciplinary survey including physico-chemical and ecotoxicological characterization of sediments, status of benthic communities, bioavailability of metals from sediments to the polychaete *Hediste diversicolor*, bioaccumulation of inorganic and organic chemicals, and biomarker responses in natural and transplanted mussels. Despite the main objective of this paper is not a detailed discussion of such results, all these data have been provided to present the elaboration procedure through weighted criteria within the integrated WOE scheme, thus highlighting the importance of advanced criteria in risk assessment procedures, ensuring both scientific reliability and synthetic indices for stakeholders.

MATERIALS AND METHODS

Sampling Activities and Experimental Design

Sampling of Sediments for Chemical Analyses, Ecotoxicological Characterization, and Bioavailability Assay

The oil field "Rospo Mare" contains three off-shore platforms, RSM-A, RSM-B and RSM-C, in the Southern Adriatic Sea, 20 km east from the city of Vasto (**Figure 1**). It's Produced Water is not discharged into the sea but transported on land for treatment. According to requirements of the Italian Ministry of Environment, chemical characterization of sediments and analyses of benthic communities were carried out on 20 samples for platform collected along two orthogonal transects, from NW to SE and from NE to SW, at distances of 25, 50, 100, 250, and 1000 m (**Figure 1**). Samples were stored at −20°C for chemical analyses, while those for benthic communities were sieved onboard at 0.5 mm, immediately fixed with Formalin Free Fixative, Accustain^{TM} (Sigma-Aldrich), and analyzed within 6 weeks.

Sediments for assessing bioavailability of metals and ecotoxicological characteristics were sampled at eight sites for each platform: of these, four were at 50 m from the structures on the same transects previously identified, while four samples were collected at 1000 m along the transects of dominant currents, two upstream and two downstream (**Figure 1**). These sediments were stored at +4°C until the preparation of elutriates (within 72 h from the collection), or their use for the bioaccumulation assay with the polychaete *H. diversicolor*.

Mussel Watch With Native and Transplanted Mussels

Natural mussels, *Mytilus galloprovincialis*, were sampled from each platform at 3 m depth, while for translocation experiments, control organisms from a local farm were maintained for 4 weeks at the same depth: mussels were deployed in net bags (80 cm height × 25 cm diameter, mesh size 1.5 × 3 cm) secured to a nylon rope, and the system was reinforced by an external net cylinder (2 m height × 80 cm diameter, mesh size 2 × 3 cm) to guarantee free circulation of seawater and protect mussels from fish predation (Gorbi et al., 2008). Approximately 200 adult mussels (6.0 ± 0.5 cm in shell length) were collected; organisms were then wrapped in humid towels, rapidly transported to laboratory and further processed: for bioaccumulation analyses,

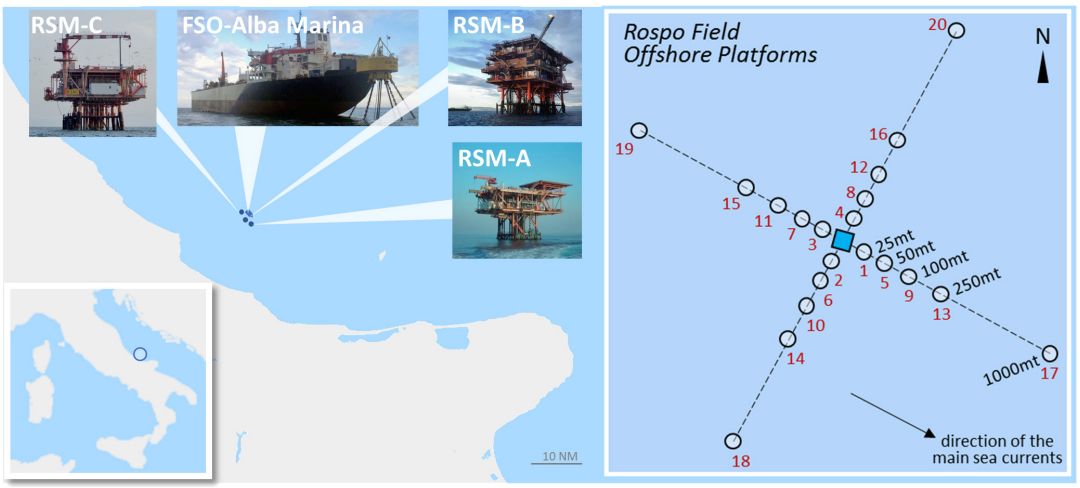


FIGURE 1 | Geographical localization of Rospo Mare field and representative experimental sampling design: scale NM, nautical miles. For each of the three off-shore platforms the following analyses (samples) were performed: chemical characterization of sediments (samples 1–20); benthic communities (samples 1–20 in triplicate); ecotoxicological bioassays (samples 5–8, 17, 17bis, 19, and 19bis); bioavailability of metals from sediments to laboratory exposed polychaetes (samples 5–8, 17, 17bis, 19, and 19bis); bioavailability of metals and organic pollutants in mussels (both native organisms and transplanted on the platforms); biomarkers in mussels (both native organisms and transplanted on the platforms).

10 replicates were prepared from every sampling site, each consisting of whole tissues from 10 organisms, flash frozen in liquid nitrogen and stored at −20°C. For biomarkers, digestive glands were rapidly removed from 30 specimens, pooled in 10 samples (each with tissues of three specimens), frozen in liquid nitrogen and maintained at −80°C; small pieces of digestive glands were rapidly excised from five mussels for histological analyses, placed on cork chucks, frozen in n-hexane precooled to −70°C in liquid nitrogen, and maintained at −80°C; hemolymph was withdrawn from the adductor mussels of 15 specimens, divided in five aliquots, each with fluids of three individuals, and immediately frozen in liquid nitrogen; genotoxic effects were measured in hemocytes of five specimens maintained in Carnoy fixative (acetic acid:methanol 1:3).

Bioaccumulation Assay With the Polychaete *Hediste diversicolor*

The bioavailability of metals in the eight sediment samples specifically collected at each platform (see above) were tested with the polychaete *H. diversicolor*. Organisms were obtained from a commercial supplier, acclimated to laboratory conditions for 72 h in aerated artificial seawater (18 ± 2°C, salinity 30 psu, prepared by dissolving InstantOcean® marine salt in reverse osmosis water), and randomly placed in 25 vessels (eight sites for platform and one control sediment), each containing 20 organisms, 600–800 g of sediment and 500 mL of artificial seawater. Water was renewed every week and no mortality was observed after 28 days, when organisms were placed for additional 72 h in artificial seawater to excrete sediment grains. For each treatment, three pools with whole tissues of 6–7 organisms were flash frozen in liquid nitrogen and stored at −20°C until analyses of trace metals.

Analytical Methodologies and Weighted Elaboration of Results

Chemical Characterization of Sediments and Organisms

Concentrations of trace metals (Al, As, Cd, Cu, Cr, Hg, Ni, Pb, Zn), polycyclic aromatic hydrocarbons and aliphatic hydrocarbons were determined in sediments and in mussel tissues, while only metals were measured in polychaetes *H. diversicolor* exposed to sediments at laboratory conditions. Analytical methods and procedures for quality assurance/quality control were previously described, based on conventional procedures of gas-chromatography with flame ionization detector (FID) for aliphatic hydrocarbons, high performance liquid chromatography (HPLC) with diode array (DAD) and fluorimetric detection for PAHs, atomic absorption spectrophotometry (AAS) for trace metals (Benedetti et al., 2014).

Obtained results have been elaborated through weighted criteria of the WOE model (Sediqualsoft) summarizing specific hazard indices for each typology of data (LOE): conceptual elaborations of the WOE model have been fully detailed elsewhere (Piva et al., 2011). The evaluation of chemical hazards in sediments (LOE-1) is based on the initial calculation, for each pollutant, of the Ratio to Reference (RTR), i.e., the ratio between concentration measured in sediments and threshold indicated by various sediment quality guidelines, SQGs (**Figure 2**); in the present investigation, SQGs were those from European Water Framework Directive 2000/60 for the achievement of a good ecological status. From the calculated ratio to reference, a RTRw is obtained by the application of a correction factor (w) which, depending to the typology of chemicals, ranges from 1 to 1.3 for "non-priority" (w = 1), "priority" (w = 1.1) or

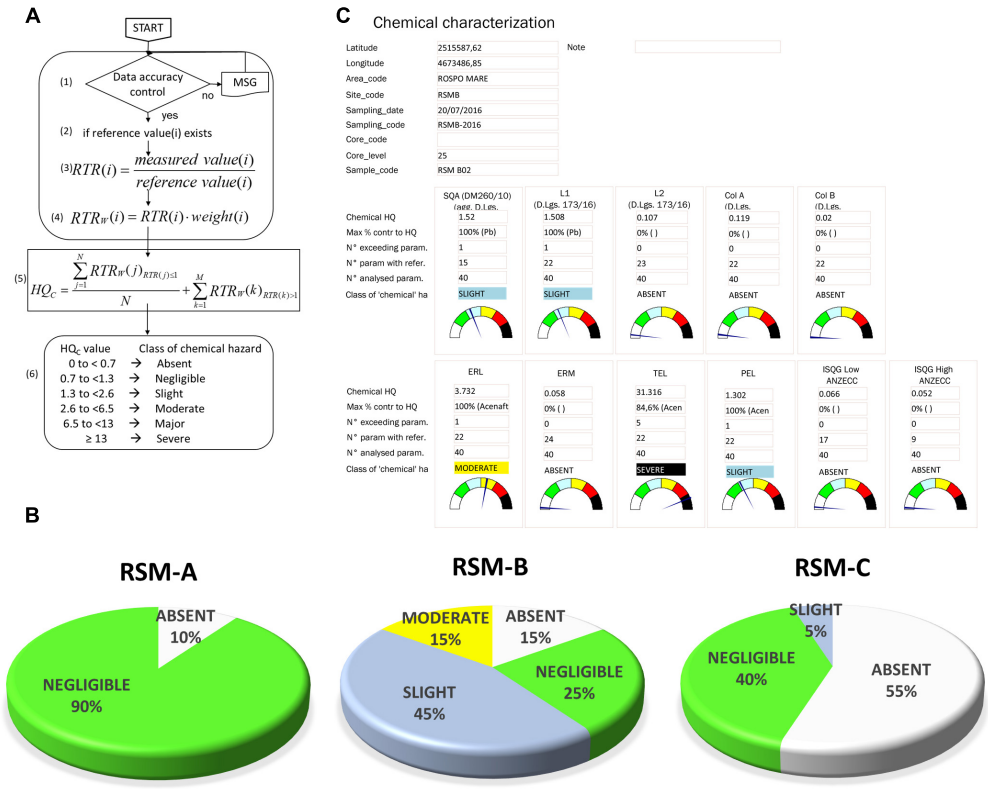


FIGURE 2 | LOE-1: elaboration of chemical results in sediments. **(A)** Flowchart and calculations of Chemical Hazard Quotient (HQ_C). **(B)** Results obtained for the three platforms indicating the percentage distribution of samples assigned to various classes of hazard considering European Sediment Quality Guidelines (EQS) as reference. **(C)** Model output of chemical characterization (Sediqualsoft).

"priority and hazardous" pollutants (w = 1.3) according to EC Directive 2008/105.

In the calculation of the specific Hazard Quotient for chemistry (HQ_C), an average RTRw is obtained for all of the parameters with RTR < 1 (i.e., values below the SQG), while for those with RTR > 1, the RTRw are individually added into the summation $\sum$:

$$HQ_C = \frac{\sum_{j=1}^{N} RTR_W(j)_{RTR(j)\leq 1}}{N} + \sum_{k=1}^{M} RTR_W(k)_{RTR(k)>1}$$

With this calculation, the HQ_C increases according to both the number and the magnitude of the exceeding parameters (for which the specific RTRw are individually added), and it is not lowered by the analysis of many "not exceeding" parameters (which are summarized in the averaged RTRw). The values of HQ_C are assigned to one class of chemical hazard (absent or negligible, slight, moderate, major, severe) depending on the number, typology, and magnitude of exceeding chemicals (see **Figure 2** and Piva et al., 2011 for details).

The results on bioaccumulation of chemicals in tissues of wild and caged mussels (LOE-2) were elaborated calculating, for each parameter, the increase of concentration compared to control organisms, corrected for the typology of pollutant and the statistical significance of the difference (Piva et al., 2011 for details). The cumulative Hazard Quotient for bioavailability (HQ_{BA}) does not consider parameters with RTRw < 1.3 (tissue concentration ≤ value in control organisms for a priority and hazardous pollutant), calculates the average for those with RTRw ranging between 1.3 and 2.6 (i.e., up to 2-fold increase compared to controls for a priority and hazardous pollutant), and adds the summation ($\sum$) of all those with RTRw ≥ 2.6 (see **Figure 3** and Piva et al., 2011 for details):

$$HQ_{BA} = \frac{\sum_{n=1}^{j} RTR_W(n)_{1.3\leq RTR_W<2.6}}{j} + \sum_{n=1}^{k} RTR_W(n)_{RTR_W\geq 2.6}$$

The level of cumulative HQ_{BA} is summarized in one class of hazard for bioavailability, from Absent to Severe, depending on the distribution of analyzed chemicals within the different classes of effect (Piva et al., 2011; Regoli et al., 2014).

Biomarkers Analyses in Native and Transplanted Mussels

Biomarkers were analyzed in native and transplanted mussels. Metallothioneins (MTs), single antioxidant defenses (catalase,

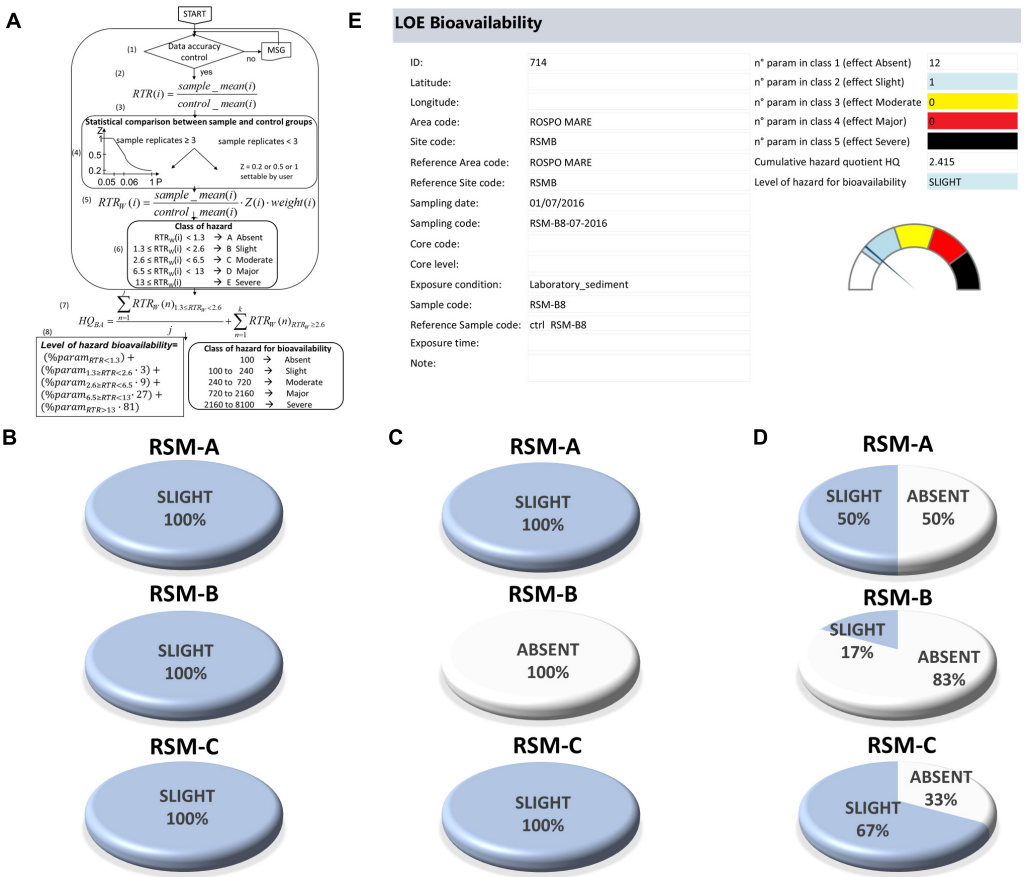


FIGURE 3 | LOE-2: elaboration of bioavailability results. **(A)** Flowchart and calculations of Bioavailability Hazard Quotient (HQ_{BA}). **(B–D)** Results obtained for the three platforms indicating the percentage distribution of samples assigned to various classes of hazard for native mussels **(B)**, transplanted mussels **(C)**, and laboratory exposed polychaetes **(D)**. **(E)** Model output for bioavailability characterization.

glutathione S-transferases, glutathione peroxidases, glutathione reductase, total glutathione), total oxyradical scavenging capacity toward peroxyl radicals (TOSC ROO•) and hydroxyl radicals (TOSC HO•), malondialdehyde content (MDA) were spectrophotometrically measured in digestive glands of mussels. Hemocytes were processed for acetylcholinesterase activity, immune-related alterations, (lysosomal membrane stability, phagocytosis activity, and granulocytes versus hyalinocytes ratio) and genotoxic effects, in terms of percentage of DNA integrity (comet assay) and micronuclei frequency (MN). Standardized protocols have been fully described elsewhere (Benedetti et al., 2014).

The elaboration of biomarkers data is based on a specifically developed algorithm (LOE-3) which assigns to each response a "weight" (based on the toxicological relevance of the endpoint) and a "threshold," indicating the minimum change above which, depending on species and tissue, the biomarker response should be considered as biologically relevant (Piva et al., 2011). For every analyzed biomarker, the measured variation is normalized by comparison to its specific threshold (effect), then corrected for the weight of the response and the statistical significance of the difference in respect to controls. The calculation of the Hazard Quotient for biomarkers (HQ_{BM}) does not consider the contribution of responses with an effect < 1 (lower than threshold), calculates the average for those with an effect up to two-fold compared to the threshold and adds the summation ($\sum$) for the responses more than 2-fold greater than the respective threshold (**Figure 4** and Regoli et al., 2014 for details):

$$HQ_{BM} = \left\{ \frac{\sum_{j=1}^{N} Effect_W(j)_{1<Effect(j)\leq 2}}{num\ biomark_{1<Effect(j)\leq 2}} + \sum_{k=1}^{M} Effect_W(k)_{Effect(j)>2} \right\}$$

According to variations measured for various biomarkers, the Sediqualsoft model summarizes the level of cumulative HQ_{BM} in one of five classes of hazard for biomarkers, from Absent to Severe (Piva et al., 2011).

Ecotoxicological Bioassays

A battery of ecotoxicological bioassays was applied to sediments (eight sites for each platform) following standardized procedures. The bioluminescence test with *Vibrio fischeri* (ISO 11348-3:

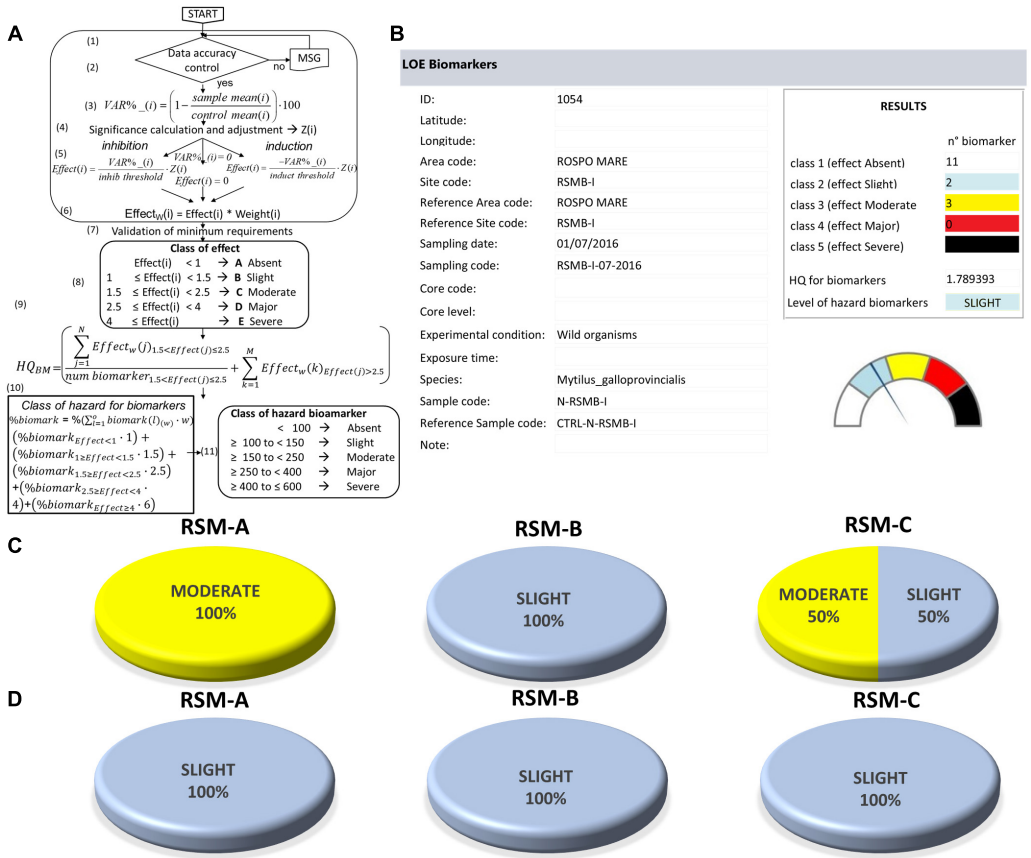


FIGURE 4 | LOE-3: elaboration of biomarker results. **(A)** Flowchart and calculations of Biomarkers Hazard Quotient (HQ_{BM}). **(B)** Model output of biomarker characterization. **(C,D)** Results obtained for the three platforms indicating the percentage distribution of samples assigned to various classes of hazard for native **(C)** and transplanted mussels **(D)**.

200/) and the larval development assay with *Acartia tonsa* (ISO 16778:2015) were selected for testing the toxicity of the solid phase, while the algal growth inhibition of *Phaeodactylum tricornutum* (ISO 10253: 2006) and the sea urchin embryotoxicity assay with *Paracentrotus lividus* (ISPRA, 2017) were used for testing the toxicity of elutriates.

In the weighted elaboration of ecotoxicological bioassays (LOE-4), each test has a weight depending on the biological relevance of the endpoint, and a threshold derived from the sensitivity of the species (Piva et al., 2011). The cumulative hazard quotient ($HQ_{Battery}$) is obtained by the summation ($\sum$) of the weighted effects ($Effect_w$), i.e., the variations measured for each test normalized to its specific thresholds, and corrected for the statistical significance of the difference (w), biological importance of the endpoint, and exposure conditions (w_2) (see **Figure 5** and Piva et al., 2011 for details):

$$HQ_{Battery} : \sum Effect_w(k)\ w_2$$

The $HQ_{Battery}$ is normalized to a scale ranging from 0 to 10, where 1 is the Battery Threshold (when all the measured bioassays exhibit an effect equal to the threshold, 10 when all the assays exhibit 100% of effect), and then assigned to one of five classes of hazard, from Absent to Severe (**Figure 5**).

Benthic Communities Analyses

Sediment samples were sorted at the stereomicroscope and the principal animal taxa were generally classified at species level. For each species, whenever possible, the corresponding biocenosis was identified to define the bionomic and ecological settlement.

For the elaboration of data on benthic communities (LOE-5), a specific module has been developed in structured query language (SQL) and Visual Basic to convert the list of identified species in several available univariate and multivariate indices for the classification of ecological quality (Borja et al., 2000; Simboura and Zenetos, 2002; Dauvin and Ruellet, 2007; Muxika et al., 2007; Mistri and Munari, 2008; Sigovini et al., 2013). Such elaborated indices include total abundance (*N*), species richness (*S*), Shannon-Weaver Diversity Index (*H'*), Margalef index (*D*), Pielou's evenness index (*J*), AZTI' Marine Biotic Index (*AMBI*), multimetric-AZTI Marine Biotic Index (*m-AMBI*), Bentic Index *(BENTIX)*, Benthic Index based on Taxonomic Sufficiency (*BITS*), and Benthic Opportunistic Polychaetes Amphipods (*BOPA* index). In this work, the AMBI index was chosen for

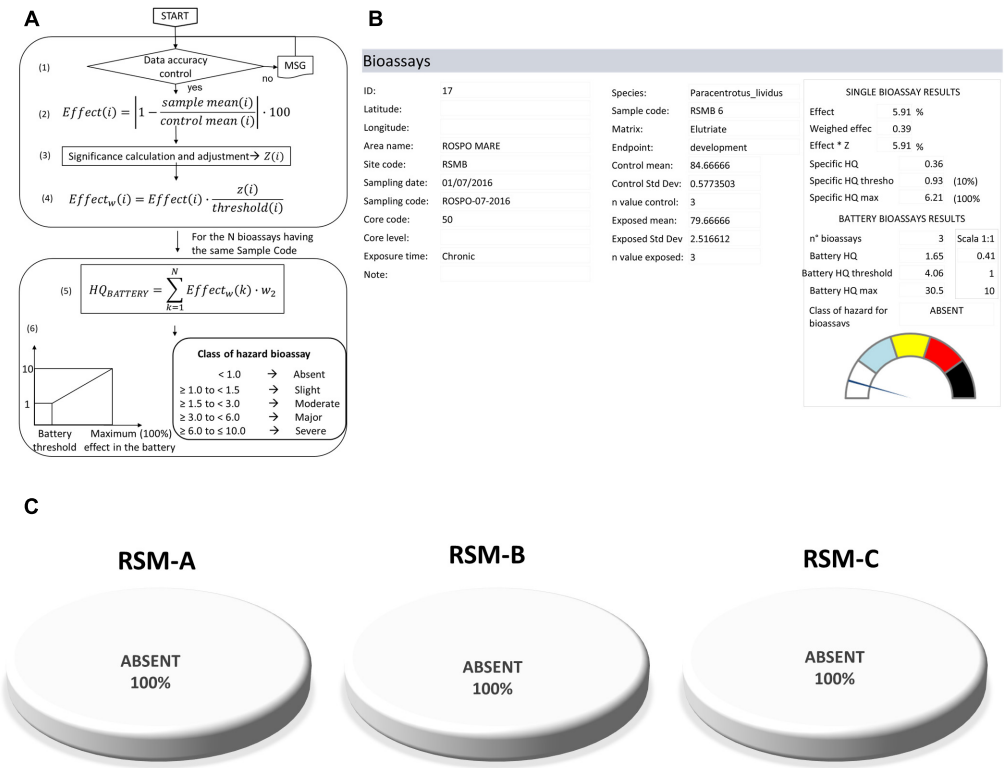


FIGURE 5 | LOE-4: elaboration of ecotoxicological bioassays. **(A)** Flowchart and calculations of the Battery of Bioassays Hazard Quotient ($HQ_{BATTERY}$). **(B)** Model output for bioassays elaborations. **(C)** Results obtained for the three platforms indicating a class of hazard assigned as absent in 100% of samples.

the integration with other LOEs in the final WOE elaboration of ecological risk.

Data Elaboration Through Weight of Evidence (WOE) Ecological Risk Assessment Model

The huge data-sets of results elaborated from the five lines of evidence have been finally integrated through a Weight of Evidence approach (WOE) based on the quantitative model Sediqualsoft. The quantitative hazard quotients (HQs) obtained for each LOEs are normalized to a common scale and given a different weight according to their ecological relevance in the assessment.

In this study, assigned weights to various LOEs were 1.0 for chemical characterization of sediments (LOE-1), 1.2 for bioavailability of chemicals in native and transplanted mussels and 0.6 for bioavailability of metals in laboratory exposed polychaetes (LOE-2), 1.0 for sub-lethal effects on biomarkers in mussels (LOE-3), 1.2 for the ecotoxicological results of the battery of bioassays (LOE-4), 1.3 for composition of benthic communities (LOE-5). An overall WOE level of risk is thus calculated and assigned to 1 of 5 classes of risk from absent to severe (Piva et al., 2011). Scientific criteria, validation of weights and thresholds, expert judgment evaluations and specific flow-charts of each LOE have been validated elsewhere (Piva et al., 2011; Benedetti et al., 2012, 2014).

RESULTS

Chemical Characterization of Sediments (LOE-1)

Chemical analyses of sediments on the three platforms included 60 sampling sites, 53 analytes (individual trace metals, PAHs and aliphatic hydrocarbons), for a total of 3180 analytical results to interpret and compare toward different normative thresholds [i.e., european quality standards (EQS), and other Italian or international sediments guidelines]. Despite the aim of this work is not to discuss the results of the monitoring study but rather the advantages of the integrated WOE elaboration procedure, nonetheless all the measured values are given in **Supplementary Materials** (**Supplementary Table S1**). Concentrations of chemical parameters did not reveal critical values, despite some metals were higher than their respective EQS; among PAHs, low molecular weight hydrocarbons largely prevailed, representing typically more than 90% of the total PAH content.

The weighted criteria based on the number, typology and the magnitude of exceeding contaminants (**Figure 2A**), easily summarized the overall significance of almost 3200 analytical results: the chemical hazard level elaborated toward the European Quality Standards for sediments, ranged mostly between Absent and Negligible in the three platforms without significant differences between sampling distances or different platforms

(**Figure 2B**). The Sediqualsoft model output provides, for all the potentially available sediment normative guidelines, the quantitative value of the calculated HQ_C, the class of hazard and other useful information on obtained results, such as the chemical contributing more to the HQ_C, or the number of exceeding chemicals (**Figure 2C**). A very large scientific information was thus summarized in an easy format for non-expert stakeholders, maintaining the possibility to get important information from the module output: the Moderate chemical hazard observed in 15% of sediments from platform RSM-B was due, for 50–75%, to the contribution of one trace metal (Cr, Cd, or Zn), slightly higher than the corresponding EQS.

Bioaccumulation of Chemicals in Polychaetes and Mussels (LOE-2)

The assessment of bioavailability was evaluated in this study on native and transplanted mussels for both inorganic and organic chemicals and, for trace metals only, also in polychaetes exposed to sediments at laboratory conditions. According to the experimental design on the three platforms and the measured analytes, a total of 2160 results were obtained which are reported in **Supplementary Materials** (**Supplementary Table S2**). Data from *H. diversicolor* revealed often a marked variability in tissue concentrations of metals between organisms exposed to various sediment samples, without clear trends according to platforms or sampling distance. Also bioaccumulation in native and transplanted mussels revealed a few variations, of limited magnitude and without differences between platforms or experimental conditions. The elaboration of bioavailability data through weighted criteria, (**Figure 3A**) allowed to summarize all the data as representing an Absent or Slight hazard in terms of bioavailability for native mussels, transplanted mussels or laboratory exposed polychaetes (**Figure 3B**). The model output clearly visualizes the number of parameters assigned to each class of effect, the quantitative HQ_{BA}, and the overall level of bioavailability hazard among 1 of 5 possible classes (**Figure 3C**).

Biomarker Responses in Mussels (LOE-3)

Biomarkers analyzed in mussels included 16 well recognized responses among metallothioneins, acetylcholinesterase, immune parameters, lysosomal responses, antioxidants and total oxyradical scavenging capacity, lipid peroxidation, DNA damage, for a total of almost 600 results to interpret (see **Supplementary Materials** and **Supplementary Table S3**). Significant variations were occasionally measured for granulocytes/hyalinocytes ratio, metallothioneins, catalase, total antioxidant capacity, malondialdehyde, DNA integrity. Beside the possibility to discuss on specific pathways and mechanisms of action, the complexity of scientific information is summarized by the flow-chart given in **Figure 4A** which elaborates data according to weight and threshold assigned to each biomarker. The overall hazard quotient for biomarkers (HQ_{BM}) is thus calculated from the number of changed biomarkers, their toxicological relevance, statistical significance and magnitude of such variations, all information which are visualized in the Sediqualsoft model output (**Figure 4B**). In this study the hazard index for subcellular effects ranged between Slight and Moderate, depending on the experimental approach and platform (**Figure 4C**), mostly influenced by variations of some immune system responses and antioxidant defenses.

Ecotoxicological Bioassays (LOE-4)

Ecotoxicological characteristics of eight sediment samples for each of the three platforms were evaluated with a battery of four bioassays for a total of almost 100 bioassays tested in triplicate. These results highlighted only a few significant responses for embryotoxicity assay (**Supplementary Materials** and **Supplementary Table S4**). The use of weighted criteria did not consider the worst result for the ecotoxicological classification but elaborated the whole battery based on the biological relevance of each endpoint, and the thresholds derived from the sensitivity of various species (**Figure 5A**). The weighted criteria resulted in an ecotoxicological hazard index which was always summarized as Absent for all the sediment samples of the three platforms: the model output provides all the details on the results obtained for individual bioassays (**Figures 5B,C**).

Status of Benthic Communities (LOE-5)

For benthic communities the analyses of 180 sediment samples from the three platforms allowed to identify 6473 organisms as belonging to 112 taxonomic groups. The WOE Sediqualsoft model did not develop any new index, but developed the informatic tool to elaborate the list of identified species in all the already available community descriptors, diversity indices and ecological indicators, with corresponding evaluations (**Figures 6A,B**). **Supplementary Materials** provide a list of observed species (**Supplementary Table S5a**) and calculated indices of abundance, richness, Margalef, Shannon, Pielou, AMBI, BENTHIX, BOPA, BITS, mAMBI (**Supplementary Table S5b**). In this study, the AMBI index, selected as the community descriptor for the investigated site, provided a hazard quotient classified as Absent or Negligible (**Figure 6C**).

Weight of Evidence Integration (WOE)

The final WOE elaboration, after normalization to a common scale, integrated the Hazard Quotients obtained from various LOEs giving them a different weight according to the ecological relevance of each typology of analyses (**Figure 7**). A total of approximately 7000 heterogeneous analytical results were summarized in a WOE evaluation of Slight risk (**Figure 7**), derived from a combination of specific HQs ranging from Absent (i.e., ecotoxicological bioassays or benthic communities) to Moderate (i.e., biomarker responses).

DISCUSSION

It is now worldwide recognized that the characterization of environmental quality and ecological risk assessment must be addressed with multidisciplinary approaches that integrate traditional chemical analyses of abiotic matrices (water and

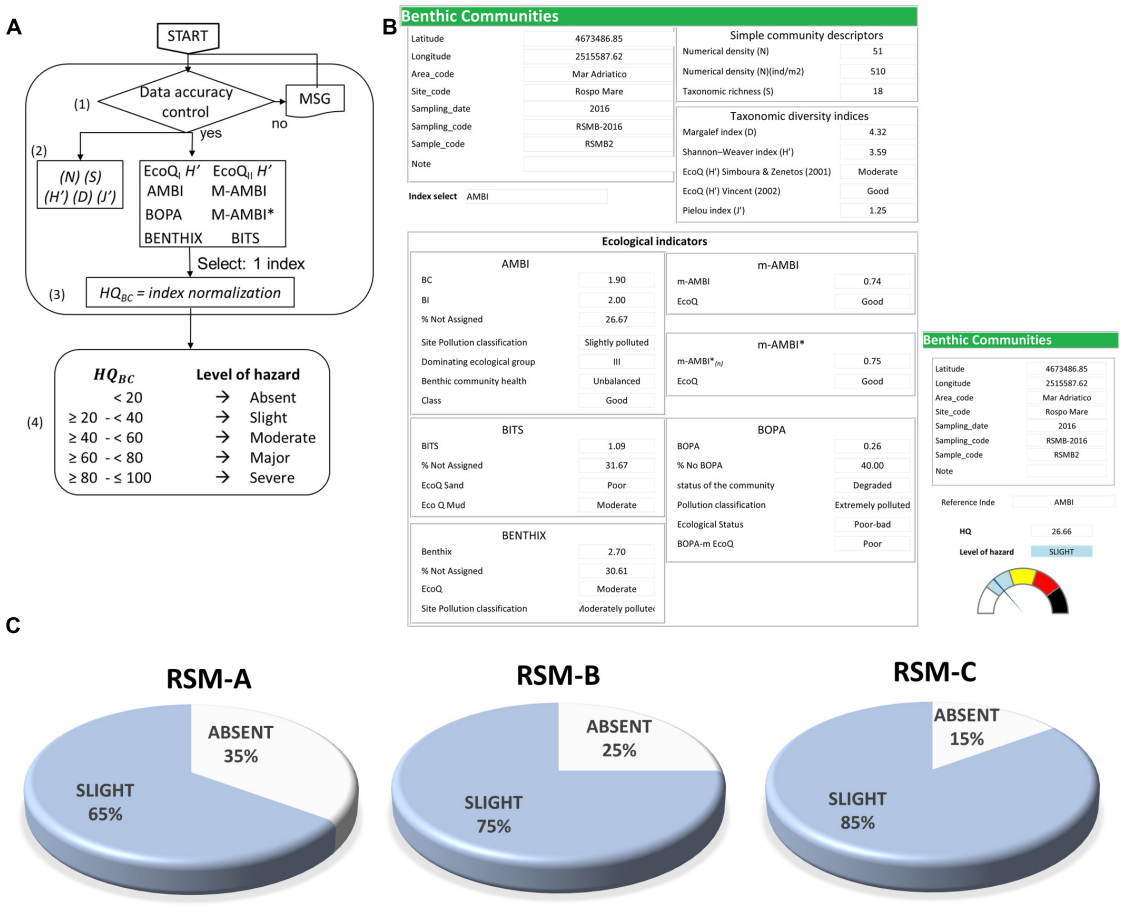


FIGURE 6 | LOE-5: elaboration of benthic communities analyses. **(A)** Flowchart for the calculation of different indices. **(B)** Model output for elaborations of benthic communities. **(C)** Results obtained for the three platforms indicating the percentage of samples assigned to various classes of hazard.

sediments), with those revealing the onset of effects at different levels of biological organization, from bioaccumulation processes and molecular alterations up to population and communities structures (Moore et al., 2004; Hylland, 2006; Chapman, 2007; Viarengo et al., 2007).

The combination of chemical and biological analyses represents an added value for monitoring and management protocols, in line with the recent European Directives which recommend the use of multiple quality indicators for aquatic ecosystems (Lyons et al., 2010, 2017; Lehtonen et al., 2014). Although multidisciplinary approaches are universally accepted from a conceptual point of view, some critical aspects still limit their practical application in standardized procedures of the risk assessment. The interpretation of heterogeneous data often requires expert evaluations, and their integration is even more difficult. The development of quantitative or qualitative scales and indicators is aimed to summarize complex scientific information for an easier interpretation by policy-makers or environmental managers but this approach is generally limited to specific classes of results (Dagnino et al., 2008; Linkov et al., 2009; Piva et al., 2011; Marigómez et al., 2013; Borja et al., 2016, Borja et al., 2017).

The innovative aspect of this study is the application of a quantitative multidisciplinary Weight Of Evidence (WOE) model (Sediqualsoft), able to process and integrate data from five lines of evidence (LOEs) including chemical characterization of sediments, bioavailability, biomarker responses, battery of ecotoxicological assays, status of benthic communities. The presented case-study was the monitoring of an off-shore platforms field in the Adriatic Sea, selected to demonstrate the practical importance of a procedure combining a scientifically sound approach with the possibility to synthesize the overall significance of obtained results.

The most relevant feature of this WOE model is the use of weighted evaluation criteria that for such a huge amount of chemical data allowed to abandon the "pass-to fail" approach, where even a single parameter slightly below or above a threshold, would have determined the chemical classification of sediments. The evaluation of a quantitative index based on the number, typology and magnitude of exceeding chemicals guarantees a more accurate discrimination between samples and comparisons between different areas or periods. Similarly, biological evaluations are not based on variations of individual biomarkers or the worst result for ecotoxicological bioassays,

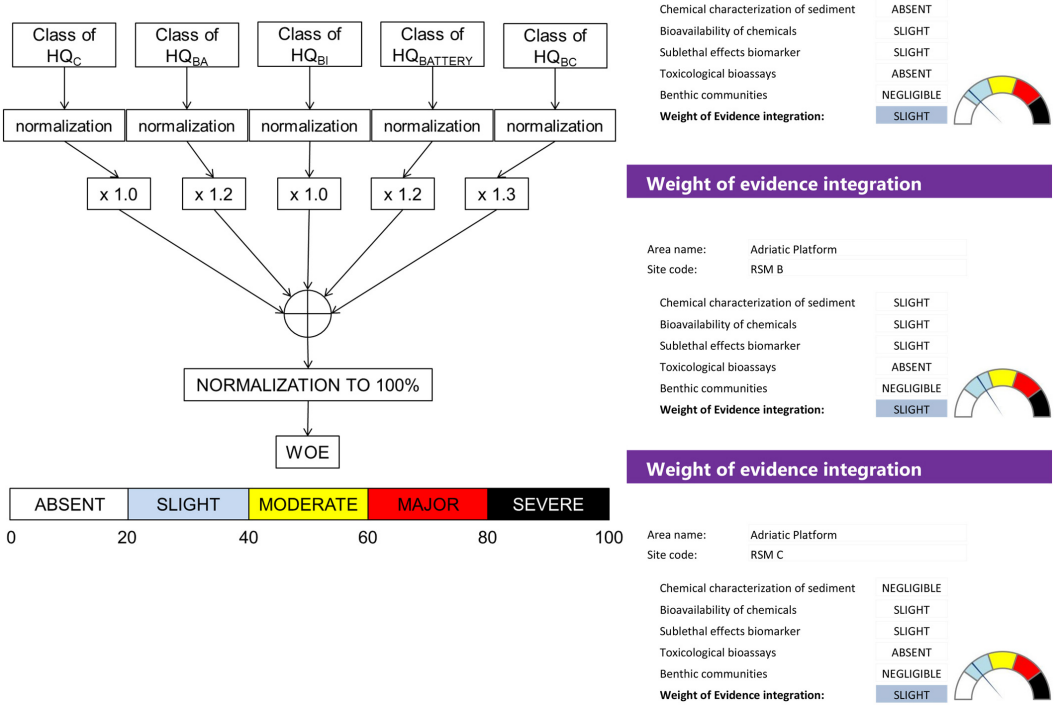


FIGURE 7 | WOE integration with normalization procedure and model output for the three platforms.

allowing a more integrated assessment of the ecotoxicological hazard. Such weighted criteria have been successfully applied in several field studies (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al., 2014; Bebianno et al., 2015; Mestre et al., 2017) and incorporated in the last Italian law for determining quality class and management options for dredged marine sediments based on their chemical and ecotoxicological characterization (DM 173, 16 July, 2016).

Concerning the different typologies of results obtained in this study, the chemical characterization of sediments around the 3 platforms produced almost 3,200 results to be interpreted according to regulatory references or guidelines. A tabular approach applied to such a massive number of data would have not allowed their overall evaluation, the comparison between different areas or periods, nor any effective synthesis and communication of their environmental significance to non-expert stakeholders. In fact, although concentrations were always within typical ranges of values reported for the Adriatic or non-impacted basins, some values exceeding corresponding EQS were measured for a few metals (Dolenec et al., 1998; Zhang et al., 2008; Tobiszewski and Namieśnik, 2012; Benedetti et al., 2014; Etiope et al., 2014). The substantial uniformity of results suggests that these values represent the typical basal levels of the area, and the weighted elaboration provided a class of chemical hazards generally ranging from Absent to Negligible toward EQS: only for three samples the summarized chemical HQ was Medium due to the concentration of Cd, Cr, or Pb reinforcing the evidence that such individual levels can not to be considered as environmental anomalies due to the presence of the platforms.

The assessment of bioavailability allows to evaluate the biological relevance of chemical data and the application of weighted evaluation criteria is of particular utility in environmental risk assessment. In fact, there are only a few EQS for biota, and their application is often too simplistic and not reliable to represent the marked variability in bioaccumulation phenomena and local geochemical features. As an example, the EQS for mercury in biota is 0.02 μg/g (fresh weight), a value that does not consider differences in tissues or the trophic position, and appears inadequate for Mediterranean organisms which, influenced by natural geochemical anomalies of the basin, typically present higher basal levels of this element. With weighted criteria, tissue concentrations in the organisms from the investigated sites are compared to values measured in control organisms, allowing to eliminate also the effect of seasonal variability. Their elaboration considers the typology of each contaminant, as well as the magnitude and the statistical significance of observed variations. In the present study, the bioaccumulation of more than 30 analytes (between metals, polycyclic aromatic

hydrocarbons and aliphatic hydrocarbons) in mussels and polychaetes resulted in approximately 2,200 analytical results that would have been impossible to evaluate based on EQS available for only a few parameters and not always appropriate for Mediterranean organisms. Although obtained results were similar to natural variability intervals already reported for these species (Bocchetti et al., 2008; Fattorini et al., 2008; Gorbi et al., 2008; Benedetti et al., 2014), nonetheless such a large number of data would have also hampered a quantitative comparison between different areas, to discriminate the effects of platforms from natural variability. Based on weighted integration, bioaccumulation data were summarized in a bioavailability hazard ranging between absent and Low depending on the species or experimental conditions, providing a user-friendly format, easy to understand also for non-expert stakeholders.

Biomarkers are recognized for their sensitivity in highlighting the early onset of molecular or cellular alterations, with potential adverse effects on the health conditions of organisms. Widely used to demonstrate the biological impact in polluted environments, they are of particular relevance also in areas with limited anthropogenic pressure since they evaluate integrated responses even when concentrations of individual contaminants are below their respective thresholds of effect (Regoli et al., 2004, 2011, 2014; Regoli and Giuliani, 2014). In this respect, variations of immune and antioxidant biomarkers are widely recognized as early warning signals; however, the complexity of the oxidative network, characterized by several interactions and cascade-effects among different pathways (Regoli and Giuliani, 2014), does not allow to consider variations of individual responses as diagnostic of cellular stress conditions. Routine application of biomarkers in environmental risk assessment procedures is thus often prevented by such difficulty in summarizing data from multiple cellular pathways in simple indices that reflect the overall severity of biological effects. In the present study, the elaboration of results obtained from almost 600 analyses of metabolic systems and biochemical pathways, weighted the toxicological relevance of analyzed biomarkers and normalized the magnitude of observed variations toward specific thresholds for each response. The application of weighted criteria was again fundamental to summarize a large data-set of complex results in a synthetic form; regardless of the significance of variations observed for biomarkers and the mechanistic interactions between immune system and oxidative responses, the overall cellular hazard elaborated from biomarkers ranged from Low to Medium in native mussels, being always Low in transplanted ones. A similar outcome of elaboration does not reduce the scientific relevance of investigated pathways and mechanisms, while being easy to be communicated to stakeholders without an in-depth molecular and cellular knowledge.

The ecotoxicological bioassays carried out with a battery of 4 different species (*Vibrio fischeri*, *Phaeodactylum tricornutum*, *Paracentrotus lividus*, *Acartia tonsa*) provided rather uniform results between different platforms and distances. The only test showing effects of toxicity was the embryo development of sea urchin (*P. lividus*), which would have conditioned the ecotoxicological classification of some sediment samples if the "worst result" approach had been followed. On the other hand, the use of weighted criteria to integrate the results of the whole battery of ecotoxicological bioassays (based on weight of endpoints and sensitivity of different species) always indicated the lack of an ecotoxicological hazard for the sediments collected around the three platforms. The usefulness of summarizing the different responsiveness of bioassays in a synthetic index of the battery, led to the adoption of such weighted criteria in Italian normative on classification and management of dredged sediments allowing to better discriminate the ecotoxicological potential of different samples.

For elaboration of data on benthic communities, there are many descriptors, indices of taxonomic diversity and ecological indicators which have been developed in the last three decades due to the ecological value of these analyses, and the need of standardized evaluation procedures. In this respect, the model used in the present study (Sediqualsoft) was set to automatically calculate all the available indices for selecting the more appropriate in the final integration with other LOEs. Different indices provided some contradictory results around Adriatic off-shore platforms because of the differences in their structure and discrepancies in their assignment of species sensitivity (Spagnolo et al., 2014). In our study, the only significant differences among the collected sediment samples, were obtained in terms of total abundance that, however, were attributable more to the granulometric composition of sediments rather than to a possible impact of the platforms. The application of the AMBI index (Borja et al., 2000) which classifies benthic species of soft substrates as belonging to one of five ecological groups with different levels of sensitivity or tolerance to environmental stress, allowed to classify as Absent or Negligible the hazard for benthic communities in all the samples collected from the three platforms.

Besides the novelty to summarize in quantitative hazard indices the different typologies of data, the presented WOE approach allowed for the first time further integration between various LOEs for a more complex level of risk assessment. In this respect, the environmental survey in the off-shore field of Campo Rospo represents a unique case study applying a quantitative WOE approach to such a large number of heterogeneous data in a complex monitoring scenario. More than 7000 analytical results from different LOEs could be summarized in a quantitative risk index, classified as Slight for the three platforms: beside the local significance of the specific result, the presented elaboration procedure has greater general importance in terms of communication and risk management, still maintaining scientifically robust info derived from the weighted elaboration of various results.

Another practical advantage of summarizing quantitative hazard indices is the possibility to suggest variations of consolidated monitoring protocols. Considering the substantial homogeneity of Hazard Quotients elaborated for different typologies of results at various distances from the three platforms, the redundancy in the number of analyzed samples was mathematically calculated from the 95% confidence interval of obtained results. By averaging the minimum sample size for

each of the three platforms to estimate the HQ values with the required accuracy, it has been calculated that the minimum, still cautionary, number of stations for chemical and benthic analyses should be eight for each platform (instead of the actual 20), sampled with a random design. For bioaccumulation, the analyses of metals in the polychaete *H. diversicolor* exposed at laboratory conditions do not appear to be of particular utility, especially in already monitored areas where such assays always gave negative results, and where control activities continue to be carried out in terms of chemical, ecotoxicological and benthic communities. At the same time, bioaccumulation and biomarker analyses in mussels allow a reliable characterization of organisms health status, and comparison between natural and transplanted, despite logistically more complex, provides useful elements to better characterize the origin and significance of the potential impacts.

At this stage, the presented WOE model (Sediqualsoft) demonstrated how to summarize and quantitatively integrate huge amounts of results data from the five analyzed LOEs, chemical characterization of sediments, bioavailability, biomarker responses, battery of ecotoxicological assays, status of benthic communities. Such typologies of analyses are typically included in most environmental quality assessments, and the outputs of the present work can thus be of value to be applied in other monitoring scenarios. In addition, this model should not be considered as a final version since, maintaining its general structure, it can be easily updated or adapted to local or national specifics to include additional parameters, normative limits, species, biomarkers or ecotoxicological bioassays. Further, additional LOEs can be developed and added as specific modules, to elaborate other typologies of data, such as those from sea water analyses or from other investigations which may depend on objectives and specificities of other case-studies.

From the overall results, this work was fundamental for drawing the following conclusions: (i) the multidisciplinary WOE approach allowed for the first time the qualitative and quantitative assessment of environmental quality in a complex monitoring scenario, represented by an off-shore field. Different typologies of data could be summarized in terms of HQs and levels of hazard, allowing an easy comparison between platforms, distances, and future temporal trends. (ii) The WOE model allows the interpretation of large data sets of scientifically complex and heterogeneous data, without the logic of tabular comparison, and thus increasing the capability to discriminate between various environmental conditions. It would have been impossible to interpret and compare almost 7000 heterogeneous data from more than 60 sites based on the pass-to-fail or worst result approach. (iii) The application of a scientifically robust elaboration to summarize synthetic hazard indices in a user-friendly format, supports a more comprehensive process of risk assessment and "site-oriented" management decisions.

ETHICS STATEMENT

Only invertebrates (bivalves and polychaetes) were analyzed for bioaccumulation and biomarkers: these species are not listed in the National or European guidelines (Directive 2010/63/EU) on the protection of animals used for scientific purposes. Also species used for ecotoxicological bioassays were tested according to standardized protocols and do not require authorization or approval by ethics committee.

AUTHOR CONTRIBUTIONS

FR conceived the study and responsible for carrying out the study, edited, and reviewed the final version of the manuscript. AN, DF, Gd'E, MB, and FR participated in the sampling activities. AN, MM, DF, MB, MDC, and SG carried out the laboratory analyses. Gd'E carried out the statistical and weighted elaboration of data. FR, SG, and DP discussed the results. AN, Gd'E, and FR wrote the manuscript. All authors approved the final version of the manuscript before submission.

FUNDING

This work has been partly supported by funds of the monitoring plan of Edison SpA offshore platforms.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2019.00377/full#supplementary-material

REFERENCES

Bebianno, M. J., Pereira, C. G., Rey, F., Cravo, A., Duarte, D., D'Errico, G., et al. (2015). Integrated approach to assess ecosystem health in harbor areas. *Sci. Total Environ.* 514, 92–107. doi: 10.1016/j.scitotenv.2015.01.050

Beliaeff, B., and Burgeot, T. (2002). Integrated biomarker response: a useful tool for ecological risk assessment. *Environ. Toxicol. Chem.* 21, 1316–1322. doi: 10.1002/etc.5620210629

Benedetti, M., Ciaprini, F., Piva, F., Onorati, F., Fattorini, D., Notti, A., et al. (2012). A multidisciplinary weight of evidence approach toward polluted sediments: integrating sediment chemistry, bioavailability, biomarkers responses and bioassays. *Environ. Int.* 38, 17–28. doi: 10.1016/j.envint.2011.08.003

Benedetti, M., Giuliani, M. E., and Regoli, F. (2015). Oxidative metabolism of chemical pollutants in marine organisms: molecular and biochemical biomarkers in environmental toxicology. *Ann. N. Y. Acad. Sci.* 1340, 8–19. doi: 10.1111/nyas.12698

Benedetti, M., Gorbi, S., Fattorini, D., Pacitti, D., D'Errico, G., Piva, F., et al. (2014). Environmental hazards from natural seepage: integrated classification of risk from sediment chemistry, bioavailability and biomarkers responses in sentinel species. *Environ. Pollut.* 185, 116–126. doi: 10.1016/j.envpol.2013.10.023

Bocchetti, R., Fattorini, D., Pisanelli, B., Macchia, S., Oliviero, L., Pilato, F., et al. (2008). Contaminant accumulation and biomarker responses in caged mussels, Mytilus galloprovincialis, to evaluate bioavailability and toxicological effects of remobilized chemicals during dredging and disposal operations

in harbour areas. *Aquat. Toxicol.* 89, 257–266. doi: 10.1016/j.aquatox.2008.07.011

Borja, A., Elliott, M., Andersen, J. H., Berg, T., Carstensen, J., Halpern, B. S., et al. (2016). Overview of integrative assessment of marine systems: the ecosystem approach in practice. *Front. Mar. Sci.* 3:20. doi: 10.3389/fmars.2016.00020

Borja, A., Elliott, M., Uyarra, M. C., Carstensen, J., and Mea, M. (2017). Editorial: bridging the gap between policy and science in assessing the health status of marine ecosystems. *Front. Mar. Sci.* 4:32. doi: 10.3389/fmars.2017.00032

Borja, A., Franco, J., and Pérez, V. (2000). A marine Biotic Index to establish the ecological quality of soft-bottom benthos within European estuarine and coastal environments. *Mar. Pollut. Bull.* 40, 1100–1114. doi: 10.1016/S0025-326X(00)00061-8

Broeg, K., and Lehtonen, K. K. (2006). Indices for the assessment of environmental pollution of the Baltic Sea coasts: integrated assessment of a multi-biomarker approach. *Mar. Pollut. Bull.* 53, 508–522. doi: 10.1016/j.marpolbul.2006.02.004

Chapman, P. M. (2007). Determining when contamination is pollution – weight of evidence determinations for sediments and effluents. *Environ. Inter.* 33, 492–501. doi: 10.1016/j.envint.2006.09.001

Dagnino, A., Sforzini, S., Dondero, F., Fenoglio, S., Bona, E., Jensen, J., et al. (2008). A "Weight-of-Evidence" approach for the integration of environmental "Triad" data to assess ecological risk and biological vulnerability. *Integr. Environ. Assess. Manag.* 4, 314–326. doi: 10.1897/IEAM_2007-067.1

Dauvin, J.-C. (2015). History of benthic research in the English channel: from general patterns of communities to habitat mosaic description. *J. Sea Res.* 100, 32–45. doi: 10.1016/j.seares.2014.11.005

Dauvin, J. C., and Ruellet, T. (2007). Polychaete/amphipod ratio revisited. *Mar. Pollut. Bull.* 55, 215–224. doi: 10.1016/j.marpolbul.2006.08.045

Davies, I. M., and Vethaak, A. D. (2012). Integrated monitoring of chemicals and their effects. *ICES Coop. Res. Rep.* 315:227.

Dolenec, T., Faganeli, J., and Pirc, S. (1998). Major, minor and trace elements in surficial sediments from the open Adriatic sea: a regional geochemical study. *Geol. Croat.* 51, 47–58.

Etiope, G., Panieri, G., Fattorini, D., Regoli, F., Vannoli, P., Italiano, F., et al. (2014). A thermogenic hydrocarbon seep in shallow Adriatic Sea (Italy): gas origin, sediment contamination and benthic foraminifera. *Mar. Petrol. Geol.* 57, 283–293. doi: 10.1016/j.marpetgeo.2014.06.006

Fattorini, D., Notti, A., Di Mento, R., Cicero, A. M., Gabellini, M., Russo, A., et al. (2008). Seasonal, spatial and inter-annual variations of trace metals in mussels from the Adriatic Sea: a regional gradient for arsenic and implications for monitoring the impact of off-shore activities. *Chemosphere* 72, 1524–1533. doi: 10.1016/j.chemosphere.2008.04.071

Gorbi, S., Virno Lamberti, C., Notti, A., Benedetti, M., Fattorini, D., Moltedo, G., et al. (2008). An ecotoxicological protocol with caged mussels, Mytilus galloprovincialis, for monitoring the impact of an offshore platform in the Adriatic sea. *Mar. Environ. Res.* 65, 34–49. doi: 10.1016/j.marenvres.2007.07.006

HELCOM (2010). *Ecosystem Health of the Baltic Sea 2003–2007: HELCOM Initial Holistic Assessment*. Helsinki: HELCOM.

Hylland, K. (2006). Biological effects in the management of chemicals in the marine environment. *Mar. Pollut. Bull.* 53, 614–619. doi: 10.1016/j.marpolbul.2006.08.010

Hylland, K., Burgeot, T., Martínez-Gómez, C., Lang, T., Robinson, C. D., Svavarsson, J., et al. (2017). How can we quantify impacts of contaminants in marine ecosystems? *ICON Project. Mar. Environ. Res.* 124, 2–10. doi: 10.1016/j.marenvres.2015.11.006

ISPRA (2017). *Saggio di fecondazione e saggio di sviluppo embrionale con il riccio di mare Paracentrotus lividus (Lamarck) (Echinodermata: Echinoidea)*. Rome: ISPRA.

Lehtonen, K. K., Sundelin, B., Lang, T., and Strand, J. (2014). Development of tools for integrated monitoring and assessment of hazardous substances and their biological effects in the Baltic Sea. *AMBIO* 43, 69–81. doi: 10.1007/s13280-013-0478-3

Linkov, I., Loney, D., Cormier, S., Satterstrom, F. K., and Bridges, T. (2009). Weight-of-evidence evaluation in environmental assessment: review of qualitative and quantitative approaches. *Sci. Total Environ.* 407, 5199–5205. doi: 10.1016/j.scitotenv.2009.05.004

Lyons, B. P., Bignell, J. P., Stentiford, G. D., Bolam, T. P. C., Rumney, H. S., Bersuder, P., et al. (2017). Determining good environmental status under the marine strategy framework directive: case study for descriptor 8 (chemical contaminants). *Mar. Environ. Res.* 124, 118–129. doi: 10.1016/j.marenvres.2015.12.010

Lyons, B. P., Thain, J. E., Hylland, K., Davis, I., and Vethaak, A. D. (2010). Using biological effects tools to define good environmental status under the marine strategy framework directive. *Mar. Pollut. Bull.* 60, 1647–1651. doi: 10.1016/j.marpolbul.2010.06.005

Marigómez, I., Garmendia, L., Soto, M., Orbea, A., Izagirre, U., and Cajaraville, M. P. (2013). Marine ecosystem health status assessment through integrative biomarker indices: a comparative study after the Prestige-oil spill "Mussel Watch". *Ecotoxicology* 22, 486–505. doi: 10.1007/s10646-013-1042-4

Mestre, N. C., Rocha, T. L., Canals, M., Cardoso, C., Danovaro, R., Dell'Anno, A., et al. (2017). Environmental hazard assessment of a marine mine tailings deposit site and potential implications for deep-sea mining. *Environ. Pollut.* 228, 169–178. doi: 10.1016/j.envpol.2017.05.027

Mistri, M., and Munari, C. (2008). BITS: a SMART indicator for soft-bottom, non-tidal lagoons. *Mar. Pollut. Bull.* 56, 587–599. doi: 10.1016/j.marpolbul.2007.12.002

Moore, M. N., Depledge, M. H., Readman, J. W., and Leonard, P. D. R. (2004). An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. *Mutat. Res.* 18, 247–268. doi: 10.1016/j.mrfmmm.2004.06.028

Moore, M. N., Icarus Allen, J., and McVeigh, A. (2006). Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. *Mar. Environ. Res.* 61, 278–304. doi: 10.1016/j.marenvres.2005.10.005

Muxika, I., Borja, Á., and Bald, J. (2007). Using historical data, expert judgement and multivariate analysis in assessing reference conditions and benthic ecological status, according to the European Water Framework Directive. *Mar. Pollut. Bull.* 55, 16–29. doi: 10.1016/j.marpolbul.2006.05.025

Newman, M. C., and Clements, W. H. (2007). *Ecotoxicology: A Comprehensive Treatment, 1st Edition. 880 pag, ISBN 9781420005011*. Boca Raton: CRC Press.

OSPAR (2013). *Technical Annex on Supporting Parameters for Biological Effects Measurements in Fish and Mussels. Background Document and Technical Annexes for Biological Effects Monitoring, Update 2013, Monitoring and Assessment Series 589/2013. ISBN 978-1-909159-22-8*. Paris: OSPAR.

Pittura, L., Avio, C. G., d'Errico, G., Keiter, S., and Cormier, B. (2018). Microplastics as vehicles of environmental PAHs to marine organisms: combined chemical and physical hazards to the Mediterranean mussels, Mytilus galloprovincialis. *Front. Mar. Sci.* 5:103. doi: 10.3389/fmars.2018.00103

Piva, F., Ciaprini, F., Onorati, F., Benedetti, M., Fattorini, D., Ausili, A., et al. (2011). Assessing sediment hazard through a Weight of Evidence approach with bioindicator organisms: a practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays. *Chemosphere* 83, 475–485. doi: 10.1016/j.chemosphere.2010.12.064

Regoli, F., Frenzilli, G., Bocchetti, R., Annarumma, F., Scarcelli, V., Fattorini, D., et al. (2004). Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, Mytilus galloprovincialis, during a field translocation experiment. *Aquat. Toxicol.* 68, 167–178. doi: 10.1016/j.aquatox.2004.03.011

Regoli, F., and Giuliani, M. E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. *Mar. Environ. Res.* 93, 106–117. doi: 10.1016/j.marenvres.2013.07.006

Regoli, F., Giuliani, M. E., Benedetti, M., and Arukwe, A. (2011). Molecular and biochemical biomarkers in environmental monitoring: a comparison of biotransformation and antioxidant defense systems in multiple tissues. *Aquat. Toxicol.* 105, 56–66. doi: 10.1016/j.aquatox.2011.06.014

Regoli, F., Pellegrini, D., Cicero, A. M., Nigro, N., Benedetti, M., Gorbi, S., et al. (2014). A multidisciplinary weight of evidence approach for environmental risk assessment at the Costa Concordia wreck: integrative indices from Mussel Watch. *Mar. Environ. Res.* 96, 92–104. doi: 10.1016/j.marenvres.2013.09.016

Semenzin, E., Critto, A., Rutgers, M., and Marcomini, A. (2008). Integration of bioavailability, ecology and ecotoxicology by three lines of evidence into

ecological risk indexes for contaminated soil assessment. *Sci. Total Environ.* 389, 71–86. doi: 10.1016/j.scitotenv.2007.08.032

Sigovini, M., Keppel, E., and Tagliapietra, D. (2013). M-AMBI revisited: looking inside a widely-used benthic index. *Hydrobiologia* 717, 41–50. doi: 10.1007/s10750-013-1565-y

Simboura, N., and Zenetos, A. (2002). Benthic indicators to use in Ecological Quality classification of Mediterranean soft bottom marine ecosystems, including a new Biotic Index. *Mediterr. Mar. Sci.* 3, 77–111. doi: 10.12681/mms.249

Spagnolo, A., Punzo, E., Santelli, A., Scarcella, G., Strafella, P., Grati, F., et al. (2014). Offshore platforms: comparison of five benthic indicators for assessing the macrozoobenthic stress levels. *Mar. Pollut. Bull.* 82, 55–65. doi: 10.1016/j.marpolbul.2014.03.023

Tobiszewski, M., and Namieśnik, J. (2012). PAH diagnostic ratios for the identification of pollution emission sources. *Environ. Pollut.* 162, 110–119. doi: 10.1016/j.envpol.2011.10.025

Vethaak, A. D., Davies, I. M., Thain, J. E., Gubbins, M. J., Martínez-Gómez, C., Robinson, C. D., et al. (2017). Integrated indicator framework and methodology for monitoring and assessment of hazardous substances and their effects in the marine environment. *Mar. Environ. Res.* 124, 11–20. doi: 10.1016/j.marenvres.2015.09.010

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., and Koehler, A. (2007). The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 146, 281–300. doi: 10.1016/j.cbpc.2007.04.011

Zhang, W., Zhang, S., Wan, C., Yue, D., Ye, Y., and Wang, X. (2008). Source diagnostics of polycyclic aromatic hydrocarbons in urban road runoff, dust, rain and canopy throughfall. *Environ. Pollut.* 153, 594–601. doi: 10.1016/j.envpol.2007.09.004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

PERSPECTIVE
published: 24 July 2019
doi: 10.3389/fmars.2019.00454

The Use of Cryopreserved Biological Material for Water Quality Assessment

Estefania Paredes[1]† and Juan Bellas[2]†*

[1] Marine Biological Resources Functional Preservation Service, Estación de Ciencias Mariñas de Toralla, Universidade de Vigo, Vigo, Spain, [2] Centro Oceanográfico de Vigo, Instituto Español de Oceanografía, Galicia, Spain

The stated aim of this perspective article is to present new developments and discuss future directions on the applications of cryopreserved organisms to marine water quality assessment. To facilitate this, the authors provide a background of essential knowledge of cryopreservation when applied to ecotoxicology, as well as, practical examples available in literature. An integrated approach with combined monitoring of chemical status plus measurements of biological effects has been recommended extensively by international institutions for the assessment of marine pollution. Among the available techniques, bioassays have been considered as sufficiently robust to be incorporated in marine pollution monitoring programs. However, the routine application of bioassays has also allowed the identification of one of the factors that limits a more extensive use of such biological methods: the availability of biological material throughout the year, regardless of natural spawning periods. A solution to this limitation is the application of cryopreservation techniques. Cryopreservation may, for instance, provide access to stable quality biological material when test species are out of the reproductive season, without the need for maintaining and conditioning organisms in the laboratory. It also guarantees access to a large variety of species that might not be available at the same time of the year and, on top of that, cryopreservation provides opportunities to laboratories that might not have the facilities to keep all these organisms in culture.

Keywords: cryopreservation, water quality assessment, cryobiology, bioassay, model organisms

OPEN ACCESS

Edited by:
Naser A. Anjum,
Aligarh Muslim University, India

Reviewed by:
Gabriela Verónica Aguirre-Martínez,
Universidad Arturo Prat, Chile
Benoit Xuereb,
University of Le Havre, France

***Correspondence:**
Estefania Paredes
eparedes@uvigo.es

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *12 December 2018*
Accepted: *08 July 2019*
Published: *24 July 2019*

Citation:
Paredes E and Bellas J (2019) The Use of Cryopreserved Biological Material for Water Quality Assessment. Front. Mar. Sci. 6:454. doi: 10.3389/fmars.2019.00454

INTRODUCTION

Water quality assessment is crucial for achieving good chemical and biological status throughout coastal waters and current approaches include the monitoring of responses at different levels of biological organization to indicate effects on the ecosystem. Integrative approaches, intended for the protection of the marine environment, are based mainly on the use of biological tools at different trophic levels in combination with chemical measures, in order to establish environmental damage thresholds (Lyons et al., 2010). In fact, the European Union Marine Strategy Framework Directive (2008/56/EC), which has the objective of achieving and maintaining the Good Environmental Status (GES) in European seas by 2020, emphasizes the need to evaluate and keep within acceptable limits the biological effects of pollutants.

Chemical analyses can identify many contaminants present in the environment, whilst biological methods permit to obtain ecologically relevant information. Among the biological tools that have been considered sufficiently robust for marine pollution assessment, ecotoxicological bioassays present several advantages such as: the detection of new pollutants for which analytical techniques have not yet been developed, provide information about the bioavailability of the pollutants (i.e., the fraction of pollutant that can be incorporated by the organism); they allow to integrate the toxic effects of the different substances present in the environment, and present a good cost/effect ratio (e.g., Stebbing et al., 1980; Calow, 1993).

As useful as they can be, the application of biological techniques using bioassays in routine monitoring has allowed to identify one of the factors that limit a more extensive use of this tools: obtaining biological material of stable high quality throughout the year, regardless of the natural spawning periods (His et al., 1999a).

A great number of response variables can be measured at different levels of biological organization and at different trophic levels in order to determine the GES of the marine environment (e.g., Lyons et al., 2010; Davies and Vethaak, 2012). A wide range of organisms have been considered for marine pollution monitoring, including microorganisms like marine bacteria (Gellert, 2000; Parvez et al., 2006), microalgae (Debelius et al., 2009; Aylagas et al., 2014; Araujo and Moreno-Garrido, 2015), marine invertebrates (Snell and Persoone, 1989; His et al., 1999a; Bellas et al., 2005; Bellas, 2008; Laranjeiro et al., 2015; Perez Fernández et al., 2015) or fish (Hutchinson et al., 1994; EPA, 2002), in all these examples the endpoints are either hatching, growth or normal development along time.

The cryopreservation and cryobanking of test organisms to be used for marine quality assessment, could ensure the accessibility to organisms or their reproductive material all year round as an alternative to either conditioning adults or continuous culture efforts for availability of biological material, which is a very time consuming and expensive process. Biobanking these test organisms in a stable manner (below −135°C) is possible, either using liquid nitrogen or ultrafreezers. At this low temperature, no chemical reactions take place and cellular metabolism is on hold. These stored cells are stopped in time and their viability would only be affected by background radiation, which at normal level will take 2000 years to become a hazard to stored cells (Glenister et al., 1984). There are not many marine cells biobanks apart from culture collections (usually microalgae and/or bacteria), but this is beginning to change (mainly at local level) as cryopreservation becomes a more popular tool and many Marine Biological Research Stations acquire biobanking equipment.

The aim of this perspective paper is to present new developments and discuss future directions on the applications of cryopreserved organisms to marine water quality assessment. To facilitate this, the authors provide a background of essential knowledge of cryopreservation when applied to ecotoxicology, as well as, practical examples available in literature.

CRYOPRESERVATION AND MARINE WATER QUALITY ASSESSMENT

The application of cryopreservation techniques to marine water quality assessment requires the development and standardization of specific cryopreservation protocols for different types of organisms. The main question that needed to be answered was if cryopreserved organisms would be sensitive enough to detect gradual increases of toxic compounds in the water. If so, they could be used to obtain dose-response curves. It was also necessary to compare and establish the differences, or lack thereof, in sensitivity when using fresh and cryopreserved biological material. Regarding the first point, as listed below it has been proved that cryopreserved organisms can be used to detect gradual increases in the concentrations of chemical compounds present in the water, both with single chemicals and with complex natural samples. Cryopreserved organisms can therefore be used to produce dose-response curves and to obtain the No Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC), or 10 and 50% Effective Concentrations (EC10 or EC50), as well as their fresh counterparts. In this paper we present a comprehensive list indicating examples of bioassays that specifically reported the use of cryopreserved organisms as an alternative to standard bioassays with fresh organisms (methodological information is indicated in **Table 1**), each case will be discussed in terms of their comparability with the standard method (toxicological information is indicated in **Table 2**).

Cryopreserved Microalgae

Microalgae are an important part of the food chain in the ocean. A disruption of the basis of the food chain would have deep long lasting effects in the ecosystems and therefore they are of high ecotoxicological relevance (Arensberg et al., 1995; Geis et al., 2000). It has been shown that microalgae are more sensitive than other test organisms to some compounds like metals (Wong and Beaver, 1980; Satoh et al., 2005; Araújo et al., 2010) detergents (Lewis, 1990) or herbicides (Pavlic et al., 2006).

Use of cryopreserved freshwater algae *Selenastrum capricornutum* in ecotoxicity testing has been evaluated by Benhra et al. (1997). Experiments compared the performance of this method, named Cryoalgotox, versus the classic microplate test using fresh algae. *S. capricornutum* was cryopreserved by slow cooling (**Table 1**) using 10% (v/w) polyvinylpyrrolidone (PVP) as a cryoprotecting agent (CPA) giving comparable toxicity results. After 72 h incubation, Cryoalgotox produced lower 50% effective concentrations (EC50s) for Cd^{2+}, Cu^{2+}, Cr^{6+}, and atrazine (i.e., higher sensitivity) than the classical microplate tests, which was explained by the periodic renewal of the test medium in the semistatic procedure. This test assay using cryopreserved microalgae produced highly repeatable results (low coefficients of variation).

Hundreds of cryopreservation protocols have been published for both freshwater and marine microalgae that could potentially be used to develop more bioassays with cryopreserved material. Despite most of the microalgae currently held in culture collections are kept cryopreserved and, therefore, most of the microalgae toxicity test are probably carried out with algae

TABLE 1 | Cryopreserved marine organisms that had been used as an alternative to fresh standard methods for evaluating marine water quality.

Species	Viability	Cryopreservation and conservation	Advantages	Comparison with standard
S. capricornutum (Benhra et al., 1997)	Toxicity tests with cryopreserved algae lead to lower EC50s than standard methods. High repeatability and reliability.	Cryoprotectant used is 10 %PVP (w/v), Addition (1:1) of the cryoprotectant to the algae and allow to equilibrate for 30 min at 22°C under light. Cells were cooled at 1.5°C min^{-1} until −30°C and faster at 10.5°C/min until −80°C. Storage in a −80°C freezer up to 90 days. Thawing by immersion in a water bath 37°C until ice melting. No washing of the PVP needed prior inoculation for culture.	Rapid method, no preculture needed. Cost-effectiveness by elimination of algal stock cultures.	Ratio between EC50s obtained by classic/cryoalgotox rages from 1.3 to 1.4
C. gigas and *T. philippinarum* (McFadzen, 1992)	They concluded that cryopreserved D-veliger larvae were sensitive to environmentally realistic levels of contaminants and can be used for water quality assessment.	Patent number PCT/GB90/01267 Filled on 13/08/1990. Cooling from 20°C to −20°C at 16°C min^{-1} and then at 45°C min^{-1} to −45°C then stored in liquid nitrogen preferably. Cryprotecting agents used were 15% Me_2SO (v/v) + 1 M Trehalose and 0.5 $mgml^{-1}$ crystallized cholesterol. Thawing in water bath at 22–28°C	Immediate access to biological material all year-round.	N/A
P. lividus (Paredes and Bellas, 2015)	Comparative bioassays with fresh/cryopreserved sea urchin embryos. Cryopreserved embryos usually yielded more sensitive results. Can be used for water quality assessment.	Cryopreservation protocol using Me_2SO 1.5 M + 0.04 M Trehalose. One milliliter of CPA solution was added in 15 equimolar steps 1 min apart. The cooling ramp started with a hold at 4°C for 2 min, cooled at a rate of 1°C min^{-1} to −12°C, followed by cooling at 1°C min^{-1} to −80°C and vials were transferred to liquid nitrogen for storage. Thawing was performed by immersion into a 17°C water bath until the ice was melted. CPAs were then removed in 12 equimolar steps.	Immediate access to biological material all year-round.	Correlation between EC50s obtained with classic/cryopreserved sea urchin embryos is $y = 0.68x+0.53$, $n = 4$
S. aurata (Fabbrocini et al., 2013)	Toxicity tests with cryopreserved sperm. Analysis of motility parameters visually and sperm velocity with CASA. Cryopreserved sperm can be sufficiently sensitive to be used for bioassays.	Cryopreservation protocol detailed in Fabbrocini et al. (2000). The Cryoprotecting agent used was 5% Me_2SO. Cooled in straws at 10–15°C min^{-1} to −150 and stored in liquid nitrogen. Thawing at 15°C min^{-1}.	Rapid and easy method.	Computer assisted analysis of the samples lead to significantly lower NOEC/LOEC values than visual examination of motility. CASA parameters produce a LOEC Coherent with other fish sperm samples.

Me_2SO stands for Dimethyl sulfoxide.

that had been cryopreserved at some point, there are no other published comparisons for cultured vs. cryopreserved marine microalgae as far as the authors know.

Cryopreserved Molluscs

Molluscs have been extensively used for several ecotoxicological tests, among which stands out the embryo-larval bioassay (International Council for the Exploration of the Sea (ICES), 1991; His et al., 1999b). The high sensitivity of early-life stages allows the detection of low pollution levels by the identification of effects in the embryonic development (delays or morphological abnormalities) after a short period of exposure/incubation in the presence of a toxicant or a water sample of unknown quality. Oysters, such as *Crassostrea gigas* (His et al., 1999b; Leverett and Thain, 2013) and mussels, such as *Mytilus edulis* (Nolan and Duke, 1983) or *Mytilus galloprovincialis* (His et al., 1997; Beiras and Bellas, 2008), are the star test species for this procedure for being well known and studies species but also for their worldwide distribution.

Cryopreserved bivalve larvae (*C. gigas* and *Tapes philippinarum* larvae) have been exposed to different water samples and shown to be sensitive to environmentally realistic levels of contaminants for field monitoring of water quality (McFadzen, 1992). This was the first attempt to use cryopreserved cells of any type for ecotoxicology studies proving that those cells retain the sensitivity to chemicals and could be used for bioassays.

Larvae were cryopreserved at 24 h for *C. gigas* and 48 h for *T. philippinarum* at the late trochophore/early D-veliger stages (**Table 1**) and stored in liquid nitrogen at (−196°C), while using 15% dimethyl sulfoxide (v/v) with 1.0 M Trehalose and

TABLE 2 | Available toxicological information for different contaminants using cryopreserved cells.

Organism	Compound	Standard method (μgL^{-1})	Cryopreserved (μgL^{-1})
S. capricornutum	Cd^{2+}	72 h EC50 ± *SD* = 43.5 ± 3.4	72 h EC50 ± *SD* = 31.8 ± 0.9
S. aurata		N/A	CASA Motility parameters LOEC = 10
S. capricornutum	Cu^{2+}	72 h EC50 ± *SD* = 28.5 ± 2.8	72 h EC50 ± *SD* = 21.7 ± 0.8
P. lividus		48 h EC50 95% c.i. = 34.1 (31.9–63.4)	96 h EC50 95% c.i. = 53.7 (51.9–55.5)
S. capricornutum	Cr^{6+}	48 h EC50 ± *SD* = 139.1 ± 31.1	96 h EC50 ± *SD* = 74.3 ± 5
S. capricornutum	Antrazine	48 h EC50 ± *SD* = 164.3 ± 37	96 h EC50 ± *SD* = 92.9 ± 2
P. lividus	Pb^{2+}	48 h EC50 95% c.i. = 425 (236.8–590.1)	96 h EC50 95% c.i. = 81 (79.1–83.0)
P. lividus	BP-3	48 h EC50 95% c.i. = 4048.6 (1950.6–6218.7)	96 h EC50 95% c.i. = 1541 (1257.5–1824.5)
P. lividus	4-MBC	48 h EC50 95% c.i. = 389.2 (254.8–523.6)	96 h EC50 95% c.i. = 300.6 (141.2–460.0)

Heavy metals like Cadmium (Cd^{2+}), Copper (Cu^{2+}), Chrome (Cr^{6+}), or Lead (Pb^{2+}), pesticides like Antrazine or emerging pollutants like UV-filters like 4-methylbenzylidene-camphor (4-MBC) and benzophenone-3 (BP-3). EC50 data provided with either the standard deviation or 95% confidence intervals (C.I.) for: microalgae (S. capricornutum),fish sperm (S. aurata), and sea urchin embryos (P. lividus). References available in ***Table 1****.*

0.5 mg/ml cholesterol as CPAs. Survival was reported as highly variable upon thawing. Despite no comparison between fresh and cryopreserved cells was carried out at the time, cryopreserved cells responded to toxicity and allowed for the calculation of toxicological parameters.

The description of cryopreservation protocols for marine invertebrates is also flourishing and protocols for molluscs like the mussels *M. galloprovincialis* (Paredes et al., 2013) and *Perna canaliculus* (Paredes et al., 2012) have been developed. Results with bivalves are promising, since the cryopreservation methods for these organisms have been proven to be reliable, repeatable and sensitive, being on an advanced stage of development. A way forward would be to test the comparison between the procedures with cryopreserved organisms and standard tests, which have not yet been performed.

Cryopreserved Echinoids

Sea urchins are other of the classic models (Bellas et al., 2005; Durán and Beiras, 2010) for water quality testing. Paredes and Bellas (2015) established for the first time a bioassay using cryopreserved sea-urchin embryos (*Paracentrotus lividus*) (Paredes et al., 2011) and provided a comparison with the already standardized sea urchin embryo larval bioassay for standard chemicals like copper and lead (**Figure 1**).

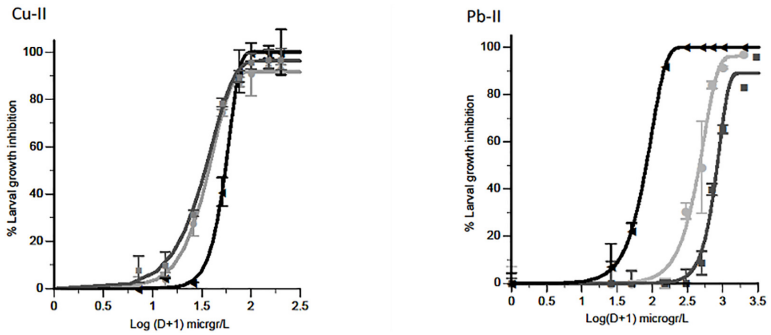
Sea urchin embryos (early blastula) were cryopreserved using 1.5 M dimethyl sulfoxide plus 0.04 M trehalose and cooled at 1°C min^{-1} (protocol in **Table 1**). Samples were then stored in liquid nitrogen. These experiments showed that there was no significant loss in sensitivity when using early blastulas instead of fresh fertilized oocytes. Paredes and Bellas (2015) did find differences in sensitivity when using cryopreserved vs. fresh cells, in some cases the differences were minimal, in other cases the cryopreserved test was clearly more sensitive (**Table 2**). This increased sensitivity may be because cryopreserved organisms are going through a recovery process after thawing, and might be more sensitive to additional stress, such as toxicant exposure.

Ribeiro et al. (2018) developed a cryopreservation protocol for *Echinometra lucunter* sperm and they are studying the cryopreservation of embryos for water quality assessment. There is a cryopreservation protocol described for *P. lividus* sperm (Fabbrocini et al., 2014) that yields good motility. Cryopreservation protocols already exist or are under development for different developmental stages for 10–14 different sea urchin species (embryos and sperm), and since sea urchins are a highly demanded model, more applications will probably be further developed soon using cryopreserved cells, including toxicology (Paredes, 2015a).

Cryopreserved Fish Sperm

The case of fish cryopreservation (but also crustaceans) is more complicated, since these organisms are very sensitive to low temperatures and have proven exceptionally difficult to cryopreserve, being fish sperm the only exception. There has been exhaustive research on marine fish sperm cryopreservation and protocols have been described for most farmed species (*Sparus aurata* by Fabbrocini et al., 2012, 2016; *Dicentrarchus labrax* by Fauvel et al., 1998; or *Mugil cephalus* by Balamurugan and Munuswamy, 2017), any of which could be used as a biomonitoring test organism.

The study by Fabbrocini et al. (2013) evaluated the feasibility of using cryopreserved *S. aurata* spermatozoa to be used in toxicity tests (**Table 1**). Sperm motility parameters were evaluated



	Embryo-larval bioassays EC50 (μg L^{-1})		
	Standard	Fresh embryo	Cryopreserved embryo
Cu^{2+}	34.1 (31.9 - 36.4)	32.2 (29.1 - 35.3)	53.7 (51.9 - 55.5)
Pb^{2+}	425 (263.8 - 590.1)	752 (688.7 - 816.2)	81 (79.1 - 83.0)

FIGURE 1 | Copper and lead toxicity tests. Larval growth inhibition at each concentration ± *SD* (*n* = 35). Light gray, dark gray, and black represent the standard bioassay, the fresh embryo bioassay and cryopreserved embryo bioassay, respectively. Table attached shows EC50s (μg L^{-1}) and 95% confidence intervals (in brackets). Figures modified from Paredes and Bellas (2015).

after thawing by a computer-assisted analysis. The sensitivity of the sperm (motility percentages and velocities) to a reference toxicant (cadmium) was comparable to what has been recorded for the fresh sperm of other aquatic species (**Table 2**). The test was found to be sensitive, rapid, easy to perform and showed good reliability.

DISCUSSION

Bioassays have been widely reported to provide a lot of information and be very useful for water quality assessment but in many cases there is either a need for maintaining breeding animals in the lab for out of season use (if possible) or some tests have a very marked seasonality (matching the spawning season of the test species). Using cryopreserved biological material is a good option to overcome this constraint, but it is crucial to be able to compare the results of the procedure with cryopreserved material to the standard tests.

According to Cairns and Pratt (1989) extrapolations from bioassays on one species to another species are not straightforward and results are only comparable in some cases. From the studies reviewed here, the same principle can be applied to the comparison between bioassays with cryopreserved cells and standard bioassays. Cryopreservation is a very useful add-on to an already developed testing methodology that could help increasing the use of bioassays. On the other hand, until an exhaustive comparison and compilation of data takes place and a robust correspondence between bioassays with cultured vs. cryopreserved organisms can be obtained with a reliable level of certainty, these results should be taken with precaution.

The advantages of using cryopreserved biological material for bioassays are many: from providing a reliable source of cells and organisms that can be stored for out of season need, to provide flexibility to the analyser. Making possible the simultaneous testing with a battery of organisms that do not reproduce at the same time of the year, without having to hold the animals in the lab for out of season production, which is costly and labor intensive. Last but not least, it also aligns with the 3R's of animal welfare principle of reduction, by allowing the storage of unused material for other experiments therefore reducing the number of animals used per trial. As more marine organisms have been successfully cryopreserved, including different cells or development stages, there is great potential for this to continue to develop (Suquet et al., 2000; Paredes, 2015b).

Many of the microalgae currently held in culture collections are kept cryopreserved, there are also available protocols for different molluscs (Wang et al., 2011; Paredes et al., 2013) and being mussels the most widely used organism for biomonitoring, this is another potential candidate for the development of a cryopreserved toxicity test in the near future. Regarding sea urchins, right now there are cryopreservation protocols developed or under development for different cells for 10–14 different sea urchin species, and being sea urchins a highly demanded model soon more applications will be developed, including toxicology (Paredes, 2015b).

Crustaceans are, as of today, not on the table as they have no reliable cryopreservation protocol. Fish are very sensitive to low temperatures and had proven exceptionally difficult to cryopreserve, being the only exception fish sperm. There has been exhaustive research on marine fish sperm cryopreservation and protocols have been described for the most farmed species (*S. aurata* by Fabbrocini et al., 2012; *D. labrax* by Fauvel et al., 1998; or *M. cephalus* by Balamurugan and Munuswamy, 2017), any of these could be used as a biomonitoring test organism and cryopreservation could enhance the possibilities of development

of this test. There is potential for the further use of this biotechnology applied to marine water quality assessment.

The parameters used as endpoints in the classic bioassays were characterized by good reliability and sensitivity but, when using cryopreserved cells those parameters might need a little adjusting in order to obtain the best results, For instance, cryopreserved cells develop slower in the first hours post-thaw, therefore experimental protocols need to be adjusted in terms of exposure duration; cryopreserved microalgae can show sensitivity to high light intensities immediately post-thaw so that light intensity needs to be lowered during the first hours of exposure. Cryopreserved samples can be easily stored and transferred, making it possible to perform bioassays in different sites or at different times and can even be part of long-term monitoring programs. Finally, the application of certain bioassays with cryopreserved material in environmental monitoring and risk assessment schemes, may allow the detection of lower concentrations of toxic substances that classical bioassays, which would offer a higher level of protection to marine ecosystems.

CONCLUSION

This is a perspective on the state of the art and critical analysis of the application of cryopreservation as a tool to improve toxicity testing. As of today, cryopreservation holds great potential as a tool to improve toxicity testing by solving, for instance, the seasonal shortage of biological material. On the other side, there is a need for extensive comparative testing in order to select those cryopreserved cells/protocols that can be more useful, either by developing new protocols for key cell types or making sure the cryopreservation outcome of the existing protocols is specifically designed to be used in a bioassay. There is also a need to obtain good and reliable correlations between methods with both fresh and cryopreserved biological material for a wide variety of chemical compounds. An extensive battery of comparisons using both methods will establish a frame of comparison that would enable researchers to use one or the other according with their practical needs and keep increasing the historical databases. Currently, the cryopreservation of *P. lividus* embryos and *S. aurata* sperm are in an advanced stage of development and present promising perspectives for their use in water quality assessment. As cryopreservation of aquatic marine resources continues to develop, the application of those preserved cells to toxicity testing will continue to expand.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the ASSEMBLE plus, grant from the European Union's Horizon 2020 Research and Innovation Programme (No. 730984).

REFERENCES

Araújo, C. V. M., Diz, F. R., Lubián, L. M., Blasco, J., and Moreno-Garrido, I. (2010). Sensitivity of cylindrotheca closterium to copper: influence of three test endpoints and two test methods. *Sci. Total Environ.* 408, 3696–3703. doi: 10.1016/j.scitotenv.2010.05.012

Araujo, C. V. M., and Moreno-Garrido, J. (2015). *Toxicity Bioassays on Benthic Diatoms*. Amsterdam: Elsevier, 539–545.

Arensberg, P., Hemmingsen, V. H., and Nyholm, N. (1995). A miniscale algal toxicity test. *Chemosphere* 30, 2103–2115. doi: 10.1016/0045-6535(95)00090-u

Aylagas, E., Menchaca, I., Laza-Martínez, A., Seoane, S., and Franco, J. (2014). Evaluation of marine phytoplankton toxicity by application of marine invertebrate bioassays. *Sci. Mar.* 78, 173–183. doi: 10.3989/scimar.03957.26c

Balamurugan, R., and Munuswamy, N. (2017). Cryopreservation of sperm in grey mullet mugil cephalus (Linnaeus, 1758). *Anim. Reprod. Sci.* 185, 205–213. doi: 10.1016/j.anireprosci.2017.08.022

Beiras, R., and Bellas, J. (2008). Inhibition of embryo development of the mytilus galloprovincialis marine mussel by organic pollutants; assessment of risk for its extensive culture in the galician rias. *Aquaculture* 277, 208–212. doi: 10.1016/j.aquaculture.2008.03.002

Bellas, J. (2008). Prediction and assessment of mixture toxicity of compounds in antifouling paints using the sea urchin embryo-larval bioassay. *Aquat. Toxicol.* 88, 309–315. doi: 10.1016/j.aquatox.2008.05.011

Bellas, J., Beiras, R., Mariño-Balsa, J. C., and Fernandez, N. (2005). Comparison between the Sea urchin embryogenesis bioassay and alternative test species. *Ecotoxicology* 14, 337–353. doi: 10.1007/s10646-004-6370-y

Benhra, A., Radetski, C. M., and Ferard, J. F. (1997). Cryoalgotox: use of cryopreserved alga in a semistatic microplate test. *Environ. Toxicol. Chem.* 16, 505–508. doi: 10.1002/etc.5620160316

Cairns, J. Jr., and Pratt, J. R. (1989). The scientific basis of bioassays. *Hydrobiologia* 18, 5–20. doi: 10.1007/bf00027769

Calow, P. (1993). *General Principles and Overview. En: Handbook of Ecotoxicology I*. Hoboken, NJ: Blackwell Scientific Publications.

Davies, I. M., and Vethaak, A. D. (2012). Integrated marine environmental monitoring of chemicals and their effects. *ICES Cooperative Res. Rep.* 315:277.

Debelius, B., Forja, J. M., DelValls, A., and Lubian, L. M. (2009). Toxicity and bioaccumulation of copper and lead in five marine microalgae. *Ecotoxicol. Environ. Saf.* 72, 1503–1513. doi: 10.1016/j.ecoenv.2009.04.006

Durán, I., and Beiras, R. (2010). Assessment criteria for using the sea-urchin embryo test with sediment elutriates as a tool to classify the ecotoxicological status of marine water bodies. *Environ. Toxicol. Chem.* 29, 1192–1198. doi: 10.1002/etc.136

EPA (2002). *Method 1004.0. Sheepshead Minnow, Cyprinodon variegatus, Larval Survival and Growth Test; Chronic Toxicity. Excerpt from: Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms*. Washington, DC: EPA.

Fabbrocini, A., D'Adamo, R., Del Prete, F., Langellotti, A. L., Barone, C. M. A., Rinna, F., et al. (2013). Motility of cryopreserved spermatozoa for the ecotoxicological evaluation of aquatic environments. chemistry and ecology: motility of cryopreserved spermatozoa for the ecotoxicological evaluation of aquatic environments. *Chem. Ecol.* 29, 660–667. doi: 10.1080/02757540.2013.810723

Fabbrocini, A., D'Adamo, R., Del Prete, F., Langellotti, A. L., Rinna, F., Silvestri, F., et al. (2012). Cryopreserved semen in ecotoxicological bioassays: sensitivity and reliability of cryopreserved *Sparus aurata* spermatozoa. *Ecotoxicol. Environ. Saf.* 84, 293–298. doi: 10.1016/j.ecoenv.2012.07.024

Fabbrocini, A., D'Adamo, R., Del Prete, F., Maurizio, D., Specchiuli, A., Oliveira, F. L., et al. (2016). The sperm motility pattern in ecotoxicological tests. The

CRYO-Ecotest as a case study. *Ecotoxicol. Environ. Saf.* 123, 53–59. doi: 10.1016/j.ecoenv.2015.08.018

Fabbrocini, A., D'Adamo, R., Pelosi, S., Oliveira, L. F. J., Silvestri, F., and Sansone, G. (2014). Gamete cryobanks for laboratory research: developing a rapid and easy to perform protocol for the cryopreservation of the sea urchin Paracentrotus lividus (Lmk, 1816) Spermatozoa. *Cryobiology* 69, 149–156. doi: 10.1016/j.cryobiol.2014.06.009

Fabbrocini, A., Lavadera, S. L., Rispoli, S., and Sansone, G. (2000). Cryopreservation of Seabream (*Sparus aurata*) spermatozoa. *Cryobiology* 40, 46–53. doi: 10.1006/cryo.1999.2220

Fauvel, C., Suquet, M., Dreanno, C., Zonno, V., and Menu, B. (1998). Cryopreservation of sea bass (*Dicentrarchus labrax*) spermatozoa in experimental and production simulating conditions. *Aquat. Living Resour.* 11, 387–394. doi: 10.1016/s0990-7440(99)80004-7

Geis, S. W., Fleming, K. L., Korthals, E. T., Searle, G., Reynolds, L., and Karner, D. A. (2000). Modifications to the algal growth inhibition test for use as a regulatory assay. *Environ. Toxicol. Chem.* 19, 36–41. doi: 10.1002/etc.5620190105

Gellert, G. (2000). Sensitivity and significance of luminescent bacteria in chronic toxicity testing based on growth and bioluminescence. *Ecotoxicol. Environ. Saf.* 45, 87–91. doi: 10.1006/eesa.1999.1849

Glenister, P. H., Whittinham, D. G., and Lyon, M. F. (1984). Further studies on the effect of radiation during the storage of frozen 8-cell mouse embryos at −196°C. *J. Reprod. Fert.* 70, 229–234. doi: 10.1530/jrf.0.0700229

His, E., Beiras, R., and Seaman, M. N. L. (1999a). "The assessment of marine pollution-bioassays with bivalve embryos and larvae," in *Advances in Marine Biology*, eds A. I. Southeward, P. A. Tyler, and C. M. Young (Cambridge, MA: Academic Press), 1–178. doi: 10.1016/s0065-2881(08)60428-9

His, E., Heyvang, I., Geffard, O., and Montaudouin, X. (1999b). A comparison between oyster (*Crassostrea gigas*) and sea urchin (*Paracentrotus lividus*) larval bioassays for toxicological studies. *Water Res.* 33, 1706–1718. doi: 10.1016/s0043-1354(98)00381-9

His, E., Seaman, M. N. L., and Beiras, R. (1997). A simplification the bivalve embryogenesis and larval development bioassay method for water quality assessment. *Water Res.* 31, 351–355. doi: 10.1016/s0043-1354(96)00244-8

Hutchinson, T. H., Williams, T. D., and Eales, G. J. (1994). Toxicity of cadmium, hexavalent chromium and copper to marine fish larvae (*Cyprinodon variegatus*) and copepods (*Tisbe battagliai*). *Mar. Environ. Res.* 38, 275–290. doi: 10.1016/0141-1136(94)90028-0

International Council for the Exploration of the Sea (ICES) (1991). Techniques in Marine Environmental Sciences, N° 11.

Laranjeiro, F., Pérez, S., Navarro, P., Carrero, J. A., and Beiras, R. (2015). The usefulness of a sediment bioassay with the gastropod *Nassarius reticulatus* in tributyltin monitoring programs. *Chemosphere* 139, 550–557. doi: 10.1016/j.chemosphere.2015.07.076

Leverett, D., and Thain, J. (2013). Oyster embryo-larval bioassay revised. *Tech. Mar. Environ. Sci.* 54:34.

Lewis, M. A. (1990). Chronic toxicities of surfactants and detergent builders to algae: a review and risk assessment. *Ecotoxicol. Environ. Saf.* 20, 123–140. doi: 10.1016/0147-6513(90)90052-7

Lyons, B. P., Thain, J. E., Stentiford, G. D., Hylland, K., Davies, I. M., and Vethaak, A. D. (2010). Using biological effects tools to define good environmental status under the european union marine strategy framework directive. *Mar. Pollut. Bull.* 60, 1647–1651. doi: 10.1016/j.marpolbul.2010.06.005

McFadzen, I. R. B. (1992). Growth and survival of cryopreserved oyster and clam larvae along a pollution gradient in the German Bight. *Mar. Ecol. Prog. Ser.* 91, 215–220. doi: 10.3354/meps091215

Nolan, C. V., and Duke, E. J. (1983). Cadmium accumulation and toxicity in mytilus edulis: involvement of metallothioneins and heavy-molecular weight protein. *Aquat. Toxicol.* 4, 153–163. doi: 10.1016/0166-445x(83)90052-8

Paredes, E. (2015b). Exploring the evolution of marine invertebrate cryopreservation – landmarks, state of the art and future lines of research. *Rev. Artic. Cryobiol.* 71, 198–209. doi: 10.1016/j.cryobiol.2015.08.011

Paredes, E. (2015a). Biobanking of a marine invertebrate model organism: the sea urchin. *J. Mar. Sci. Eng.* 3:7. doi: 10.3390/jmse4010007

Paredes, E., Adams, S. L., Tervit, H. R., Smith, J. F., McGowan, L. T., Gale, S. L., et al. (2011). Cryopreservation of GreenshellTM mussel (*Perna canaliculus*) trochophore larvae. *Cryobiology* 65, 256–262. doi: 10.1016/j.cryobiol.2012.07.078

Paredes, E., and Bellas, J. (2015). The use of cryopreserved sea urchin embryos (*Paracentrotus lividus*) in marine quality assessment. *Chemosphere* 128, 278–283. doi: 10.1016/j.chemosphere.2015.02.007

Paredes, E., Bellas, J., and Adams, S. L. (2012). Comparative cryopreservation study of trochophore larvae from two species of bivalves: pacific oyster (*Crassostrea gigas*) and Blue mussel (*Mytilus galloprovincialis*). *Cryobiology* 67, 274–279. doi: 10.1016/j.cryobiol.2013.08.007

Paredes, E., Bellas, J., and Adams, S. L. (2013). Comparative cryopreservation study of trochophore larvae from two species of bivalves: pacific oyster (*Crassostrea gigas*) and blue mussel (*Mytilus galloprovincialis*). *Cryobiology* 67, 274–279. doi: 10.1016/j.cryobiol.2013.08.007

Parvez, S., Venkataraman, C., and Mukherji, S. (2006). A review on advantages of implementing luminescence inhibition test (*Vibrio fischeri*) for acute toxicity prediction of chemicals. *Environ. Int.* 32, 265–268. doi: 10.1016/j.envint.2005.08.022

Pavlic, Z., Stjepanovic, B., Horvatic, J., Persic, V., Puntaric, D., and Culig, J. (2006). Comparative sensitivity of green algae to herbicides using erlenmeyer flask and microplate growth-inhibition assays. *Bull. Environ. Contam. Toxicol.* 76, 883–890. doi: 10.1007/s00128-006-1001-3

Perez Fernández, S., Rial, D., and Beiras, R. (2015). Acute toxicity of selected organic pollutants to saltwater (mysid Siriella armata) and freshwater (cladoceran Daphnia magna) ecotoxicological models. *Ecotoxicology* 24, 1229–1238. doi: 10.1007/s10646-015-1489-6

Ribeiro, R. C., Caroline da Silva Veronez, A., Tristão Tovar, T., Adams, S. L., Bartolomeu, D. A., Peronico, C., et al. (2018). Cryopreservation: extending the viability of biological material from sea urchin (*Echinometra lucunter*) in ecotoxicity tests. *Cryobiology* 80, 139–143. doi: 10.1016/j.cryobiol.2017.10.002

Satoh, A., Vudikaria, L. Q., Kurano, N., and Miyachi, S. (2005). Evaluation of the sensitivity of marine microalgal strains to the heavy metals. Cu, As, Sb, Pb and Cd. *Environ. Int.* 31, 713–722. doi: 10.1016/j.envint.2005.01.001

Snell, T. W., and Persoone, G. (1989). Acute toxicity bioassays using rotifers. I. A test for brackish and marine environments with *Brachionus plicatiliss*. *Aquat. Toxicol.* 14, 65–80. doi: 10.1016/0166-445x(89)90055-6

Stebbing, A. R. D., Akesson, B., Calabrese, A., Gentile, J. H., Jensen, A., and Lloyd, R. (1980). The role of bioassays in marine pollution monitoring. bioassay panel report, rapp. P.-v. Réuni. *Cons. Int. Explor. Mer.* 179, 322–332.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., and Billard, R. (2000). Cryopreservation of sperm in marine fish. *Aquacult. Res.* 31, 231–243. doi: 10.1046/j.1365-2109.2000.00445.x

Wang, H., Li, X., Wang, M., Clarke, S., Gluis, M., and Zhang, Z. (2011). Effects of larval cryopreservation on subsequent development of the blue mussels, mytilus galloprovincialis Lamarck. *Aqua. Res.* 42, 1816–1823. doi: 10.1111/j.1365-2109.2010.02782.x

Wong, S. L., and Beaver, J. L. (1980). Algal bioassays to determine toxicity of metal mixtures. *Hydrobiologia* 74, 199–208. doi: 10.1007/bf00008753

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 02 August 2019
doi: 10.3389/fenvs.2019.00120

Effect-Directed Analysis of Ah Receptor-Mediated Potencies in Microplastics Deployed in a Remote Tropical Marine Environment

Christine Schönlau[1], Maria Larsson[1], Florian Dubocq[1], Anna Rotander[1], Rene van der Zande[2], Magnus Engwall[1] and Anna Kärrman[1]*

[1] MTM Research Centre, School of Science and Technology, Örebro University, Örebro, Sweden, [2] Coral Reef Ecosystems Lab and Global Change Institute, The University of Queensland, St. Lucia, QLD, Australia

To facilitate the study of potential harmful compounds sorbed to microplastics, an effect-directed analysis using the DR CALUX® assay as screening tool for Aryl hydrocarbon receptor (AhR)-active compounds in extracts of marine deployed microplastics and chemical analysis of hydrophobic organic compounds (HOCs) was conducted. Pellets of three plastic polymers [low-density polyethylene (LDPE), high-density polyethylene (HDPE) and high-impact polystyrene (HIPS)] were deployed at Heron Island in the Great Barrier Reef, Australia, for up to 8 months. Detected AhR-mediated potencies (bio-TEQs) of extracted plastic pellets ranged from 15 to 100 pg/g. Contributions of target HOCs to the overall bioactivities were negligible. To identify the major contributors, remaining plastic pellets were used for fractionation with a gas chromatography (GC) fractionation platform featuring parallel mass spectrometric (MS) detection. The bioassay analysis showed two bioactive fractions of each polymer with bio-TEQs ranging from 5.7 to 14 pg/g. High resolution MS was used in order to identify bioactive compounds in the fractions. No AhR agonists could be identified in fractions of HDPE or LDPE. Via a multivariate statistical approach the polystyrene (PS) trimer 1e- Phenyl-4e-(1- phenylethyl)-tetralin was identified in fractions of HIPS and in fractions of the blank polymer of HIPS.

Keywords: polyethylene, polystyrene, PCBs, reporter gene assay, fractionation

OPEN ACCESS

Edited by:
Juan Bellas, Spanish Institute of Oceanography, Spain

Reviewed by:
Begoña Jiménez, Spanish National Research Council (CSIC), Spain
Pauline Pannetier, Heidelberg University, Germany

****Correspondence:***
Christine Schönlau
christine.schonlau@oru.se

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Environmental Science

Received: *15 February 2019*
Accepted: *18 July 2019*
Published: *02 August 2019*

Citation:
Schönlau C, Larsson M, Dubocq F, Rotander A, van der Zande R, Engwall M and Kärrman A (2019) Effect-Directed Analysis of Ah Receptor-Mediated Potencies in Microplastics Deployed in a Remote Tropical Marine Environment. Front. Environ. Sci. 7:120. doi: 10.3389/fenvs.2019.00120

INTRODUCTION

Hydrophobic organic compounds (HOCs) are able to sorb to plastics from the ambient environment, especially polyethylene (PE) has been recognized as a good sorbent for HOCs and has been utilized as a passive sampler in various studies (Zabiegala et al., 2010). Polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, such as chlordanes, dichlorodiphenyltrichloroethane (DDT) and its breakdown products, and polybrominated diphenyl ethers (PBDEs) have been frequently detected on marine microplastics (Mato et al., 2001; Rios et al., 2007; Hirai et al., 2011; Van et al., 2012). Furthermore, plastics often contain additives to provide the end-products with the desired properties. One concern is that additives leach out from the plastics, as well as unreacted monomers or oligomers from the plastics themselves (Teuten et al., 2009). The importance of plastics as a vector for transferring HOCs to organisms is yet unclear,

but has been debated by scientists in the past decade. In contrast to increasing the exposure to HOCs upon ingestion of microplastics, it has been hypothesized that ingested microplastics rather act as passive samplers for HOCs, therefore reflecting tissue concentrations of such compounds (Herzke et al., 2016) or can even act as a sink for pollutants, hence decreasing the bioaccumulation of pollutants (Koelmans et al., 2016). A better knowledge of identities and concentrations of sorbed HOCs in marine plastics would help in risk assessment of plastics. This knowledge can best be gained under field conditions in which microplastics are exposed to real life scenarios with stressors that microplastics have to face in the marine environment.

The Aryl hydrocarbon receptor (AhR) mediates toxic effects of several HOCs, including polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs), coplanar non-di-ortho-substituted PCBs, and some PAHs (Denison and Heath-Pagliuso, 1998; Marlowe and Puga, 2005). In a previous study we assessed AhR-mediated potencies for the first time for extracts of plastic pellets of low-density polyethylene (LDPE), polypropylene (PP), polyethylene terephthalate (PET), and polyvinylchloride (PVC) (Schönlau et al., 2019). The samples in the previous study have been deployed in an urban marina which was highly impacted by anthropogenic activity. In some samples the potency could mainly be attributed to quantified PAHs, but in other samples the analyzed PAHs only contributed partially to the overall AhR-mediated efficacies. Therefore, it is of interest to assess also the contribution of other AhR agonist, such as coplanar non-di-ortho-substituted PCBs and PCDD/Fs.

The challenge with environmental samples, including marine microplastics, is that they usually contain complex mixtures of chemicals and chemical analysis can often not detect all toxicologically relevant compounds. In effect-directed analysis (EDA), the complexity of a mixture is sequentially reduced by separating the components into different fractions. The potential toxicity of these fractions is thereafter tested in a relevant bioassay. Fractions that demonstrate great bioactivities are then subjected to chemical analysis to identify bioactive compounds, or can be further fractionated if the fraction is still too complex. In general, liquid chromatography (LC) is used for fractionation of samples in EDA studies (Lubcke-von Varel et al., 2008; Weiss et al., 2009; Grung et al., 2011), but gas chromatography (GC) is the preferred separation technique for analysis of non-polar organic pollutants in environmental samples (Poster et al., 2006; Tombesi et al., 2017; Bjurlid et al., 2018). In recent years, a new GC-based fractionation platform with a post-column introduction of a trap solvent for fraction collection has been developed (Pieke et al., 2013; Jonker et al., 2016, 2017). This platform allows for fractionation during the entire time of a GC run, thus covering a complete chromatogram. Recently, the GC fractionation platform has been coupled to mass spectrometric (MS) detection for parallel analysis of compounds, and was successfully evaluated for the fractionation of a mixture of n-alkanes (C_7–C_{30}), and a mixture of two pesticides, vinclozolin and p,p'-DDE (Jonker et al., 2017).

The aim of this study was to assess the levels of AhR-mediated potencies in extracts of three types of mass produced pristine plastic pellets deployed in a remote site and to estimate the contribution of sorbed HOCs to the overall biological effect. Additionally, for the first time a GC-based fractionation platform was utilized for the identification of HOCs sorbed to marine microplastics and tested for its suitability. Pristine plastic pellets, which are plastic pellets that are usually used as raw material for manufacturing of plastic products [LDPE, high-density polyethylene (HDPE) and high-impact polystyrene (HIPS)], were deployed at Heron Island in the Great Barrier Reef, Australia, and were analyzed for their potential to elicit an AhR- mediated activity in the DR CALUX® (Dioxin Receptor Chemical Activated LUciferase gene eXpression) assay. In order to identify the compounds causing the AhR-mediated activity a two-step approach was conducted. Firstly potency balance calculations were conducted, comparing predicted activities of the target HOCs, including PCBs, PBDEs, pesticides, and PCDD/Fs, in the microplastics to the observed activities in the DR-CALUX® bioassay. If the observed bioassay activities were only partially explained, the second step was GC-MS fractionation followed by bioassay analysis and chemical analysis of the bioactive fractions using high resolution GC-Orbitrap™ MS.

MATERIALS AND METHODS

Chemicals

SupraSolv® n-hexane (≥98%) (Merck, Darmstadt, Germany) was used for the extractions and dimethyl sulfoxide (DMSO) (99.9%) (Sigma Aldrich, Stockholm, Sweden) was used for dissolving extracts in bioassay test medium. Toluene (>99.5) and Heptane (≥99%), were used for fraction collection (Honeywell, Riedel-de Haën, Steinheim, Germany). Tetradecane for chemical analysis was purchased from Sigma Aldrich (Stockholm, Sweden).

The bioassay reagent Steady Lite plus™ was purchased from Perkin Elmer (Hägersten, Sweden). A 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard, with a purity of 99.1%, was from AccuStandard Inc. (New Haven, USA).

Target chemicals included PCBs, PBDEs, pesticides and PCDD/Fs. Target chemicals after fractionation also included PAHs. Information regarding native and labeled chemical standards are presented in the **Supplementary Material**.

Deployment of Plastics

Three pristine plastic pellets, LDPE, HDPE and HIPS were deployed on the 14th of February 2015 for 8 months at Heron Island, Great Barrier Reef, Australia (For information about coordinates and sampling dates see **Table S1** of the Supplementary Material). HDPE samples were spherical with a diameter of 3 mm, HIPS and LDPE samples were cylindrical with a length of 4 mm and a height of 2–3 mm. HDPE granules contained an unknown UV stabilizer. The samples were deployed in two stainless steel cages which contained larger mesh bags (10 mm mesh size) to separate each plastic polymer from another (**Figure S1**). The larger mesh bags contained smaller mesh bags (2–3 mm mesh size) with approximately 4 g of a polymer for every sampling time point. The metal cages were anchored in an open sandy patch, between coral rubble in a shallow part of

Heron Island lagoon, using sand pegs. At low tide the water depth at the site was approximately 0.5 m, and between 1.5 and 2.5 m depth at high tide, depending on the weather conditions. In order to avoid too much sand accumulation on the samples and secure them against tidal movements, two layers of a black knitted shade cloth were used to cover the samples. The shade cloth was cleaned once a month from sand. Samples were taken after 1, 3, 6, and 8 months of deployment, wrapped in alumina foil and stored at −20°C until analysis. For all LDPE samples replicates were available, one from each cage. For HDPE and HIPS a replicate sample was only available for the first month of deployment, after that only one sample of each polymer was collected from the first cage.

Extraction of Microplastics

Prior to extraction of real samples, pellets of LDPE and HDPE were spiked with labeled PCBs, pesticides, OCDD and PBDE#47 standards to test the extraction efficiency of the compounds (**Table S2**). Samples were rinsed with ultrapure water to remove sand and bigger particles, and left to dry in the fume hood. Approximately 3 g of plastic pellets were extracted with 4 mL of n-hexane by ultrasonication for 60 min. The extracts were transferred to brown glass vials and extraction was repeated with 3 mL of n-hexane. Both extracts were pooled together and 1–2 mL of concentrated sulfuric acid (98%) was added to the extracts to eliminate organic matter. Non-persistent organic chemicals were also degraded in this step. After vortex mixing, and incubation at room temperature for 3 h and centrifugation at 4,500 rpm for 10 min, the supernatant was transferred to brown glass vials. Finally, 2 mL of n-hexane were pipetted into the centrifuge vials, vortexed and centrifuged at 4,500 rpm for 10 min. The remaining supernatant was transferred to the brown glass vials. Extracts were evaporated under a gentle nitrogen flow to 1 mL of extract. Subsequently extracts were split into two aliquots. One aliquot was solvent exchanged to DMSO for bioassay testing. The other aliquot was spiked with internal standard (IS) and recovery standard (RS), solvent exchanged to tetradecane and evaporated under a gentle nitrogen flow to a volume of 25 μL for chemical analysis. Additionally to the deployed samples, pristine plastic pellets of each polymer type were extracted as polymer blanks. The samples are named hereafter by their polymer acronym, LDPE, HDPE and HIPS, the cage number written as I or II, and the time period of deployment written as 1, 3, 6, or 8 m.

In order to decrease detection limits for the fractionation of real samples, the remaining 1 g of each of the plastics collected at 1, 3, 6, and 8 months of deployment and available replicates were pooled according to the type of polymer, resulting in 3 samples. Hereafter the pooled samples are named by the polymer acronym and the time span of deployment from 1 to 8 months written as 1–8 m. For LDPE 1–8 m 8.56 g, for HDPE 1–8 m 5.04 g and for HIPS 1–8 m 5.56 g were extracted. Besides two process blanks without polymers, 10 g of each plastic pellet were extracted as polymer blank samples. The extraction procedure was the same as for the individual, non-pooled samples with the exception that 20 mL of extraction solvent was used and the obtained extracts were evaporated to a volume of 5–6 mL in a rotary evaporator. All extracts were split into two aliquots, one aliquot was solvent exchanged to DMSO for bioassay testing and the second aliquot was solvent exchanged to toluene and evaporated to a volume of 300 μL for further fractionation.

Chemical Analysis

For analysis of target compounds in individual plastic samples an Agilent 7890 A GC system (Agilent Technologies, Santa Clara, USA) coupled to atmospheric pressure chemical ionization tandem quadrupole mass spectrometer Xevo TQ-S (APCI-MS/MS), (Waters Corporation, Milford, USA), was used. GC separation was achieved using a 30 m × 0.25 mm, 0.25 μm film thickness, DB-5MS capillary column (J & W Agilent Technologies, Santa Clara, USA). All samples were injected at 280°C in splitless mode. Measurements were done in multiple reaction mode (MRM).

Quantification of target compounds in individual plastic samples was done by use of the software MassLynx V4.1 (Waters Cooperation, Milford, USA). Target compounds were quantified by use of a one-point calibration and a quantification standard containing target compounds. The quantification standard was analyzed in the beginning and in the end of a run. The limit of detection (LOD) was defined as the concentration of the native compounds in the quantification standard divided by three times the signal-to-noise ratio, and the limit of quantification (LOQ) was defined as the concentration of the compound divided by ten times the signal-to-noise ratio.

A GC fractionation platform with parallel MS detection, as described by Jonker et al. (2017), was used for fractionation of pooled plastic samples. Prior to fractionation of real samples, the platform was evaluated for its performance by injection and qualitative analysis of five individual standards, and a mixture of 134 known compounds, including PAHs (5 ng/μL), PCBs (20–40 pg/μL), PCDD/Fs (20–100 pg/μL), PBDEs (20 pg/μL), and pesticides (20 pg/μL). The GC fractionation platform consisted of an Agilent 7890 B gas chromatograph coupled to a 5977 B series low-resolution mass spectrometer (GC/LRMS) and a multipurpose sampler (GERSTEL GmbH & Co. KG, Mühlheim an der Ruhr, Germany). An Agilent 1260 Infinity II LC pump was connected to an inert two-way splitter (Agilent Technologies, Santa Clara, USA) that allowed the post-GC column split. One part (∼1%) of the eluate was guided toward the MS via a 1 m × 0.1 mm internal diameter, deactivated fused silica column (Agilent Technologies). The other part was guided toward fraction collection via a 1.2 m × 0.32 mm internal diameter deactivated fused silica column. The instrument components were controlled by the Agilent Masshunter software. The multipurpose sampler, used for injection and fraction collection, was controlled by the DVLS GC fractionation software, version 1.0.2.6. (Da Vinci Laboratory Solutions, Rotterdam, The Netherlands). Separation of compounds was achieved on a capillary column (30 m × 0.25 mm, 0.15 μm film thickness) (Select PAH; Agilent Technologies). Measurements of the mixture and real extracts were performed in full scan mode from m/z 50 to 550. Measurements of single native standards were done in selected

ion monitoring mode (SIM). Seventeen fractions were collected between 4 and 54 min with 15 consecutive injections per sample. It was fractionated with 60 s/well into glass inserts. Fractions of three sequential inserts were pooled into 1.5 mL brown glass vials, which resulted in 17 fractions. The last fraction, fraction 17, consisted of a pool of two inserts. For each sample two batches of 17 fractions were obtained, one batch was subjected to bioassay analysis and the second batch was used for further targeted and non-targeted chemical analysis.

Qualitative chemical analysis of the obtained fractions was performed on a ThermoFisher Scientific Q Exactive™ Quadrupole-Orbitrap™ Mass Spectrometer at a resolution of 60,000 in full scan mode with a scan range of m/z 53.4 to 800 and a simultaneous SIM mode, for 49 selected m/z (**Table S3**). Suspect screening for additives, which are commonly used in the specific plastic polymers, was done on blank and deployed plastic samples (**Table S4**). XCMS online (The Scripps Research Institute, La Jolla, USA) was used to preprocess (peak detection, filtering and alignment) and post process (ANOVA statistical test) raw datasets. Graphical tools resulting from the statistical test as principal component analysis (PCA) were used to detect outliers. In addition, mass spectra of the most abundant signals were extracted from the chromatogram and formulae and structures were tentatively determined using library searches including National Institute for Standards and Technology (NIST) library or by literature search. All the m/z extracted from the non-target analysis were below two parts- per-million (ppm) accuracy compared with the theoretical m/z.

For more details regarding the used GC temperature programs on each instrument refer to the **Supplementary Material**.

DR CALUX® Assay

AhR-mediated potencies were measured by use of the DR CALUX® assay, using a recombinant rat hepatoma cell line with a stably transfected luciferase reporter gene (H4IIepGudluc1.1cells) (Murk et al., 1996). The cell line was obtained from BioDetection Systems (BDS) (Amsterdam, The Netherlands). The DR CALUX® assay was performed as previously described for the H4IIE-*luc* assay in Larsson et al. (2013). Extracts of individual plastic samples were tested for 24 h and 72 h exposure. Sample extracts were prepared as 3-fold serial dilutions in culture medium and added to the test plates in six different test concentrations. Dilutions of fractionated samples were prepared as 4-fold serial dilutions. Cells were microscopically examined for cytotoxicity before and after exposure. Luciferase activity in each well was measured in a luminometer (Fluostar, Omega). Concentration-response curves for TCDD and extracts were obtained by use of a sigmoidal concentration-response (variable slope) equation (GraphPad Prism® 5.0 software). All individual plastic samples were tested in three independent experiments. Bioactive fractions were tested twice.

Data Analysis of Bioassay Results

Bioassay derived TCDD equivalents (bio-TEQs), were calculated from concentration-response curves by relating the luciferase induction potencies of the samples to that of the TCDD standard as described in Larsson et al. (2013). Samples that had a TCDD EC_{50} value between 6 and 18 pM and a maximal induction factor (MIF) between 7 and 17 were used in TEQ calculations. Limit of detection (LOD) in the assay is the mean of the solvent control (DMSO) plus three times the standard deviation.

Chemically derived TCDD equivalents (chem-TEQs) were calculated as the sum of the product of individual concentrations of PCBs and PCDD/Fs multiplied with their H4IIE-*luc* or CALUX assay specific relative potency factors (REP) based on EC_{50} or EC_{20-80} values. The applied REP values were taken from Lee et al. (2013) and USEPA (2014). Potency balance calculations between bio- TEQs and chem-TEQs were conducted to determine the contribution of analyzed chemicals to the total induced AhR-mediated potency.

Statistical analysis was done by using GraphPad Prism® 5.0 software and the significance level was set to $p < 0.05$.

RESULTS

AhR-Mediated Potencies of Deployed Plastics Over Time

A total of 21 samples were examined for their AhR-mediated potency, including three blank samples of each polymer. In the 24 h assay, most of the samples had responses <50% of the TCDD maximum induction (TMI). Bio-TEQs for LDPE and HDPE samples were calculated at the 25% effect level (EC_{25}) of TMI (**Figure 1**) (Villeneuve et al., 2000). Cytotoxicity was observed for all deployed HIPS samples at the greatest concentration tested in the assay. For some replicates also the second greatest concentration tested showed cytotoxicity. Therefore, bio-TEQs for HIPS samples were calculated based on the EC_{20} level of TMI, where no cytotoxicity was observed. Among the three polymers HDPE showed the greatest AhR-mediated potency with a bio-TEQ of 100 pg/g after 8 months of deployment, followed by HIPS with 69 pg/g (EC_{20}), and LDPE with 57 pg/g (LDPE I 8 m) and 41 pg/g (LDPE II 8 m), respectively (**Table S6**). There was, however, no significant difference among the three polymers (ANOVA, $p = 0.53$), while there were significant differences in the deployment times for HDPE ($p < 0.0001$), and LDPE ($p = 0.001$). The polymer blank of HDPE had an AhR-mediated activity with a bio-TEQ of 7.6 pg/g (EC_{20}). Polymer blanks of LDPE and HIPS did not induce measurable AhR-mediated activities. The blank sample of HIPS was observed to be cytotoxic in the highest concentration tested in the bioassay.

In order to assess if the AhR-mediated potencies observed in the 24 h assay were due to compounds resistant to cellular metabolism, like PCDD/Fs, the AhR-mediated potencies of the extracts of plastic samples were also investigated following 72 h of exposure. None of the samples induced measurable AhR-mediated potencies after an exposure time of 72 h.

Occurrence of HOCs on Deployed Plastics

The chemical analysis showed low concentrations of target HOCs, which exceeded polymer blank concentrations in a few samples. All polymer blanks contained measurable levels of PCBs ranging from 84 pg/g in HIPS to 355 pg/g in HDPE (**Table S7**). HDPE samples demonstrated the greatest

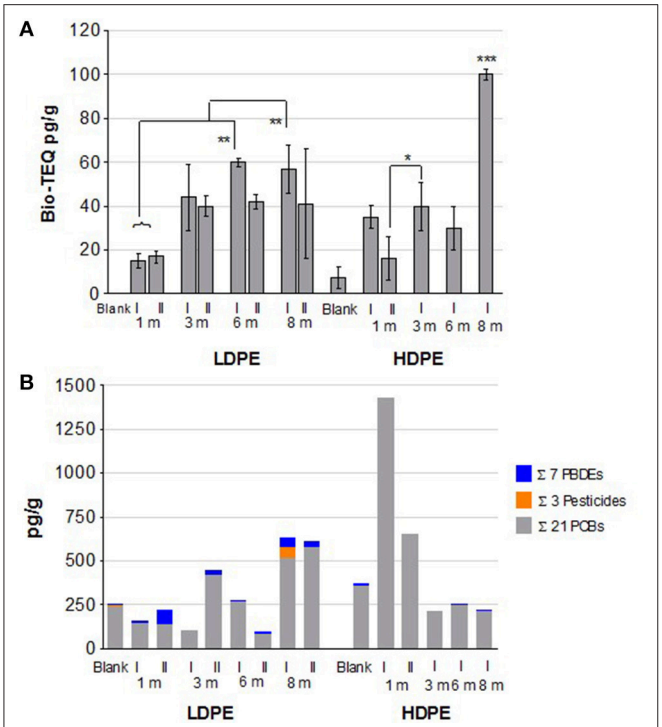


FIGURE 1 | Bioassay (DR CALUX®) and chemical analysis of extracts of deployed LDPE and HDPE pellets. **(A)** Bio-TEQs (EC_{25}) in pg/g calculated following a 24 h exposure given as a mean of n replicates ($n = 3$–4) and the standard deviation as error bars. Significance levels of ANOVA followed by Tuckey's *post hoc* test are indicated as $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$. **(B)** Sum of concentrations of detectable HOCs above LOQ in pg/g in extracts of plastic pellets. PBDEs included congeners 28, 47, 85, 99, 100, 154, and 183; pesticides included heptachlor, t-chlordane, and p, p'-DDD; PCBs included congeners 44, 49, 52, 66, 70, 74, 79, 87, 95, 99, 101, 105, 110, 118, 138, 149, 153, 155, 156, 157, and 162.

total PCB concentrations of 1,430 pg/g and 652 pg/g after 1 month of deployment, followed by a decrease of concentrations in the following sampling months (**Figure 1**). LDPE samples demonstrated an increase in total PCB concentrations from a polymer blank concentration of 239 pg/g to 519 pg/g and 577 pg/g after 8 months of deployment. The quantification of HOCs in HIPS samples with a one-dimensional GC was not always possible due to the complex matrix of this polymer, which also has been noticed in another study (Rochman et al., 2013b). Therefore, the results for HIPS were excluded from the data set. While in the blank polymer of LDPE PCB #81 had the greatest concentration among detected PCBs (81 pg/g), PCB #153 and #138 had the greatest concentrations in blank polymer of HDPE (155 pg/g and 111 pg/g, respectively). In the deployed LDPE pellets PCB #138 and #153 had the greatest concentrations among detected PCBs, except for the sample LDPE II 1 m in which PCB #49 had the greatest concentration among PCBs. Also in deployed samples of HDPE the PCB congeners #138 and #153 were the major components among detected PCBs. The seven target PBDEs #28, #47, #85, #99, #100, #154, #183 were detected in different deployed LDPE and HDPE samples. BDE #47 had the greatest concentrations among detected PBDEs in all samples, including blank polymers of LDPE and HDPE, except for LDPE I 3 m in which PBDE #28 was most abundant. PBDE #28 was detected in all deployed samples and blank HDPE as well, although the concentration in HDPE I 1m was below the LOQ (**Table S7**). Among the three detected pesticides, heptachlor, p, p'-DDD, and t-chlordane, only heptachlor had a concentration above the LOQ in sample LDPE I 8 m, and p, p'-DDD in the polymer blank of LDPE.

Potency Balance Calculations

Chem-TEQ values were calculated based on the analyzed PCBs and PCDD/Fs to assess the contribution of these compounds to the overall bioassay activities. Among those two compound classes only the known AhR agonists PCB 105, 118, 156, 157, and OCDD had detectable concentrations in some of the samples. The contribution of these five compounds to the measured bio-TEQs was negligible, calculated chem-TEQs could only explain 1.3×10^{-6} % (LDPE I 6m) to 0.001% (HDPE II 1m) (**Table S6**). Also concentrations which were below LOQ but above LOD levels were considered for the calculation of chem-TEQs.

Effect-Directed Analysis of Pooled Plastic Extracts

Performance of the GC-MS Fractionation Platform

To further investigate and identify the AhR-active compounds in deployed plastic pellets a GC-MS fractionation platform was used to collect 17 fractions of each polymer extract. As a proof of principle, the fractionation performance was evaluated by repeated injections, and collecting fractions, of a known mixture of 134 compounds containing PAHs, PCBs, PBDEs, PCDD/Fs and pesticides. The consistency of retention times was proven to be good, <0.1 min variation.

Most compounds of the mixture, except for some pesticides (Aldrin, dieldrin, methoxychlor, isodrin, heptachlor, endrin, heptachlor exo- and endo epoxide, alpha and beta endosulfan, oxy chlordane), PCB #111, and four PBDEs (#99, #85, #138, and #183), were detectable in collected fractions (**Table S7**). Recoveries of the 118 detected compounds were calculated using theoretical concentrations and the quantified concentrations in fractions by analysis with GC-HRMS. The recoveries of the detected compounds, excluding the 16 PAHs, ranged from 4 % (hexachlorobenzene) to 85 % (PCB #138, and #153) and recoveries were <50% for 96 out of 102 compounds. A total of 41 compounds were close to instrumental detection limits. Recoveries for the low molecular weight PAHs were above theoretical concentrations (144–455%), while the high molecular weight PAHs benzo[a]anthracene, benzo[b]fluoranthene, benzo[a]pyrene, benzo[g,h,i]perylene and dibenz[a,h]anthracene had acceptable recoveries between 85 and 106%.

AhR-Mediated Activities

In order to increase concentrations of AhR agonists present in the samples, plastic pellets from the different time points, 1 to 8 months, were pooled according to polymer type and 17 fractions were collected of 15 consecutive injections. AhR-mediated responses were observed in fractions 12 and 14 of

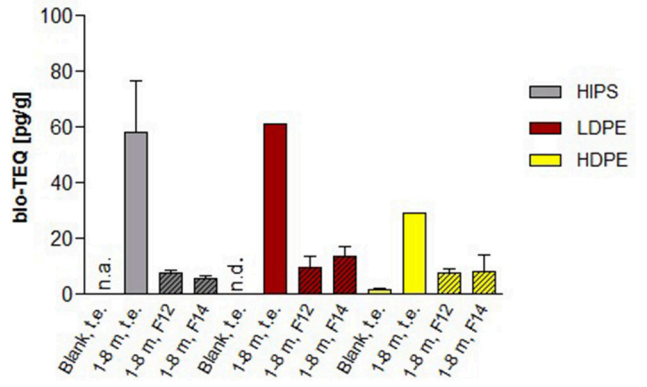


FIGURE 2 | Overview of calculated bio-TEQs (EC_{10}) in pg/g in total and fractionated extracts of pooled plastics. n.a. = not analyzed; n.d. = AhR-mediated potency less than LOD in the assay; t.e. = total extract, unfractionated.

all deployed polymers. Their maximum responses were <20% of TMI for most of the fractions, therefore bio-TEQs were calculated based on EC_{10}. Only fraction 14 of LDPE 1–8 m induced a response up to 20% of TMI. Calculated bio-TEQs ranged from 5.7 pg/g in fraction 14 of HIPS 1–8 m to 14 pg/g in fraction 14 of LDPE 1–8 m. In fraction 12 bio-TEQs ranged from 7.6 pg/g (HIPS 1–8 m) to 9.8 pg/g (LDPE 1–8 m). Fraction 12 of the HIPS blank showed a bioactivity with a bio-TEQ of 4.5 pg/g, while fractionated polymer blanks of LDPE and HDPE did not induce measurable AhR-mediated activities. Fraction 11 of HIPS 1–8 m was observed to be cytotoxic. However, fraction 11 of the HIPS blank showed also cytotoxicity.

Additionally, non-fractionated total extracts of pooled pellets were examined for AhR-mediated potencies. Total extracts had greater bioactivities than individual fractions (**Figure 2**). Total extracts of HDPE and LDPE induced AhR-mediated responses up to 25% of TMI. Bio-TEQs (EC_{10}) for total extracts were 29 pg/g in HDPE 1–8 m, 58 pg/g in HIPS 1–8 m, and 61 pg/g in LDPE 1–8 m. Total extracts of polymer blanks were tested as well, except for HIPS. The total extract of blank HDPE showed an AhR-mediated potency of 1.6 pg/g, whereas the potency of blank LDPE was less than the LOD in the assay. Although the total blank extract of HIPS was not tested in this batch of samples, a HIPS blank was tested in the analysis of individual samples of each time point of deployment. A cytotoxicity was observed before in the HIPS blank and was assumed to also occur in the second HIPS blank since the extraction procedure was the same and only the amount of extracted plastic pellets was higher.

Chemical Analysis

The compounds were fractionated according to their octanol-water partition coefficients (log K_{OW}) with more hydrophobic compounds in later eluting fractions. The cytotoxic fraction 11 of HIPS 1–8 m corresponded to the retention time of 34.12–37.12 min and comparison with fraction 11 of the standard mixture (see **Table S7**) showed that compounds with a log K_{OW} between 6.73 (BDE 66) and 8 (PCB 208) were eluted in this fraction. The bioactive fractions 12 and 14 corresponded to the retention times of 37.12–40.12 min and 43.12–46.12 min, respectively. Fraction 12 corresponded to compounds with a log K_{OW} between 6.92 (2,3,4,6,7,8-HxCDF) and 8.18 (PCB 209). Fraction 14 corresponded to compounds with a log K_{OW} between 5.97 (benzo[a]pyrene) and 8.2 (OCDD).

As last part of the conducted effect-directed analysis in this study (**Figure 3**), bioactive fractions of deployed plastic pellets, the non-fractionated total extracts, and corresponding blank polymer fractions were subjected to a qualitative target, suspect, and non-target analysis (**Tables S3–S5**). Neighboring fractions of fractions 12 and 14 were analyzed as well.

The target analysis revealed naphthalene, fluorene, phenanthrene, anthracene, pyrene, and fluoranthene in different fractions of HIPS 1–8 m and blank HIPS. The intensities of detected PAHs were observed to be similar in blank HIPS compared to deployed HIPS, except for fluorene which was 2 orders of magnitude higher in the deployed sample compared to the blank polymer. The suspect screening of known plastic additives (**Table S4**), did not result in any detected compounds in blank or deployed plastic samples.

In order to identify unknown bioactive compounds multivariate statistics, PCA, were used on full scan MS data for both bioactive and non-bioactive fractions. Fraction 11 of HIPS 1–8 m and blank HIPS were segregated from other HIPS fractions in the score plot (**Figure S2**). In fraction 11 m/z 129.1 with the retention time of 23.6 min (HIPS 1–8 m) and 24.8 min (blank HIPS), and m/z 91.1 with the retention time of 23.7 min (HIPS 1–8 m) and 24.6 min (blank HIPS) were found to be predominant ions. No difference among fractions of HDPE or among fractions of LDPE was observed in the score plot.

Additional spectral analysis of the major peaks (>10% of base peak) was performed (**Table 1**). In fraction 9 to 15 of HIPS 1–8 m and blank HIPS the peak at a retention time of 23.63 min was tentatively identified as 1e-Phenyl-4e-(1-phenylethyl)-tetralin (accurate mass 312.1878, error 0 ppm) (**Figure 4**). The abundance in fraction 11 was one or two orders of magnitude higher than in the other fractions.

DISCUSSION

AhR-Mediated Potencies of Deployed Plastics

All extracts of deployed plastic pellets induced AhR-mediated potencies, but replicate samples of LDPE had no significantly increasing AhR-mediated potencies during time of deployment. However, a sorption of AhR agonists onto deployed plastic pellets was observed compared to polymer blank samples. The blank polymer of HDPE demonstrated a weak AhR-mediated potency as well, if the activity was due to contained additives could not be verified by chemical analysis. AhR-mediated potencies were only observed following a 24 h exposure to extracts of deployed plastic pellets. This indicates that AhR agonists, present in the samples, were degraded after 72 h due to cellular metabolism in the assay. Previous studies have demonstrated that more metabolic persistent chemicals, like PCDD/Fs and coplanar PCBs, are stable

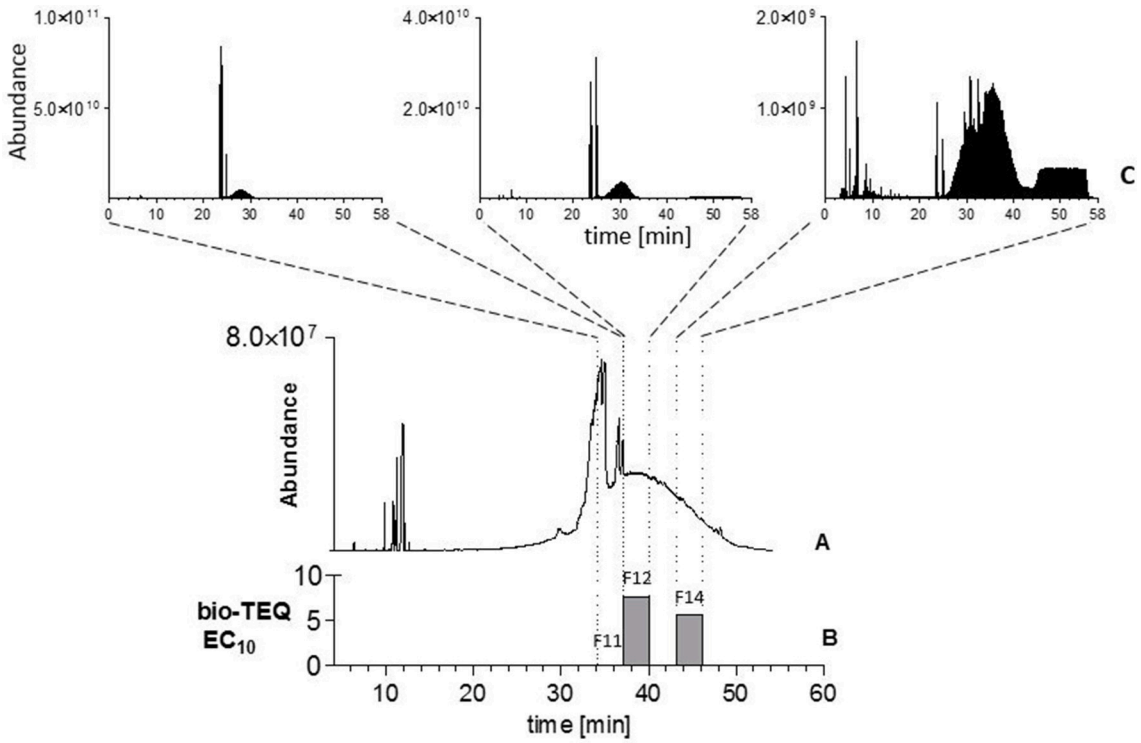


FIGURE 3 | Effect-directed analysis conducted in pooled plastic pellets of marine deployed HIPS. **(A)** GC- MS chromatogram of the pooled HIPS sample. **(B)** Measured bio-TEQs in pg/g (EC_{10}) of 17 fractions of pooled HIPS. Dashed lines represent fractions 11, 12, and 14, which induced AhR-mediated potencies in the DR CALUX® (fraction 11 was cytotoxic). **(C)** GC-MS chromatograms of fractions 11, 12, and 14 of pooled HIPS from left to right analyzed with HRMS. Y-axis represents the abundance of detected peaks.

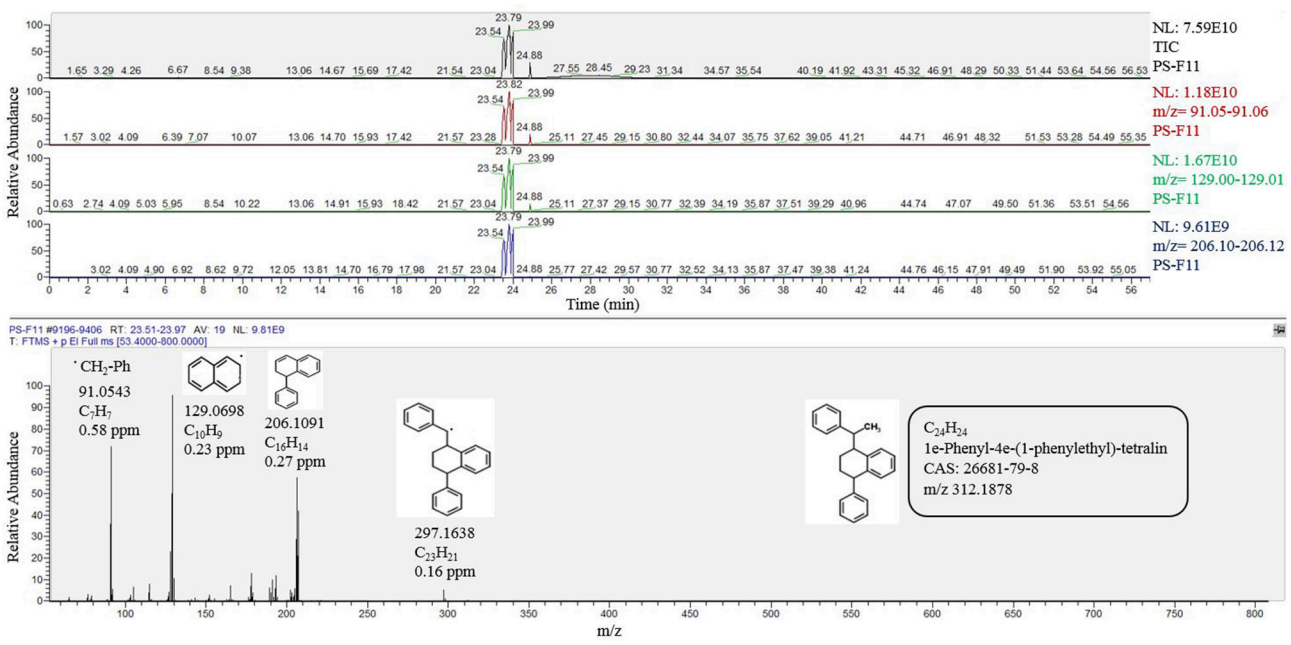


FIGURE 4 | Total ion chromatogram (TIC) and extracted masses of fragment ions of 1e- Phenyl- 4e- (1- phenylethyl)-tetralin (top) and mass spectra for 1e- Phenyl- 4e- (1- phenylethyl)- tetralin (bottom) in HIPS fraction 11.

TABLE 1 | Detected major peaks (>10% base peak) in the total ion chromatograms (m/z 53.4–800) of fractions of deployed and blank plastic polymers detected by GC-HRMS.

Sample	Retention time [min]	m/z	Formula	Mass error (ppm)	Molecular ion	Identification confidence degree*
F 1-17 HIPS 1-8 m; F 1-17 blank HIPS	6.39	162.1403 148.1246 133.1012 105.0699	$C_{12}H_{18}$ $C_{11}H_{16}$ $C_{10}H_{13}$ C_8H_9	−0.02 −0.00 0.01 0.06	Hexamethylben-zene	2 a)
F 10-13 HIPS 1-8 m; F 10-13 blank HIPS	14.67	204.0934 203.0854 101.0387	$C_{16}H_{12}$ $C_{16}H_{11}$ C_8H_5	0.22 −0.57 1.17		4
F 9-15 HIPS 1-8 m; F 9-15 blank HIPS	23.7	312.18782 97.1639 206.1091 129.0698 91.0543	$C_{24}H_{24}$ $C_{23}H_{21}$ $C_{16}H_{14}$ $C_{10}H_9$ C_7H_7	ND 0.43 0.38 −0.23 0.60	1e-Phenyl-4e-(1-phenylethyl)-tetralin	2 b)

The m/z is given in descending order from molecular ion to detected fragment ions.
**Identification confidence degree as proposed by Schymanski et al. (2014); ND, non-detected.*

inducers of the AhR pathway over time compared to more readily degradable compounds, such as PAHs (Lee et al., 2013; Larsson et al., 2014). Therefore, AhR agonists in plastic extracts were probably not PCDD/Fs and coplanar PCBs. It is possible that the sulfuric acid reaction time of 3 h used in this study was not sufficient to completely break down acid labile compounds, like PAHs. A previous study demonstrated that a sulfuric acid treatment of 1 h was able to reduce the AhR-mediated activity of a mixture of the 16 EPA PAHs by 50 %, while no response was induced after 10 h of acid treatment of the mixture (Villeneuve et al., 2002). Consequently, acid-degradable compounds, such as PAHs, could still have been present in the sample extracts. Since Heron Island is also used for recreational purposes, it is likely that PAHs were emitted into the water by boat traffic and sorbed to the plastic pellets.

LDPE, HDPE, and HIPS samples had similar bio-TEQs for the different deployment times. Similar sorption capacities have been reported before for PAHs to plastic pellets of these polymers (Rochman et al., 2013b), and for PCBs for PE polymer types (Rochman et al., 2013a). Sorption of other compound classes to LDPE and HDPE is therefore likely to be similar as well, due to the similar polymer structure.

The observed cytotoxicity in deployed HIPS pellets and in the HIPS polymer blank indicates that the cause of the cytotoxicity originated from the pristine pellet itself. Indeed, the styrene monomer is known to be disruptive and carcinogenic to the endocrine system (Ohyama et al., 2001; Lithner et al., 2011), and some styrene dimers and trimers have been reported to act as weak AhR agonists (Hong et al., 2016). However, cytotoxic effects of polystyrene (PS) monomers or oligomers have not been reported in H4IIepGudluc1.1cells, to the best of our knowledge, although hepatotoxicity of styrene and styrene oxide has been reported in mice (Gadberry et al., 1996).

Due to the relative remote area of the deployment site in the present study a rather small anthropogenic impact was expected. Therefore, the obtained bio-TEQs might act as benchmarks of control sites for AhR-mediated potencies in similar studies. In the previous study by Schönlau et al. (2019) the greatest AhR-mediated potency (277 pg/g) was observed in extracts of LDPE pellets, which had been deployed for 9 months in an urban harbor with industrial and recreational activity. The greatest AhR-mediated potency in the current study for LDPE was almost five times less (57 pg/g LDPE I 6 m) and almost three times less in HDPE (100 pg/g HDPE I 8 m). The difference in AhR-mediated activities between the two study sites is quite small considering the fact of the two very different locations and that in the previous study no acid treatment was conducted. The herein analyzed plastic pellets were only deployed for 8 months and a longer deployment time at the present study location could lead to greater AhR-mediated potencies because elevated levels of especially octa-chlorinated dibenzo-p-dioxins have been reported in sediment and seagrass samples from the Great Barrier reef (McLachlan et al., 2001). In general higher substituted congeners need longer times to sorb to microplastics, as sorption kinetics decrease with increasing hydrophobicity (Endo et al., 2013). It has been suggested that marine microplastics will be at equilibrium with most HOCs by 2 years (Koelmans et al., 2016). However, it has also been demonstrated that AhR-mediated potencies in 12 months deployed plastic pellets were lower compared to 9 months deployed plastic pellets (Schönlau et al., 2019). This suggests that there is not a steady state of compounds sorbed to marine microplastics and peak concentrations might be reached much earlier than expected and/or vary throughout the residence time in the marine system.

Although observed AhR-mediated potencies were small in the analyzed samples, for environmental implications it is, however, noteworthy that pristine pellets of HDPE and the fractionated HIPS elicited a measurable AhR-mediated response before deployment. The unfractionated blank sample of HIPS even led to cytotoxicity in the bioassay. These results suggest that the polymers themselves have a potential to

activate the AhR-mediated pathway, thus might be of greater concern than other polymers regarding an exposure in the marine environment.

Occurrence of HOCs on Deployed Plastics

Because PE has been widely used in passive sampling devices (Zabiegala et al., 2010), sorption of compounds to LDPE and HDPE pellets was expected. In general, relatively low levels of PCBs, organochlorine pesticides and PAHs have been reported in sediment and surface water samples from the Great Barrier Reef, with the exception of sites that are close to human activity, such as harbors and sites adjacent to agriculture (Haynes and Johnson, 2000; Haynes et al., 2000). In fact, anthropogenic impact via agricultural and industrial runoff were the major sources of contamination (Haynes and Johnson, 2000; Packett et al., 2009). Therefore, the deployed plastic pellets were expected to contain low concentrations of such HOCs. This was reflected by the relatively small concentrations of analyzed target compounds in deployed samples. The predominance of hexa-chlorinated PCB congeners is similar to patterns observed for beached plastic particles of PE (Hirai et al., 2011). Due to the lower recoveries of PCB# 138 (66%) and PCB #153 (42%) in LDPE pellets, which were observed in the spiked recovery test (see **Table S2**), it indicates that the herein reported values for both congeners are underestimations. While PBDEs can be expected to be present already in the pristine pellets as additives (Hirai et al., 2011), it was unexpected to find PCBs in measurable concentrations in pristine plastic pellets, and has not been reported before. For HDPE pellets it was notable that the total PCB concentration increased after 1 month of deployment compared to the blank level, and then declined in concentration with time of deployment. It is unclear whether this was due to desorption into the water phase after an initial adsorption or absorption into the polymer matrix. In deployed LDPE samples concentrations of the predominant congeners PCB #138 and PCB #153 suggest a sorption from the surrounding environment to the pellets because the congeners were not detected in the blank polymer.

The predominance of the PBDE congener #47 in deployed samples is not surprising because this congener is commonly detected in seawater (Mizukawa et al., 2009; Wang et al., 2011). In deployed LDPE samples concentrations of BDE47 were higher compared to the measured concentration in the blank polymer which suggests a sorption from seawater. However, in deployed HDPE samples the concentrations were slightly lower than in the blank polymer which suggest that no BDE47 sorbed from the surrounding water to the pellets. The spiked recovery of BDE47 from LDPE pellets was 43% (**Table S2**) which indicates that measured values of BDE47 in LDPE samples were underestimated.

Overall, while the biological activities showed an increase over the time of deployment, this could not be seen in total concentrations of detected HOCs in all samples. LDPE samples expressed a fluctuation of total concentrations of analyzed compounds during the time of deployment with a final increase and HDPE samples showed firstly an increase followed by a decrease with a rather constant concentration at all three subsequent sampling dates. The average water temperature during the time of deployment was reported to decrease from 27°C at the time of deployment to 20°C in August, and increased again up to 22°C in October when the last samples were taken (http://weather.aims.gov.au/#/station/130). Because the water temperature does not reflect the observed patterns in bioactivities and measured chemicals over the time of deployment, it suggest that the water temperature did not have a significant influence on the uptake of AhR agonist nor analyzed HOCs. However, the longer the plastic pellets were deployed in the water, the more have they been impacted by weathering. The degree of weathering has been reported to be positively correlated with increasing concentrations of PCBs (Endo et al., 2005), therefore concentrations of other compounds might also be positively correlated with more weathered plastic pellets which was only observed in the increasing bio-TEQs over time.

The calculated chem-TEQ values demonstrated a negligible contribution of the quantified PCBs and OCDD to the total induced AhR-mediated activities (<0.001%) even though concentrations below LOQ were also considered. Although the occurrence of several halogenated natural compounds produced by algae, sponges and other marine biota in the Great Barrier Reef is well-known (Vetter et al., 2018), they were not likely to be the inducers of the observed AhR-mediated responses. Halogenated dimethyl bipyrroles (HDBPs), for example, have been detected as AhR agonists, but they are relatively persistent and even elicited greater AhR-mediated potencies following 48 h of exposure (Tittlemier et al., 2003). This would suggest that HDBPs also show an AhR-mediated activity following a 72 h exposure and herein analyzed plastic extracts did not elicit an AhR-mediated response following a 72 h exposure.

In the present study we used a quick and more exhaustive extraction technique compared to, for example, leaching, to screen for total concentrations of AhR-active compounds in marine microplastics. This method does not reflect the bioavailable fraction of the compounds, but if bio-TEQs of total extracts are already low, it might not be necessary to further investigate the bioavailability of the compounds causing the activity since the bioavailable fraction can be expected to be even lower. The calculated bio-TEQs in the present study were smaller than previously measured bio-TEQs in LDPE plastic pellets that were deployed in an urban marine harbor (Schönlau et al., 2019). Furthermore, since no measurable AhR-mediated activities were induced following a 72 h exposure, compounds in the present study were readily metabolized, thus probably present a smaller risk to organisms. It has been hypothesized that the contribution of ingested microplastics in transferring HOCs to organisms is rather negligible and might be much greater from other marine sources, such as colloids and dissolved organic carbon, because they hold greater amounts of sorbed HOCs (Koelmans et al., 2016). In addition, the dietary intake of HOCs via prey is likely a more important exposure pathway for many marine organisms compared to microplastics, and has so far been shown in northern fulmars (Herzke et al., 2016). However, the exposure to microplastics will most certainly vary according to different marine habitats,

and accumulation zones or point sources of microplastics might be more important sites for the exposure to plastic-derived HOCs.

Performance of the GC-MS Fractionation Platform

By chemical analysis of the fractionated standard mixture with a HRMS system, 88% of the compounds in the mixture were detected. The calculated recoveries were, however, small for the majority of compounds. For identification of bioactive compounds the small recoveries might not demonstrate a problem as long as the concentration of a compound in the fraction is sufficient for obtaining a measurable signal in the bioassay and chemical analysis. But for the assessment of the contribution of a compound to the overall potency in the bioassay a quantification of the compound is necessary. For more certainty of the calculated recoveries it would be beneficial to spike the fractions with labeled internal standards which also accounts for matrix effects. Nevertheless, for further studies it needs to be investigated if a loss of compounds already occurs in the fractionation step or if the subsequent transfer and evaporation of the eluate in fractionation vials leads to a loss of compounds. The high recoveries of lower molecular weight PAHs need to be further investigated, but are likely due to background contamination during fractionation since the compounds are present in air, especially naphthalene (Buckpitt et al., 2010; Jia and Batterman, 2010), and colleting fractions from 15 injections leaves time for airborne contamination.

Among the analyzed pesticides only methoxychlor and p, p'-DDT have been reported to be weak AhR agonists (Takeuchi et al., 2008), but methoxychlor could not be identified in fractions of the known standard mixture.

However, the implications for the present study can be expected to be negligible because high recoveries of low molecular weight PAHs would not contribute to AhR-mediated activities in the samples since they are not AhR agonists. Also non-fractionated total plastic extracts were investigated qualitatively for target, suspect, and non-target chemicals and results were compared with results of fractionated samples. The same compounds were found in total and fractionated extracts in similar intensities.

Bioassay Testing of Fractionated Pooled Plastic Extracts

Bio-TEQs derived from individual plastic pellets were greater compared to bio-TEQs obtained by pooled samples. This was not expected and could implicate that concentrations of AhR agonists in pooled samples were not high enough for identification, even though collecting 15 repeated injections.

That total pooled extracts demonstrated greater bioactivities than fractionated samples be explained by higher tested plastic concentrations of total extracts compared to plastic concentrations in the individual fractions. The amount of plastic that was tested in the bioassay in fractions of HDPE 1–8 m was about 50% of the amount of tested plastic in the total extract of HDPE 1–8 m, and in fractions of LDPE 1–8 m the amount of plastic was around 40% of the amount of the total extract of LDPE 1–8 m. This means that the concentration of some agonists in the fractions could have been too low to even reach EC_{10} levels, thus did not contribute to the calculated bio-TEQs. Furthermore, total extracts integrate the entire bioactivity of compounds present in the mixture. This means that AhR agonists present in the mixture can act, for instance, in a synergistic or additive manner. Separated in fractions, AhR agonists will only elicit AhR-mediated responses based on their potencies and concentrations in each fraction. For instance, weak AhR agonists that did not induce a measurable bioactivity in single fractions could have added up to the bioactivity in the total extracts. The AhR-mediated potencies of fractions 12 and 14 added up together induced 54 and 38% of the potency measured in total extracts of HDPE 1–8 m and LDPE 1–8 m, respectively. Additionally, antagonists might have been present in the extracts which can possibly bind easier to the AhR in individual fractions due to less competition with AhR agonists, thus decreasing the bioactivity in fractions.

Overall, the GC-MS fractionation platform was efficient in reducing the complexity and isolating the bioactive drivers in the samples. This was especially noted for HIPS for which only one fraction showed cytotoxicity. The detected bioactivities in later eluting fractions (fraction 12 and 14) indicate that the AhR-mediated activities in extracts of plastic samples were caused by more hydrophobic compounds. Since the AhR-mediated activities were observed in the same fractions, regardless of the type of polymer, it is most likely the same compounds that were causing the bioactivities.

Chemical Analysis of Fractionated Pooled Plastic Extracts by HRMS

The conducted target analysis of fractionated pooled plastic samples revealed a few PAHs in earlier fractions (fraction 2–6). Because all of the detected PAHs have been reported to be present in polystyrene pristine pellets (Rochman et al., 2013b), and all PAHs, except for fluorene, had similar intensities in the TICs of deployed HIPS compared to blank HIPS, the PAHs were most likely present from the manufacturing of the HIPS pellets. Of all the detected PAHs only pyrene is a weak AhR agonist, while the others have not been observed to act as AhR agonists (Larsson et al., 2012). Pyrene was detected in fraction 5 of HIPS 1–8 m and in blank HIPS, and not in a fraction which demonstrated a measurable AhR activity.

The tentatively identified compound 1e-Phenyl-4e-(1-phenylethyl)-tetralin, in fractions 9 to15 of pooled HIPS and blank HIPS, was found to be a styrene trimer of polystyrene and has been detected in extracts of sediments as residues of PS contamination (Hong et al., 2016), as well as in food packaging materials (Choi et al., 2005; Nakai et al., 2014). Some styrene trimers have been reported as weak AhR agonists (Hong et al., 2016), and it is possible that 1e-Phenyl-4e-(1-phenylethyl)-tetralin contributed to the AhR-mediated response in fractions 12 and 14 of pooled HIPS, and fraction 12 of blank HIPS. However, it is unclear which isomer of 1e-Phenyl-4e-(1-phenylethyl)-tetralin was present in the herein analyzed HIPS

fractions. A cytotoxicity of 1e-Phenyl-4e-(1-phenylethyl)-tetralin has not been reported in the literature. The occurrence of this compound in the blank, as well as in the deployed samples, corroborates the hypothesis that the plastic polymer itself was the source of this compound.

Furthermore, hexamethylbenzene was detected in fractions of deployed and blank HIPS, which suggests that also this compound was originating from the HIPS pellets itself. Various substituted benzenes have been identified in PS food packaging material (Nerin et al., 1998), but no AhR-mediated potency of hexamethylbenzene was found in the literature.

Compounds specific for the bioactive fractions 12 and 14 of HDPE 1-8 m and LDPE 1-8 m could not be identified using the conducted effect-directed approach. This was probably due to low concentrations of compounds in the fractions and a greater number of repeated injections would have been necessary to enable a detection. In addition, the bioactive compounds might not form stable ions in the used electron impact ionization of the mass spectrometer and inhibit a detection.

However, AhR-mediated potencies of individual plastic pellets compared to AhR-mediated potencies of blank polymers indicated that AhR agonists were sorbed from the marine environment during the time of deployment (**Table S6** and **Figure 1**), and the used DR CALUX® assay was observed to be a more sensitive tool in the detection of AhR agonists compared to chemical analysis.

CONCLUSIONS

Bio-TEQs in the present study were similar for LDPE, HDPE, and HIPS pellets and most likely reflect background levels of AhR-mediated activities from the marine environment, because of the supposed small anthropogenic impact at the deployment site reflected in the low bio-TEQs of the deployed plastics. HIPS might be of greater concern for the environment due to the observed cytotoxicity of the pristine pellet. The contribution of PCB 105, 118, 156, 157 and OCDD to the measured bioactivities was negligible (<0.001%), therefore indicating the presence of other AhR agonists. However, the conducted effect-directed approach was not successful in identifying the bioactive compounds in deployed LDPE and HDPE samples. First and foremost, the concentrations of AhR agonist in the samples were small, as indicated by low bio-TEQs of pooled plastics, and likely below detection limits of the used instrumentation. The used GC-MS fractionation platform was, however, successful in separating 118 out of 134 HOCs, but recoveries for most compounds were low and present a difficulty for samples with low levels of pollution. The fractionation set up reduced the complexity of the samples, and only three fractions out of 17 showed an AhR-mediated activity. 1e-Phenyl-4e-(1-phenylethyl)-tetralin, was discovered by non-target analysis in fractions of both deployed HIPS and blank HIPS, indicating the compound originated from the pellets itself. This compound is suspected to having contributed to the observed AhR-mediated potencies in fraction 12 and 14 of HIPS samples as it has been reported to be an AhR agonist. Overall, the bioassay presented to be a sensitive tool in the detection of AhR agonist sorbed to marine microplastics and demonstrates the importance of bioassay analysis along with chemical analysis in order to estimate risks of sorbed compounds.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because of the size of the data set, especially the non-target data. Requests to access the datasets should be directed to Christine Schönlau [Christine.Schonlau@oru.se].

AUTHOR CONTRIBUTIONS

AR, CS, ML, and AK designed the study. AR and RvdZ carried out the deployment and sampling of microplastics. CS carried out the laboratory work including target chemical and bioassay analysis. FD did the non-target and suspect chemical analysis. CS and FD carried out the data analysis. CS wrote the manuscript and ML, FD, ME, AR, and AK contributed to the manuscript by commenting on the manuscript.

FUNDING

This study was funded by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (223-2014-1064) and by the EnForce research project (20160019), funded by the Knowledge foundation.

ACKNOWLEDGMENTS

CS would like to thank Jochen Mueller for use of laboratory facilities at the Queensland Alliance for Environmental Health Sciences, Brisbane, Australia, and Rebecca Bülow for help with the bioassay testing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2019.00120/full#supplementary-material

REFERENCES

Bjurlid, F., Roos, A., Ericson Jogsten, I., and Hagberg, J. (2018). Temporal trends of pbdd/fs, pcdd/fs, pbdes and pcbs in ringed seals from the baltic sea (*pusa hispida botnica*) between 1974 and 2015. *Sci. Total Environ.* 616–617:1374–1383. doi: 10.1016/j.scitotenv.2017.10.178

Buckpitt, A., Kephalopoulos, S., Koistinen, K., Kotzias, D., Morawska, L., and Sagunski, H. (2010). "Who guidelines for indoor air quality: selected pollutants," in *Who Guidelines for Indoor Air Quality: Selected Pollutants*.

Choi, J. O., Jitsunari, F., Asakawa, F., and Lee, D. (2005). Migration of styrene monomer, dimers and trimers from polystyrene to food simulants. *Food Addit. Contaminants* 22, 693–699. doi: 10.1080/02652030500160050

Denison, M. S., and Heath-Pagliuso, S. (1998). The ah receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals. *B Environ. Contam. Tox* 61, 557–568. doi: 10.1007/PL00002973

Endo, S., Takizawa, R., Okuda, K., Takada, H., Chiba, K., Kanehiro, H., et al. (2005). Concentration of polychlorinated biphenyls (pcbs) in beached resin pellets: variability among individual particles and regional differences. *Mar. Pollut. Bull.* 50, 1103–1114. doi: 10.1016/j.marpolbul.2005.04.030

Endo, S., Yuyama, M., and Takada, H. (2013). Desorption kinetics of hydrophobic organic contaminants from marine plastic pellets. *Mar. Pollut. Bull.* 74, 125–131. doi: 10.1016/j.marpolbul.2013.07.018

Gadberry, M. G., DeNicola, D. B., and Carlson, G. P. (1996). Pneumotoxicity and hepatotoxicity of styrene and styrene oxide. *J. Toxicol. Env. Health* 48, 273–294. doi: 10.1080/009841096161339

Grung, M., Naes, K., Fogelberg, O., Nilsen, A. J., Brack, W., Lubcke-von Varel, U., et al. (2011). Effects-directed analysis of sediments from polluted marine sites in norway. *J. Toxicol. Env. Heal. A* 74, 439–454. doi: 10.1080/15287394.2011.550555

Haynes, D., and Johnson, J. E. (2000). Organochlorine, heavy metal and polyaromatic hydrocarbon pollutant concentrations in the great barrier reef (australia) environment: a review. *Mar. Pollut. Bull.* 41, 267–278. doi: 10.1016/S0025-326X(00)00134-X

Haynes, D., Müller, J., and Carter, S. (2000). Pesticide and herbicide residues in sediments and seagrasses from the great barrier reef world heritage area and queensland coast. *Mar. Pollut. Bull* 41, 279–287. doi: 10.1016/S0025-326X(00)00097-7

Herzke, D., Anker-Nilssen, T., Nost, T. H., Gotsch, A., Christensen-Dalsgaard, S., Langset, M., et al. (2016). Negligible impact of ingested microplastics on tissue concentrations of persistent organic pollutants in northern fulmars off coastal norway. *Environ. Sci. Technol.* 50, 1924–1933. doi: 10.1021/acs.est.5b04663

Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., et al. (2011). Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. *Mar. Pollut. Bull* 62, 1683–1692. doi: 10.1016/j.marpolbul.2011.06.004

Hong, S., Lee, J., Lee, C., Yoon, S. J., Jeon, S., Kwon, B.-O., et al. (2016). Are styrene oligomers in coastal sediments of an industrial area aryl hydrocarbon-receptor agonists? *Environ. Pollut.* 213, 913–921. doi: 10.1016/j.envpol.2016.03.025

Jia, C., and Batterman, S. (2010). A critical review of naphthalene sources and exposures relevant to indoor and outdoor air. *Int. J. Environ. Res. Public Health* 7, 2903–2939. doi: 10.3390/ijerph7072903

Jonker, W., Clarijs, B., de Witte, S. L., van Velzen, M., de Koning, S., Schaap, J., et al. (2016). Gas chromatography fractionation platform featuring parallel flame-ionization detection and continuous high-resolution analyte collection in 384-well plates. *J. Chromatogr. A* 1462, 100–106. doi: 10.1016/j.chroma.2016.07.068

Jonker, W., Zwart, N., Stockl, J. B., de Koning, S., Schaap, J., Lamoree, M. H., et al. (2017). Continuous fraction collection of gas chromatographic separations with parallel mass spectrometric detection applied to cell-based bioactivity analysis. *Talanta* 168, 162–167. doi: 10.1016/j.talanta.2017.02.067

Koelmans, A. A., Bakir, A., Burton, G. A., and Janssen, C. R. (2016). Microplastic as a vector for chemicals in the aquatic environment: critical review and model-supported reinterpretation of empirical studies. *Environ. Sci. Technol.* 50, 3315–3326. doi: 10.1021/acs.est.5b06069

Larsson, M., Hagberg, J., Giesy, J. P., and Engwall, M. (2014). Time-dependent relative potency factors for polycyclic aromatic hydrocarbons and their derivatives in the h4iie-luc bioassay. *Environ. Toxicol. Chem.* 33, 943–953. doi: 10.1002/etc.2517

Larsson, M., Hagberg, J., Rotander, A., van Bavel, B., and Engwall, M. (2013). Chemical and bioanalytical characterisation of pahs in risk assessment of remediated pah-contaminated soils. *Environ. Sci. Pollut. Res.* 20, 8511–8520. doi: 10.1007/s11356-013-1787-6

Larsson, M., Orbe, D., and Engwall, M. (2012). Exposure time-dependent effects on the relative potencies and additivity of pahs in the ah receptor-based h4iie-luc bioassay. *Environ. Toxicol. Chem.* 31, 1149–1157. doi: 10.1002/etc.1776

Lee, K. T., Hong, S., Lee, J. S., Chung, K. H., Hilscherova, K., Giesy, J. P., et al. (2013). Revised relative potency values for pcdds, pcdfs, and non-ortho-substituted pcbs for the optimized h4iie-luc *in vitro* bioassay. *Environ. Sci. Pollut. Res.* 20, 8590–8599. doi: 10.1007/s11356-013-1770-2

Lithner, D., Larsson, A., and Dave, G. (2011). Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. *Sci. Total Environ.* 409, 3309–3324. doi: 10.1016/j.scitotenv.2011.04.038

Lubcke-von Varel, U., Streck, G., and Brack, W. (2008). Automated fractionation procedure for polycyclic aromatic compounds in sediment extracts on three coupled normal-phase high-performance liquid chromatography columns. *J. Chromatogr. A* 1185, 31–42. doi: 10.1016/j.chroma.2008.01.055

Marlowe, J. L., and Puga, A. (2005). Aryl hydrocarbon receptor, cell cycle regulation, toxicity, and tumorigenesis. *J. Cell. Biochem.* 96, 1174–1184. doi: 10.1002/jcb.20656

Mato, Y., Isobe, T., Takada, H., Kanehiro, H., Ohtake, C., and Kaminuma, T. (2001). Plastic resin pellets as a transport medium for toxic chemicals in the marine environment. *Environ. Sci. Technol.* 35, 318–324. doi: 10.1021/es0010498

McLachlan, M. S., Haynes, D., and Müller, J. F. (2001). Pcdds in the water/sediment–seagrass–dugong (dugong dugon) food chain on the great barrier reef (australia). *Environ. Pollut.* 113, 129–134. doi: 10.1016/S0269-7491(00)00172-X

Mizukawa, K., Takada, H., Takeuchi, I., Ikemoto, T., Omori, K., and Tsuchiya, K. (2009). Bioconcentration and biomagnification of polybrominated diphenyl ethers (pbdes) through lower-trophic-level coastal marine food web. *Mar. Pollut. Bull.* 58, 1217–1224. doi: 10.1016/j.marpolbul.2009.03.008

Murk, A. J., Legler, J., Denison, M. S., Giesy, J. P., vandeGuchte, C., and Brouwer, A. (1996). Chemical-activated luciferase gene expression (calux): a novel *in vitro* bioassay for ah receptor active compounds in sediments and pore water. *Fund. Appl. Toxicol.* 33, 149–160. doi: 10.1093/toxsci/33.1.149

Nakai, M., Tsubokura, M., Suzuki, M., Fujishima, S., Watanabe, Y., Hasegawa, Y., et al. (2014). Genotoxicity of styrene oligomers extracted from polystyrene intended for use in contact with food. *Toxicol. Rep.* 1, 1175–1180. doi: 10.1016/j.toxrep.2014.11.007

Nerin, C., Rubio, C., Cacho, J., and Salafranca, J. (1998). Parts-per-trillion determination of styrene in yoghurt by purge-and-trap gas chromatography with mass spectrometry detection. *Food Additives Contaminants* 15, 346–354. doi: 10.1080/02652039809374650

Ohyama, K. I., Nagai, F., and Tsuchiya, Y. (2001). Certain styrene oligomers have proliferative activity on mcf-7 human breast tumor cells and binding affinity for human estrogen receptor α. *Environ. Health Persp.* 109, 699–703. doi: 10.1289/ehp.01109699

Packett, R., Dougall, C., Rohde, K., and Noble, R. (2009). Agricultural lands are hot-spots for annual runoff polluting the southern great barrier reef lagoon. *Mar. Pollut. Bull.* 58, 976–986. doi: 10.1016/j.marpolbul.2009.02.017

Pieke, E., Heus, F., Kamstra, J. H., Mladic, M., van Velzen, M., Kamminga, D., et al. (2013). High-resolution fractionation after gas chromatography for effect-directed analysis. *Anal. Chem.* 85, 8204–8211. doi: 10.1021/ac401384q

Poster, D. L., Schantz, M. M., Sander, L. C., and Wise, S. A. (2006). Analysis of polycyclic aromatic hydrocarbons (pahs) in environmental samples: a critical review of gas chromatographic (gc) methods. *Anal. Bioanal. Chem.* 386, 859–881. doi: 10.1007/s00216-006-0771-0

Rios, L. M., Moore, C., and Jones, P. R. (2007). Persistent organic pollutants carried by synthetic polymers in the ocean environment. *Mar. Pollut. Bull.* 54, 1230–1237. doi: 10.1016/j.marpolbul.2007.03.022

Rochman, C. M., Hoh, E., Hentschel, B. T., and Kaye, S. (2013a). Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: implications for plastic marine debris. *Environ. Sci. Technol.* 47, 1646–1654. doi: 10.1021/es303700s

Rochman, C. M., Manzano, C., Hentschel, B. T., Simonich, S. L., and Hoh, E. (2013b). Polystyrene plastic: a source and sink for polycyclic aromatic hydrocarbons in the marine environment. *Environ. Sci. Technol.* 47, 13976–13984. doi: 10.1021/es403605f

Schönlau, C., Larsson, M., Lam, M. M., Engwall, M., Giesy, J. P., Rochman, C., et al. (2019). Aryl hydrocarbon receptor-mediated potencies in field-deployed plastics vary by type of polymer. *Environ. Sci. Pollut. Res.* 26, 9079–9088. doi: 10.1007/s11356-019-04281-4

Schymanski, E. L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H. P., et al. (2014). Identifying small molecules via high resolution mass spectrometry: communicating confidence. *Environ. Sci. Technol.* 48, 2097–2098. doi: 10.1021/es5002105

Takeuchi, S., Iida, M., Yabushita, H., Matsuda, T., and Kojima, H. (2008). *In vitro* screening for aryl hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line, dr-ecoscreen cells, and *in vivo* mouse liver cytochrome p450-1a induction by propanil, diuron and linuron. *Chemosphere* 74, 155–165. doi: 10.1016/j.chemosphere.2008.08.015

Teuten, E. L., Saquing, J. M., Knappe, D. R., Barlaz, M. A., Jonsson, S., Bjorn, A., et al. (2009). Transport and release of chemicals from plastics to the environment and to wildlife. *Philos. T. R. Soc. B* 364, 2027–2045. doi: 10.1098/rstb.2008.0284

Tittlemier, S. A., Kennedy, S. W., Hahn, M. E., Reddy, C. M., and Norstrom, R. J. (2003). Naturally produced halogenated dimethyl bipyrroles bind to the aryl hydrocarbon receptor and induce cytochrome p4501a and porphyrin accumulation in chicken embryo hepatocytes. *Environ. Toxicol. Chem.* 22, 1622–1631. doi: 10.1002/etc.5620220726

Tombesi, N., Pozo, K., Alvarez, M., Pribylova, P., Kukucka, P., Audy, O., et al. (2017). Tracking polychlorinated biphenyls (pcbs) and polybrominated diphenyl ethers (pbdes) in sediments and soils from the southwest of buenos aires province, argentina (south eastern part of the grulac region). *Sci. Total. Environ.* 575, 1470–1476. doi: 10.1016/j.scitotenv.2016.10.013

USEPA (2014). *Method 4435-Screening for Dioxin-Like Chemical Activity in Soil and Sediments Using the Calux® Bioassay and Teq Determinations.* Available online at: https://www.epa.gov/sites/production/files/2015-12/documents/4435.pdf

Van, A., Rochman, C. M., Flores, E. M., Hill, K. L., Vargas, E., Vargas, S. A., et al. (2012). Persistent organic pollutants in plastic marine debris found on beaches in san diego, california. *Chemosphere* 86, 258–263. doi: 10.1016/j.chemosphere.2011.09.039

Vetter, W., Kaserzon, S., Gallen, C., Knoll, S., Gallen, M., Hauler, C., et al. (2018). Occurrence and concentrations of halogenated natural products derived from seven years of passive water sampling (2007–2013) at normanby island, great barrier reef, australia. *Mar. Pollut. Bull.* 137, 81–90. doi: 10.1016/j.marpolbul.2018.09.032

Villeneuve, D. L., Blankenship, A. L., and Giesy, J. P. (2000). Derivation and application of relative potency estimates based on *in vitro* bioassay results. *Environ. Toxicol. Chem.* 19, 2835–2843. doi: 10.1002/etc.5620191131

Villeneuve, D. L., Khim, J. S., Kannan, K., and Giesy, J. P. (2002). Relative potencies of individual polycyclic aromatic hydrocarbons to induce dioxinlike and estrogenic responses in three cell lines. *Environ. Toxicol.* 17, 128–137. doi: 10.1002/tox.10041

Wang, J., Lin, Z., Lin, K., Wang, C., Zhang, W., Cui, C., et al. (2011). Polybrominated diphenyl ethers in water, sediment, soil, and biological samples from different industrial areas in zhejiang, china. *J. Hazard. Mater.* 197, 211–219. doi: 10.1016/j.jhazmat.2011.09.078

Weiss, J. M., Hamers, T., Thomas, K. V., van der Linden, S., Leonards, P. E., and Lamoree, M. H. (2009). Masking effect of anti-androgens on androgenic activity in european river sediment unveiled by effect-directed analysis. *Anal. Bioanal. Chem.* 394, 1385–1397. doi: 10.1007/s00216-009-2807-8

Zabiegala, B., Kot-Wasik, A., Urbanowicz, M., and Namiesnik, J. (2010). Passive sampling as a tool for obtaining reliable analytical information in environmental quality monitoring. *Anal. Bioanal. Chem.* 396, 273–296. doi: 10.1007/s00216-009-3244-4

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

ORIGINAL RESEARCH
published: 08 August 2019
doi: 10.3389/fmars.2019.00488

The Use of the Marine Microalga *Tisochrysis lutea* (*T-iso*) in Standard Toxicity Tests; Comparative Sensitivity With Other Test Species

Tania Tato[1]* *and Ricardo Beiras*[2]

[1] ECIMAT, Universidade de Vigo, Vigo, Spain, [2] Departamento de Ecoloxía e Bioloxía Animal, Universidade de Vigo, Vigo, Spain

The marine flagellate *Tisochrysis lutea* (*T-iso*), with a global distribution, is one of the most common microalgae used as natural food in aquaculture. In recent years *T-iso* has also been increasingly used in toxicity testing, although it is not included in current international protocols such as ISO that relies on *Phaeodactylum tricornutum* and *Skeletonema costatum* as marine species, and *Raphidocelis subcapitata* as freshwater species. Sensitivity of *Isochrysis galbana* to selected model toxicants was compared with that of those recommended species using the 72 h growth-rate inhibition response. Internationally accepted standard methods with fixed test conditions (light intensity, photoperiod, temperature, nutrients concentrations, initial cell density, time of exposure and endpoint) were followed to allow this comparison. Toxicity of model chemicals representative of the main environmental toxicants—a trace metal (zinc), a polyaromatic hydrocarbon (fluoranthene), an herbicide (benzalkonium chloride), an insecticide (chlorpyrifos), a surfactant (4-nonylphenol), and a microbiocide (triclosan)—were evaluated to determine EC_{50} and EC_{10} values. In general, *T-iso* showed higher sensitivity for most groups of toxicants, meeting the acceptability criteria in terms of control growth set in standard protocols. For example, EC_{50} and EC_{10} of *T-iso* for chlorpyrifos were 246 $\mu g\ L^{-1}$ and 132 $\mu g\ L^{-1}$, whereas for *P. tricornutum* these values were ca. fivefold higher: 1117 and 746 $\mu g\ L^{-1}$ respectively. Therefore, the use of *T-iso* in marine toxicity testing as a standard model representative of photosynthetic organisms is recommended.

Keywords: growth rate, zinc, benzalkonium chloride, fluoranthene, chlorpyrifos, nonylphenol, triclosan, toxicity testing

OPEN ACCESS

Edited by:
Julian Blasco, Spanish National Research Council (CSIC), Spain

Reviewed by:
Adam Lillicrap, Norwegian Institute for Water Research (NIVA), Norway
Ignacio Moreno-Garrido, Institute of Marine Sciences of Andalusia (ICMAN), Spain

***Correspondence:**
Tania Tato
taniatato@uvigo.es

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *31 October 2018*
Accepted: *18 July 2019*
Published: *08 August 2019*

Citation:
Tato T and Beiras R (2019) The Use of the Marine Microalga Tisochrysis lutea (T-iso) in Standard Toxicity Tests; Comparative Sensitivity With Other Test Species. Front. Mar. Sci. 6:488. doi: 10.3389/fmars.2019.00488

INTRODUCTION

Unicellular planktonic algae are primary producers that support oceanic food webs. Microalgae are well suited for toxicity bioassays because they are easily cultured and sensitive to organic and inorganic pollutants (Klaine and Lewis, 1995). Algal tests are chronic and sublethal tests conducted with several generations of organisms and have the advantage of a short duration (48–96 h). The significance of the algal toxicity test has been recognized internationally and numerous test guidelines have been published (ASTM, 2004; ISO, 2006a; OECD, 2011) and incorporated

in various regulatory procedures. The Organization for Economic Cooperation and Development (OECD) recommended a growth-inhibition test with microalgae in the base set of ecotoxicological tests, and it is legally demanded for all the substances produced or imported in Europe exceeding 10 t/year (REACH annexes VII-X). Green algae (Division Chlorophyta) and diatoms (Division Chromophyta, Class Bacillariophyceae) are the most commonly used microalgae in toxicity tests (Walsh, 1993). However standard methods did not take into account the sensitivity differences between algae species. In the marine environment, the International Standardization Organization (ISO) propose *Phaeodactylum tricornutum* and *Skeletonema costatum*, both worldwide spread marine diatoms. In recent years, *Isochrysis galbana,* culture strain isolated from Tahiti (hereafter *T-iso*), recently renamed *Tisochrysis lutea* (Bendif et al., 2013), has been one of the most used marine microalgae in toxicity tests (Moreno-Garrido et al., 2000; Hampel et al., 2001; Yap et al., 2004; Satoh et al., 2005; Campa-Córdova et al., 2006; Correa-Reyes et al., 2007; Garrido-Perez et al., 2008; Debelius et al., 2009; Pérez et al., 2010a; Liu et al., 2011; Fisher et al., 2014; Suratno et al., 2015; Trenfield et al., 2015). This species was selected on the basis of its sensitivity and the ease to culture (Shaw and Chadwick, 1998; Pérez et al., 2009) as well as its economic importance in aquaculture (Renaud et al., 1991). Due to its nutritive value, it is used in marine aquaculture to feed early life stages of mollusks and crustaceans, and in fish larvae culture (Sukenik and Wahnon, 1991). *T-iso* cells are round shaped, 3–7.5 μm in diameter, typically covered with several layers of organic scales, and have two apical flagella (Heimann and Huerlimann, 2015). They have a fast growth rate and wide physicochemical tolerance ranges (O'Shea et al., 2008).

Marine organisms are increasingly exposed to a suite of natural and synthetic anthropogenic pollutants, including trace metals, polycyclic aromatic hydrocarbons, pesticides, antifouling biocides, and other emerging pollutants like components of plastic and cosmetic products.

Metals, mostly accumulated in bottom sediment and later released into water bodies, have long been recognized as major marine pollutants (Ansari et al., 2004). Although zinc is not regarded as being especially toxic, it is sometimes present in seawater and sediment interstitial water in estuaries at quantities exceeding the toxicity thresholds to some species (Stauber and Florence, 1990).

Fluoranthene (FLU) is a low molecular weight polycyclic aromatic hydrocarbons (PAH). According to Neff and Stubblefield (1995), FLU, naphthalene, phenanthrene and pyrene are the most toxic components of oil for marine biota in the short term. It has also been reported to be toxic to photosynthetic aquatic organisms (Spehar et al., 1999; Pérez et al., 2010b).

The organophosphate (OP) broad spectrum insecticide chlorpyrifos (CPF) is extensively used in agriculture and residential pest control (Saulsbury et al., 2009). OPs are considered hazardous environmental pollutants since they are persistent, non-biodegradable and bioaccumulative (Readman et al., 1992; Bondarenko et al., 2009). Schimmel et al. (1983) found CPF to be the most hydrophobic pesticide of six pesticides studied in his work, reporting a Log K_{ow} = 5.2, a solubility in seawater of 73 μg L^{-1} and a half-life of 24 days in seawater. Bioconcentration factor (BCF) in *Mytilus galloprovincialis* was 400 ± 119 L kg^{-1} (Serrano et al., 1997). Thus, contamination by pesticides is a serious water pollution issue, and CPF is listed as a priority substance according to Annex I of the Directive 2013/39/EU.

Benzalkonium chloride (BAC) is a quaternary ammonium compound broadly used in disinfection treatments in aquaculture species (Hoskins and Dalziel, 1984; Lio-Po and Sanvictores, 1986), as an algaecide (Lee et al., 1994) and as an antifouling component (His et al., 1996; Parr et al., 1998; Smith et al., 2002). Effects on phytoplankton species were studied in Pérez et al. (2009).

Nonylphenols (NP) are degradation products of detergents (NP ethoxylates), cleaning products and are also directly used as pesticides and as plasticizers for high-density polyethylene (HDPE), polyethylene terephthalate (PET) and polyvinyl chloride (PVC) (Loyo-Rosales et al., 2004). It poses remarkable environmental risk in marine environments (Tato et al., 2018), and the EU has included 4-NP on the list of priority hazardous substances for surface waters, with chronic and acute environmental quality standards of 0.3 and 2 mg L^{-1}, respectively (Directive 2013/39/EU).

Triclosan (TCS) is a broad spectrum antimicrobial used in pharmaceutical and personal-care products (Singer et al., 2002) and as a preservative in textile fibers and plastics (Dann and Hontela, 2011). TCS has been detected in rivers, lakes and coastal marine waters of several European countries, United States, Canada, Australia, Japan and Hong Kong (EC, 2010; Dann and Hontela, 2011; Pintado-Herrera et al., 2014a,b). Previous work identified TCS as highly toxic for marine microalgae (Tato et al., 2018).

The aim of this study was to assess the sensitivity of *T-iso* in comparison with other microalgal species recommended in standard methods: *P. tricornutum* and *R. subcapitata*, using selected model toxicants representative of the main groups of aquatic pollutants. With that aim, the standard 72 h growth inhibition test was used, and effective concentrations that cause a decrease of 10 and 50% on the biological variable (EC_{10} and EC_{50}) were estimated for each chemical for *T-iso* and *P. tricornutum* and for zinc, 4-n-nonylphenol and triclosan for *R. subcapitata*.

MATERIALS AND METHODS

Test Chemicals

All standards were of the highest commercially available purity (>98%): $ZnCl_2$ (CAS 7646-85-7) obtained from Merck (Germany), FLU (CAS 206-44-0), CPF (CAS 2921-88-2), BAC (CAS 63449-41-2), 4-NP (CAS 104-40-5) and TCS (CAS number 3380-34-5) obtained from Sigma-Aldrich (Madrid, Spain). FLU, CPF, 4-NP and TCS stock solutions were prepared in dimethyl sulfoxide (DMSO, CAS 67-68-5). $ZnCl_2$ and BAC stocks were made in water of ultrapure quality, obtained by means of Milli-Q apparatus (Millipore, Bedford, MA, United States), ultrapure water hereinafter. Exposure solutions were made up by diluting the stock solutions in 0.22 μm-filtered sea water (FSW) of oceanic characteristics (34 ± 2 psu salinity, 8.2 ± 0.1 pH, 8.0 ± 0.1 mg L^{-1}

dissolved oxygen) from an uncontaminated area in the outer part of Ría de Vigo (Galicia, NW Iberian Peninsula) for the marine species, or ultrapure water for *R. subcapitata*.

Test Organisms

Isochrysis affinis *galbana* recently renamed *Tisochrysis lutea* clone Tahiti (*T-iso*) (strain ECC038) and *Phaeodactylum tricornutum* Bohlin (strain ECC028), and the freshwater microalga *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, Kristiansen and Skulbertg (formerly known as *Selenastrum capricornutum* or *Pseudokirchneriella subcapitata*) strain ECC029 were obtained from the ECIMAT (University of Vigo) microalgae collection.

Algal Growth Inhibition Test

Toxicity tests were performed under strict quality assurance/quality control tools following internationally standard methods. Growth-rate inhibition tests followed ISO (2006a) for *T-iso* and *P. tricornutum* and ISO (2012) for *R. subcapitata*. Experimental solutions and the inoculum for the algal culture (10,000 ± 2,000 cells mL^{-1}) were added to 250 mL borosilicate conical flasks in triplicate and six additional flasks as control cultures. For tests with FLU, CPF, 4-NP and TCS controls were DMSO solutions in FSW (0.1% v/v), below NOEC for *T-iso* (0.4%) and *R. subcapitata* 1% (El Jay, 1996). Flasks were kept in an isothermal room at 20°C with a 24 h light period (cool daylight lamps Osram L36W/865, emission spectrum range 380–780 nm, and light intensity 80–100 μE m^{-2} s^{-1}; Biospherical Instruments Inc., QSL2101). No agitation was provided during incubation and pH was recorded initially and after 72 ± 2 h. Cells were counted at the beginning and after the 72 ± 2 h of exposure with a Z2 Coulter Counter particle size analyzer (Beckman-Coulter, United States). Growth rate (GR) was calculated as:

$$\mathrm{GR}(\mathrm{d}^{-1}) = \frac{[\ln(\text{final cell number})] - [\ln(\text{intial cell number})]}{\mathrm{d}}$$

The response (GR) was expressed as growth inhibition (I) using the following equation:

$$\mathrm{I}(\%) = \frac{GR_c - GR_i}{GR_c} \times 100$$

Where GR_c is the average growth rate in control cultures and GR_i is the growth rate in each flask.

Validity criteria described in ISO (2006a) and ISO (2012) were adopted (see **Table 1**). The zinc assay required a modification of the protocol (ISO, 2006b) that implies the use of growth medium lacking EDTA, and reducing incubation time to 48 ± 2 h. In addition, as an internal quality control, the marine algae bioassays were also performed on the reference chemical 3,5-dichlorophenol (ISO, 2006a).

Statistical Analyses

The EC_{50} and EC_{10} values and their 95% confidence intervals were calculated by fitting data to a Probit dose-response model using IBM SPSS statistics version 22.0.

TABLE 1 | Summary of test conditions for the microalgae growth-rate inhibition bioassay.

Test type	Chronic toxicity test; static
Temperature	20 ± 2°C
Salinity	34 ± 0.5 psu
Light quality	Cool white fluorescent lighting
Light intensity	60 μE m^{-2} s^{-1}
Photoperiod	Continuous illumination
Test chamber	Borosilicate glass Erlenmeyer flasks of 250 mL
Test volume	200 mL
Age of test organism	5–7 days (Log growth phase)
Initial algae density	10,000 ± 2,000 cells mL^{-1}
Number of replicates	3
Control replicates	6
Test medium	Natural 0.22 μm filtered seawater + 2 mL L^{-1} of f/2 medium (*T-iso*) Natural 0.22 μm filtered seawater + culture media ISO (2006a) (*P. tricornutum*) Ultrapure (conductivity < 10 μS cm^{-1}) water + culture media ISO (2012) (*R. subcapitata*)
Test duration	72 ± 2 h
Test end-point	Growth-rate inhibition (I)
Validity criteria*	Growth rate (GR) in the control replicates at least 0.9 d^{-1} (1.4 d^{-1}) Variation coefficient of the GR in the control replicates shall not exceed 7% (5%) pH in the control shall not increase more than 1 relative of the growth medium (1.5)

**In brackets values for R. subcapitata.*

RESULTS

Dose-response curves and the effective concentrations derived for the different chemicals tested are shown in **Figure 1** and **Table 2**. For all the chemicals evaluated, *T-iso* was more sensitive than *P. tricornutum* and also than *R. subcapitata* for 4-n-NP and TCS. For the three chemicals tested with the three algal species, the ranking of toxicity according to the EC_{50} values was the same: 4-n-NP > TCS > Zn. For the extra three chemicals tested with the marine species only, the ranking of toxicity was also the same: BAC > CPF > FLU. Therefore the choice of test species will not affect toxicity rankings provided if a single species is used. In contrast, sensitivity was consistently higher for *T-iso* compared to *P. tricornutum*, and the EC_{50} values for the former were on average fivefold lower than for the latter.

Validity criteria were met in all the assays and values of reference chemical 3,5-dichlorophenol were EC_{50} = 2.31 (2.26–2.36) mg L^{-1} and EC_{50} = 1.63 (1.53–1.74) mg L^{-1} for *P. tricornutum* and *T-iso*, respectively.

DISCUSSION

Methods standardization is crucial to ensure comparability of results, and in the case of microalgae tests, numerous recent publications use different methodologies (see **Table 3**). Incubation temperatures ranging from 24 to 30°C

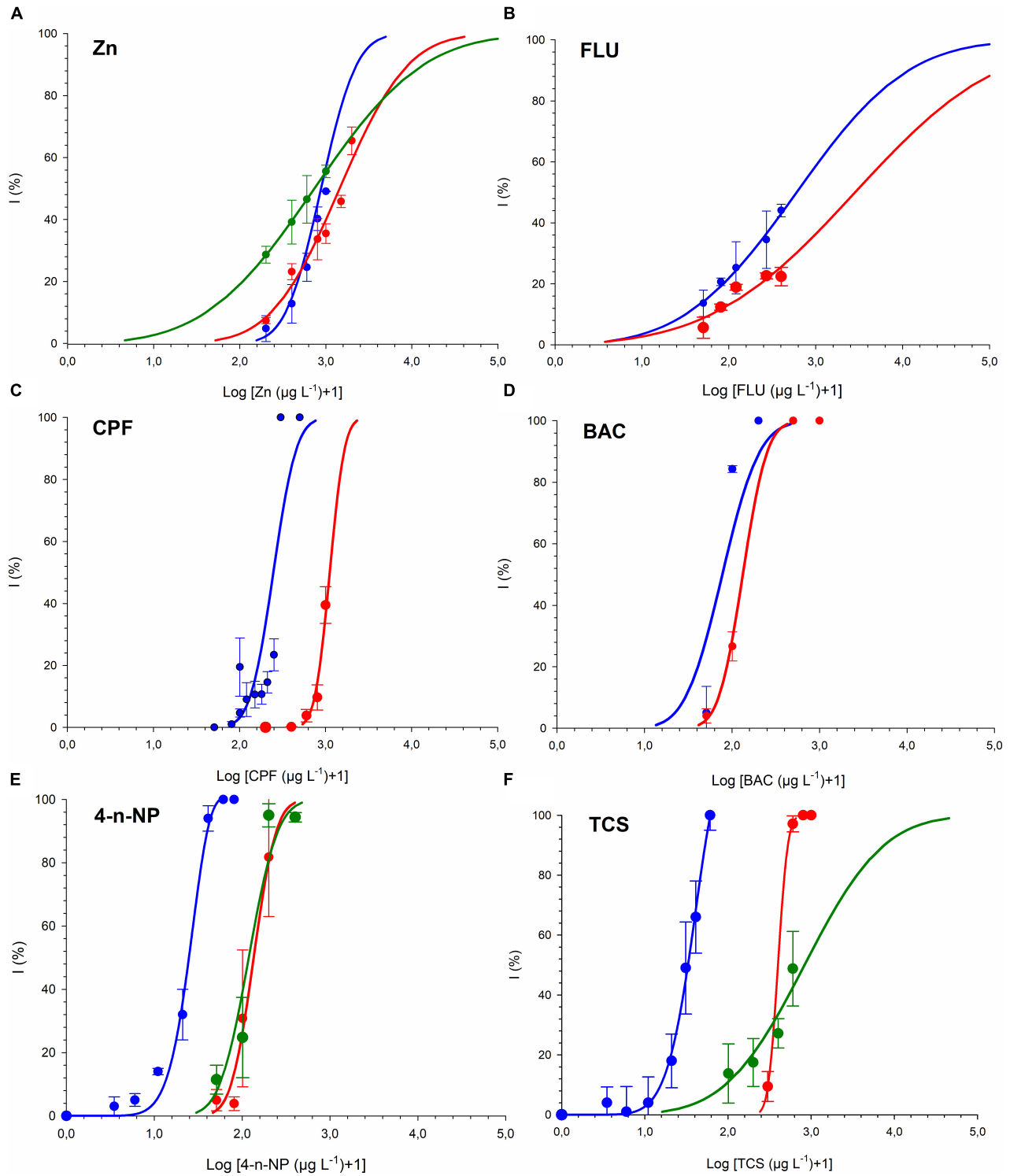


FIGURE 1 | Growth inhibition of *T-iso* (blue), *P. tricornutum* (red) and *R. subcapitata* (green) exposed to **(A)** Zinc **(B)** Fluoranthene **(C)** Chlorpyrifos **(D)** Benzalkonium chloride **(E)** 4-n-Nonylphenol. **(F)** Triclosan. Blue curve from panel **(E,F)** published in Tato et al. (2018).

(Trenfield et al., 2015), culture media and cellular density changes to improve sensitivity (Moreno-Garrido et al., 2000), *in vivo* fluorescence endpoint (Pérez et al., 2010a) and microplate test (Satoh et al., 2005) are some examples of sources of interassay and interlaboratory variability. Interlaboratory calibrations and definition of strict acceptability criteria are needed in order to

TABLE 2 | Effective concentrations ($\mu g\ L^{-1}$) obtained in the growth inhibition test for zinc (Zn), fluoranthene (FLU), chlorpyrifos (CPF), Benzalkonium chloride (BAC), 4-n-nonylphenol (4-n-NP) and triclosan (TCS).

		Zn	FLU	CPF	BAC	4-n-NP	TCS
P. tricornutum	EC_{10}	228.8 (169.0–286.2)	62.2 (37.6–84.3)	746.3 (697.2–784.6)	69.0 (63.3–74.2)	73.8 (49.7–90.8)	302.3 (289.2–314.5)
	EC_{50}	1442 (1265–1692)	2838 (1406–10394)	1117 (1047–1229)	131.9 (122.2–144.5)	134.1 (111.0–176.6)	397.9 (384.3–412.5)
T-iso	EC_{10}	323.1 (255.8–378.0)	31.0 (13.9–48.4)	132.0 (102.8–154.9)	57.1 (30.0–71.7)	11.1* (8.2–14.0)	14.6* (11.8–17.4)
	EC_{50}	1054.9 (911.0–1306.8)	602.2 (413.6–1163)	246.3 (213.0–301.0)	86.0 (67.2–109.9)	24.1* (20.9–27.3)	34.0* (27.7–40.3)
R. subcapitata	EC_{10}	39.3 (14.3–69.7)	n.t.	n.t.	n.t.	54.4 (33.7–71.6)	144.4 (80.2–198.9)
	EC_{50}	733.5 (613.2–945.6)				117.7 (93.7–146.8)	387.3 (309.0–476.8)

*95% confidence intervals in brackets. n.t, not tested. *Tato et al. (2018).*

increase robustness of results and improve the reliability of the toxicity thresholds obtained, used in risk assessment and for derivation of environmental quality regulations.

Standard Test Conditions

Test guidelines incorporated in regulatory procedures (ASTM, 2004; ISO, 2006a, 2012; OECD, 2011) are strict protocols that seek robust and reproducible results. The recommended test format is glass Erlenmeyer flasks and polycarbonate for tests with metals. The comparability of toxicity responses between tests in microplates and standard flask has been the subject of discussion (Ismail et al., 2002; Eisentraeger et al., 2003; Blaise and Vasseur, 2005; Pavlic et al., 2006). Microplate tests have advantages such as reduction of the volume of sample needed and the possibility to perform high throughput analyses, reducing also costs in glassware and reagents. However, problems of adsorption of substances in microplate walls (Araújo et al., 2010), evaporation of volatile chemicals that can interfere with neighbor wells, and cell density increase due to evaporation are some of the significant interferences that can occur (Pavlic et al., 2006).

Microalgae require constant light conditions which supports exponential growth. This can be accomplished providing light above saturation intensity, applying mechanical mixing and lowering the culture volume and density. Continuous light is proposed in these standard methods with the advantages to allow constant growth conditions, the obtaining of desynchronized algal populations and the practice in routine testing (Nyholm and Källqvist, 1989). The algal growth rate exponentially increases with temperature until an optimum temperature is reached and rapidly declines thereafter. Nyholm and Källqvist (1989) highlighted that the main cause of temperature differences is created by the heat evolved from light tubes. Again, standard methods recommend using cool white fluorescent lighting that avoids this heating effect and has the main peak in the wavelengths corresponding to blue and green (20.8 and 24.7%), decreasing progressively to 8.5% for the red waveband (Sánchez-Saavedra and Voltolina, 2006), and the temperature of 20 ± 2°C is proposed. Algae growth limitation should be avoided to obtain comparable data (Klaine and Ward, 1983). Growth rates are related to intracellular nutrient concentrations and not to extracellular concentrations, and uptake of nutrients is not coupled with growth under nutrient-deficient conditions (Nyholm and Källqvist, 1989). Results obtained by Garrido-Perez et al. (2008) show that test medium composition is a determining factor in the EC_{50} value of alkylbenzene sulfonate (LAS) in *I. galbana*. Therefore a strict nutrient composition in the incubation medium is recommended. In seawater tests, the medium is usually synthetic or natural filtered seawater enriched with salts, nutrients (nitrates and phosphates, trace metals) and vitamins. ISO gives a formulation for *P. tricornutum* and *S. costatum*. The present study demonstrates that in the case of *T-iso*, f/2 nutritive medium (Guillard and Ryther, 1962) allows obtaining reproducible growth rate results. CO_2 is the microalgae carbon source for photosynthesis and airtight vessels should be avoided in order to allow flow of CO_2. ISO recommends to continuously shake, stir and aerate the culture flasks to maintain the cells in suspension and ease CO_2 mass transfer form air to water, and reduce pH drift. However, test solutions in our case were not shaken, stirred or aerated and ISO validity criteria for marine species were met for both *T-iso* and *P. tricornutum*. In the case of the freshwater species *R. subcapitata*, in contrast, pH occasionally increased more than the prescribed 1.5 units in control flasks for the 72 h test. Unlike freshwater, the carbonate content of seawater provides an efficient pH buffer. Therefore, we recommend for the marine test static conditions in order to reduce the laboratory equipment required.

Algal toxicity tests are more sensitive when initial cell density is low (Moreno-Garrido et al., 2000; Trenfield et al., 2015). Standard protocols recommend a maximum of 10,000 cells mL^{-1}, the lowest cell density suitable to be counted with the Neubauer chamber, but electronic particle counters, such as the Coulter counter, enable counting lower cell concentrations. Again, in order to establish a strict protocol and control sensitivity of the test due to cell density, initial cell density should be fixed, and we propose 10,000 ± 2,000 cells mL^{-1} as acceptable values.

Algae tests are restricted to the initial period of exponential growth, between 2 and 4 days of duration. This short duration

TABLE 3 | Overview of algal toxicity test conditions established by different authors.

Medium	Test format	Test species	Duration	Endpoint	Test conditions	References
Marine	250 mL Erlenmeyer flask	*R. salina*	24 h	Oxygen production	24°C	Moreno Garrido et al., 1999
Marine	250 mL Erlenmeyer flask	*C. autotrophyca* *N. atomus* *P. tricornutum* *I. galbana*	72 h	Growth rate inhibition	24 ± 0.1°C Continuous illumination 10,000 cells/mL 5,000 cells/mL 1,000 cells/mL	Moreno-Garrido et al., 2000
Marine	Erlenmeyer flask	*N. gaditana* *T. suecica* *R. salina* *I. galbana*	72 h	Growth rate inhibition Esterase activity Flow cytometry	OECD procedure	Hampel et al., 2001
Marine	250 mL Erlenmeyer flask	*I. galbana*	5 day; shaking	Growth rate (cell counting)	Continuous illumination	Yap et al., 2004
Marine	250 mL Erlenmeyer flask	*I. galbana* *C. gracilis*	15 days	Growth rate (spectrometer, readings at 750 nm)	24 ± 0.5°C	Campa-Córdova et al., 2006
Marine	250 mL Erlenmeyer flask (200 mL)	*I. galbana*	72 h	Growth rate (cell counting)	20°C Continuous illumination 10,000 cells/mL	Mhadhbi et al. (2012a, 2012b)
Marine	250 mL Erlenmeyer flask (200 mL)	*I. galbana*	72 h	Fluorescence (Fast Repetition Rate Fluorometry)	20°C Continuous illumination 5,000 cells/mL	Pérez et al. (2006, 2010a)
Marine	250 mL Erlenmeyer flask	*I. galbana*	72 h	Growth rate	Protocol ASTM E 1218–04	Fisher et al., 2014
Marine	100 mL Erlenmeyer flask (50 mL)	*I. galbana*	72 h; shaking	Growth rate	24–31°C Continuous illumination 3000 cells/mL	Trenfield et al., 2015
Marine	Microplate	*T. suecica* *S. Costatum* *P. lima*	5 h	Esterase activity	20°C 12:12 light:dark	Galgani et al., 1992; Gilbert et al., 1992
Marine	Microplate (24-well)	*C. calcitrans* *I. galbana* *T. tetrathele* Tetraselmis sp.	96 h	Chlorophyll fluorescence intensity	28 ± 1°C Continuous illumination	Ismail et al., 2002
Marine	Microplate (96-well; 270 μL/well)	*C. littorale* *Chlorococcum* sp. *I. galbana* *Heterocapsa* sp. *Prasinococcus* sp. *Cylindrotheca* sp. *Synechococcus* sp. *T. tetrathele*	72 h	Chlorophyll fluorescence intensity	22°C Continuous illumination	Satoh et al., 2005
Marine	Sterile polystyrene 6-well cell culture clusters (10 mL)	*I. galbana* *T. chuii*	n.d.	Motility	28 ± 1°C Natural daylight 550,000–750,000 cell/mL	Liu et al., 2011
Marine	Glass pyrex tubes (10 mL)	*T. chuii,* *R. salina* *Chaetoceros* sp. *I. galbana* (*T-iso*) *N. gaditana*	72 h	Toxic cellular quota Flow cytometry	20 ± 1°C Continuous illumination	Debelius et al., 2009
Marine/ Freshwater	Microplate (96-well, 270 μL/well)	*P. tricornutum* *C. vulgaris* *D. subspicatus*	Five saturation pulses at 90 s intervals	Inhibition of photosynthesis using fluorometry	n.d.	Schreiber et al., 2007
Freshwater	Microplate (96-well; 220 μL)	*Chlamydomonas* sp.	5 h	Motility inhibition	n.d.	Kusui and Blaise, 1995
Freshwater	Erlenmeyer flask Microplate (96-well and 24-well; 200 μL and 2 mL)	*D. subspicatus*	72 h	Chlorophyll fluorescence	23°C	Eisentraeger et al., 2003

(Continued)

TABLE 3 | Continued

Medium	Test format	Test species	Duration	Endpoint	Test conditions	References
Freshwater	Microplate (96-well; 200 μL)	*S. capricornutum*	72 h; static	Growth rate (hemacytometer)	24 ± 2°C 10,000 cells/mL	Blaise and Vasseur, 2005
Freshwater	15 mL tube (5 mL)	*S. capricornutum* *C. vulgaris* *C. reinhardtii* *S. quadricauda,* *Microcystis* sp. *A. flosque*	96 h	Chlorophyll fluorescence	25°C 16:8 light:dark 20,000 cells/mL	Fairchild et al., 1998
Freshwater	20 mL glass scintillation vials (2.5 mL)	*S. capricornutum*	48 h; shaking	Growth rate	22 ± 1°C 9,000–21,000 cells/mL	Arensberg et al., 1995

n.d, no description available.

avoids the nutrient limitation effect in control flasks, loss of toxicity by adsorption of chemicals on algal cells and detoxification caused by excretion of organics and volatilization (Nyholm and Källqvist, 1989). ISO and OECD established 72 ± 2 h for all algae tests with the exception of metal testing, where 48 ± 2 h is recommended in order to restrict the final algal biomass density.

A common endpoint in microalgae bioassays is growth rate, assessed in terms of cell density, recommended in several protocols and guidelines dealing with the standardization of these assays (ASTM, 2004; ISO, 2006a, 2012; OECD, 2011). Many authors discuss this issue and several other endpoints are suggested (see **Table 3**). Growth rate may also be assessed through chlorophyll content, and has also been applied to microscale assay using microplates (Ismail et al., 2002; Satoh et al., 2005). Pérez et al. (2010a) found fluorescence more suitable for assays with fuel but Othman et al. (2012) found greater sensibility in the population density endpoint at 72 h (absorbance at 650 nm) than the photosynthetic endpoint at 24 h (fluorescence of chlorophyll at 450 and 680 nm) for benz(a)anthracene and fluoranthene. Furthermore, fluorescence endpoint also requires previous measurement of chlorophyll a (Chl a) concentration and establishment of a relation between Chl a and fluorescence intensity. Hampel et al. (2001) compared esterase activity endpoint with growth inhibition resulting in 2 and 5 times less sensitivity than growth inhibition rate. The need for a standardization of the toxicological endpoints was evident by the large disparity between the EC values found in literature for the same chemicals. Algal biomass growth rate, recorded through counts of cell densities, is the toxicity test endpoint proposed in ISO and OECD, and we recommend using an electronic particle counter for rapid and objective measurements of this endpoint.

Test Organism

The excessive variety in results from algal toxicity tests can be decreased by suitable standardization/harmonization of test methods, and this includes the selection of standard test species. Several test species and strains were studied in the past (Walsh et al., 1988), and published guidelines (ASTM, 2004; ISO, 2006a, 2012; OECD, 2011) recommend the diatoms *P. tricornutum* and *S. costatum* for marine environment and the green algae *R. subcapitata* (formerly *S. capricornutum* or *P. subcapitata*) for freshwater ecosystems. OECD also recommends for freshwater *Desmodesmus subspicatus* (green algae), *Navicula pelliculosa* (diatoms), *Anabaena flos-aquae* and *Synechococcus leopoliensis* (cyanobacteria).

There are diverse factors that influence algae species' susceptibility to pollutants, including cell size, cell wall type and thickness and taxonomic group characteristics. Small cells have large surface area to volume ratios which increase their potential uptake rates for solutes and in turn increase their sensitivity to pollutants, reported for copper (Quigg et al., 2006) and PAHs (Echeveste et al., 2010; Othman et al., 2012). In the case of metals, there is not a clear tendency. Levy et al. (2007) found the wall-less *D. tertiolecta* more tolerant to copper than *M. pusilla* and *Tetraselmis* sp., both with cell wall. On the other hand, Macfie et al. (1994) compared a naked clone and clone with cell wall of the freshwater green alga *C. reinhardtii* and reported more sensitivity to cadmium, copper and nickel in the naked strain. Regarding detergents, Corre et al. (1996) found higher sensitivity in *Chlorella* species with different outer layer composition and Lewis (1990) contemplated the thickness of the cell wall as a key factor to explain the damage caused by a surfactant to microalgae. Later Pérez et al. (2009) reported differences in toxicity response to BAC regarding the penetration of the surfactant into the cell in two species with different outer layer characteristics, *T-iso* and *C. gracilis*. According to the EC_{50} values, *T-iso* shows more sensibility to all tested chemicals than *P. tricornutum* (with outer frustule). The absence of a cell wall in *T-iso* (membrane covered with body scale), compared to the outer frustule of the diatom, may account for these differences. In fact, *T-iso* was the most sensitive species for all tested chemicals except for the Zn where, as expected, the freshwater species was the most sensitive. In seawater, toxicity of divalent metal ions is reduced because these ions compete for cell binding sites with the non-toxic Ca^{2+} and Mg^{2+} (Penttinen et al., 1998; Heijerick et al., 2002, 2009).

Marine Species

Several studies compare the sensitivity of algae related to the taxonomic group. Hampel et al. (2001) evaluated the toxicity of alkylbenzene sulfonate (LAS) on *N. gaditana*, *T. suecica*, *R. salina*, and *I. galbana*, reporting the highest sensibility for *R. salina*.

Satoh et al. (2005) studied the toxicity of five heavy metals (Cu, As, Sb, Pb, and Cd) using nine marine microalgal species of five divisions and seven genera) and a fluorometric growth-inhibition assay with microplates resulting in haptophyta *I. galbana*, the most sensitive species for all metals except for Sb. Using Cu as a reference toxicant and 11 algal species, Levy et al. (2007) did not find any large taxonomic class of algae was more sensitive than another. Values of 72 h $EC_{50} < 5\ \mu g\ L^{-1}$ were reported in five different classes of algae (a diatom, a prasinophytes, a cryptomonad, a prymnesiophyte and a dinoflagellate). Again using Cu and 16 marine diatoms, Stauber and Florence (1990) only highlighted the high tolerance of *D. tertiolecta*. Algae from the genera *Dunaliella* and *Tetraselmis* have been classified as very tolerant species. For example the euryhaline *D. salina* and *T. chuii* are also tolerant to metals and have a competitive advantage against other algal species in metal-stressed conditions (Moreno-Garrido et al., 2005; Millán de Kuhn et al., 2006).

Freshwater Species

Blanck et al. (1984) showed with 13 freshwater species and 19 compounds that a universally more sensitive species of alga could not be identified, but suggested that some species are more regular and others more selective in their response to pollutants, recommending *Scenedesmus obtusiusculus*, *Chlorella emersonii* and *Raphidocelis subcapitata*. Later, Larsen et al. (1986) studied the toxicity of Atrazine to eight different algal species and identified *R. subcapitata*, *Scenedesmus obliquus* and *Chlamydomonas reinhardtii* as the most sensitive species. This agrees with Fairchild et al. (1998) who studied the toxicity of four pesticides to six species of algae and found that green algae (*R. subcapitata*, *Chlorella vulgaris*, *Chlamydomonas reinhardtii* and *Scenedesmus quadricauda*) were more sensitive than the blue-green algae (*Microcystis* sp. and *Anabaena flosque*).

CONCLUSION

The marine microalgae *Tisochrysis lutea* (*T-iso*), formerly *Isochrysis galbana*, has the ideal characteristics to be used as a model in toxicity testing. It is easily available because of its worldwide use in aquaculture, is suitable for culture in low volumes displaying high growth rates in standard culture media, and shows equal or higher sensitivity to a broad range of toxic chemicals compared to alternative marine species.

AUTHOR CONTRIBUTIONS

RB designed the experiment. TT conducted the toxicity tests and data analysis. Both authors wrote the manuscript.

FUNDING

This work was supported by the Spanish Government through the ARPA-ACUA (CTM2016-77945-C3) and EPHEMARE (PCIN-2015-187-C03-03) projects, the latter under the framework of JPI Oceans.

ACKNOWLEDGMENTS

The authors acknowledge Damián Costas and all the staff of ECIMAT for their helpful technical support.

REFERENCES

Ansari, T. M., Marr, I. L., and Tariq, N. (2004). Heavy metals in marine pollution perspective–a mini review. *J. Appl. Sci.* 4, 1–20. doi: 10.3923/jas.2004.1.20

Araújo, C. V. M., Diz, F. R., Lubián, L. M., Blasco, J., and Moreno-Garrido, I. (2010). Sensitivity of *Cylindrotheca closterium* to copper: influence of three test endpoints and two test methods. *Sci. Total Environ.* 408, 3696–3703. doi: 10.1016/j.scitotenv.2010.05.012

Arensberg, P., Hemmingsen, V. H., and Nyholm, N. (1995). A miniscale algal toxicity test. *Chemosphere* 30, 2103–2115. doi: 10.1016/0045-6535(95)00090-U

ASTM (2004). *E1218-04e1 Standard Guide for Conducting Static Toxicity Tests with Microalgae*. West Conshohocken, PA: American Society for Testing and Materials.

Bendif, E. M., Probert, I., Schroeder, D. C., and de Vargas, C. (2013). On the description of *Tisochrysis lutea* gen. nov. sp. nov. and *Isochrysis nuda* sp. nov. in the *Isochrysidales*, and the transfer of Dicrateria to the Prymnesiales (Haptophyta). *J. Appl. Phycol.* 25, 1763–1776.

Blaise, C., and Vasseur, P. (2005). "Algal Microplate Toxicity Test," in *Small-Scale Freshwater Toxicity Investigations*, eds C. Blaise and J. Férard (Dordrecht: Springer).

Blanck, H., Wallin, G., and Wängberg, S. -Å. (1984). Species-dependent variation in algal sensitivity to chemical compounds. *Ecotoxicol. Environ. Saf.* 8, 339–351. doi: 10.1016/0147-6513(84)90003-90004

Bondarenko, S., Gan, J., Haver, D. L., and Kabashima, J. N. (2009). Persistence of selected organophosphate and carbamate insecticides in waters from a coastal watershed. *Environ. Toxicol. Chem.* 23, 2649–2654. doi: 10.1897/03-436

Campa-Córdova, A. I., Luna-González, A., Ascencio, F., Cortés-Jacinto, E., and Cáceres-Martínez, C. J. (2006). Effects of chloramphenicol, erythromycin, and furazolidone on growth of *Isochrysis galbana* and *Chaetoceros gracilis*. *Aquaculture* 260, 145–150. doi: 10.1016/j.aquaculture.2006.06.014

Corre, G., Templier, J., Largeau, C., Rousseau, B., and Berkaloff, C. (1996). Incluence of cell wall composition on the resistance of two *Chlorella* species (Chlorophyta) to detergents. *J. Phycol.* 32, 584–590. doi: 10.1111/j.0022-3646.1996.00584.x

Correa-Reyes, G., Viana, M. T., Marquez-Rocha, F. J., Licea, A. F., Ponce, E., and Vazquez-Duhalt, R. (2007). Nonylphenol algal bioaccumulation and its effect through the trophic chain. *Chemosphere* 68, 662–670. doi: 10.1016/j.chemosphere.2007.02.030

Dann, A. B., and Hontela, A. (2011). Triclosan: environmental exposure, toxicity and mechanisms of action. *J. Appl. Toxicol.* 31, 285–311. doi: 10.1002/jat.1660

Debelius, B., Forja, J. M., DelValls, Á., and Lubián, L. M. (2009). Toxicity and bioaccumulation of copper and lead in five marine microalgae. *Ecotoxicol. Environ. Saf.* 72, 1503–1513. doi: 10.1016/j.ecoenv.2009.04.006

EC (2010). *Preliminary Opinion on Triclosan-Antimicrobial Resistance*. Brussels: Scientific Committee on Consumer Safety, European Commission.

Echeveste, P., Agustí, S., and Dachs, J. (2010). Cell size dependent toxicity thresholds of polycyclic aromatic hydrocarbons to natural and cultured phytoplankton populations. *Environ. Pollut.* 158, 299–307. doi: 10.1016/j.envpol.2009.07.006

Eisentraeger, A., Dott, W., Klein, J., and Hahn, S. (2003). Comparative studies on algal toxicity testing using fluorometric microplate and Erlenmeyer flask growth-inhibition assays. *Ecotoxicol. Environ. Saf.* 54, 346–354. doi: 10.1016/S0147-6513(02)00099-94

El Jay, A. (1996). Toxic effects of organic solvents on the growth of *Chlorella vulgaris* and *Selenastrum capricornutum*. *Bull. Environ. Contam. Toxicol.* 57, 191–198. doi: 10.1007/s001289900174

Fairchild, J. F., Ruessler, D. S., and Carlson, A. R. (1998). Comparative sensitivity of five species of macrophytes and six species of algae to atrazine, metribuzin, alachlor, and metolachlor. *Environ. Toxicol. Chem.* 17, 1830–1834. doi: 10.1002/etc.5620170924

Fisher, D., Yonkos, L., Ziegler, G., Friedel, E., and Burton, D. (2014). Acute and chronic toxicity of selected disinfection byproducts to *Daphnia magna*, *Cyprinodon variegatus*, and *Isochrysis galbana*. *Water Res.* 55, 233–244. doi: 10.1016/j.watres.2014.01.056

Galgani, F., Cadiou, Y., and Gilbert, F. (1992). Simultaneous and iterative weighted regression analysis of toxicity tests using a microplate reader. *Ecotoxicol. Environ. Saf.* 23, 237–243. doi: 10.1016/0147-6513(92)90061-90067

Garrido-Perez, M. C., Perales-VargasMachuca, J. A., Nebot-Sanz, E., and Sales-Márquez, D. (2008). Effect of the test media and toxicity of LAS on the growth of *Isochrysis galbana*. *Ecotoxicology* 17, 738–746. doi: 10.1007/s10646-008-0223-z

Gilbert, F., Galgani, F., and Cadiou, Y. (1992). Rapid assessment of metabolic activity in marine microalgae: application in ecotoxicological tests and evaluation of water quality. *Mar. Biol.* 112, 199–205. doi: 10.1007/BF00702462

Guillard, R. R., and Ryther, J. H. (1962). Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt, and Detonula confervacea (cleve) Gran. *Can. J. Microbiol.* 8, 229–239. doi: 10.1139/m62-029

Hampel, M., Moreno-Garrido, I., Sobrino, C., Lubián, L. M., and Blasco, J. (2001). Acute toxicity of LAS homologues in marine microalgae: esterase activity and inhibition growth as endpoints of toxicity. *Ecotoxicol. Environ. Saf.* 48, 287–292. doi: 10.1006/eesa.2000.2028

Heijerick, D. G., De Schamphelaere, K. A. C., and Janssen, C. R. (2002). Biotic ligand model development predicting Zn toxicity to the alga *Pseudokirchneriella subcapitata*: possibilities and limitations. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 133, 207–218. doi: 10.1016/S1532-0456(02)00077-77

Heijerick, D. G., De Schamphelaere, K. A. C., and Janssen, C. R. (2009). Predicting acute zinc toxicity for *Daphnia magna* as a function of key water chemistry characteristics: development and validation of a biotic ligand model. *Environ. Toxicol. Chem.* 21, 1309–1315. doi: 10.1002/etc.5620210628

Heimann, K., and Huerlimann, R. (2015). "Chapter 3 - microalgal classification: major classes and genera of commercial microalgal species," in *Handbook of Marine Microalgae*, ed. S.-K. Kim (Boston, MA: Academic Press), 25–41. doi: 10.1016/b978-0-12-800776-1.00003-0

His, E., Beiras, R., Quiniou, F., Parr, A. C. S., Smith, M. J., Cowling, M. J., et al. (1996). The non-toxic effects of a novel antifouling material on oyster culture. *Water Res.* 30, 2822–2825. doi: 10.1016/S0043-1354(96)00167-164

Hoskins, G. E., and Dalziel, F. C. (1984). Survival of chinook fry (*Oncorhynchus tschawytscha*) following exposure to benzalkonium chloride in soft water. *Progr. Fish. Cult.* 46, 98–101. doi: 10.1577/1548-8640(1984)46<98:socfot>2.0.co;2

Ismail, M., Phang, S.-M., Tong, S.-L., and Brown, M. T. (2002). A modified toxicity testing method using tropical marine microalgae. *Environ. Monit. Assess.* 75, 145–154. doi: 10.1023/A:1014483713719

ISO (2006a). *ISO 10253:2006 Water quality - Marine Algal Growth Inhibition Test with Skeletonema costatum and Phaeodactylum tricornutum*. Geneva: ISO.

ISO (2006b). *ISO 14442:2006 Water Quality - Guidelines for Algal Growth Inhibition Tests with Poorly Soluble Materials, Volatile Compounds, Metals and Waste Water*. Geneva: ISO.

ISO (2012). *ISO 8692:2012 Water quality - Fresh Water Algal Growth Inhibition Test with Unicellular Green Algae*. Geneve: ISO.

Klaine, S. J., and Lewis, M. A. (1995). "Algal and plant toxicity testing," in *Handbook of Ecotoxicology*, eds D. J. Hoffman and B. A. Rattner (Boca Raton, FL: CRC Press), 163–184.

Klaine, S. J., and Ward, C. H. (1983). Growth optimized algal bioassays for toxicity evaluation. *Environ. Toxicol. Chem.* 2, 245–249. doi: 10.1002/etc.5620020212

Kusui, T., and Blaise, C. (1995). "Acute exposure phytotoxicity assay based on motility inhibition of *Chlamydomonas variabilis*," in *Environmental Toxicology Assessment*, ed. M. Richardson (London: Taylor & Francis), 125–135.

Larsen, D. P., Stay, F., Shiroyama, T., and Frank, D. (1986). Comparisons of single-species, microcosm and experimental pond responses to atrazine exposure. *Environ. Toxicol. Chem.* 5, 179–190. doi: 10.1002/etc.5620050209

Lee, C.-L., Chen, I. M., and Fong, S.-C. (1994). Effects of benzalkonium chloride on growth of phytoplankton. *J. Fish. Soc. Taiwan* 21, 69–77. doi: 10.29822/JFST

Levy, J. L., Stauber, J. L., and Jolley, D. F. (2007). Sensitivity of marine microalgae to copper: the effect of biotic factors on copper adsorption and toxicity. *Sci. Total Environ.* 387, 141–154. doi: 10.1016/j.scitotenv.2007.07.016

Lewis, M. A. (1990). Chronic toxicities of surfactants and detergent builders to algae: a review and risk assessment. *Ecotoxicol. Environ. Saf.* 20, 123–140. doi: 10.1016/0147-6513(90)90052-90057

Lio-Po, G. D., and Sanvictores, E. G. (1986). Tolerance of Penaeus monodon eggs and larvae to fungicides against *Lagenidium sp. and Haliphthoros philippinensis*. *Aquaculture* 51, 161–168. doi: 10.1016/0044-8486(86)90305-90304

Liu, G., Chai, X., Shao, Y., Hu, L., Xie, Q., and Wu, H. (2011). Toxicity of copper, lead, and cadmium on the motility of two marine microalgae *Isochrysis galbana* and *Tetraselmis chui*. *J. Environ. Sci.* 23, 330–335. doi: 10.1016/S1001-0742(10)60410-X

Loyo-Rosales, J. E., Rosales-Rivera, G. C., Lynch, A. M., Rice, C. P., and Torrents, A. (2004). Migration of nonylphenol from plastic containers to water and a milk surrogate. *J. Agric. Food Chem.* 52, 2016–2020. doi: 10.1021/jf0345696

Macfie, S. M., Tarmohamed, Y., and Welbourn, P. M. (1994). Effects of cadmium, cobalt, copper, and nickel on growth of the green alga *Chlamydomonas reinhardtii*: the influences of the cell wall and pH. *Arch. Environ. Con. Tox.* 27, 454–458. doi: 10.1007/BF00214835

Mhadhbi, L., Fumega, J., and Beiras, R. (2012a). Toxicological effects of three polybromodiphenyl ethers (BDE-47, BDE-99 and BDE-154) on growth of marine algae *Isochrysis galbana*. *Water Air Soil Pollut.* 223, 4007–4016. doi: 10.1007/s11270-012-1167-1168

Mhadhbi, L., Rial, D., Pérez, S., and Beiras, R. (2012b). Ecological risk assessment of perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) in marine environment using *Isochrysis galbana*, *Paracentrotus lividus*, *Siriella armata* and *Psetta maxima*. *J. Environ. Monit.* 14, 1375–1382. doi: 10.1039/C2EM30037K

Millán de Kuhn, R., Streb, C., Breiter, R., Richter, P., Neeße, T., and Häder, D.-P. (2006). Screening for unicellular algae as possible bioassay organisms for monitoring marine water samples. *Water Res.* 40, 2695–2703. doi: 10.1016/j.watres.2006.04.045

Moreno Garrido, I., Lubián, L. M., and Soares, A. M. V. M. (1999). Oxygen production rate as a test for determining toxicity of copper to *Rhodomonas salina* hill and wehterbee (Cryptophyceae). *Bull. Environ. Contam. Toxicol.* 62, 776–782. doi: 10.1007/s001289900939

Moreno-Garrido, I., Campana, O., Lubián, L. M., and Blasco, J. (2005). Calcium alginate immobilized marine microalgae: experiments on growth and short-term heavy metal accumulation. *Mar. Pollut. Bull.* 51, 823–829. doi: 10.1016/j.marpolbul.2005.06.008

Moreno-Garrido, I., Lubián, L. M., and Soares, A. M. V. M. (2000). Influence of cellular density on determination of EC50 in microalgal growth inhibition tests. *Ecotoxicol. Environ. Saf.* 47, 112–116. doi: 10.1006/eesa.2000.1953

Neff, J. M., and Stubblefield, W. A. (1995). "Chemical and toxicological evaluation of water quality following the Exxon Valdez oil spill," in *Exxon Valdez Oil Spill: Fate and Effects in Alaskan Waters*, eds P. G. Wells, J. N. Butler, and J. S. Hughes (Philadelphia, PA: ASTM), 141–177.

Nyholm, N., and Källqvist, T. (1989). Methods for growth inhibition toxicity tests with freshwater algae. *Environ. Toxicol. Chem.* 8, 689–703. doi: 10.1002/etc.5620080807

OECD (2011). *Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD Guidelines for the Testing of Chemicals, Section 2*. París: OECD Publishing.

O'Shea, S. K., Holland, F., and Bilodeau, A. (2008). Modeling the effects of salinity and pH on the cadmium bioabsorptive properties of the microalgae *Isochrysis galbana (T-Iso)* in coastal waters. *J. Coast. Res.* 26, 59–66. doi: 10.2112/08-1073.1

Othman, H. B., Leboulanger, C., Le Floc'h, E., Hadj Mabrouk, H., and Sakka Hlaili, A. (2012). Toxicity of benz(a)anthracene and fluoranthene to marine phytoplankton in culture: does cell size really matter? *J. Hazardous Mater.* 243, 204–211. doi: 10.1016/j.jhazmat.2012.10.020

Parr, A. C. S., Smith, M. J., Beveridge, C. M., Kerr, A., Cowling, M. J., and Hodgkiess, T. (1998). Optical assessment of a fouling-resistant surface (PHEMA/benzalkonium chloride) after exposure to a marine environment.

Adv. Mater. Opt. Electr. 8, 187–193. doi: 10.1002/(sici)1099-0712(1998070)8:4<187::aid-amo353>3.0.co;2-7

Pavlic, Z., Stjepanovic, B., Horvatic, J., Persic, V., Puntaric, D., and Culig, J. (2006). Comparative sensitivity of green algae to herbicides using erlenmeyer flask and microplate growth-inhibition assays. *Bull. Environ. Contam. Toxicol.* 76, 883–890. doi: 10.1007/s00128-006-1001-1003

Penttinen, S., Kostamo, A., and Kukkonen, J. V. K. (1998). Combined effects of dissolved organic material and water hardness on toxicity of cadmium to *Daphnia magna*. *Environ. Toxicol. Chem.* 17, 2498–2503. doi: 10.1002/etc.5620171217

Pérez, P., Estévez-Blanco, P., Beiras, R., and Fernández, E. (2006). Effect of copper on the photochemical efficiency, growth, and chlorophyll-a biomass of natural phytoplankton assemblages. *Environ. Toxicol. Chem.* 25, 137–143. doi: 10.1897/04-392R1.1

Pérez, P., Fernández, E., and Beiras, R. (2009). Toxicity of benzalkonium chloride on monoalgal cultures and natural assemblages of marine phytoplankton. *Water Air Soil Pollut.* 201, 319–330. doi: 10.1007/s11270-008-9947-x

Pérez, P., Fernández, E., and Beiras, R. (2010a). Fuel toxicity on *Isochrysis galbana* and a coastal phytoplankton assemblage: growth rate vs. *variable fluorescence*. *Ecotoxicol. Environ. Saf.* 73, 254–261. doi: 10.1016/j.ecoenv.2009.11.010

Pérez, P., Fernández, E., and Beiras, R. (2010b). Use of fast repetition rate fluorometry on detection and assessment of PAH toxicity on microalgae. *Water Air Soil Pollut.* 209, 345–356. doi: 10.1007/s11270-009-0203-209

Pintado-Herrera, M. G., González-Mazo, E., and Lara-Martín, P. A. (2014a). Atmospheric pressure gas chromatography–time-of-flight-mass spectrometry (APGC–ToF-MS) for the determination of regulated and emerging contaminants in aqueous samples after stir bar sorptive extraction (SBSE). *Anal. Chim. Acta* 851, 1–13. doi: 10.1016/j.aca.2014.05.030

Pintado-Herrera, M. G., González-Mazo, E., and Lara-Martín, P. A. (2014b). Determining the distribution of triclosan and methyl triclosan in estuarine settings. *Chemosphere* 95, 478–485. doi: 10.1016/j.chemosphere.2013.09.101

Quigg, A., Reinfelder, J. R., and Fisher, N. S. (2006). Copper uptake kinetics in diverse marine phytoplankton. *Limnol. Oceanogr.* 51, 893–899. doi: 10.4319/lo.2006.51.2.0893

Readman, J. W., Liong Wee Kwong, L., Mee, L. D., Bartocci, J., Nilve, G., Rodriguez-Solano, J. A., et al. (1992). Persistent organophosphorus pesticides in tropical marine environments. *Mar. Pollut. Bull.* 24, 398–402. doi: 10.1016/0025-326X(92)90500-90506

Renaud, S. M., Parry, D. L., Thinh, L.-V., Kuo, C., Padovan, A., and Sammy, N. (1991). Effect of light intensity on the proximate biochemical and fatty acid composition of *Isochrysis sp.* and *Nannochloropsis oculata* for use in tropical aquaculture. *J. Appl. Phycol.* 3, 43–53. doi: 10.1007/BF00003918

Sánchez-Saavedra, M. D. P., and Voltolina, D. (2006). The growth rate, biomass production and composition of *Chaetoceros sp*. Grown with different light sources. *Aquacult. Eng.* 35, 161–165. doi: 10.1016/j.aquaeng.2005.12.001

Satoh, A., Vudikaria, L. Q., Kurano, N., and Miyachi, S. (2005). Evaluation of the sensitivity of marine microalgal strains to the heavy metals, Cu, As, Sb, Pb and Cd. *Environ. Int.* 31, 713–722. doi: 10.1016/j.envint.2005.01.001

Saulsbury, M. D., Heyliger, S. O., Wang, K., and Johnson, D. J. (2009). Chlorpyrifos induces oxidative stress in oligodendrocyte progenitor cells. *Toxicology* 259, 1–9. doi: 10.1016/j.tox.2008.12.026

Schimmel, S. C., Garnas, R. L., Patrick, J. M., and Moore, J. C. (1983). Acute toxicity, bioconcentration, and persistence of AC 222,705, benthiocarb, chlorpyrifos, fenvalerate, methyl parathion, and permethrin in the estuarine environment. *J. Agric. Food Chem.* 31, 104–113. doi: 10.1021/jf00115a027

Schreiber, U., Quayle, P., Schmidt, S., Escher, B. I., and Mueller, J. F. (2007). Methodology and evaluation of a highly sensitive algae toxicity test based on multiwell chlorophyll fluorescence imaging. *Biosens. Bioelectron.* 22, 2554–2563. doi: 10.1016/j.bios.2006.10.018

Serrano, R., López, F. J., Hernández, F., and Peña, J. B. (1997). Bioconcentration of chlorpyrifos, chlorfenvinphos, and methidathion in *Mytilus galloprovincialis*. *Bull. Environ. Contam. Toxicol.* 59, 968–975. doi: 10.1007/s001289900577

Shaw, I. C., and Chadwick, J. (1998). *Principles of Environmental Toxicology*. London: CRC Press.

Singer, H., Müller, S., Tixier, C., and Pillonel, L. (2002). Triclosan: occurrence and fate of a widely used biocide in the aquatic environment: field measurements in wastewater treatment plants, surface waters, and lake sediments. *Environ. Sci. Technol.* 36, 4998–5004. doi: 10.1021/es025750i

Smith, M. J., Adam, G., Duncan, H. J., and Cowling, M. J. (2002). The effects of cationic surfactants on marine biofilm growth on hydrogels. *Estuarine Coast. Shelf Sci.* 55, 361–367. doi: 10.1006/ecss.2001.0910

Spehar, R. L., Poucher, S., Brooke, L. T., Hansen, D. J., Champlin, D., and Cox, D. A. (1999). Comparative toxicity of fluoranthene to freshwater and saltwater species under fluorescent and ultraviolet light. *Arch. Environ. Contam. Toxicol.* 37, 496–502. doi: 10.1007/s002449900544

Stauber, J. L., and Florence, T. M. (1990). Mechanism of toxicity of zinc to the marine diatom *Nitzschia closterium*. *Mar. Biol.* 105, 519–524. doi: 10.1007/BF01316323

Sukenik, A., and Wahnon, R. (1991). Biochemical quality of marine unicellular algae with special emphasis on lipid composition. *I. Isochrysis galbana*. *Aquaculture* 97, 61–72. doi: 10.1016/0044-8486(91)90279-G

Suratno, S., Puspitasari, R., Purbonegoro, T., and Mansur, D. (2015). Copper and cadmium toxicity to marine phytoplankton, *Chaetoceros gracilis* and *Isochrysis sp*. *Indones. J. Chem.* 15, 172–178. doi: 10.22146/ijc.21211

Tato, T., Salgueiro-González, N., León, V. M., González, S., and Beiras, R. (2018). Ecotoxicological evaluation of the risk posed by bisphenol A, triclosan, and 4-nonylphenol in coastal waters using early life stages of marine organisms (*Isochrysis galbana*, *Mytilus galloprovincialis*, *Paracentrotus lividus*, and *Acartia clausi*). *Environ. Pollut.* 232(Suppl. C), 173–182. doi: 10.1016/j.envpol.2017.09.031

Trenfield, M. A., van Dam, J. W., Harford, A. J., Parry, D., Streten, C., Gibb, K., et al. (2015). Aluminium, gallium, and molybdenum toxicity to the tropical marine microalga *Isochrysis galbana*. *Environ. Toxicol. Chem.* 34, 1833–1840. doi: 10.1002/etc.2996

Walsh, G. E. (1993). "Marine producers," in *Handbook of Ecotoxicology*, ed. Calow (Oxford: Blackwell Scientic).

Walsh, G. E., McLaughlin, L. L., Yoder, M. J., Moody, P. H., Lores, E. M., Forester, J., et al. (1988). *Minutocellus polymorphus*: a new marine diatom for use in algal toxicity tests. *Environ. Toxicol. Chem.* 7, 925–929. doi: 10.1002/etc.5620071109

Yap, C. K., Ismail, A., Omar, H., and Tan, S. G. (2004). Toxicities and tolerances of Cd, Cu, Pb and Zn in a primary producer (*Isochrysis galbana*) and in a primary consumer (*Perna viridis*). *Environ. Int.* 29, 1097–1104.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

REVIEW
published: 11 September 2019
doi: 10.3389/fmars.2019.00532

Less-Studied Technology-Critical Elements (Nb, Ta, Ga, In, Ge, Te) in the Marine Environment: Review on Their Concentrations in Water and Organisms

*Ana Romero-Freire[1], Juan Santos-Echeandía[2], Patricia Neira[1] and Antonio Cobelo-García[1]**

[1] Marine Biogeochemistry Group, Instituto de Investigacións Mariñas-CSIC, Vigo, Spain, [2] Marine Pollution and Biological Effects, Instituto Español de Oceanografía, San Pedro del Pinatar, Spain

The development in recent years of new technological and energy-related applications has increased the use and demand of a specific group of trace elements (Technology-Critical Elements, TCEs). Among the TCEs, there are a number of elements (Nb, Ta, Ga, In, Ge, Te) for which their biogeochemical cycles and their ecotoxicology and uptake by biota has been scarcely studied; they are known as Less-Studied TCEs (LSTCEs). Here we present a review on the concentrations of LSTCEs in marine waters and biota. We show that whereas oceanic profiles have been reported for all LSTCEs, and their geochemical behavior is well-constrained for some of them (e.g., Ga, In, Ge), only very few studies are available on the concentrations and behavior of these elements in estuarine and coastal waters which makes impossible the assessment of their status in environmentally impacted coastal areas. In marine biota, despite of the fact that concentrations have been reported in several organisms, information on the factors controlling the LSTCEs uptake or their potential to be biomagnified through the food web is mostly missing. It is therefore encouraged further research in order to have a better assessment on the impact of the uses of these metals on the concentrations of LSTCEs in such sensitive coastal zones, including their concentrations, bioavailability, thresholds for non-lethal endpoints and their capability of biomagnification.

Keywords: technology critical elements, seawater, organisms, concentrations, bioaccumulation

OPEN ACCESS

Edited by:
Ketil Hylland,
University of Oslo, Norway

Reviewed by:
Giuseppe Bonanno,
University of Catania, Italy
Yongfen Du,
Nanjing University, China

****Correspondence:***
Antonio Cobelo-García
acobelo@iim.csic.es

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *30 January 2019*
Accepted: *13 August 2019*
Published: *11 September 2019*

Citation:
Romero-Freire A, Santos-Echeandía J, Neira P and Cobelo-García A (2019) Less-Studied Technology-Critical Elements (Nb, Ta, Ga, In, Ge, Te) in the Marine Environment: Review on Their Concentrations in Water and Organisms. Front. Mar. Sci. 6:532. doi: 10.3389/fmars.2019.00532

INTRODUCTION

The Earth's crust is composed of a great variety of igneous, metamorphic and sedimentary rocks, mainly dominated by a few elements (e.g., Si, Al, Fe, Ca, Na, K, Mg) and their oxides, comprising approximately 99.7% of the crust (Rudnick and Gao, 2003). The majority of the naturally occurring chemical elements (minor and trace elements) account only for the remaining 0.3%. The deleterious effects of some of the trace elements to living organisms have been well documented and have led to their concentrations in the aquatic environment to be regulated by protocols and directives (i.e., EU Water Framework Directive 2000/60/CE and 2008/56/CE) that determine their appropriate environmental concentrations.

A significant number of trace elements are, however, excluded in these studies. This is due to: (i) their low ambient concentrations, generally below the detection limits of the analytical procedures employed, and (ii) the absence of any significant industrial role in the past, thus having no apparent environmental implications (Cobelo-García et al., 2015). This situation is currently changing, since several of these non-regulated trace elements are now key components in the development of new technologies, including electronic displays, semiconductors, energy–related technologies or telecommunications technology. They are defined as Technology-Critical Elements (TCEs[1]), and include tellurium (Te), germanium (Ge), gallium (Ga), indium (In), niobium (Nb), tantalum (Ta), the platinum group elements (PGEs: Pt, Pd, Rh, Os, Ir, Ru) and the rare earth elements (REEs: La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Yb, Lu). The extent of the environmental impact of the increasing anthropogenic use of these technology–critical elements needs therefore to be further assessed. Among the TCEs, the group of the less-studied TCEs (LSTCEs) – comprising Te, Ge, Ga, In, Nb and Ta – was defined by Filella and Rodríguez-Murillo (2017) for those TCEs for which, from an environmental perspective, have been less studied than the REEs or PGEs. A major reason for this scarcity in environmental studies of LSTCEs relies in the fact that their analytical determination is still challenging (e.g., Filella and Rodushkin, 2018).

Here we present a review on the available information existing to date on the concentrations of the LSTCEs in the marine environment, specifically in the water column and marine organisms. Based on the data collected, we provide an evaluation of the current gaps in our knowledge of these elements in marine systems and indicate directions for future research.

OCCURRENCE IN NATURE AND ANTHROPOGENIC APPLICATIONS

Niobium and Tantalum

Niobium and Ta are metals belonging to the Group 5 of the Periodic Table of Elements, and together with the adjacent metals Zr, Hf, Mo and W are known as 'high field strength elements' (HFSE; Rudnick and Gao, 2003). Both elements form compounds with a variety of oxidation states, but the + 5 is the only environmentally relevant (Filella, 2017). Niobium has only one natural stable isotope (^{93}Nb), but several artificial unstable isotopes that are found in radioactive wastes such as ^{95}Nb (35 days half-life) and ^{94}Nb (20,300 years half-life) (Astrom et al., 2008). Tantalum has two natural isotopes, the stable ^{181}Ta (99.988%) and the metastable ^{180}Ta (0.012%) (Filella, 2017). According to Rudnick and Gao (2003), the average concentrations of Nb and Ta in the upper continental crust are 12 and 0.9 $\mu g\ g^{-1}$, respectively. Niobium and Ta are considered as chemical twins due to their similar chemical properties (i.e., ionic charge and radius) generating a coherent behavior during magmatic differentiation; as a results, the Nb/Ta ratios are expected to be constant and of chondritic value (∼17) in mantle and mantle-derived rocks (Hui et al., 2011; Niu, 2012). However, several studies have observed a large Nb/Ta fractionation in rocks from the world ocean floor; the causes for such unexpected fractionation are still under debate (Niu, 2012). The strongest fractionation observed for the Nb/Ta occurs in the aquatic environment, particularly in seawater; in general, the Nb/Ta ratios increase in the following order: continental crust < river water < coastal water < open ocean (Firdaus et al., 2008).

These elements are usually found together in nature, with coltan (short for columbite-tantalites) as the most commonly known ore of Nb–Ta. The world mine production for these metals has significantly increased in the past two decades, currently accounting for roughly 60,000 (Nb) and 1,200 (Ta) tons per year (Filella and Rodríguez-Murillo, 2017). Both elements are used in a variety of high-tech applications, including high-grade structural steel and superalloys (e.g., aerospace industry), and capacitators in several electronic devices (e.g., medical appliances, portable electronics, flat-screen TVs, etc.; Cobelo-García et al., 2015; Filella, 2017).

Gallium and Indium

Gallium and In are close neighbors in the periodic table and both have the + 3 as their only oxidation state in the environment. They have two natural isotopes each, ^{69}Ga (60.11%) and ^{71}Ga (39.89%), and ^{115}In (95.72%) and ^{113}In (4.28%). Their typical concentrations in the upper continental crust are 17.5 (Ga) and 0.056 (In) μg/g (Rudnick and Gao, 2003).

The use of Ga in semiconductors has made it a high-tech metal in the past decades. The two main application fields are integrated circuits and optoelectronic devices. The most commonly used Ga compound is gallium-arsenide (GaAs), followed by gallium-nitride (GaN); other compounds (e.g., gallium-antimonide – GaSb – and gallium-phosphide – GaP) are used in much smaller amounts. By mid-2010's, the global Ga consumption was around 285 t, representing a 70% increase compared to late 2000's, and an increasing demand is foreseeing during the coming years (Rongguo et al., 2016). The In production by mid-2010's was estimated to be around 1,500 t, being the liquid crystal display (LCD) industry in the manufacturing of flat-panel, touch-screen, and plasma displays for televisions, computers, and handheld electronic devices the main In application (>50% of the total consumption) in the form of ITO (Indium Tin Oxide). By 2020, the In demand is expected to increase over 2000 t (Lokanc et al., 2015).

Germanium

Germanium (Ge) has 5 naturally occurring isotopes: ^{70}Ge (20.8%), ^{72}Ge (27.5%), ^{73}Ge (7.7%), ^{74}Ge (36.3%), and ^{76}Ge (7.6%). This element shows a similar geochemical behavior to that of Si, as it has similar electronic configuration and ionic radii (Lewis et al., 1988), but unlike Si it has no known essential function for organisms (Höll et al., 2007). It has an abundance in the upper continental crust of ∼1.4 μg/g (Rudnick and Gao, 2003), and the common oxidation state in natural systems is +4, forming at natural pHs in solution the hydroxide $Ge(OH)_4$ as its main species.

[1]www.costnotice.net

Germanium is used in fiber-optic systems, infrared optics and in electronics and solar electric applications. It is mainly obtained as a by-product during Zn mining and production; by mid-1990's, the Ge world production was ∼60 tons per year, and has increased since then to reach around 160 tons per year by 2015 (Filella and Rodríguez-Murillo, 2017).

Tellurium

Tellurium has eight natural isotopes (^{120}Te, ^{122}Te, ^{123}Te, ^{124}Te, ^{125}Te, ^{126}Te, ^{128}Te, ^{130}Te), the most abundant being ^{130}Te (34.1%) and ^{128}Te (31.7%). Tellurium (Te) belongs to the chalcogen group, which also includes O, S and Se. Unlike the other chalcogen elements, Te has not any known biological role (Nolan et al., 1991; Belzile and Chen, 2015). Te can exist in nature in various redox states: telluride (−2), elemental Te (0), tellurite (+ 4) and tellurate (+ 6). Under typical environmental conditions (e.g., oxygen, pH, etc.), the most abundant redox form of Te are Te^{4+} and Te^{6+} (Filella et al., 2019). They exist mainly as oxyanions and/or hydroxides, although their main chemical species are still a matter of controversy (Filella et al., 2019). The classical publications on elemental crustal abundance do not provide values for Te; values given for the Te terrestrial abundance display a range from 2 to 27 ng/g (Filella et al., 2019).

The main use of Te is for the production of cadmium–telluride (CdTe), which is used in semiconductor materials due to its optical and electrical properties especially in solar cells (Ramos-Ruiz et al., 2016). The solar cells industry is estimated to account for 40% of the global Te demand, followed by the thermoelectric production (30%; USGS, 2018). The estimation of the global Te production in 2017 was 410 tons, which is a 3–4 fold increase with respect to two decades ago – this increase being driven by its demand in the photovoltaic and thermoelectric applications (Filella et al., 2019).

CONCENTRATIONS AND BEHAVIOR IN THE MARINE ENVIRONMENT

Concentrations of dissolved Nb in the world's oceans range from ∼1 to 7 pmol/kg (**Table 1**), and from ∼ 0.02 to 0.3 pmol/kg for Ta. Both elements are depleted in surface waters and show a deep water enrichment (**Table 1**; Firdaus et al., 2011, 2018), indicating a removal in the photic zone by carrier phases and bottom water enrichment due to sediment resuspension (Firdaus et al., 2008). Contrary to open ocean waters, higher concentrations of Nb and Ta were observed in surface waters of coastal stations (Andaman Sea and Gulf of Thailand; Firdaus et al., 2018) and decreasing toward deep waters. This was attributed to the dissolution effect of atmospheric particles and riverine inputs (Firdaus et al., 2018).

The main dissolved inorganic forms of Ga and In in seawater are the hydroxylated species $Me(OH)_3^0$ and $Me(OH)_4^-$, as derived from thermodynamic calculations (Byrne et al., 1988). The hydrolysis products of trivalent metals display a significant particle-reactivity in the oceans, thus leading to short residence times (Alibo et al., 1999; Obata et al., 2007). Accordingly, Alibo et al. (1999) found the acid-soluble particulate fraction of In the Mediterranean to average 47 ± 11% (n = 6), which is typical of other particle-reactive metals as Ce or Th. Thus, their concentrations are generally maintained at low concentrations due to removal from seawater by scavenging onto particles (Alibo et al., 1999). Both elements are geochemically similar to aluminum, although less particle-reactive (Al/Ga and Al/In in seawater are two orders of magnitude smaller than that of shale; Amakawa et al., 1996), and have the potential to provide information on dust input to the surface ocean that complements that obtained using Al as a tracer (Shiller and Bairamadgi, 2006).

Elevated In concentrations (up to 300 pM) compared to open ocean waters were reported for Japanese coastal waters (**Table 1**), especially those close to industrialized ports (Miyazaki et al., 2012). Also, evidence for In contamination was found in several estuaries in Japan (Tama, Ara, and Tone estuaries; Nozaki et al., 2000b); here dissolved In followed a smooth decrease with salinity and was significantly correlated with Gd – which presented a positive anomaly due to its use of Gd-DTPA as a medical agent in magnetic resonance imaging in hospitals. Nozaki et al. (2000b) suggested that In could be anthropogenically derived in a soluble form similar to that of Gd and pointed to the use of $In(DTPA)^{2-}$ as a diagnostic medical agent as a possible explanation. For Ga, concentrations of 11,000–19,000 pM were obtained for a coastal stream in Taiwan receiving wastewater treatment plant discharges impacted by a science park housing semiconductor, electronics and optoelectronic manufacturing (Hsu et al., 2011), and is therefore a potential Ga source to the neighboring coastal waters.

Criteria continuous concentrations (CCC) for Ga and In – defined as the highest concentration of a toxicant to which aquatic organisms can be exposed indefinitely without causing unacceptable effects –were predicted by use of quantitative ion characteristic-activity relationships-species sensitivity distributions (QUICAR-SSD; Mu et al., 2014; Qie et al., 2017); this method, used to predict the biological activity of metal ions, is based on the assumption that similar electronic configurations should have similar functions and therefore several physico-chemical descriptors are applied for such calculations. For In, the calculated CCC of 5 nM (Qie et al., 2017) to 120 nM (Mu et al., 2014) are at least one order of magnitude higher than the highest dissolved In observed in the marine environment (0.3 nM in Japanese coastal waters, see above). In the case of Ga, a predicted CCC of 200 nM was reported (Mu et al., 2014). However, based on chronic toxicity data for several marine species, van Dam et al. (2018) proposed a marine water quality guideline (WQG) for this element of 11.5 μM, i.e., 2 orders of magnitude higher than the value proposed by Mu et al. (2014). In any case, the highest reported Ga concentrations in impacted estuarine-coastal areas (<20 nM; see above) are below both guideline values.

The Ga/In ratios in seawater are in the range of those in shale, indicating a similar chemical reactivity in the oceans, although some fractionation during takes place during scavenging resulting in different vertical profiles (Amakawa et al., 1996). Both Ga and In show a large interoceanic variation, with concentrations in the Atlantic considerably higher than in the Pacific (**Table 1**; Alibo et al., 1999; Shiller and Bairamadgi, 2006). Solubilization of In from aeolian dust – rather than riverine

TABLE 1 | Concentrations (pmol/kg) of dissolved TCEs in natural seawater samples.

Element	Location	Depth	Concentration	References
Niobium	North East Pacific	0–5000 m (range)	2.6–4.2	Sohrin et al., 1998
		0–200 m (average)	3.0 ± 0.3 (*n* = 10)	
		>2000 (average)	3.8 ± 0.1 (*n* = 5)	
	Western North Pacific	0–5000 (range)	4.0–7.2	Firdaus et al., 2008
		0–200 m (average)	4.8 ± 0.5 (*n* = 31)	
		>2000 (average)	6.4 ± 0.4 (*n* = 28)	
	North Atlantic	0–5000 m (range)	0.9–3.1	Firdaus et al., 2018
		0–200 m (average)	1.3 ± 0.2 (*n* = 5)	
		>2000 (average)	2.8 ± 0.2 (*n* = 10)	
Tantalum	North East Pacific	0–5000 m (range)	0.06–0.29	Sohrin et al., 1998
		0–200 m (average)	0.12 ± 0.03 (*n* = 7)	
		>2000 (average)	0.20 ± 0.06 (*n* = 5)	
	Western North Pacific	0–5000 (range)	0.08–0.29	Firdaus et al., 2008
		0–200 m (average)	0.11 ± 0.02 (*n* = 31)	
		>2000 (average)	0.20 ± 0.03 (*n* = 28)	
	North Atlantic	0–5000 m (range)	0.024–0.088	Firdaus et al., 2018
		0–200 m (average)	0.033 ± 0.007 (*n* = 5)	
		>2000 (average)	0.071 ± 0.011 (*n* = 10)	
Indium	Chao Phraya Estuary (Thailand)	Surface (range)	0.03–0.42*	Nozaki et al., 2000a
	Tone, Tama and Ara Estuaries (Japan)	Surface (range)	1.0–14.7*	Nozaki et al., 2000b
	Coastal Waters of Japan	Surface (range)	2–300	Miyazaki et al., 2012
		Surface (average)	25 ± 55 (*n* = 38)	
	Western North Pacific	0–3500 m (range)	0.047–0.101	Amakawa et al., 1996
		0–200 m (average)	0.069 ± 0.031 (*n* = 2)	
		>2000 (average)	0.064 ± 0.003 (*n* = 3)	
	North Atlantic	0–2100 m (range)	0.59–1.62	Alibo et al., 1999
		0–200 m (average)	0.65 ± 0.07 (*n* = 3)	
		>2000 (average)	1.56 ± 0.09 (*n* = 2)	
	Mediterranean Sea	0–2000 m (range)	3.2–4.7	
		0–2000 m (average)	3.8 ± 0.6 (*n* = 8)	
	Eastern Indian Ocean and SE Asian Seas	0–5500 m (range)	0.06–3.4*	Obata et al., 2004
		0–200 m (average)	1.2 ± 0.6* (*n* = 30)	
		>2000 m (average)	1.3 ± 0.6* (*n* = 65)	
	Japan Sea and Sea of Okhotsk	0–3500 m (range)	0.05–0.58*	Obata et al., 2007
		0–200 m (average)	0.27 ± 0.15* (*n* = 15)	
		>2000 m (average)	0.13 ± 0.06* (*n* = 19)	
Gallium	North East Pacific	0–5300 m (range)	2–30	Orians and Bruland, 1988
		0–200 m (average)	9.5 ± 4.7 (*n* = 24)	
		>2000 m (average)	17 ± 7 (*n* = 16)	
	South and Central Atlantic	0–5200 m (range)	6–40	Shiller and Bairamadgi, 2006
		0–200 m (average)	25 ± 6 (*n* = 45)	
		>2000 m (average)	33 ± 4 (*n* = 40)	
	North West Pacific	0–4500 m (range)	3–30	
		0–200 m (average)	14 ± 5 (*n* = 44)	
		>2000 m (average)	22 ± 5 (*n* = 8)	
Germanium[a]	Western Indian	0–5000 m (range)	2–99	Froelich et al., 1989
		0–200 m (average)	7 ± 4 (*n* = 24)	
		>2000 m (average)	92 ± 6 (*n* = 26)	
	North Atlantic	0–5400 m (range)	<4–37	
		0–200 m (average)	<4 (*n* = 2)	
		>2000 m (average)	22 ± 7 (*n* = 8)	
	South Pacific	0–5300 m (range)	5–97	
		0–200 m (average)	8 ± 3 (*n* = 6)	

(Continued)

TABLE 1 | Continued

Element	Location	Depth	Concentration	References
		>2000 m (average)	84 ± 9 (*n* = 23)	
	Northwest Pacific	0–5900 m (range)	5–117	
		0–200 m (average)	7 ± 3 (*n* = 2)	
		>2000 m (average)	107 ± 5 (*n* = 16)	
	Southwest Pacific	0–4100 m (range)	0.7–97	Sutton et al., 2010
		0–200 m (average)	1.6 ± 0.7 (*n* = 20)	
		>2000 m (average)	87 ± 8 (*n* = 9)	
	Atlantic	0–4800 m (range)	1.5–45	
		0–200 m (average)	4 ± 2 (*n* = 18)	
		>2000 m (average)	32 ± 6 (*n* = 15)	
	Southern Ocean	0–4000 m (range)	0.4–106	
		0–200 m (average)	3 ± 2 (*n* = 7)	
		>2000 m (average)	86 ± 13 (*n* = 12)	
Tellurium	Pacific Ocean	0–300 m	1.39 ± 0.19 (*n* = 7)	Yoon et al., 1990
		301–1000 m	1.01 ± 0.10 (*n* = 7)	
		1001–2000 m	0.67 ± 0.12 (*n* = 4)	
		2001–4000 m	0.46 ± 0.08 (*n* = 6)	
	Atlantic Ocean	0–1000 m	1.03 ± 0.12 (*n* = 10)	
		1001–2000 m	0.74 ± 0.03 (*n* = 5)	
		2001–3000 m	0.64 ± 0.05 (*n* = 4)	
		3001–5000 m	0.56 ± 0.04 (*n* = 7)	
	Atlantic Ocean	Surface water	∼1.3–1.7	Lee and Edmond, 1985
		>2500 m	∼0.5–0.6	

**A filtration through 0.04 μm was reported instead of the more commonly used 0.2 or 0.45 μm.* [a]*Concentrations refer to inorganic Ge only.*

and/or coastal waters – has been suggested as the main source of In to the oceans, and would explain the elevated concentrations observed in the Mediterranean Sea (**Table 1**) due to the higher dust flux in this basin. Aeolian dust deposition also represents the main Ga delivery to the surface ocean at a global scale, although inputs from river plumes may be important in certain coastal areas (e.g., Columbia river plyme; McAlister and Orians, 2012).

In natural waters dissolved Ge is present as inorganic Ge (Ge_i) in the form of $Ge(OH)_4$, monomethyl germanium (MMGe) and dimethylgermanium (DMGe) (Lewis et al., 1988). The geochemical behavior of Ge_i closely resembles that of Si, showing a strong correlation (Froelich et al., 1989; Santosa et al., 1997; Ellwood and Maher, 2003), as they are cycled in seawater by diatoms. However, fractionation between Si and Ge has been observed during uptake by and regeneration from phytoplankton, which may explain the slight positive Ge intercepts seen for the global Ge versus Si relationships (Ellwood and Maher, 2003; Sutton et al., 2010). In estuaries, Ge_i displays a non-conservative behavior, showing both net inputs and removal following the seasonal Si cycle (Andreae et al., 1983; Froelich et al., 1985a,b). Unlike Ge_i, MMGe and DMGe display and apparent stable and inert behavior, producing conservative profiles in estuaries and the ocean (Lewis et al., 1988) in contrast with methylated species for other metals such as As (e.g., monomethylarsonic acid and dimethylarsinic acid) which show maximum concentrations in surface waters (Santosa et al., 1997). Experiments conducted by Lewis et al. (1989) suggested that methylgermanium species are produced on the continents during methanogenensis and derived to the oceans where they remain unreactive; however, Ge_i enrichment and methylgermanium deplation was observed in marine anoxic waters indicating that anaerobic processes are capable of demethylating marine organogermanium (Lewis et al., 1989).

Ge_i concentrations typically range from 0.5 to 120 pM, with lower concentrations in the surface layers reflecting diatom uptake. For MMGe and DMGe, a global average concentration of 330 ± 15 pM (MMGe) and 120 ± 20 pM (DMGe) has been proposed, and account for more than 70% of total dissolved Ge in seawater (Lewis et al., 1988). In estuaries, conservative behavior of MMGe and DMGE – reflecting their unreactive characteristics – was observed, with low concentrations in the freshwater end-member compared to seawater. For Ge_i, its distribution in estuaries is non-conservative (Froelich et al., 1985a,b) and also showing a similar behavior to Si as observed for the ocean.

A criteria continuous concentrations (CCC; see above) around 1 μM was predicted for Ge in the marine environment (Mu et al., 2014). Concentrations reported for Ge_i in estuaries and coastal areas (Andreae et al., 1983; Froelich et al., 1985a,b) are in the range of those found for the ocean, and are thus several orders of magnitude lower that these quality limits. Elevated Ge concentrations in coastal watersheds were only reported in areas impacted by effluents by the leather industry, which contains high amounts of Ge (Zhang and Zhang, 2007).

The only two studies reporting an oceanic Te profile provided a total dissolved Te ranging from ∼0.4 to ∼2 pM (**Table 1**),

showing a scavenged-type behavior profile in the water column. The two studies cited in **Table 1** were published in 1985 and 1990 (no other oceanic profiles has been reported since) and, as noted in the extensive review of Filella et al. (2019), their reported concentrations are 2–3 orders of magnitude lower than those provided in more recent studies for seawater. Accordingly, a total Te concentration of 305 pM was given for a coastal water in the English Channel (Biver et al., 2015), with 70% as Te(VI). Concentrations of 85–263 pM were obtained for coastal seawater close to cities in China, with Te(VI) representing 70–87% of total Te (Huang and Hu, 2008). For the Changjiang Estuary and nearby coastal waters, Wu et al. (2014) reported concentrations of Te(IV) ranging from 3 to 60 pM, and from 5 to 330 for Te(VI), observing a predominance of Te(IV) in surface waters and Te(VI) in bottom waters. A similar Te(VI) predominance over Te(IV) was found throughout the water column of the Atlantic and Pacific oceans (Yoon et al., 1990). Te(IV) predominance over Te(VI) was observed at the redox boundary of the Saanich Inlet (Canada) indicating that the reduced form may prevail under oxygen-depleted conditions; however, these inorganic Te forms were not the dominant species in this basin, and accounted only less than 30% of total Te, which suggests the formation of organotellurium species in these waters (Yoon et al., 1990).

CONCENTRATIONS AND UPTAKE BY MARINE ORGANISMS

There are few data on Nb and Ta concentrations in marine organisms, although there is some evidence of a high accumulation coefficient for these elements by zooplankton in aquatic systems (Chebotina et al., 2011). Sánchez-Rodríguez et al. (2001) reported Ta concentrations ranging from 5 to 350 ng/g in different seaweed species collected in Loreto Bay (Gulf of California, Mexico). Using a dissolved Ta concentration in surface coastal waters of ∼0.2 pmol/kg (0.036 ng/kg), results in an enrichment factor (EF) of 10^5–10^7, indicating an elevated accumulation of this element by seaweed. Concentrations of Ta in invertebrates and fishes from coastal zones of Chile representing different climatic zones were reported by Espejo et al. (2018). In macroinvertebrates they found concentrations ranging from 0.17 to 7.8 ng/g, with a median value of 0.51 ng/g (n = 24), whereas for fishes ranged from 0.61 to 14.0 ng/g and a median of 2.4 ng/g (n = 22). Importantly, they observed a correlation between the Ta concentrations and the trophic levels for each of the coastal areas sampled indicating a Ta biomagnification through the aquatic food webs. In mussels (*Mytilus Galloprovincialis*) cultivated in rafts from the Galician rias (NW Iberian Peninsula), concentrations up to 211 ng/g were obtained, although average values were normally below the detection limit (<8 ng/g; Costas-Rodríguez et al., 2010). In this same study, average Nb concentrations ranged from <6 to 29 ng/g, with values as high as 235 ng/g observed. In the CRM DORM-2 (dog-fish muscle), Engström et al. (2004) provided a concentration of 2.8 ± 0.3 ng/g.

For In, we only found one published study reporting concentrations in marine organisms, namely the marine mussel tissue CRM NIST2976 (24 ± 5 ng/g; Krishna and Arunachalam, 2004) – probably reflecting the analytical difficulties in its determination and the fact that this metal is commonly used as an internal standard during the analytical determination by ICPMS. The situation is somewhat different for Ga, for which there are several studies reporting concentrations in biota. For example, Costas-Rodríguez et al. (2010) obtained concentrations ranging from 20 to 580 ng/g in mussels (*Mytilus Galloprovincialis*), with average values from 60 (Pontevedra Ria) to 240 ng/g (Vigo Ria). In two species of clams collected in the coastal Ganzirri Lake (Messina, Italy), Di Bella et al. (2013) reported concentrations of 610 ± 370 ng/g (range 380–1500 ng/g) for *Venerupis aurea laeta* and 370 ± 300 ng/g (range 140–880 ng/g) for *Cerastoderma edule glaucum*, with no significant differences in the concentrations between both species. In 32 fish species from the French market, Guérin et al. (2011) obtained an average Ga concentration of 2 ± 1 ng/g, and an average of 7 ± 12 ng/g in other seafood and products (n = 20). It is interesting noting that the value obtained by Guérin et al. (2011) for mussels (3 ng/g) is 1–2 orders of magnitude than those obtained from the Galician rias (Costas-Rodríguez et al., 2010); suggesting possible geographical or species-specific reasons explaining such differences in concentrations, although analytical bias cannot be excluded.

In fish from Malaysian coastal waters, Agusa et al. (2005) obtained Ga concentrations ranging from 9 to 469 ng/g in liver, and from <1 to 104 ng/g in muscle; here, the authors observed a strong positive correlation between concentrations in liver and muscle. Gallium concentrations in liver samples of dolphins collected in the Brazilian coast reported by Kunito et al. (2004) were 5 ± 2 ng/g (n = 20; *Sotalia guianensis*) and 3 ± 4 ng/g (n = 23; *Pontoporia blainvillei*), with no significant differences between both species and between immature and mature specimens. Campbell et al. (2005) analyzed a range of trace elements in a pelagic Arctic marine food web (North water Polynya, Baffin Bay, Canada), including primary organisms (e.g., ice algae), invertebrates, fish (cod), seabirds and mammals (ringed seal); for Ga they observed no relationship in its concentrations with the trophic position indicating that it was not biomagnified or biodiluted through the food web. They found, however, significant correlations of Ga between liver and muscle in ringed seals and seabirds – in accordance with the results given by Agusa et al. (2005) for fish –, indicating that this element is proportionally distributed throughout the body tissues.

Germanium concentrations in fish and seafood from French market ranged from 1 to 5 ng/g (Guérin et al., 2011), with an average value of 2 ± 2 ng/g (n = 52). One order of magnitude higher concentration (68 ± 5 ng/g) was reported for the marine mussel tissue CRM NIST2976 (Krishna and Arunachalam, 2004). For Te, Guérin et al. (2011) reported concentrations from 1 to 12 ng/g, with a mean value of 2–3 ng/g (n = 52) in fish. These values are in the range of those observed for mussels from the Galician rias, with concentration ranging from <1.4 to 5.9 ng/g (Costas-Rodríguez et al., 2010). Similar concentrations were reported in an historical Te record (1984–2017) in wild oysters from the Gironde Estuary (France); here Gil-Díaz et al. (2019) obtained an average concentration of 2.08 ng/g, with values ranging from

1.33 to 2.89 ng/g with no clear temporal trend unlike other anthropogenically released metals (e.g., Cd, Ag, Pt). These values are within those obtained in wild oysters for the Arcachon Bay (France; 1.18 ± 0.52 ng/g, n = 20) or the Bilbao Estuary (Spain; 3.48 ± 1.39 ng/g, n = 20) (Gil-Díaz et al., 2019).

Data on Te for two CRMs have been reported; accordingly, Engström et al. (2004) provided a concentration of 1.8 ± 0.1 ng/g for DORM-2 (dog-fish muscle) and Filella et al. (2019) a value of 5.5 ± 0.9 ng/g for BCR-414 (plankton). Tellurium was also analyzed in different tissues of the squid Todarodes pacificus in the Korean East Coast (Waska et al., 2008), with concentrations of (n = 10) 0.9 ± 0.6 ng/g in muscle, 2.4 ± 4.1 ng/g in stomach, 0.9 ± 0.1 ng/g in gills and 3.4 ± 2.5 ng/g in hepatopancreas. These results indicate a moderate Te bioaccumulation, with values in the range of 6×10^3 to 2×10^4 (Waska et al., 2008).

CONCLUSION AND FUTURE RESEARCH PRIORITIES

Research on the biogeochemical cycles of the less-studied technology-critical elements (Nb, Ta, Ga, In, Ge, Te) and their ecotoxicology and uptake by biota has been, in general, scarce to date. Therefore the are still important open questions on their environmental behavior and potential impact. The absence of a known biological role for these LSTCEs and the common time-consuming and complicated analytical procedures employed to analyze their trace to ultra-trace concentrations – and the presence of salt normally adds another issue, especially when using spectroscopic techniques – have greatly discouraged marine scientists working on trace elements to include them in their studies. The recent use of these LSTCEs in a number of new technologies – e.g., electronic displays, semiconductors, energy–related technologies or telecommunications technology – has considerably increased the production of these metals at a global scale. Therefore, studies on their biogeochemical behavior and ecotoxicology are expected to increase in the coming years (e.g., Cobelo-García et al., 2015) especially if new analytical procedures, making easier their determination, are developed (e.g., Poehle et al., 2015). Such studies would greatly benefit from the availability of appropriate certified reference materials (in water and biota), which do not exist at present for these elements, in order to guarantee the quality and, therefore, the comparability of the results provided by different authors.

Oceanic profiles have been reported for all the LSTCEs, especially for those that display similar geochemical behavior than 'key' oceanic elements. For example, Ga and In have the potential to provide information on the dust input to the surface ocean complementing that obtained using Al, whereas interest in Ge relies on its close similarity with Si as both are subject to cycling by diatoms. From an environmental perspective it is surprising, however, that only very few studies – especially in the recent years – are available on the concentrations and behavior of LSTCEs in estuarine and coastal waters which makes impossible the assessment of their status in environmentally impacted coastal areas. From the available literature, contamination has been observed in waters close to industrialized areas in Japan (In) and Taiwan (Ga), although their concentrations are still below the predicted WQGs for these elements. In marine biota, despite of the fact that concentrations have been reported in several organisms (with the exception of In), information on the factors controlling the LSTCEs uptake (e.g., metal speciation) or their potential to be biomagnified through the food web is mostly missing; accordingly, only two papers reported a study on the trophic transfer – specifically for Ga and Ta – showing a significant biomagnification for Ta but not for Ga.

A scientific effort is therefore encouraged regarding the determination of LSTCEs in different estuarine and coastal areas under varying degrees of anthropogenic influence in order to provide: (i) a better assessment on the impact of the uses of these metals on the concentrations of LSTCEs in such sensitive zones. (ii) Information on the bioavailability of the different chemical forms (e.g., speciation) of the LSTCEs to marine biota, and the factors controlling such speciation (i.e., pH, salinity). (iii) A determination of concentration thresholds for non-lethal endpoints (e.g., stress markers). (iv) A degree of biomagnification through the aquatic food web in order to evaluate potential long-term effects.

AUTHOR CONTRIBUTIONS

AR-F and PN made the bibliographic search and the first draft of the manuscript. JS-E and AC-G critically revised the manuscript. AC-G made the final revision and English editing.

FUNDING

This work was partially funded by the *Xunta de Galicia* (ref. IN607A 2016/11).

ACKNOWLEDGMENTS

We acknowledge the support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

REFERENCES

Agusa, T., Kunito, T., Yasunaga, G., Iwata, H., Subramanian, A., Ismail, A., et al. (2005). Concentrations of trace elements in marine fish and its risk assessment in Malaysia. *Mar. Pollut. Bull.* 51, 896–911. doi: 10.1016/j.marpolbul.2005.06.007

Alibo, D. S., Nozaki, Y., and Jeandel, C. (1999). Indium and yttrium in North Atlantic and Mediterranean waters : comparison to the Pacific data. *Geochim. Cosmochim. Acta* 63, 1991–1999. doi: 10.1016/S0016-7037(99)00080-0

Amakawa, H., Alibo, D. S., and Nozaki, Y. (1996). Indium concentration in Pacific seawater. *Geophys. Res. Lett.* 23, 2473–2476. doi: 10.1029/96gl02300

Andreae, M. O., Byrd, J. T., and Froelich, P. N. (1983). Arsenic, antimony, germanium and tin in the Tejo Estuary, Portugal: modelling a polluted estuary. *Environ. Sci. Technol.* 17, 731–737. doi: 10.1021/es00118a008

Astrom, M. E., Peltola, P., Virtasalo, J. J., Kotilainen, A. T., and Salminen, R. (2008). Niobium in boreal stream waters and brackish-water sediments. *Geochem. Exp. Environ. Anal.* 8, 139–148. doi: 10.1144/1467-7873/07-155

Belzile, N., and Chen, Y. (2015). Tellurium in the environment : a critical review focused on natural waters, soils, sediments and airborne particles. *Appl. Geochem.* 63, 83–92. doi: 10.1016/j.apgeochem.2015.07.002

Biver, M., Quentel, F., and Filella, M. (2015). Direct determination of tellurium and its redox speciation at the low nanogram level in natural waters by catalytic cathodic stripping voltammetry. *Talanta* 144, 1007–1013. doi: 10.1016/j.talanta.2015.07.010

Byrne, R. H., Kump, L. R., and Cantrell, K. J. (1988). The influence of pH and temperature on trace metal speciation in seawater. *Mar. Chem.* 25, 163–181. doi: 10.1016/0304-4203(88)90062-x

Campbell, L. M., Norstrom, R. J., Hobson, K. A., Muir, D. C. G., Backus, S., and Fisk, A. T. (2005). Mercury and other trace elements in a pelagic Arctic marine food web (Northwater Polynya, Baffin Bay). *Sci. Total Environ.* 35, 247–263. doi: 10.1016/j.scitotenv.2005.02.043

Chebotina, M. J., Polyakov, E. V., Guseva, V. P., Khlebnikov, N. A., and Surikov, V. T. (2011). The geochemical role of phyto- and zooplankton in the extraction of chemical elements from water. *Doklady Earth Sci.* 439, 1138–1140. doi: 10.1134/s1028334x11080113

Cobelo-García, A., Filella, M., Croot, P., Frazzoli, C., Du Laing, G., Ospina-Alvarez, N., et al. (2015). COST action TD1407: network on technology-critical elements (NOTICE)—from environmental processes to human health threats. *Environ. Sci. Pollut. R.* 22, 15188–15194. doi: 10.1007/s11356-015-5221-0

Costas-Rodríguez, M., Lavilla, I., and Bendicho, C. (2010). Classification of cultivated mussels from Galicia (Northwest Spain) with European protected designation of origin using trace element fingerprint and chemometric analysis. *Anal. Chim. Acta* 664, 121–128. doi: 10.1016/j.aca.2010.03.003

Di Bella, G., Lo Turco, V., Potorti, A. G., Rando, R., Licata, P., and Dugo, G. (2013). Statistical analysis of heavy metals in Cerastoderma edule glaucum and *Venerupis aurea* laeta from Ganzirri Lake, Messina (Italy). *Environ. Monit. Assess.* 185, 7517–7525. doi: 10.1007/s10661-013-3116-4

Ellwood, M. J., and Maher, W. A. (2003). Germanium cycling in the waters across a frontal zone: the Chatham Rise. *N. Z. Mar. Chem.* 80, 145–159. doi: 10.1016/s0304-4203(02)00115-9

Engström, E., Stenberg, A., Senioukh, S., Edelbro, R., Baxter, D. C., and Rodushkin, I. (2004). Multi-elemental characterization of soft biological tissues by inductively coupled plasma-sector field mass spectrometry. *Anal. Chim. Acta* 521, 123–135. doi: 10.1016/j.aca.2004.06.030

Espejo, W., Kitamura, D., Kidd, K. A., Celis, J. E., Kashiwada, S., Galbán-Malagón, C., et al. (2018). Biomagnification of tantalum through diverse aquatic food webs. *Environ. Sci. Technol. Lett.* 5, 196–201. doi: 10.1021/acs.estlett.8b00051

Filella, M. (2017). Tantalum in the environment. *Earth Sci. Rev.* 173, 122–140. doi: 10.1016/j.earscirev.2017.07.002

Filella, M., Reimann, C., Biver, M., Rodushkin, I., and Rodushkina, K. (2019). Tellurium in the environment: current knowledge and identification of gaps. *Environ. Chem.* 16, 215–228. doi: 10.1071/EN18229

Filella, M., and Rodríguez-Murillo, J. C. (2017). Less-studied TCE: are their environmental concentrations increasing due to their use in new technologies? *Chemosphere* 182, 605–616. doi: 10.1016/j.chemosphere.2017.05.024

Filella, M., and Rodushkin, I. (2018). A concise guide for the determination of less-studied technology-critical elements (Nb, Ta, Ga, In, Ge, Te) by inductively coupled plasma mass spectrometry in environmental samples. *Spectrochim. Acta Part B Atom. Spect.* 141, 80–84. doi: 10.1016/j.sab.2018.01.004

Firdaus, M. L., Mashio, A. S., Obata, H., McAlister, J. A., and Orians, K. J. (2018). Distribution of zirconium, hafnium, niobium and tantalum in the North Atlantic Ocean, northeastern Indian Ocean and its adjacent seas. *Deep Sea Res. Part I* 140, 128–135. doi: 10.1016/j.dsr.2018.08.008

Firdaus, M. L., Minami, T., Norisuye, K., and Sohrin, Y. (2011). Strong elemental fractionation of Zr – Hf and Nb – Ta across the Pacific Ocean. *Nat. Geosci.* 4, 227–230. doi: 10.1038/ngeo1114

Firdaus, M. L., Norisuye, K., Nakagawa, Y., Nakatsuka, S., and Sohrin, Y. (2008). Dissolved and labile particulate Zr, Hf, Nb, Ta, Mo and W in the Western North Pacific Ocean. *J. Oceanogr.* 64, 247–257. doi: 10.1007/s10872-008-0019-z

Froelich, P. N., Hambrick, G. A., Kaul, L. W., Byrd, J. T., and Lecointe, O. (1985a). Geochemical behavior of inorganic germanium in an unperturbed estuary. *Geochim. Cosmochim. Acta* 49, 519–524. doi: 10.1016/0016-7037(85)90043-2

Froelich, P. N., Kaul, L. W., Byrd, J. T., Andreae, M. O., and Roe, K. K. (1985b). Arsenic, barium, germanium, tin, dimethylsulfide and nutrient biogeochemistry in Charlotte Harbor, Florida, a phosphorous-enriched estuary. *Estuar. Coast. Shelf Sci.* 20, 239–264. doi: 10.1016/0272-7714(85)90041-1

Froelich, P. N., Mortlock, R. A., and Shemesh, A. (1989). Inorganic germanium and silica in the Indian Ocean: biological fractionation during (Ge/Si)opal formation. *Glob. Biogeochem. Cycle* 3, 79–88. doi: 10.1029/gb003i001p00079

Gil-Díaz, T., Schäfer, J., Dutruch, L., Bossy, C., Pougnet, F., Abdou, M., et al. (2019). Tellurium behaviour in a major European fluvial-estuarine system (Gironde, France): fluxes, solid/liquid partitioning and bioaccumulation in wild oysters. *Environ. Chem.* 16, 229–242. doi: 10.1071/EN18226

Guérin, T., Chekri, R., Vastel, C., Sirot, V., Volatier, J.-L., Leblanc, J.-C., et al. (2011). Determination of 20 trace elements in fish and other seafood from the French market. *Food Chem.* 127, 934–942. doi: 10.1016/j.foodchem.2011.01.061

Höll, R., Kling, M., and Schroll, E. (2007). Metallogenesis of germanium-A review. *Ore. Geol. Rev.* 30, 145–180. doi: 10.1016/j.oregeorev.2005.07.034

Hsu, S. C., Hsieh, H. L., Chen, C. P., Tseng, C. M., Huang, S. C., Huang, C. H., et al. (2011). Tungsten and other heavy metal contamination in aquatic environments receiving wastewater from semiconductor manufacturing. *J. Hazard. Mater.* 18, 193–202. doi: 10.1016/j.jhazmat.2011.02.020

Huang, C., and Hu, B. (2008). Speciation of inorganic tellurium from seawater by ICP-MS following magnetic SPE separation and preconcentration. *J. Sep. Sci.* 31, 760–767. doi: 10.1002/jssc.200700405

Hui, H., Niu, Y., Zhidan, Z., Huixin, H., and Dicheng, Z. (2011). On the enigma of Nb-Ta and Zr-Hf fractionation - a critical review. *J. Earth Sci.* 22, 52–66. doi: 10.1007/s12583-011-0157-x

Krishna, M. V. B., and Arunachalam, J. (2004). Ultrasound-assisted extraction procedure for the fast estimation of major, minor and trace elements in lichen and mussel samples by ICP-MS and ICP-AES. *Anal. Chim. Acta* 522, 179–187. doi: 10.1016/j.aca.2004.07.006

Kunito, T., Nakamura, S., Ikemoto, T., Anan, Y., Kubota, R., Tanabe, S., et al. (2004). Concentration and subcellular distribution of trace elements in liver of small cetaceans incidentally caught along the Brazilian coast. *Mar. Pollut. Bull.* 49, 574–587. doi: 10.1016/j.marpolbul.2004.03.009

Lee, D. S., and Edmond, J. M. (1985). Tellurium species in seawater. *Nature* 313, 782–785. doi: 10.1038/313782a0

Lewis, B. L., Andreae, M. O., and Froelich, P. N. (1989). Sources and sinks of methylgermanium in natural waters. *Mar. Chem.* 27, 179–200. doi: 10.1016/0304-4203(89)90047-9

Lewis, B. L., Andreae, M. O., Froelich, P. N., and Mortlock, R. A. (1988). A review of the biogeochemistry of germanium in natural waters. *Sci. Total Environ.* 73, 107–120. doi: 10.1016/0048-9697(88)90191-x

Lokanc, M., Eggert, R., and Redlinger, M. (2015). *The Availability of Indium: the Present, Medium Term, and Long Term*. Denver, CO: National Renewable Energy Laboratory.

McAlister, J., and Orians, K. (2012). Calculation of river-seawater endmembers and differential trace metal scavenging in the Columbia River plume. *Estuar. Coast. Shelf Sci.* 99, 31–41. doi: 10.1016/j.ecss.2011.12.013

Miyazaki, A., Kimura, A., and Tao, H. (2012). Distribution of indium, thallium and bismuth in the environmental water of Japan. *Bull. Environ. Contam. Toxicol.* 89, 1211–1215. doi: 10.1007/s00128-012-0851-0

Mu, Y., Wu, F., Chen, C., Liu, Y., Zhao, X., and Liao, H. (2014). Predicting criteria continuous concentrations of 34 metals or metalloids by use of quantitative ion character-activity relationships-species sensitivity distributions (QUICAR-SSD) model. *Environ. Pollut.* 188, 50–55. doi: 10.1016/j.envpol.2014.01.011

Niu, Y. (2012). Earth processes cause Zr-Hf and Nb-Ta fractionations, but why and how? *RSC Adv.* 2, 3587–3591.

Nolan, C., Whitehead, N., and Teyssie, J. (1991). Tellurium speciation in seawater and accumulation by marine phytoplankton and Crustaceans. *J. Environ. Radioactiv.* 13, 217–233. doi: 10.1016/0265-931x(91)90062-k

Nozaki, Y., Lerche, D., Alibo, D. S., and Snidvongs, A. (2000a). The estuarine biogeochemistry of rare earth elements and indium in the Chao Phraya River, Thailand. *Geochim. Cosmochim. Acta* 64, 3983–3994. doi: 10.1016/s0016-7037(00)00473-7

Nozaki, Y., Lerche, D., Alibo, D. S., and Tsutsumi, M. (2000b). Dissolved indium and rare earth elements in three Japanese rivers and Tokyo Bay: evidence for anthropogenic Gd and In. *Geochim. Cosmochim. Acta* 64, 3975–3982. doi: 10.1016/s0016-7037(00)00472-5

Obata, H., Alibo, D. S., and Nozaki, Y. (2007). Dissolved aluminum, indium, and cerium in the Sea of Japan and the Sea of Okhotsk : comparison to the marginal seas of the western North Pacific. *J. Geophys. Res.* 112, 1–10.

Obata, H., Nozaki, Y., Alibo, D. S., and Yamamoto, Y. (2004). Dissolved Al, In, and Ce in the eastern Indian Ocean and the Southeast Asian Seas in comparison with the radionuclides 210 Pb and 210 Po. *Geochim. Cosmochim. Acta* 68, 1035–1048. doi: 10.1016/j.gca.2003.07.021

Orians, K. J., and Bruland, K. W. (1988). The marine geochemistry of dissolved gallium: a comparison with dissolved aluminium. *Geochim. Cosmochim. Acta* 52, 2955–2962. doi: 10.1016/0016-7037(88)90160-3

Poehle, S., Schmidt, K., and Koschinsky, A. (2015). Determinatin of Ti, Zr, Nb, V, W and Mo in seawater by a new online-preconcentration method and subsequent ICP-MS analysis. *Deep Sea Res. I* 98, 83–93. doi: 10.1016/j.dsr.2014.11.014

Qie, Y., Chen, C., Guo, F., Mu, Y., Sun, F., Wang, H., et al. (2017). Predicting criteria continuous concentrations of metals or metalloids for protecting marine life by use of quantitative ion characteristic-activity relationships-species sensitivity distributions (QUICAR-SSD). *Mar. Pollut. Bull.* 124, 639–644. doi: 10.1016/j.marpolbul.2017.02.055

Ramos-Ruiz, A., Zeng, C., Sierra-Alvarez, R., Teixeira, L. H., and Field, J. A. (2016). Microbial toxicity of ionic species leached from the II-VI semiconductor materials, cadmium telluride (CdTe) and cadmium selenide (CdSe). *Chemosphere* 162, 131–138. doi: 10.1016/j.chemosphere.2016.07.081

Rongguo, C., Juan, G., Liwen, Y., Huy, D., and Liedtke, M. (2016). *Supply and Demand of Lithium and Gallium*. Hannover: Federal Institute for Geosciences and Natural Resources.

Rudnick, R. L., and Gao, S. (2003). Composition of the continental crust. *Treat. Geochem.* 1, 1–64. doi: 10.1016/b0-08-043751-6/03016-4

Sánchez-Rodríguez, I., Huerta-Díaz, M. A., Choumiline, E., Holguín-Quiñones, O., and Zertuche-González, J. A. (2001). Elemental concentrations in different species of seaweeds from Loreto Bay, Baja California Sur, Mexico: implications for the geochemical control of metals in algal tissue. *Environ. Pollut.* 114, 145–160. doi: 10.1016/s0269-7491(00)00223-2

Santosa, S. J., Wada, S., Mokudai, H., and Tanaka, S. (1997). The contrasting behaviour of arsenic and germanium species in seawater. *App. Organometal. Chem.* 11, 403–414. doi: 10.1002/(sici)1099-0739(199705)11:5<403::aid-aoc596>3.3.co;2-w

Shiller, A. M., and Bairamadgi, G. R. (2006). Dissolved gallium in the northwest Pacific and the south and central Atlantic Oceans: implications for aeolian Fe input and a reconsideration of profiles. *Geochem. Geophys. Geosyst.* 7, 1–14. doi: 10.1029/2005GC001118

Sohrin, Y., Fujishima, Y., Ueda, K., Akiyama, S., Mori, K., Hasegawa, H., et al. (1998). Dissolved niobium and tantalum in the North Pacific. *Geophys. Res. Lett.* 25, 999–1002. doi: 10.1029/98gl00646

Sutton, J., Ellwood, M. J., Maher, W. A., and Croot, P. L. (2010). Oceanic distribution of inorganic germanium relative to silicon: germanium discrimination by diatoms. *Glob. Biogeochem. Cycl.* 24:GB2017. doi: 10.1029/2009GB003689

USGS (2018). *Selenium and Tellurium. Statistics and Information*. Available at: https://minerals.usgs.gov/minerals/pubs/commodity/selenium (accessed June, 2019).

van Dam, J. W., Trenfield, M. A., Streten, C., Harford, A. J., Parry, D., and van Dam, R. A. (2018). Water quality guideline values for aluminium, gallium and molybdenum in marine environments. *Environ. Sci. Pollut. Res.* 25, 26592–26602. doi: 10.1007/s11356-018-2702-y

Waska, H., Kim, S., Kim, G., Kang, M. R., and Kim, G. B. (2008). Distribution patterns of chalcogens (S, Se, Te, and 210Po) in various tissues of a squid, *Todarodes pacificus*. *Sci. Total Environ.* 392, 218–224. doi: 10.1016/j.scitotenv.2007.12.003

Wu, X., Song, J., and Li, X. (2014). Occurrence and distribution of dissolved tellurium in Changjiang River estuary. *Chin. J. Oceanol. Limnol.* 32, 444–454. doi: 10.1007/s00343-014-3161-z

Yoon, B. M., Shim, S. C., Pyun, H. C., and Lee, D. S. (1990). Hydride generation atomic absorption determination of tellurium species in environmental samples with in situ concentration in a graphite furnace. *Anal. Sci.* 6, 561–566. doi: 10.2116/analsci.6.561

Zhang, M., and Zhang, M. (2007). Assessing the impact of leather industries on the quality of water discharged into the East China Sea from Wenzhou Watersheds. *J. Environ. Manag.* 85, 393–403. doi: 10.1016/j.jenvman.2006.10.016

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 18 September 2019
doi: 10.3389/fenvs.2019.00135

Multi-Laboratory Hazard Assessment of Contaminated Microplastic Particles by Means of Enhanced Fish Embryo Test With the Zebrafish (*Danio rerio*)

Bettie Cormier[1,2], Annika Batel[3], Jérôme Cachot[2], Marie-Laure Bégout[4], Thomas Braunbeck[3], Xavier Cousin[5,6] and Steffen H. Keiter[1]**

[1] Man-Technology-Environment Research Centre, School of Science and Technology, Örebro University, Örebro, Sweden, [2] University of Bordeaux, EPOC UMR CNRS 5805, Pessac, France, [3] Aquatic Ecology and Toxicology Group, Center for Organismal Studies, University of Heidelberg, Heidelberg, Germany, [4] Laboratoire Ressources Halieutiques, IFREMER, L'Houmeau, France, [5] IFREMER, L3AS, UMR MARBEC, Palavas-les-Flots, France, [6] UMR GABI INRA, AgroParisTech, University Paris-Saclay, Jouy-en-Josas, France

OPEN ACCESS

Edited by:
Mariana Teles,
University of Porto, Portugal

Reviewed by:
Gabriela Verónica Aguirre-Martínez,
Universidad Arturo Prat, Chile
Lluis Tort,
Autonomous University of Barcelona, Spain

***Correspondence:**
Bettie Cormier
bettie.cormier@u-bordeaux.fr
Steffen H. Keiter
steffen.keiter@oru.se

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Environmental Science

Received: 15 February 2019
Accepted: 02 September 2019
Published: 18 September 2019

Citation:
Cormier B, Batel A, Cachot J, Bégout M-L, Braunbeck T, Cousin X and Keiter SH (2019) Multi-Laboratory Hazard Assessment of Contaminated Microplastic Particles by Means of Enhanced Fish Embryo Test With the Zebrafish (Danio rerio). Front. Environ. Sci. 7:135. doi: 10.3389/fenvs.2019.00135

As wide-spread pollutants in the marine environment, microplastics (MPs) have raised public concern about potential toxic effects in aquatic organisms, and, among others, MPs were suspected to act as a vector for organic pollutants to biota. The purpose of the present study was to investigate effects by three model pollutants, oxybenzone (BP3), benzo[a]pyrene (BaP), and perfluorooctane sulfonate (PFOS) adsorbed to polyethylene MPs on the basis of a standard assay, the acute fish embryo toxicity test (FET; OECD TG 236) with zebrafish (*Danio rerio*) supplemented by additional endpoints such as induction of ethoxyresorufin-O-deethylase (EROD) activity, modification of *cyp1a* gene transcription and changes in larval swimming behavior. FET assays were performed in three laboratories using slightly different husbandry and exposure conditions, which, however, were all fully compatible with the limits defined by OECD TG 236. This allowed for testing of potential changes in the FET assay due to protocol variations. The standard endpoints of the FET (acute embryotoxicity) did not reveal any acute toxicity for both virgin MPs and MPs spiked with BP3, BaP, and PFOS. With respect to sublethal endpoints, EROD activity was increased after exposure to MPs spiked with BP3 (3 h pulse) and MPs spiked with BaP (96 h continuous exposure). *Cyp1a* transcription was increased upon exposure to MPs spiked with BP3 or BaP. For the selected combination of MPs particles and contaminants, the basic FET proved not sensitive enough to reveal effects of (virgin and spiked) MPs. However, given that the FET can easily be supplemented by a broad variety of more subtle and sensitive endpoints, an enhanced FET protocol may provide a relevant approach with developmental stages of a vertebrate animal model, which is not protected by current EU animal welfare legislation (Directive EU 2010/63).

Keywords: fish embryotoxicity test (FET), swimming behavior, EROD, *cyp1a*, perfluorooctane sulfonate, benzo[a]pyrene, oxybenzone

INTRODUCTION

Plastics have become an indispensable part of our daily life and yield important societal benefits (Andrady and Neal, 2009). Due to poor waste management, however, large portions of the manufactured plastic end up in the oceans and will eventually show up as macroplastics (>5 mm) or microplastics (MPs), i.e., particles between 1 μm and 5 mm in size (Jambeck et al., 2015). The latter consist of either plastic granules designed for purpose in e.g., cosmetics or small plastic fragments derived from the breakdown of macroplastics (Derraik, 2002; Thompson et al., 2004). MPs of both types are considered as a potential threat for aquatic ecosystems (Thompson et al., 2009; Cole et al., 2011).

Plastic materials are not restricted to the polymers themselves, but often also contain numerous additives designed to produce their specific physicochemical properties and to protect MPs from damage by heat, UV light, oxidative processes as well as from microbiological degradation (Andrady, 2011). Many additives are *per se* known as putative toxicants and, upon release from plastic materials, they may potentially cause adverse health effects (Bakir et al., 2014). MPs particles have been shown to adsorb significant amounts of organic pollutants from the surrounding environment (Mato et al., 2001; Rios et al., 2007; Barnes et al., 2009; Koelmans et al., 2016), and weathering processes as well as mechanical fragmentation of MPs lead to smaller particles and may increase the specific reactive surface area of particles and thus facilitate further the sorption of pollutants (Teuten et al., 2007; Koelmans et al., 2013; Lee et al., 2014). Under both laboratory and field conditions, different MPs have been shown to adsorb various classes of organic pollutants such as per- and polyfluorinated compounds, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides and personal care products (Rochman et al., 2013a,b; Ziccardi et al., 2016), and the relative contribution of MPs to the transfer of organic pollutants to biota has for long been subject to controversy (Bakir et al., 2016; Koelmans et al., 2016; Lohmann, 2017).

Polyethylene (PE) represents the polymer with the greatest global production for plastic manufacturing, and—for its highly diverse uses—PE contains a wide variety of additives. PE was selected as experimental MPs for the present study because of its predominance in marine ecosystem (Andrady, 2011). As a polymer, PE *per se* is supposed to bear a relatively low hazard (Lithner et al., 2011; Karami et al., 2017); however, due to its physicochemical properties and high abundancy in aquatic environments, PE MPs have a high potential to adsorb and transfer organic pollutants (Rochman et al., 2013a; Wang F. et al., 2015). Together, chemical contaminants and PE may represent a higher risk to aquatic organisms compared to other polymers (Karami et al., 2017).

Benzophenone-3 (oxybenzone, BP3) is commonly used as a UV-filter in cosmetics such as sunscreens, but also as a light stabilizer in plastics such as PE. In addition, BP3 has repeatedly been reported to act as an endocrine disrupting chemical interfering with reproduction and sex hormone signaling in fish and mammals (Kim and Choi, 2014; Kinnberg et al., 2015; Rodríguez-Fuentes et al., 2015). BP3 shows low degradation rates in surface waters (Kim and Choi, 2014) and—due to its lipophilicity (log Kow 4.0)—may bioaccumulate in biota.

Benzo[a]pyrene (BaP) is formed by incomplete combustion of organic materials (Collins et al., 1991) and has become the most frequently studied representative of carcinogenic PAHs and a model molecule for the investigation of PAHs as inducers of aryl hydrocarbon receptor (AhR)-based metabolization pathways. BaP is ubiquitously distributed in coastal and offshore environments (Antunes et al., 2013; Liu et al., 2015; Châtel et al., 2017), and PAHs including BaP are known to adsorb at high rates to different types of MPs in seawater, particularly to PE (Rochman et al., 2013a; Ziccardi et al., 2016; Schönlau et al., 2019). Furthermore, BaP adsorbed to MPs has been documented to be transferred *via* trophic transfer to biota (Batel et al., 2016, 2018; Bour et al., 2018; O'Donovan et al., 2018; Pittura et al., 2018).

Given its wide distribution, perfluorooctane sulfonate (PFOS) has attracted most attention among perfluorinated substances, since it represents the main perfluorinated alkylated compounds detected in aquatic environments (Keiter et al., 2016). PFOS is an extremely persistent fluorinated pollutant that does not hydrolyze, photolyase or biodegrade. PFOS is also known to be toxic and bioaccumulative and undergo extensive transportation across all environmental media. As a consequence, PFOS has been documented in soil, sediment, sludge, and water bodies (Giesy and Kannan, 2002). Since, in ocean surface waters, PFOS and its precursors have been estimated at 235–1,770 metric tons (Paul et al., 2009), PFOS was added to the list of the Stockholm convention on Persistent Organic Pollutants in 2010.

A wide array of bioassays has been developed to assess the toxicity of chemicals or environmental samples. Given that early developmental stages of vertebrates that depend on the use of yolk are considered non-protected according to current European legislation on the protection of animals used for scientific purposes (EU, 2010; Strähle et al., 2012) and that fish embryonic stages have been demonstrated to be equally sensitive to chemical pollutants as intact fish (Nagel, 2002; Braunbeck et al., 2005, 2015; Lammer et al., 2009; Belanger et al., 2013). The fish embryo acute toxicity test (FET) has been designed as an alternative to conventional fish acute toxicity testing [e.g., OECD TG 203; (OECD, 1992)] and has been approved as OECD Test Guideline 236 (OECD, 2013). According to the guideline, the FET has successfully been applied to a wide range of substances exhibiting diverse modes of action, solubilities, volatilities, and hydrophobicities. However, given the particulate nature of MPs, the question arises whether the FET might equally be suited for the assessment of MPs toxicity. In fact, there are major differences between soluble pollutants and discrete objects such as MPs including, e.g., dispersion, homogeneity, localized desorption events of additives or adsorbed pollutants. The purpose of the present study was, therefore, to use the standard FET procedure to document the acute toxicity of MPs and MPs spiked with selected organic pollutants (BP3, BaP, and PFOS). Since OECD TG 236 provides a general framework and allows for substantial flexibility in test conditions, the present study was designed to test for potential differences in FET sensitivity toward MPs due to, e.g., variability in fish pre-conditioning, dark-light scenarios

or exposure conditions. Protocols used by the three laboratories involved in the present comparative study presented some differences but were all within the limits defined by OECD TG 236. In order to enhance resolution and sensitivity of FET, we also monitored additional endpoints such as EROD activity and *cyp1a* transcription as well as larval swimming behavior to reveal potential sublethal effects.

MATERIALS AND METHODS

Materials and Chemicals

Polyethylene (PE; order no. MPP-635 G) with a size range of 11–13 μm and a density of 0.96 kg/m^3 purchased from Micro Powders (New York, USA) was kindly provided within the JPI Oceans project EPHEMARE as a dry powder by Marina Albentosa (Instituto Español de Oceanografía, Centro Oceanografico de Murcia, Spain). Benzo[a]pyrene (BaP; CAS 50-32-8; purity ≥ 96%), benzophenone-3 [(2-hydroxy-4-methoxyphenyl)-phenylmethanone, BP3; CAS 131-57-7; purity ≥ 98%] and PFOS (1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluorooctane-1-sulfonic acid; CAS 2785-37-3; purity ≥ 98%) were purchased from Sigma Aldrich (Stockholm, SE). All other chemicals and reagents were purchased at the highest purity available from Sigma-Aldrich, unless stated otherwise.

Spiking of Microplastic Particles

Setups for the spiking, e.g., MPs and chemical concentrations were previously determined with preliminary experiments in order to determine all parameters to reach aimed concentrations, based on fish toxicity data, e.g., BP3 (Blüthgen et al., 2012; Paredes et al., 2014); BaP (Weigt et al., 2011) and PFOS (Yamashita et al., 2005). Preliminary studies demonstrated that to minimize loss of chemicals, PFOS exposure has to be prepared in polypropylene bottles (Lamaplast; Sesto San Giovanni, Italy), while for BaP and BP3, glass bottles (Thermo Scientific, Lund, SE) were the most efficient to avoid loss of compound.

According to the pretests, for the sorption of BP3 and BaP to PE, 125 g/L PE were added in 400 mL septa bottles (Thermo Scientific, Lund, SE) and bottles were filled to the maximum with double-deionized water, to avoid gas headspace in the bottle, and so avoid volatilization of compound. BP3 and BaP concentrations in the solutions were 20 and 2,500 μg/L, respectively. For the sorption of PFOS on PE, 50 g/L of plastic were weighed into 1 L polypropylene bottles (Lamaplast; Sesto San Giovanni, Italy) filled with double-deionized water and PFOS was added to a final concentration of 20 mg/L. All bottles were placed on a rotary shaker for 48 h (BP3 and BaP) or 7 days (PFOS) at 20 rpm and at room temperature (20 ± 1°C). Based on preliminary experiments, after 48 h, concentrations of BaP and BP3 adsorbed on MPs were higher than samples shaken for more than 48 h due to volatilization of compound, and an increase of the adsorption to glass bottle. While, for PFOS, the sorption time of 7 days was maintained because of the lowest standard deviation of the results and the highest concentration of PFOS adsorbed on particles, compared to other time points.

Chemical Analyses and Quality Assurance/Quality Control (QA/QC)

Concentrations of chemicals (BP3, BaP, and PFOS) were measured in corresponding spiked MPs as well as in virgin MPs to determine background contamination of each chemical.

After filtration of the MP preparations with a funnel equipped with a 1 μm Whatman® glass microfiber filter (GE Healthcare Life Sciences; Uppsala, SE), the filters were rinsed with double deionized water and dried by vacuum evaporation on a ceramic funnel.

The analysis of BP3 was performed by Eurofins Germany (Pforzheim, DE) by using an 0.3 g aliquot of the sample spiked with internal standards (i.e., 3,4,5,6-pentadeuteriobenzoic acid, 1-bromo-4-phenylbenzene, 1,2,3,4,5,6,7,8-octadeuterionaphthalene, 1,2,3,4,5-pentachloro-6-(2,3,4,5,6-pentachlorophenyl)benzene, and 1,2,3,4,5,6,7,8,9,10-decadeuteriophenanthrene) and concentrated with a factor of 10. The samples were extracted with a 1:1 solvent mixture of ethyl acetate and cyclohexane. Additionally, an external calibration standard and a blank sample were prepared. The extracts were measured with GC/MS/MS, electron impact ionization (GC: Agilent7890 A, MS/MS: Agilent 7000 multiple-reaction monitoring mode, Agilent Technologies, Kista, SE) using a split-less injection of 2 μl (column DB5-MS, 30 m × 0.25 mm × 0.25 μm). Identification and quantification were performed against the retention time and the external calibration using the BP3-specific selected reaction monitoring (one quantifier and one qualifier).

BaP analysis has been conducted according to Larsson et al. (2013): MPs were extracted in hexane (≥98%, SupraSolv; Merck, Darmstadt, DE) after addition of an internal standard (500 ng BaP D12 in toluene; Chiron, Tuttlingen, DE; prepared standard: 10 ng/μl in toluene) by sonication and centrifugation at 2,000 rpm. Extracts were filtered through fiberglass and transferred to toluene (purity 96%, SOLVECO; Rosersberg, SE). GC vials for analysis were filled with 100 ng recovery standard perylene D12 (Chiron; 2 ng/μl in toluene, 50 μl added) and 500 μl of extract. The sample volume was reduced to 500 μl using a nitrogen stream. Concentrations of BaP were quantified using a Micromass Autospec Ultima high-resolution GC/MS system, separation on a 30 meters (0.25 mm internal diameter, 25 μm film thickness) DB-5MS column (J&W Scientific, Folsom, USA). Procedure blanks were included in all batches. Target BaP was quantified by using a five-point calibration curve. Relative standard deviation of the relative response factor values was <15% for PAHs. Quantification standard replicates were analyzed after every tenth sample. Concentrations of BaP were calculated by use of the internal standard method. The limit of detection (LOD) was defined as mean concentration in blanks plus three times the standard deviations and was 5 pg/g. Samples which had concentrations exceeding the range of the calibration curve were diluted and reanalyzed. Procedure blanks were included in all batches.

Chemical analysis of PFOS has been conducted according to Eriksson et al. (2016): PFOS (linear and isomers) adsorbed to MPs were extracted in methanol (>99.9% purity, Fisher

TABLE 1 | Husbandry conditions of zebrafish in the three participating laboratories.

Laboratory	Day/night cycle [h]	Water parameters			Group sizes	Tank size	Zebrafish strain	Artificial food
		Temperature	pH	Conductivity				
Örebro University	14/10	26 ± 1°C	7.2 ± 0.2	380 ± 50 μS	100 ± 10	60 L	AB	TetraMin
IFREMER	14/10	27 ± 1°C	7.8 ± 0.2	485 ± 130 μS	28 ± 5	10 L	TU	Inicio + Biomar
Heidelberg University	16/8	26 ± 1°C	7.2 ± 0.1	360 ± 20 μS	≥150	160 L	West-aquarium	TetraMin

Provider for flake food: TetraMin (Tetra, Sweden), Tetra (Melle, Germany), Inicio + 0.5 mm (BioMar, France).

Scientific) by ultra-sonication following by centrifugation (7,000 rpm). Extracts were filtered using a 5 mL PE syringe (Norm-Ject®; Henke Sass Wolf, Tuttlingen, DE) with a filter of 0.2 μm (13 mm, 0.2 μm AcrodiscGHP; Pall, Dreieich, DE). LC vials were prepared with 2 ng of recovery standard (mass-labeled PFOS). Samples were diluted 100 times using 40% methanol (v/v) and 60% ammonium acetate (v/v). Analysis was performed on an Acquity UPLC system coupled to a Xevo TQ-S quadrupole MS (Waters, Milford, USA). A guard column (PFC isolator, Waters) was inserted between the pump and the injector to prevent contamination from the system. PFOS were separated on 100 mm Acquity BEH C18 column (2.1, 1.7 mm) using methanol and water as mobile phases, both with 2 mM ammonium acetate. Quantification of total PFOS was performed by summarization of linear PFOS and individual branched PFOS calculated against an external calibration curve using technical PFOS as standard. Recoveries were assessed using labeled internal standards. The mean recoveries of labeled PFOS internal standards was 83–96%. The LOD was calculated as above was 10 pg/g. Procedure blanks were included in all batches.

TABLE 2 | Overview about the test conditions and solutions for the zebrafish embryo toxicity test used by the three different laboratories.

	Örebro University	IFREMER	Heidelberg University
MPs size	PE 11–13 μm		
MPs concentrations	10, 100 mg/L	10 mg/L	100 mg/L
Exposure vessels	Glass vials		
Vessel volumes	100 mL	60 mL	2 mL
Solution volumes	20 mL	10 mL	1.2 mL
Embryos per assay	5	20	20*
Shaking during exposure	No shaking	No shaking	Gentle agitation
Positive control	3,4-Dichloraniline (4 mg/L)		
Temperature	26 ± 1°C	27 ± 1°C	25 ± 1°C
Dark/light rhythm	10/14 h	10/14 h	8/16 h

**One embryo per 2 mL flat bottom argon glass vials (NeoLab, Heidelberg, DE), which were placed in 20 wells of a 24-well plate (n = 20; TPB, Trasadingen, Switzerland). Each glass vial was covered with a cap (unknown composition, caps were washed before use, but did not get into contact with the medium) to prevent evaporation.*

Zebrafish Husbandry and Egg Production

Fish husbandry in the three participating laboratories conditions fully complied with OECD TG 236 (Westerfield, 2007; OECD, 2013), but maintenance of adult zebrafish (*Danio rerio*) differed slightly regarding dark/light cycles, size of the rearing tanks, size of heterosexual groups, and water conditions including temperature, pH and conductivity (**Table 1**). In each laboratory, feeding was twice daily *ad libitum* with commercially available artificial diets and freshly hatched brine *Artemia* nauplii. Constant filtering or permanent flow-through conditions guaranteed that ammonia, nitrite, and nitrate were kept below detection limits (5, 1, and 140 mg/L, respectively). Zebrafish eggs were collected according to OECD TG 236 (OECD, 2013).

Toxicity Testing

Zebrafish Embryo Acute Toxicity Test

The acute hazard potential of MPs and contaminated MPs was determine on the basis of the FET guideline (OECD 236) which provides a framework for the test, but allows for some variation in parental fish breeding and exposure conditions. In order to analyze the potential impact of minor modifications of the protocol, the three laboratories participating in the present comparative study performed the test with minor differences in the rearing temperature and the dark/light cycle of the parent fish, as well as in the volume of vessels used and the shaking of the test solutions (**Table 2**); care was taken that the protocols of all three laboratories were clearly within the limits set by OECD TG 236. All other parameters of the tests themselves were identical and followed OECD TG 236.

Embryo exposure started at a maximum of 4 hours post-fertilization (hpf) and was performed in glass containers covered with lids, under semi-static conditions with a daily renewal of exposure medium. Exposures were performed using either MPs or MPs spiked with one pollutant at 10 and/or 100 mg/L (**Table 3**). An additional treatment, later called 3POPs, was an even mixture of MPs spiked with BP3, BaP, and PFOS, also used at a final concentration of MPs of 10 or 100 mg/L. The exposure design included negative controls (virgin MPs) and positive controls (water-borne 3,4-dichloraniline, 4 mg/L). In each laboratory, all exposures were performed in triplicate. Embryos were analyzed every day for lethal (OECD, 2013) and sublethal effects as well as hatching rate.

As an additional control, the three pollutants (BP3, BaP, and PFOS) were also tested *via* waterborne exposure at final concentrations equivalent to complete desorption in the case of an exposure to 10 mg/L of MPs. Actual concentrations for BP3, BaP, and PFOS were 0.8, 170, and 700 ng/L, respectively. For waterborne exposure, DMSO was used as a solvent at a final concentration of 0.01%; as a solvent control, 0.01% DMSO was run as an additional negative control.

TABLE 3 | Exposure scenarios tested by the three laboratories for MPs, adsorbed MPs [MP + pollutant(s)] and pollutants alone.

	10 mg/L MP	100 mg/L MP	MP	MP + BP3	MP + BaP	MP + PFOS	MP + 3POPs	BP3	BaP	PFOS
Örebro University	×	×	×	×	×	×	×	×	×	×
IFREMER	×		×	×	×	×	×	–	–	–
Heidelberg University		×	×	×	×	×	–	–	–	–

TABLE 4 | Concentrations of MPs and pollutants (mean ± SD) in solution and the resulting concentration of the pollutants on the PE particles ($n = 1$ for BP3 and $n = 3$ for BaP and PFOS).

	Preparation solution		Pollutant conc. spiked to MPs (μg/g)
	PE (g/L)	Pollutants (μg/L)	
BP3	125	20	0.08
BaP	125	2,500	16.87 ± 0.22
PFOS	50	20,000	70.22 ± 12.41

In vivo EROD Assay

In vivo ethoxyresorufin-*O*-deethylase (EROD) induction in zebrafish embryos was determined according to Kais et al. (2018): Tests was performed at 96 hpf embryos, which were either incubated for 96 h with 100 mg/L of MPs (0–96 h) with daily exchange of medium as previously described, or for a 3 h short-term exposure (93–96 h) with 100 mg/L of MPs. In short, 20 embryos per treatment group and replicate were washed twice for 5 min using dilution water and incubated for 20 min in 0.6 mg/L 7-ethoxyresorufin (Sigma Aldrich, Deisenhofen, Germany). After anesthesia with 0.016% tricaine (MS-222), embryos were mounted left side down in 1% low-melting agarose (SeaKem HGT Agarose; Cambrex BioScience, Rockland, USA) supplemented with 0.016% tricaine. The fluorescence by resorufin formed from 7-ethoxyresorufin *via Cyp1a* catalysis was measured under a Nikon ECLIPSE 90i epifluorescence microscope (Nikon Instruments; Tokyo, Japan) equipped with a 10× Nikon CFI Plan Fluor water immersion objective (NA 0.17, WD 16.0 mm) at an excitation of 560 ± 20 nm (Texas Red HYQ filter) and an emission of 630 ± 30 nm with a dichroic beam splitter of 595 nm. A Nikon DS-Ri-1 camera was used for imaging. Image processing was performed with the NIS-Elements 4.0 imaging software (Nikon Instruments). As a positive control, embryos were incubated at 96 hpf for 3 h in 75 μg/L β-naphthoflavone. Three replicates were run for each test and treatment group ($n = 3 \times 20$).

Cyp1a Gene Transcription Analysis

For analyzes of *cyp1a* induction, 96 hpf embryos ($n = 15$ per exposure group) were exposed for 3 h to MPs as described above. Exposures were performed in triplicate ($n = 3 \times 15$). Ninety-six hours post-fertilization embryos incubated for 3 h in 75 μg/L β-naphthoflavone (Boehler et al., 2018) were used as a positive control. After exposure, embryos were euthanized in 500 mg/L benzocaine, thoroughly rinsed in deionized water and stored in RNALater (Qiagen, Les Ulis, France). Prior to extraction, RNALater was removed, and embryos were placed in a lysis buffer (Qiagen) before being mechanically disrupted using Beadblaster (Benchmark Scientific, Dutscher, France). The total RNA extraction followed the protocols of the RNeasy Plus Universal Mini Kit (Qiagen). Quality and quantity of the extracts were checked using electrophoresis migration and spectrophotometric dosing. Subsequently, cDNA was synthesized from 2 μg of the total RNA, using 2 μl of Superscript III Reverse Transcriptase (Invitrogen, Fisher Scientific, Illkirch, France) according to the manufacturer's protocol in a final volume of 21 μl. Prior to analysis, all cDNA was diluted 5 × in Milli-Q water. The qPCR experiments were run in a final volume of 20 μL, including an optimized primer concentration ranging between 300 and 600 nM (Eurofins, Ebersberg, Germany) and 2X Fast SYBR Green Master Mix (Applied Biosystems, Fisher Scientific, Illkirch, France). The amplification protocol used was as follows: initial denaturation (20 s at 95°C) followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. Technical triplicates were run for each biological replicate. The analysis software Relative Expression Software Tool (REST; Pfaffl, 2001; Pfaffl et al., 2002) automatically calculated fold changes in expression relative to negative controls (unexposed embryos) using the 3 most stable references genes between all groups, and combined them into an index via the free access BestKeeper software (Pfaffl et al., 2004). Reference genes were *g6pd*, *actb1,* and *rpl13a.* Sequences and characteristics of all primers used are listed in **Table S1**.

Larval Behavior

Larval behavior was monitored at 120 hpf using the larval photomotor response (LPMR) test as described by Vignet et al. (2014). The morning before the LPMR, single embryos were individually transferred into one well of a 24-well plate (Krystal 24, opaque wall and clear bottom microplate, Dutscher), and plates were transferred to an enlighten incubator at the same temperature as in the testing room. Plates were successively placed into DanioVision™ (Noldus, Wageningen, NL) in the dark for 10 min of acclimation before the LPMR test which included the following 5-min steps: Light on−1 (LON1, 70 lux), Light off (LOFF, <1 lux) and Light on−2 (LON2, 70 lux) with constant infra-red light maintained during video recording. Distance traveled (cm) was recorded and summed for each 5-min step. Four embryos from each treatment were used in each plate ($n = 6$) to avoid potential bias. Embryos with tracking issues were removed resulting in 16–24 embryos analyzed per treatment. After LPMR recording, embryos were anesthetized with benzocaine (50 mg/L) and photographed in lateral view.

Larval standard length was measured using ImageJ (Schneider et al., 2012).

Statistics

For both the FET and the *in vivo* EROD assay, three independent runs were performed, and data were tested for normality with the Shapiro-Wilk and Kolmogorov-Smirnov tests. If data were normally distributed, one-way analysis of variance (ANOVA) was run in combination with a *post-hoc* Dunnett's test; otherwise, a non-parametric Kruskal-Wallis, Mann-Whitney U or Wilcoxon's matched-pairs tests were used for statistical comparison.

Data were first analyzed for differences between runs (biological replicates). Since there were no significant differences between independent runs, single data sets were merged for each laboratory, and tests on different exposure groups were performed.

In the case of LPMR, a repeated-measure ANOVA was performed to take into account the three successive periods of the test.

A p-value of 0.05 was considered statistically significant for all analyses. Graphical illustrations and statistical tests were performed with Sigma Plot 12.5 (Jandel-Systat, Erkrath, Germany).

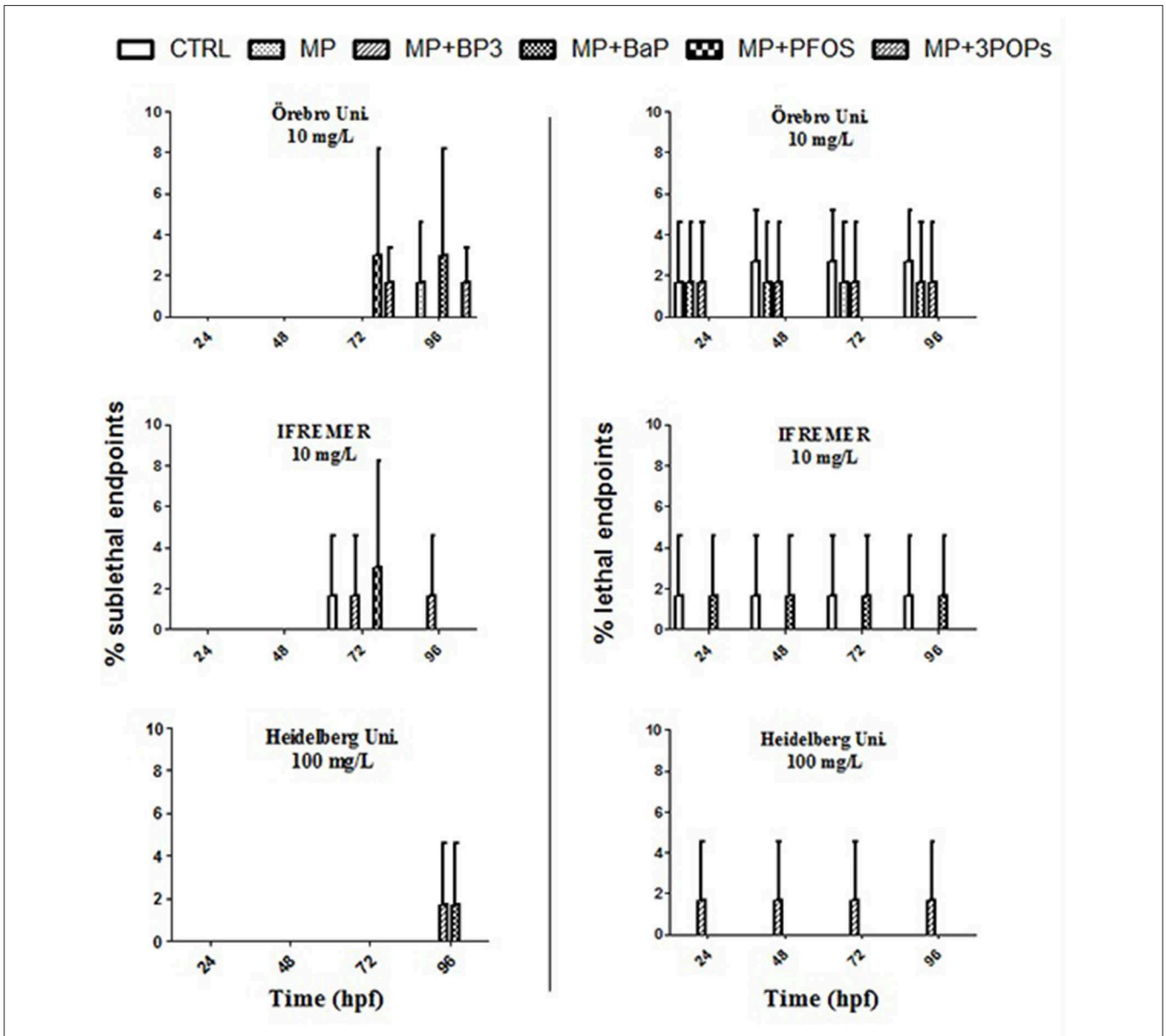


FIGURE 1 | Sublethal and lethal endpoints of zebrafish (*Danio rerio*) embryos after 24, 48, 72, and 96 h exposure to microplastics (MP) and microplastics spiked with benzophenone 3 (MP + BP3), benzo(a)pyrene (MP + BaP), perfluorooctane sulfonate (MP + PFOS) as well as a mixture of the three pollutants (MP + 3 POPs) relative to the negative control (CTRL). Endpoints are given as means ± SD from three independent experiments.

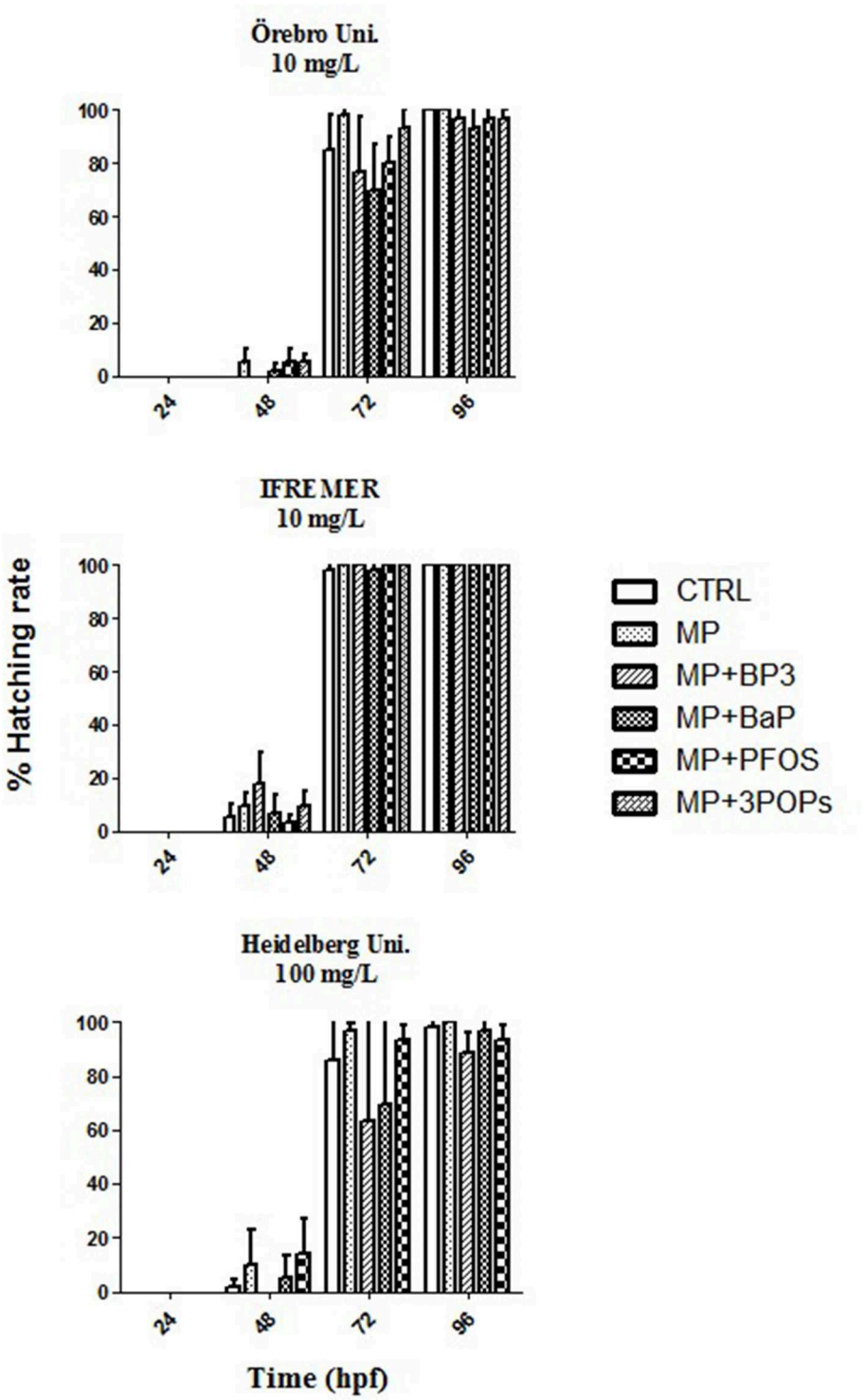


FIGURE 2 | Hatching rate of zebrafish (*Danio rerio*) embryos after 24, 48, 72, and 96 h exposure to microplastics (MP) and microplastics spiked with benzophenone 3 (MP + BP3), benzo[a]pyrene (MP + BaP), perfluorooctane sulfonate (MP + PFOS) as well as a mixture of the three pollutants (MP + 3 POPs) in comparison to the control (CTRL). Hatching rate is given as means ± SD from three independent experiments. Kruskal-Wallis analysis: $p = 0.05$.

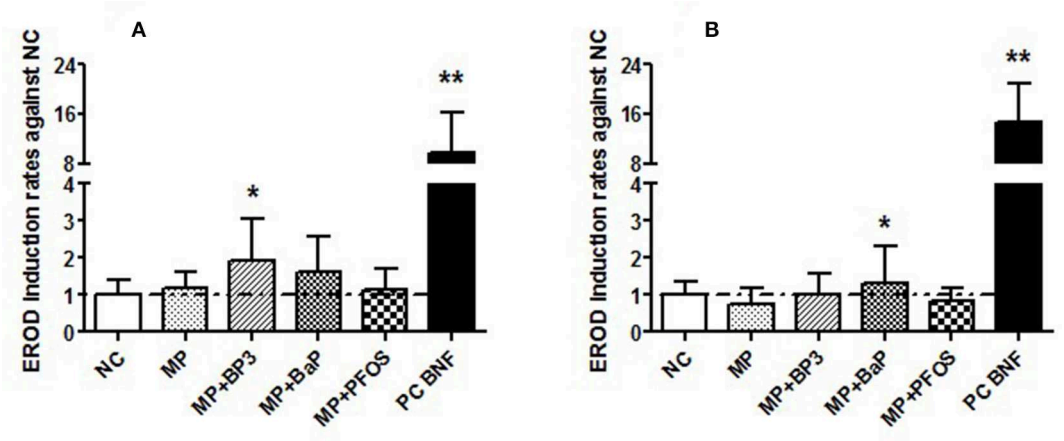


FIGURE 3 | EROD activity in 96 h old zebrafish (*Danio rerio*) embryos exposed to microplastic (MP) and microplastic spiked with benzophenone 3 (MP + BP3), benzo[a]pyrene (MP + BaP), perfuorooctane sulfonate (MP + PFOS), and β-naphthoflavone as a positive control (PC BNF) after 3 h pulse **(A)** or 96 h continuous exposure **(B)**. EROD activities are given relative to negative controls (NC) as means ± SD ($n = 3$; ANOVA on ranks: $^{*}p < 0.05$, $^{**}p < 0.01$).

RESULTS

Background Contamination and Spiking of Microplastics by the Model Pollutants

Background contamination by all three pollutants was measured on virgin microplastics. Results showed no presence of BP3 (<0.05 ng/g, LOD) and BaP (<5 pg/g, LOD). However, PFOS was found on the PE particles at a concentration of 12.1 ± 3.5 pg/g. This background concentration was taken into consideration when analyzing concentrations of PFOS adsorbed to MPs particles.

Chemical quantification of the three pollutants on PE showed that each pollutant has specific sorption efficiencies: 51% (BP3), 84% (BaP), and 18% (PFOS) of the total amount of the pollutants in the solution adsorbed to the PE particles, respectively. Concentrations of BP3, BaP and PFOS on MPs are reported in **Table 4**.

Embryotoxic and Teratogenic Effects

Embryotoxicity and teratogenicity of the selected pollutants diluted in water and DMSO without MPs were evaluated using a FET supplemented by various sublethal endpoints. The results did not show any significant effect (lethal, sublethal effects as malformations and hatching rate) between the negative control and the exposed individuals ($p > 0.05$).

The spiked MPs particles with BP3, BaP, and PFOS caused neither acute toxicity nor any teratogenic effects in the embryos using particle concentrations of 10 and 100 mg/L ($p > 0.05$; **Figure 1**). The examination of embryos exposed to the different MPs particles showed minor changes of the hatching rate at 72 hpf. Results were, however, statistically not different at any time point measured ($p > 0.05$; **Figure 2**). Moreover, measurements of the larvae standard length at 5 dpf showed no significant differences between all conditions tested ($p > 0.05$; **Figure S1**).

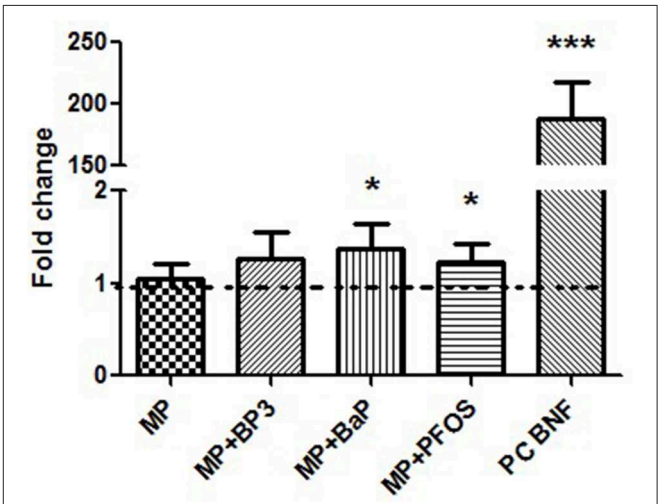


FIGURE 4 | Induction of *cyp1a* gene transcription in 96 hpf zebrafish (*Danio rerio*) embryos after exposure to 10 mg/L of microplastics (MP), microplastics spiked with benzophenone 3 (MP + BP3), microplastics spiked with benzo[a]pyrene (MP + BaP), microplastics spiked with perfuorooctane sulfonate (MP + PFOS) as well as a positive control (75 μg/L β-naphthoflavone). Transcription of *cyp1a* is given as fold-change relative to the negative control (dashed line) after 3 h exposure to MP + pollutant starting at 96 hpf. Data are given as means ± SD ($n = 4$; ANOVA on ranks: $^{*}p < 0.05$, $^{***}p < 0.0001$).

EROD Activity

EROD activity was measured in 96 hpf embryos incubated to 100 mg/L of the different MPs for either a 3 h pulse or 96 h continuously. After the 3 h pulse (**Figure 3A**), only MPs spiked with BP3 (MP + BP3) induced a significant increase in EROD activity ($p < 0.05$), whereas after 96 h of continuous exposure to MP + BP3 there was no induction of EROD activity (**Figure 3B**). In contrast, 96 h of continuous exposure to MPs spiked with BaP

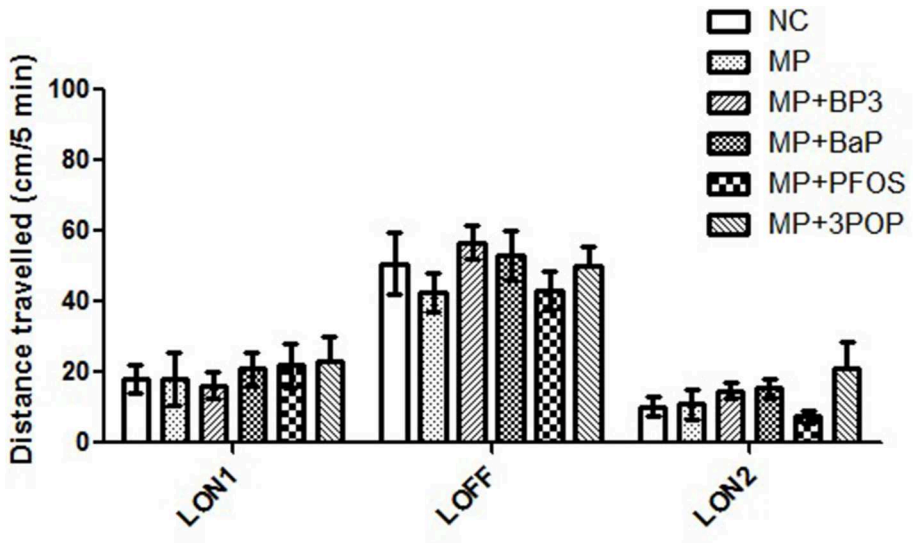


FIGURE 5 | Larval photomotor response in 5 days old zebrafish (*Danio rerio*) embryos after exposure to 10 mg/L of microplastics. Distance traveled over 5 min periods including two light-on periods (LON-1, LON-2) and one light off period (LOFF). Data are given as means ± SD (n = 16–24 larvae per treatment; repeated–measure ANOVA).

(MP + BaP) produced a significant increase in EROD activity ($p < 0.05$). MPs spiked with PFOS did not show any change in EROD activity whatever condition tested.

Gene Transcription Analysis of *Cyp1a*

Gene transcription of *cyp1a* was measured in zebrafish embryos after 3 h of exposure starting at 96 hpf. **Figure 4** shows normalized fold-change in expression of *cyp1a* relative to negative controls. Virgin MPs particles did not show any significant change of *cyp1a* transcription ($p = 0.569$). In contrast, exposure to MP + BaP and MP + PFOS induced a mild, but significant increase in *cyp1a* transcription ($p = 0.013$ and $p = 0.039$, respectively), while in the case of MP + BP3 the significance was just above the threshold ($p = 0.059$).

Larval Behavior

The larval photomotor response (LPMR) was performed at 5 dpf to monitor early behavioral disruption. Independent of the treatment, LPMR showed an increase in distance traveled during the dark phase (LOFF), if compared to both lights-on phases (LON1 and LON2; ANOVA $F_{(2,321)} = 38.07$, $p < 0.001$). Between treatments, however, there was no difference [repeated-measures ANOVA $F_{(15,281.98)} = 0.96$, $p = 0.496$; **Figure 5**].

DISCUSSION

The drastic increase in the amount of MPs in marine environments has raised concern about their potential impact on aquatic organisms (Rochman et al., 2013b; Karami et al., 2017; O'Donovan et al., 2018; Pittura et al., 2018). The adsorption of chemical pollutants from aquatic environments to MPs and the transfer to biota (Antunes et al., 2013; Bakir et al., 2016; Koelmans et al., 2016) is an additional important aspect of the potential toxicity of MPs (Batel et al., 2016; O'Donovan et al., 2018). The present study was designed to evaluate the potential hazard of contaminated PE MPs for zebrafish embryos by using the standard protocol for the acute fish embryo toxicity test (OECD TG 236) as well as enhanced protocols supplemented by additional (sublethal) endpoints such as cytochrome P450 induction and behavioral changes. In comparison to MPs levels found in aquatic environments, the microplastics concentrations selected were relatively high (10 and 100 mg/L). For ocean surface water, microplastics densities range from 0.2 to 320 μg/L (Nerland et al., 2014), and similar levels were found in freshwater ecosystems (Peng et al., 2017; Triebskorn et al., 2019). Data on environmental concentrations of BP3, BaP, and PFOS adsorbed to microplastics are either not available or scarce. Therefore, in the present study concentrations of pollutants adsorbed by PE were based on fish toxicity data from the literature, e.g., BP3 (Blüthgen et al., 2012; Paredes et al., 2014); BaP (Weigt et al., 2011), and PFOS (Yamashita et al., 2005).

In coastal waters, BP3 has been measured at relatively high concentrations, more than 2,000 ng/L, i.e., 2,500 times higher than the concentration used in the present study (Bratkovics and Sapozhnikova, 2011; Bratkovics et al., 2015). However, BaP and PFOS concentrations used in the present study were higher than quantified concentration in aquatic environment. Despite its high affinity to sediments, BaP can be found in the water column, however, the concentration used, i.e., 170 ng/L, was five times higher than concentrations found in seawater (El Nemr and Abd-Allah, 2003; Hong et al., 2016). Concentration of PFOS was also several order of magnitudes higher than concentrations detected in seawater (Ahrens et al., 2009).

Acute Embryo Toxicity and Teratogenicity

Embryotoxic and teratogenic effects were investigated for the single pollutants without MPs assuming that 100% of the

pollutants desorb from 10 mg/L of spiked MPs. Therefore, concentrations tested for BP3, BaP, and PFOS were assumed to be 0.0008, 0.17, and 0.7 μg/L, respectively. For none of the compounds, any embryotoxic effects were observed, which is in agreement with the fact that the concentrations tested were several magnitudes lower than concentrations inducing toxicity reported in the literature for different aquatic organisms. For instance, immobilization tests with *Daphnia magna* at 48 h showed an EC_{50} for BP3 between 1.67 and 1.9 mg/L (Fent et al., 2010; Sieratowicz et al., 2011). Sublethal effects of BP3 in zebrafish, rainbow trout (*Oncorhynchus mykiss*) and Japanese medaka (*Oryzias latipes*) were observed from 132 μg/L (Coronado et al., 2008; Blüthgen et al., 2012; Kim and Choi, 2014). A previous study showed that a 72 h exposure to BaP induced teratogenic and lethal effects zebrafish embryos at 63 and 126 μg/L, respectively (Weigt et al., 2011). PFOS produced sublethal and lethal effects in zebrafish embryos at 10 mg/L (Shi et al., 2008).

Likewise, PE particles up to 100 mg/L (with a diameter between 11 and 13 μm) without pollutants did not show any embryotoxic or teratogenic effects after 96 h of exposure. Similar results were found after exposure of marine medaka (*Oryzias melastigma*) using 10 mg/L of 11–13 μm PE particles (Beiras et al., 2018). Lithner et al. (2011) ranked polymers based on monomer hazard classification, and PE was classified as one of the less hazardous polymers for humans and the environment.

Regarding MPs spiked with pollutants, in the only comparable experiment so far, embryonic stages of marine medaka were exposed to 10 mg/L PE spiked with BP3 at the same concentration as in present study. Exposure to MP + BP3 induced premature hatching and a significant decrease in hatching rate (Beiras et al., 2018). The same authors also demonstrated a direct contact of MPs to villi at the surface of the marine medaka chorion, which may facilitate the uptake of toxicants. Although villi or similar structures do not exist on the zebrafish chorion, Batel et al. (2018) have shown that small MPs (1–5 μm) were able to adhere to the outer surface of the zebrafish chorion and to transfer BaP to embryos across the chorion. In order to force contact of the embryos with MPs, the exposure protocol by Ifremer used a smaller water volume than the other laboratories; yet this exposure protocol failed to induce toxic effects.

Differences have been observed between zebrafish and medaka, and beside the difference in egg surface (Hart and Donovan, 1983; Hart et al., 1984), which may interfere with MPs contact, additional factors such as exposure time might also account for the different responses observed between marine medaka and zebrafish. Indeed, different sensitivities in lethal and sublethal endpoints have been demonstrated between early life stages of Japanese medaka and zebrafish exposed to sediments spiked with two models PAHs (Perrichon et al., 2014). In the frame of FET test, one major difference between these two species is the duration of embryonic stage. For Japanese medaka, the embryonic stage lasts until days 10–11 dpf (Padilla et al., 2009), whereas this period is restricted to 48–72 hpf in zebrafish, depending on temperature (Kimmel et al., 1995). Similarly to Japanese medaka, mean hatching time for marine medaka is 11 dpf (Beiras et al., 2018). Therefore, future investigations on MPs effects in zebrafish might consider prolonged or even chronic exposure; however, this would be an animal experiment clearly requiring authorization under Directive 2010/63/EU.

Cytochrome P450 Induction and Biotransformation

CYP1A is involved in phase I of xenobiotic metabolism and is often used as a biomarker of exposure. For the investigation of sublethal effects due to MP exposures, changes in CYP1A expression was evaluated by analyzing (a) EROD activity in 96 h old zebrafish embryos exposed to 100 mg/L of MPs for 3 h (93–96 hpf) or 96 h (0–96 hpf) and (b) *cyp1a* gene transcription. Since previous studies documented short–term (3 h pulse) exposure to reference molecules to cause a more prominent EROD induction than 72 h continuous exposure (Kais et al., 2017, 2018) and has been shown to be long enough to induce *cyp1* transcription (Larcher et al., 2014), the present study tested *cyp1a* transcription levels after short-term exposure to 10 mg/L MPs for 3 h only. Independent of MP concentrations, MPs alone did not induce EROD activity nor *cyp1a* transcription.

For BP3, only the short-term exposure (93–96 hpf) to 100 mg/L MPs + BP3 significantly induced EROD activity ($p < 0.05$), while *cyp1a* transcription was slightly upregulated ($p < 0.1$) after exposure to MP + BP3 at 10 mg/L. To our knowledge, BP3 induction of EROD activity has never been shown before in embryonic stages of aquatic organisms. In adult zebrafish, Blüthgen et al. (2012) documented an increase in *cyp1a* transcription in the brain of adult zebrafish after 14 days exposure to 312 μg/L BP3.

BaP is well-known to modulate EROD activity and to induce *cyp1a* transcription in various species (Jönsson et al., 2009; Rochman et al., 2013b; Batel et al., 2016). In the present study, MPs spiked with BaP caused an induction of EROD activity after 96 h of exposure. Meanwhile, a significant induction ($p < 0.05$) of *cyp1a* transcription was also shown after 3 h of continuous exposure to BaP-spiked MPs.

MPs spiked with PFOS did not modify EROD activity, but induced *cyp1a* transcription upregulation. Both in adult marine medaka and zebrafish, previous studies showed that PFOS modifies *cyp1a* transcription after exposure to 1–16 mg/L PFOS (Fang et al., 2012; Jantzen et al., 2016).

Albeit with some differences in response with respect to exposure duration—which may reflect the need for translation or enzyme accumulation—an overall triggering of CYP1A (either as EROD activities and/or as *cyp1a* transcription) was observed after exposure to MPs spiked with pollutants, whereas virgin MPs failed to do so. Results indicate that pollutants spiked on MPs are able to desorb and reach concentrations sufficient to induce biotransformation.

Larval Behavior

An increasing number of studies uses behavioral responses to monitor sublethal effects of pollutants such as PFOS or BPA on aquatic organisms (Huang et al., 2010; Wang J. et al., 2015), and changes in, e.g., the photomotor response are commonly interpreted as evidence of potential neurotoxicity (Le Bihanic

et al., 2015; Legradi et al., 2018). Yet, our results did not show any alteration of the LPMR by any of the different MPs preparations. Similar results were shown after exposure of zebrafish embryos to pure polystyrene MPs particles by Chen et al. (2017), who spiked polystyrene MPs with EE2 (17α-ethinylestradiol) and found hypoactivity at elevated concentrations only. However, previous studies showed that microplastics may reduce other behavioral parameters such as predatory performance of fish depending on the shape of plastic particles (de Sá et al., 2015; Choi et al., 2018).

As water-borne pollutants, both PFOS (Huang et al., 2010) and BaP (Knecht et al., 2017) were shown to be able to modify the behavior of zebrafish. However, concentrations applied in these studies were several magnitudes higher than those used in this study and most likely lacked any environmental relevance. Therefore, further investigations are required to assess the toxicity of microplastics on behavioral aspects considering different endpoints and different kind of particles.

CONCLUSIONS

The current study shows that both virgin MPs and MPs spiked with BP3, BaP, or PFOS at given concentrations do not cause acute toxic effects in zebrafish embryos using the standardized protocol of OECD TG 236 (FET). Thus, the standard FET may not be sensitive enough to investigate acute embryotoxic effects of spiked MPs. Likewise, behavioral endpoints such as the LPMR were not altered under any conditions tested, even though existing literature indicate that embryonic and larval behavior of fish may be altered by MPs. Yet, given that the FET can easily be supplemented with additional (more sensitive) endpoints, enhanced FET protocols may be designed to record more subtle sublethal endpoints such as biotransformation. For the identification of adverse effects due to exposure to MPs and associated pollutants, more sensitive biomarkers or other fish models with a longer embryonic stage (i.e., Japanese or marine medaka) may thus be needed. Results indicate that sublethal toxic effects are more likely caused by pollutants associated with MPs than by the MPs themselves. This lends some support to the view that MPs might play a certain role as a vector of pollutants. Further research at more environmentally relevant MPs concentrations is needed with different types and sizes of MPs, other pollutants and adapted exposures scenarios including increased exposure duration.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AB, BC, SK, TB, and XC designed the experiment. AB, BC, and XC performed the experiments and analyzed the data. AB conducted P450 biomarker analyses. XC conducted *cyp1a* gene transcription and behavioral analyses. BC prepared and characterized the contaminated MPs, performed the chemical analyses. BC drafted the manuscript and AB, JC, M-LB, SK, TB, and XC corrected the manuscript.

FUNDING

This work was developed under the EPHEMARE project (Ecotoxicological effects of microplastics in marine ecosystems), supported by national funding agencies within the framework of JPI Oceans (FCT JPIOCEANS/0005/2015; FORMAS, 2015-01865; ANR-15-JOCE-0002-01; German BMBF 03F0735A). BC was directly supported by an IdEx grant from the University of Bordeaux; AB was awarded a scholarship by the German Academic Scholarship Foundation (Studienstiftung des Deutschen Volkes).

ACKNOWLEDGMENTS

The authors would like to thank (1) Anna Karmann and Leo Yeung for their skillful collaboration to the PFOS analyses; (2) Maria Larsson for her help and supervision with the BaP analyses; (3) Patrick Van Hees and EUROFINS Germany for their collaboration to the BP3 analyses; (4) Ezvin Le Roux for performing some FET experiments at Örebro University; and (5) Lucette Joassard at IFREMER for technical support in running the qPCR analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2019.00135/full#supplementary-material

REFERENCES

Ahrens, L., Felizeter, S., and Ebinghaus, R. (2009). Spatial distribution of polyfluoroalkyl compounds in seawater of the German Bight. *Chemosphere* 76, 179–184. doi: 10.1016/j.chemosphere.2009.03.052

Andrady, A. L. (2011). Microplastics in the marine environment. *Mar. Pollut. Bull.* 62, 1596–1605. doi: 10.1016/j.marpolbul.2011.05.030

Andrady, A. L., and Neal, M. A. (2009). Applications and societal benefits of plastics. *Philos. Trans. R. Soc. B. Biol. Sci.* 364, 1977–1984. doi: 10.1098/rstb.2008.0304

Antunes, J. C., Frias, J. G. L., Micaelo, A. C., and Sobral, P. (2013). Resin pellets from beaches of the Portuguese coast and adsorbed persistent organic pollutants. *Estuar. Coast. Shelf Sci.* 130, 62–69. doi: 10.1016/j.ecss.2013.06.016

Bakir, A., O'Connor, I. A., Rowland, S. J., Hendriks, A. J., and Thompson, R. C. (2016). Relative importance of microplastics as a pathway for the transfer of hydrophobic organic chemicals to marine life. *Environ. Pollut.* 219, 56–65. doi: 10.1016/j.envpol.2016.09.046

Bakir, A., Rowland, S. J., and Thompson, R. C. (2014). Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. *Environ. Pollut.* 185, 16–23. doi: 10.1016/j.envpol.2013.10.007

Barnes, D. K., Galgani, F., Thompson, R. C., and Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *Philos. Trans. R. Soc. B. Biol. Sci*. 364, 1985–1998. doi: 10.1098/rstb.2008.0205

Batel, A., Borchert, F., Reinwald, H., Erdinger, L., and Braunbeck, T. (2018). Microplastic accumulation patterns and transfer of benzo[a]pyrene to adult zebrafish (*Danio rerio*) gills and zebrafish embryos. *Environ. Pollut.* 235, 918–930. doi: 10.1016/j.envpol.2018.01.028

Batel, A., Linti, F., Scherer, M., Erdinger, L., and Braunbeck, T. (2016). Transfer of benzo[a]pyrene from microplastics to *Artemia nauplii* and further to zebrafish via a trophic food web experiment: CYP1A induction and visual tracking of persistent organic pollutants. *Environ. Toxicol. Chem.* 35, 1656–1666. doi: 10.1002/etc.3361

Beiras, R., Bellas, J., Cachot, J., Cormier, B., Cousin, X., Engwall, M., et al. (2018). Ingestion and contact with polyethylene microplastics does not cause acute toxicity on marine zooplankton. *J. Hazard. Mater*. 360, 452–460. doi: 10.1016/j.jhazmat.2018.07.101

Belanger, S. E., Rawlings, J. M., and Carr, G. J. (2013). Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemicals. *Environ. Toxicol. Chem.* 32, 1768–1783. doi: 10.1002/etc.2244

Blüthgen, N., Zucchi, S., and Fent, K. (2012). Effects of the UV filter benzophenone-3 (oxybenzone) at low concentrations in zebrafish (*Danio rerio*). *Toxicol. Appl. Pharmacol.* 263, 184–194. doi: 10.1016/j.taap.2012.06.008

Boehler, S., Lörracher, A. K., Schubert, J., and Braunbeck, T. (2018). Comparative live-imaging of *in vivo* EROD (ethoxyresorufin-O-deethylase) induction in zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*) embryos after exposure to PAHs and river sediment extracts. *Sci. Total Environ.* 621, 827–838. doi: 10.1016/j.scitotenv.2017.11.101

Bour, A., Haarr, A., Keiter, S., and Hylland, K. (2018). Environmentally relevant microplastic exposure affects sediment-dwelling bivalves. *Environ. Pollut.* 236, 652–660. doi: 10.1016/j.envpol.2018.02.006

Bratkovics, S., and Sapozhnikova, Y. (2011). Determination of seven commonly used organic UV filters in fresh and saline waters by liquid chromatography-tandem mass spectrometry. *Anal. Methods*. 3, 2943–2950. doi: 10.1039/c1ay05390f

Bratkovics, S., Wirth, E., Sapozhnikova, Y., Pennington, P., and Sanger, D. (2015). Baseline monitoring of organic sunscreen compounds along South Carolina's coastal marine environment. *Mar. Pollut. Bull*. 101, 370–377. doi: 10.1016/j.marpolbul.2015.10.015

Braunbeck, T., Boettcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., et al. (2005). Towards an alternative for the acute fish LC_{50} test in chemical assessment: The zebrafish (*Danio rerio*) embryo toxicity test - an update. *ALTEX* 22, 87–102. Available online at: http://www.altex.ch/resources/altex_2005_2_87_102_Braunbeck.pdf

Braunbeck, T., Kais, B., Lammer, E., Otte, J., Schneider, K., Stengel, D., et al. (2015). The fish embryo test (FET): origin, applications, and future. *Environ. Sci. Pollut. Res. Int.* 22, 16247–16261. doi: 10.1007/s11356-014-3814-7

Châtel, A., Bruneau, M., Lièvre, C., Goupil, A., and Mouneyrac, C. (2017). Spermatozoa: a relevant biological target for genotoxicity assessment of contaminants in the estuarine bivalve *Scrobicularia plana*. *Mar. Pollut. Bull*. 116, 488–490. doi: 10.1016/j.marpolbul.2016.12.062

Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D., et al. (2017). Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. *Sci. Total Environ.* 584–585, 1022–1031. doi: 10.1016/j.scitotenv.2017.01.156

Choi, J. S., Jung, Y.-J., Hong, N.-H., Hong, S. H., and Park, J.-W. (2018). Toxicological effects of irregularly shaped and spherical microplastics in a marine teleost, the sheepshead minnow (*Cyprinodon variegatus*). *Mar. Pollut. Bull*. 129, 231–240. doi: 10.1016/j.marpolbul.2018.02.039

Cole, M., Lindeque, P., Halsband, C., and Galloway, T. S. (2011). Microplastics as contaminants in the marine environment: a review. *Mar. Pollut. Bull*. 62, 2588–2597. doi: 10.1016/j.marpolbul.2011.09.025

Collins, J. F., Brown, J. P., Dawson, S. V., and Marty, M. A. (1991). Risk assessment for benzo[a]pyrene. *Regul. Toxicol. Pharmacol*. 13, 170–184. doi: 10.1016/0273-2300(91)90020-V

Coronado, M., De Haro, H., Deng, X., Rempel, M. A., Lavado, R., and Schlenk, D. (2008). Estrogenic activity and reproductive effects of the UV-filter oxybenzone (2-hydroxy-4-methoxyphenyl-methanone) in fish. *Aquat. Toxicol.* 90, 182–187. doi: 10.1016/j.aquatox.2008.08.018

de Sá, L. C., Luís, L. G., and Guilhermino, L. (2015). Effects of microplastics on juveniles of the common goby (*Pomatoschistus microps*): confusion with prey, reduction of the predatory performance and efficiency, and possible influence of developmental conditions. *Environ. Pollut.* 196, 359–362. doi: 10.1016/j.envpol.2014.10.026

Derraik, J. G. (2002). The pollution of the marine environment by plastic debris: a review. *Mar. Pollut. Bull*. 44, 842–852. doi: 10.1016/S0025-326X(02)00220-5

El Nemr, A., and Abd-Allah, A. M. A. (2003). Contamination of polycyclic aromatic hydrocarbons (PAHs) in microlayer and subsurface waters along Alexandria coast, Egypt. *Chemosphere* 52, 1711–1716. doi: 10.1016/S0045-6535(03)00300-X

Eriksson, U., Roos, A., Lind, Y., Hope, K., Ekblad, A., and Kärrman, A. (2016). Comparison of PFASs contamination in the freshwater and terrestrial environments by analysis of eggs from osprey (*Pandion haliaetus*), tawny owl (*Strix aluco*), and common kestrel (*Falco tinnunculus*). *Environ. Res.* 149, 40–47. doi: 10.1016/j.envres.2016.04.038

EU (2010). Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes. *Off. J. EU*. L276, 33–79. Available online at: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF

Fang, C., Wu, X., Huang, Q., Liao, Y., Liu, L., Qiu, L., et al. (2012). PFOS elicits transcriptional responses of the ER, AHR and PPAR pathways in *Oryzias melastigma* in a stage-specific manner. *Aquat. Toxicol.* 106–107, 9–19. doi: 10.1016/j.aquatox.2011.10.009

Fent, K., Kunz, P. Y., Zenker, A., and Rapp, M. (2010). A tentative environmental risk assessment of the UV-filters 3-(4-methylbenzylidene-camphor), 2-ethyl-hexyl-4-trimethoxycinnamate, benzophenone-3, benzophenone-4 and 3-benzylidene camphor. *Mar. Environ. Res.* 69, S4–S6. doi: 10.1016/j.marenvres.2009.10.010

Giesy, J. P., and Kannan, K. (2002). Perfluorochemical surfactants in the environment. *Environ. Sci. Technol*. 36, 146A—152A. doi: 10.1021/es022253t

Hart, N. H., and Donovan, M. (1983). Fine structure of the chorion and site of sperm entry in the egg of *Brachydanio*. *J. Exp. Zool.* 227, 277–296. doi: 10.1002/jez.1402270212

Hart, N. H., Pietri, R., and Donovan, M. (1984). The structure of the chorion and associated surface filaments in Oryzias—evidence for the presence of extracellular tubules. *J. Exp. Zool.* 230, 273–296. doi: 10.1002/jez.1402300213

Hong, W.-J., Jia, H., Li, Y.-F., Sun, Y., Liu, X., and Wang, L. (2016). Polycyclic aromatic hydrocarbons (PAHs) and alkylated PAHs in the coastal seawater, surface sediment and oyster from Dalian, Northeast China. *Ecotoxicol. Environ. Saf.* 128, 11–20. doi: 10.1016/j.ecoenv.2016.02.003

Huang, H., Huang, C., Wang, L., Ye, X., Bai, C., Simonich, M. T., et al. (2010). Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctanesulphonicacid (PFOS). *Aquat. Toxicol.* 98, 139–147. doi: 10.1016/j.aquatox.2010.02.003

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., et al. (2015). Plastic waste inputs from land into the ocean. *Science* 347, 768–771. doi: 10.1126/science.1260352

Jantzen, C. E., Annunziato, K. A., Bugel, S. M., and Cooper, K. R. (2016). PFOS, PFNA, and PFOA sub-lethal exposure to embryonic zebrafish have different toxicity profiles in terms of morphometrics, behavior and gene expression. *Aquat. Toxicol.* 175, 160–170. doi: 10.1016/j.aquatox.2016.03.026

Jönsson, M. E., Brunström, B., and Brandt, I. (2009). The zebrafish gill model: induction of CYP1A, EROD and PAH adduct formation. *Aquat. Toxicol.* 91, 62–70. doi: 10.1016/j.aquatox.2008.10.010

Kais, B., Ottermanns, R., Scheller, F., and Braunbeck, T. (2018). Modification and quantification of *in vivo* EROD live-imaging with zebrafish (*Danio rerio*) embryos to detect both induction and inhibition of CYP1A. *Sci. Total Environ.* 615, 330–347. doi: 10.1016/j.scitotenv.2017.09.257

Kais, B., Schiwy, S., Hollert, H., Keiter, S. H., and Braunbeck, T. (2017). *In vivo* EROD assays with the zebrafish (*Danio rerio*) as rapid screening tools for the detection of dioxin-like activity. *Sci. Total Environ.* 590–591, 269–280. doi: 10.1016/j.scitotenv.2017.02.236

Karami, A., Groman, D. B., Wilson, S. P., Ismail, P., and Neela, V. K. (2017). Biomarker responses in zebrafish (*Danio rerio*) larvae exposed to pristine low-density polyethylene fragments. *Environ. Pollut.* 223, 466–475. doi: 10.1016/j.envpol.2017.01.047

Keiter, S., Burkhard-Medicke, K., Kais, B., Färber, H., Skutlarek, D., Engwall, M., et al. (2016). Does perfluorooctane sulfonate (PFOS) act as chemosensitizer in zebrafish embryos? *Sci. Total Environ*. 548–549, 317–324. doi: 10.1016/j.scitotenv.2015.12.089

Kim, S., and Choi, K. (2014). Occurrences, toxicities, and ecological risks of benzophenone-3, a common component of organic sunscreen products: a mini-review. *Environ. Int.* 70, 143–157. doi: 10.1016/j.envint.2014.05.015

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn*. 203, 253–310. doi: 10.1002/aja.1002030302

Kinnberg, K. L., Petersen, G. I., Albrektsen, M., Minghlani, M., Awad, S. M., Holbech, B. F., et al. (2015). Endocrine-disrupting effect of the ultraviolet filter benzophenone-3 in zebrafish, *Danio rerio*. *Environ. Toxicol. Chem.* 34, 2833–2840. doi: 10.1002/etc.3129

Knecht, A. L., Truong, L., Simonich, M. T., and Tanguay, R. L. (2017). Developmental benzo[a]pyrene (B[a]P) exposure impacts larval behavior and impairs adult learning in zebrafish. *Neurotoxicol. Teratol*. 59, 27–34. doi: 10.1016/j.ntt.2016.10.006

Koelmans, A. A., Bakir, A., Burton, G. A., and Janssen, C. R. (2016). Microplastic as a vector for chemicals in the aquatic environment: critical review and model-supported reinterpretation of empirical studies. *Environ. Sci. Technol*. 50, 3315–3326. doi: 10.1021/acs.est.5b06069

Koelmans, A. A., Besseling, E., Wegner, A., and Foekema, E. M. (2013). Plastic as a carrier of POPs to aquatic organisms: a model analysis. *Environ. Sci. Technol.* 47, 7812–7820. doi: 10.1021/es401169n

Lammer, E., Carr, G. J., Wendler, K., Rawlings, J. M., Belanger, S. E., and Braunbeck, T. (2009). Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? *Comp. Biochem. Physiol. C*. 149, 196–209. doi: 10.1016/j.cbpc.2008.11.006

Larcher, T., Perrichon, P., Vignet, C., Ledevin, M., Le Menach, K., Lyphout, L., et al. (2014). Chronic dietary exposure of zebrafish to PAH mixtures results in carcinogenic but not genotoxic effects. *Environ. Sci. Pollut. Res.* 21:13833. doi: 10.1007/s11356-014-2923-7

Larsson, M., Hagberg, J., Rotander, A., van Bavel, B., and Engwall, M. (2013). Chemical and bioanalytical characterisation of PAHs in risk assessment of remediated PAH-contaminated soils. *Environ. Sci. Pollut. Res*. 20, 8511–8520. doi: 10.1007/s11356-013-1787-6

Le Bihanic, F., Sommard, V., Perrine de, L., Pichon, A., Grasset, J., Berrada, S., et al. (2015). Environmental concentrations of benz[a]anthracene induce developmental defects and DNA damage and impair photomotor response in Japanese medaka larvae. *Ecotoxicol. Environ. Saf*. 113, 321–328. doi: 10.1016/j.ecoenv.2014.12.011

Lee, H., Shim, W. J., and Kwon, J.-H. (2014). Sorption capacity of plastic debris for hydrophobic organic chemicals. *Sci. Total Environ.* 470–471, 1545–1552. doi: 10.1016/j.scitotenv.2013.08.023

Legradi, J. B., Di Paolo, C., Kraak, M. H. S., van der Geest, H. G., Schymanski, E. L., Williams, A. J., et al. (2018). An ecotoxicological view on neurotoxicity assessment. *Environ. Sci. Eur*. 30:46. doi: 10.1186/s12302-018-0173-x

Lithner, D., Larsson, Å., and Dave, G. (2011). Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. *Sci. Total Environ.* 409, 3309–3324. doi: 10.1016/j.scitotenv.2011.04.038

Liu, T., Pan, L., Jin, Q., and Cai, Y. (2015). Differential gene expression analysis of benzo(a)pyrene toxicity in the clam, *Ruditapes philippinarum*. *Ecotoxicol. Environ. Saf.* 115, 126–136. doi: 10.1016/j.ecoenv.2015.02.007

Lohmann, R. (2017). Microplastics are not important for the cycling and bioaccumulation of organic pollutants in the oceans—but should microplastics be considered POPs themselves? *Integ. Environ. Assess. Manag*. 13, 460–465. doi: 10.1002/ieam.1914

Mato, Y., Isobe, T., Takada, H., Kanehiro, H., Ohtake, C., and Kaminuma, T. (2001). Plastic resin pellets as a transport medium for toxic chemicals in the marine environment. *Environ. Sci. Technol.* 35, 318–324. doi: 10.1021/es0010498

Nagel, R. (2002). DarT: The embryo test with the zebrafish *Danio rerio* - a general model in ecotoxicology and toxicology. *ALTEX* 19, 38–48. Available online at: http://www.altex.ch/resources/altex_2002_Supp_1_38_48_Nagel.pdf

Nerland, I. L., Halsband, C., Allan, I., and Thomas, K. V. (2014). *Microplastics in Marine Environments: Occurrence, Distribution and Effects*. Oslo: NIVA. Available online at: https://www.miljodirektoratet.no/globalassets/publikasjoner/M319/M319.pdf

O'Donovan, S., Mestre, N. C., Abel, S., Fonseca, T. G., Carteny, C. C., Cormier, B., et al. (2018). Ecotoxicological effects of chemical contaminants adsorbed to microplastics in the clam *Scrobicularia plana*. *Front. Mar. Sci*. 5:143. doi: 10.3389/fmars.2018.00143

OECD (1992). "OECD guidelines for the testing of chemicals," in *Section 2: Effects on Biotic Systems OECD Test Guideline 203: Fish, Acute toxicity Test* (Paris: OECD publishing). Available online at: https://www.oecd-ilibrary.org/environment/test-no-203-fish-acute-toxicity-test_9789264069961-en

OECD (2013). "OECD Guidelines for the Testing of Chemicals," in *Section 2: Effects on Biotic Systems Test No 236: Fish Embryo Acute Toxicity Test* (Paris: OECD publishing). Available online at: https://www.oecd-ilibrary.org/environment/test-no-236-fish-embryo-acute-toxicity-fet-test_9789264203709-en

Padilla, S., Cowden, J., Hinton, D. E., Yuen, B., Law, S., Kullman, S. W., et al. (2009). Use of medaka in toxicity testing. *Curr. Protoc. Toxicol*. 39, 1.10.11–11.10.36. doi: 10.1002/0471140856.tx0110s39

Paredes, E., Perez, S., Rodil, R., Quintana, J. B., and Beiras, R. (2014). Ecotoxicological evaluation of four UV filters using marine organisms from different trophic levels *Isochrysis galbana*, *Mytilus galloprovincialis*, *Paracentrotus lividus*, and *Siriella armata*. *Chemosphere* 104, 44–50. doi: 10.1016/j.chemosphere.2013.10.053

Paul, A. G., Jones, K. C., and Sweetman, A. J. (2009). A first global production, emission, and environmental inventory for perfluorooctane sulfonate. *Environ. Sci. Technol.* 43, 386–392. doi: 10.1021/es802216n

Peng, J., Wang, J., and Cai, L. (2017). Current understanding of microplastics in the environment: Occurrence, fate, risks, and what we should do. *Integr. Environ. Asses*. 13, 476–482. doi: 10.1002/ieam.1912

Perrichon, P., Le Bihanic, F., Bustamante, P., Le Menach, K., Budzinski, H., Cachot, J., et al. (2014). Influence of sediment composition on PAH toxicity using zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) embryo-larval assays. *Environ. Sci. Pollut. Res*. 21, 13703–13719. doi: 10.1007/s11356-014-3502-7

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, 2002–2007. doi: 10.1093/nar/29.9.e45

Pfaffl, M. W., Horgan, G. W., and Dempfle, L. (2002). Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 30:10. doi: 10.1093/nar/30.9.e36

Pfaffl, M. W., Tichopad, A., Prgomet, C., and Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - excel-based tool using pair-wise correlations. *Biotechnol. Lett.* 26, 509–515. doi: 10.1023/B:BILE.0000019559.84305.47

Pittura, L., Avio, C. G., Giuliani, M. E., d'Errico, G., Keiter, S. H., Cormier, B., et al. (2018). Microplastics as vehicles of environmental PAHs to marine organisms: combined chemical and physical hazards to the mediterranean mussels, *Mytilus galloprovincialis*. *Front. Mar. Sci.* 5:103. doi: 10.3389/fmars.2018.00103

Rios, L. M., Moore, C., and Jones, P. R. (2007). Persistent organic pollutants carried by synthetic polymers in the ocean environment. *Mar. Pollut. Bull*. 54, 1230–1237. doi: 10.1016/j.marpolbul.2007.03.022

Rochman, C. M., Hoh, E., Hentschel, B. T., and Kaye, S. (2013a). Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: implications for plastic marine debris. *Environ. Sci. Technol.* 47, 1646–1654. doi: 10.1021/es303700s

Rochman, C. M., Hoh, E., Kurobe, T., and Teh, S. J. (2013b). Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Sci. Rep.* 3:3263. doi: 10.1038/srep03263

Rodríguez-Fuentes, G., Sandoval-Gío, J. J., Arroyo-Silva, A., Noreña-Barroso, E., Escalante-Herrera, K. S., and Olvera-Espinosa, F. (2015). Evaluation of the estrogenic and oxidative stress effects of the UV filter 3-benzophenone in zebrafish (*Danio rerio*) eleuthero-embryos. *Ecotoxicol. Environ. Saf.* 115, 14–18. doi: 10.1016/j.ecoenv.2015.01.033

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9:671. doi: 10.1038/nmeth.2089

Schönlau, C., Larsson, M., Lam, M. M., Engwall, M., Giesy, J. P., Rochman, C., et al. (2019). Aryl hydrocarbon receptor-mediated potencies in field-deployed plastics vary by type of polymer. *Environ. Sci. Pollut. Res.* 26, 9079–9088. doi: 10.1007/s11356-019-04281-4

Shi, X., Du, Y., Lam, P. K., Wu, R. S., and Zhou, B. (2008). Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS. *Toxicol. Appl. Pharmacol.* 230, 23–32. doi: 10.1016/j.taap.2008.01.043

Sieratowicz, A., Kaiser, D., Behr, M., Oetken, M., and Oehlmann, J. (2011). Acute and chronic toxicity of four frequently used UV filter substances for *Desmodesmus subspicatus* and *Daphnia magna*. *J. Environ. Sci. Health A. Tox. Hazard Subst. Environ. Eng.* 46, 1311–1319. doi: 10.1080/10934529.2011.602936

Strähle, U., Scholz, S., Geisler, R., Greiner, P., Hollert, H., Rastegar, S., et al. (2012). Zebrafish embryos as an alternative to animal experiments–a commentary on the definition of the onset of protected life stages in animal welfare regulations. *Reprod. Toxicol.* 33, 128–132. doi: 10.1016/j.reprotox.2011.06.121

Teuten, E. L., Rowland, S. J., Galloway, T. S., and Thompson, R. C. (2007). Potential for plastics to transport hydrophobic contaminants. *Environ. Sci. Technol.* 41, 7759–7764. doi: 10.1021/es071737s

Thompson, R. C., Moore, C. J., vom Saal, F. S., and Swan, S. H. (2009). Plastics, the environment and human health: current consensus and future trends. *Philos. Trans. R. Soc. B. Biol. Sci.* 364, 2153–2166. doi: 10.1098/rstb.2009.0053

Thompson, R. C., Olsen, Y., Mitchell, R. P., Davis, A., Rowland, S. J., John, A. W. G., et al. (2004). Lost at sea: where is all the plastic? *Science* 304, 838–838. doi: 10.1126/science.1094559

Triebskorn, R., Braunbeck, T., Grummt, T., Hanslik, L., Huppertsberg, S., Jekel, M., et al. (2019). Relevance of nano- and microplastics for freshwater ecosystems: a critical review. *TrAC.* 110, 375–392. doi: 10.1016/j.trac.2018.11.023

Vignet, C., Le Menach, K., Lyphout, L., Guionnet, T., Frère, L., Leguay, D., et al. (2014). Chronic dietary exposure to pyrolytic and petrogenic mixtures of PAHs causes physiological disruption in zebrafish—part II: behavior. *Environ. Sci. Pollut. R.* 21, 13818–13832. doi: 10.1007/s11356-014-2762-6

Wang, F., Shih, K. M., and Li, X. Y. (2015). The partition behavior of perfluorooctanesulfonate (PFOS) and perfluorooctanesulfonamide (FOSA) on microplastics. *Chemosphere* 119, 841–847. doi: 10.1016/j.chemosphere.2014.08.047

Wang, J., Wang, X., Xiong, C., Liu, J., Hu, B., and Zheng, L. (2015). Chronic bisphenol A exposure alters behaviors of zebrafish (*Danio rerio*). *Environ. Pollut.* 206, 275–281. doi: 10.1016/j.envpol.2015.07.015

Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., and Broschard, T. H. (2011). Zebrafish (*Danio rerio*) embryos as a model for testing proteratogens. *Toxicology* 281, 25–36. doi: 10.1016/j.tox.2011.01.004

Westerfield, C. (2007). *The Zebrafish Book: A Guid for the Labortary Use of Zebrafish (Danio rerio)*. Eugene, OR: University of Oregon Press.

Yamashita, N., Kannan, K., Taniyasu, S., Horii, Y., Petrick, G., and Gamo, T. (2005). A global survey of perfluorinated acids in oceans. *Mar. Pollut. Bull.* 51, 658–668. doi: 10.1016/j.marpolbul.2005.04.026

Ziccardi, L. M., Edgington, A., Hentz, K., Kulacki, K. J., and Driscoll, S. K. (2016). Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: a state-of-the-science review. *Environ. Toxicol. Chem.* 35, 1667–1676. doi: 10.1002/etc.3461

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

ORIGINAL RESEARCH
published: 25 September 2019
doi: 10.3389/fmars.2019.00602

Levels, Sources and Potential Risks of Polychlorinated Biphenyls and Organochlorine Pesticides in Sediments of Qingduizi Bay, China: Does Developing Mariculture Matter?

Xiaolong Yang[1], Lili Wang[1], Anguo Zhang[1], Xing Liu[1], Gorka Bidegain[2], Humin Zong[1], Chunjiang Guan[1], Mingshan Song[1], Liang Qu[1], Wei Huang[3] and Xiutang Yuan[1]**

[1] National Marine Environmental Monitoring Center, Ministry of Ecology and Environment, Dalian, China, [2] Department of Applied Mathematics/Research Centre for Experimental Marine Biology and Biotechnology, University of the Basque Country, Bilbao, Spain, [3] Key Laboratory of Marine Ecosystem and Biogeochemistry, State Oceanic Administration and Second Institute of Oceanography, Ministry of Natural Resources, Hangzhou, China

OPEN ACCESS

Edited by:
Xiaoshou Liu,
Ocean University of China, China

Reviewed by:
Yuxin Sun,
South China Sea Institute of Oceanology (CAS), China
Wenzhe Xu,
Tianjin University of Science and Technology, China

***Correspondence:**
Wei Huang
willhuang@sio.org.cn
Xiutang Yuan
xtyuan@nmemc.org.cn

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *28 December 2018*
Accepted: *11 September 2019*
Published: *25 September 2019*

Citation:
Yang X, Wang L, Zhang A, Liu X, Bidegain G, Zong H, Guan C, Song M, Qu L, Huang W and Yuan X (2019) Levels, Sources and Potential Risks of Polychlorinated Biphenyls and Organochlorine Pesticides in Sediments of Qingduizi Bay, China: Does Developing Mariculture Matter? Front. Mar. Sci. 6:602. doi: 10.3389/fmars.2019.00602

Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), including hexachlorocyclohexanes (HCHs) and dichlorodiphenyltrichloroethanes (DDTs), were studied in the surface sediments of Qingduizi Bay (Yellow Sea, China). The goal was to identify whether their distribution and levels can be influenced by aquaculture activities in ponds. The total concentration of PCBs in mariculture ponds (MP) and the outer bay (OB) ranged from 0.61 ng g^{-1} to 2.10 ng g^{-1} dry weight (dw) and from 0.79 to 2.41 ng g^{-1} dw, respectively. The concentrations of hexachlorocyclohexanes (HCHs) and dichlorodiphenyltrichloroethanes (DDTs), ranged from 0.03 to 0.18 ng g^{-1} dw and from 0.24 to 31.14 ng g^{-1} dw, respectively. The levels of PCBs were significantly different between MP and OB, whereas no significant differences of DDTs and HCHs were detected between the two areas. Among the PCB congeners, tri-PCB and tetra-PCB were dominant, implying a historical input and atmospheric deposition for PCBs. The isomeric ratios of the compositions implicated mixture sources of new and historical inputs for HCHs. However, DDT-containing feed and pesticides in aquaculture activities could be the primary source of newly inputted DDTs. A parallel ecological risk assessment analysis showed the impact of DDTs on marine organisms. In conclusion, aquaculture in ponds influences the occurrence and distribution of persistent halogenated compounds in coastal areas, causing potential ecological impacts. This result should be a baseline for the management of aquaculture ponds regarding these compounds.

Keywords: persistent organic pollutants, pond mariculture, sea reclamation, sediment, Northern Yellow Sea, ecological impact

INTRODUCTION

Coastal ecosystems are the environments most vulnerable to pollution due to anthropogenic stresses worldwide (Ricciardi and Atkinson, 2004). Large amounts of contamination, including persistent organic pollutants (POPs), nutrients and heavy metals, are introduced into estuarine and coastal systems because of rapid increases in agricultural, industrial and domestic activities, which are causing a series of toxicological impacts on marine species worldwide (Islam and Tanaka, 2004). POPs are widely used in agricultural and industrial processes and have attracted considerable interest because of their ubiquity, persistence, bio-accumulation, and biotoxicity (Sharma et al., 2014).

Polychlorinated biphenyls (PCBs), comprising 10 homologs and 209 congeners with various numbers and positions of chlorine atoms, are commonly used as a heat transfer medium in capacitors and transformers and are also perfect additives in industrial materials, such as dyed paper, plastics and paints, because of their high thermoresistance and low electrical conductivity (Ishikawa et al., 2007; Yu et al., 2014). Organochlorine pesticides (OCPs) encompass a wide range of chemicals, including hexachlorocyclohexanes (HCHs), dichlorodiphenyltrichloroethanes (DDTs), dieldrin, endrin, and aldrin. They have also been widely used for pest management in agriculture and aquaculture because of their high efficiency and low cost (Wu et al., 2015). More than 10,000 tons of PCBs, 0.4 million tons of DDTs, and 4.5 million tons of HCHs were used in China from the 1950s to 1980s owing to the population explosion and the increasing requirements for food (Wei et al., 2007; Bao et al., 2012). Though, in China, the manufacture and agricultural usage of most PCBs and OCPs were banned in 1974 and 1983, respectively (Wang et al., 2005), these organic chemicals can still be routinely detected in various environmental compartments (i.e., in air, water, soil, sediments, and organisms) (Adeleye et al., 2016; Li et al., 2017; Zhao et al., 2018). Due to the slow degradation of PCBs and OCPs, the residues and their catabolites would persist in the ecosystem for a long time (Yu et al., 2014; Wu et al., 2015). Therefore, these pollutants in environmental mediums could provide valuable records and represent potential ecological risks that should be seriously considered. However, currently, there are few available reports focusing on the levels and risk assessments of PCBs and OCPs in riverine bays.

Organochlorine pesticides and PCB residues tend to attach to suspended particulates and end up in the sediment by deposition and sedimentation because of their refractory and hydrophobic characteristics in the marine environment (Miglioranza et al., 2004; Zimmerman et al., 2004). Thus, sediments act as an important reservoir for OCP and PCB contaminations and as an efficient indicator for monitoring the pollution levels of the benthic system (Okay et al., 2009; Barhoumi et al., 2014; Yang et al., 2018). The absorbed organic pollutants can also be released and resuspended in seawater via biological and physicochemical processes, which can result in secondary pollution and can even threaten associated biota and ecosystems through bio-accumulation and bio-amplification (Gui et al., 2014; Tsygankov et al., 2015). Therefore, the investigation of PCBs and OCPs in surface sediments can provide a valuable record of contamination in an aquatic environment.

With the increasing demand for animal protein worldwide, the marine aquaculture industry in China has rapidly developed, and its aquatic products are acknowledged as high-quality protein (Cao et al., 2015). However, over-intensive aquaculture activity may lead to serious marine pollution due to the unreasonable use of pesticides and feeds (Primavera, 2006). Qingduizi Bay is a well-known and important pond culture zone and is among the few estuarine bays along the northern Yellow Sea. It receives runoff inputs from three rivers, making identification of the pollution sources difficult. Thus, understanding the sources and ecological impacts of xenobiotics in typical coastal areas, especially in dense mariculture areas, is crucial to protect and maintain habitat quality and ecosystem health (Barbier et al., 2011). Investigations of PCBs and OCPs in estuarine and costal environments have been conducted worldwide (Lebeuf and Nunes, 2005; Wang et al., 2010; Yuan et al., 2015; Montuori et al., 2016). However, studies focusing on the PCBs and OCPs in Qingduizi Bay are limited. The primary objectives of this study are to (i) perform a comparative study concerning the occurrence and distribution of PCBs and OCPs in the surface sediments of mariculture ponds and the adjacent outer areas in a bay; (ii) clarify whether the increasing aquaculture activities cause a fresh input of the pollutants; and (iii) assess the potential ecotoxicological risks of PCBs and OCPs in sediments for benthic organisms. The obtained data could provide important baseline information for environmental monitoring and could be helpful in policy-making on sustainable development in dense mariculture areas.

MATERIALS AND METHODS

Study Areas

Qingduizi Bay (39°41′59″–39°49′31″ N, 123°11′41″–123°26′06″ E) is an important estuarine bay in the northern Yellow Sea of China, receiving runoff inputs from the Inna, Huli and Diyin rivers, with an average flow of 7.9×10^8 m^3 yr^{-1}, and forms the present complex deltaic system, which covers a tidal flat area of 130 km^2 (Editorial Board of China Bay Survey, 1991). It is a semi-enclosed bay and is among the most developed bays for mariculture in China. It is exposed to stress from anthropogenic activity, including dockyards, sea farming along the coastline, and sewage discharge from pond culture (Wu et al., 2011). The main cultured species include *Fenneropenaeus chinensis*, *Rhopilema esculentum*, *Meretrix meretrix,* and *Apostichopus japonicus*. Sea reclamation had been carried out, with the expanded farming areas ranging from 5958 to 8356 hm^2 between 1990 and 2013, representing about 53% of the total area of Qingduizi Bay (Miao et al., 2014; Liu et al., 2018). The pond culture areas cover approximately 5384 hm^2 by 2013 (Yin, 2013). Substantial amounts of organic pollutants (e.g., residual feeds and pesticides) generated from aquaculture ponds are discharged into the seawater (Wang et al., 2019).

Field Sampling and Sample Preparation

The study area was divided into two parts: one is a mariculture pond (MP), which is occupied by a number of aquaculture ponds, and the other is the outer bay (OB), where no mariculture activities occur. Five and four sections perpendicular to the bay mouth were set in MP and OB, respectively (**Figure 1**). A total of 30 sampling sites (14 in MP and 16 in OB) were investigated in August 2012. For each site, the undisturbed surface sediments were gently collected using a stainless-steel grab sampler. The uppermost 5 cm of sediment was accurately collected and mixed, and then placed in pre-cleaned glass jars that had been prewashed with *n*-hexane and distilled water. The samples were initially stored in a freezer until delivery to the laboratory and kept at −20°C for further analysis. All samples were freeze-dried under conditions of −52°C and <20 Pa with a vacuum freeze dryer (FD-1C-50, Beijing Biocool Instrument, Co., Ltd., Beijing, China) for 36 h to a constant weight. Then, the samples were sieved through a 100-mesh sieve, homogenized, and stored at 4°C for further analysis.

Reagents and Standards

All the standards and chemicals used in the study were purchased from Chem Service (West Chester, PA, United States). The standard solutions for PCBs contain 27 homolog compositions, including PCB8, PCB18, PCB28, PCB52, PCB44, PCB66, PCB101, PCB77, PCB81, PCB123, PCB118, PCB114, PCB153, PCB105, PCB138, PCB126, PCB187, PCB128, PCB167, PCB156, PCB169, PCB180, PCB157, PCB170, PCB189, PCB195, and PCB206. The standard solutions for OCPs contain α-, β-, γ- and δ-HCH, heptachlor, heptachlor epoxide, aldrin, dieldrin, endrin, endrin aldehyde, *p,p′*-dichlorodiphenyltrichloroethane (*p,p′*-DDT), *p,p′*-dichlorodiphenyldichloroethane (*p,p′*-DDD), *p,p′*-dichlorodiphenyldichloroethylene (*p,p′*-DDE), methoxychlor, α-chlordane, γ-chlordane, endosulfan I, and endosulfan II. All standard solutions were diluted using appropriate volumes of isooctane.

Analysis of PCBs and OCPs

The extraction procedures for PCBs and OCPs in freeze-dried sediment samples were carried out using the 33 mL stainless-steel vessels of a Dionex ASE 200 accelerated solvent extractor system (Dionex GmbH, Idstein, Germany) following the description of Richter et al. (1996). In brief, exactly 5.0 g of single sample was homogenized with a 1.0 g mixture of 2:1 Florisil/Al_2O_3 and 10 μL of surrogate standards (^{13}C-PCB 28, ^{13}C-PCB 111, and ^{13}C-PCB178) in each vessel. Extraction was performed using hexane/dichloromethane in a 1:1 ratio under 100°C and 10.3 × 10^6 Pa. Two static cycles (each cycle maintained for 5 min) were performed for extraction. At the end, the extraction cell was flushed with solvent (60% of the cell volume) and purged with nitrogen (100 s). The fraction was evaporated to 2 mL using a rotary evaporator. Then, 1.0 g activated copper was added for the clean-up. The extracts were transferred to a chromatographic column packed with silica gel (4.0 g), alumina-N (6.0 g) and sodium sulfate (1.0 g). The column was eluted with 80 mL of *n*-hexane/dichloromethane (v:v, 3:2). The eluates were evaporated to 1.0 mL, then to 100.0 μL under a gentle stream of nitrogen at 50°C, and transferred for analysis. 10.0 μL of the internal standard (^{13}C-PCB141) solution was spiked before the instrumental analysis.

GC–MS/MS was used for PCB and OCP analysis. A 7890A GC (Agilent, United States) equipped with a 30 m × 0.25 mm × 0.25

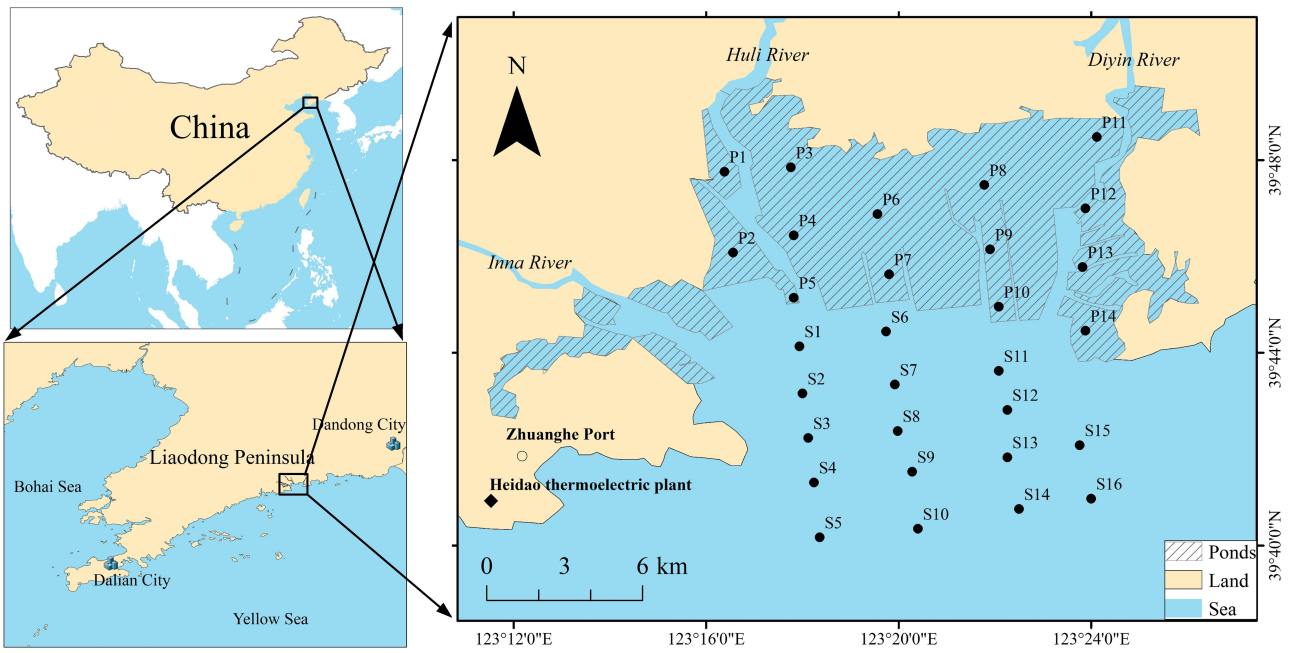


FIGURE 1 | Study area and sampling sites location in Qingduizi Bay.

μm HT8 column (SGE, United States) coupled to a 7000B MS (Agilent, United States) operated in EI + SRM was used. Injection was pulsed splitless at 280°C with helium as carrier gas at 1.0 mL/min. The GC temperature program was 60°C (1 min hold), then 40°C/min to 120°C, and finally 5°C/min to 300°C, held for 3 min. The concentrations of target elements were quantified by a six-point calibration curve and were corrected by surrogates.

Physio-Chemical Parameters of the Sediments

Six bulk physio-chemical parameters, including the percentage of total carbon (TC), total nitrogen (TN), total organic carbon (TOC), organic matter (OM), mud fractions (silt–clay) and ratios of TC and TN (C/N) of sediments, were estimated from the composite sub-sample in each site. For TOC, the samples were pretreated with 0.1 mol L^{-1} HCl solution for 18 h to remove inorganic carbon. Then the acidified sediments were rinsed with deionized water and freeze-dried before analysis. The sedimental TN, TC, and TOC were determined using a Vario Macro CHN element analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). C/N values were calculated using TN and TC ratios. OM was determined through loss on ignition (24 h at 450°C) according to the method described by Craft et al. (1991). The proportions of mud (silt–clay fractions, <0.63 μm) were determined by sieving the dried samples following the method described by Soares et al. (1999).

Quality Control and Quality Assurance

Procedure blanks, solvent blanks, spiked samples with standards, and duplicate samples were applied to analyze the cross contaminations and check the quality of chemical compounds. The losses involved during the analysis were also compensated by comparing surrogate standards. The target compounds were not detected in the method blanks. The average surrogate recoveries were 82 ± 9, 86 ± 12, and 92 ± 11% for ^{13}C-PCB 28, ^{13}C-PCB 111, and ^{13}C-PCB178, respectively. The limit of detection (LOD) of individual PCB and OCP compounds has been listed in **Supplementary Material**. The relative standard deviations of repeatability and reproducibility were < 5%, which showed the acceptable repeatability and reproducibility of the measurement.

Potential Risk Assessments

To evaluate the adverse ecological effects caused by PCBs and OCPs, two commonly used sediment quality guidelines (SQGs) were applied. These compared the concentrations of PCBs and OCPs, namely, the effects range-low (ERL) and effects range-median (ERM) value guidelines from the United States Environmental Protection Agency (Usepa, 1998) and the threshold effects level (TEL) and probable effects level (PEL) guidelines from the Canadian Council of Ministers of the Environment (Ccme, 2002). The ERL was derived from the low 10th percentile of the effect data for the organic contaminants, and the ERM was derived from the median of the effect data (Long et al., 1995, 1998). The ERL and ERM values separated the concentrations of particular chemicals into three ranges to represent adverse biological effects: "rarely," "occasionally," and "frequently observed." Both the TEL and PEL guidelines are based on the benthic community metrics and toxicity test results. The TEL was derived by calculating the geometric mean of the 15th percentile concentrations of the toxic effects dataset and the median of the no-effect dataset, while the PEL was calculated as the geometric mean of the 50th percentile of the effect dataset and the 85th percentile of the no-effect dataset (Long et al., 1998). The TEL and PEL values separated concentrations of specified chemicals into "no-effect range," "possible effect range," and "probable effect range."

Statistical Analysis

ArcGIS 10.0 was used to reflect the sampling locations and spatial distribution of the sedimental indicators, PCBs and OCPs across the study area. The Kriging interpolation method was applied to predict the unknown attribute values at certain sites and to describe the distribution pattern of six sedimental indicators. Pearson correlation analysis was conducted with R statistical software (R Core Team, 2016) using the "ggcorrplot" package to estimate the relationships between organic pollutants and sedimental indicators. Principal component analysis (PCA) using IBM SPSS Statistics 19.0, was applied to the correlation matrix with Varimax rotation and Kaiser Normalization for POP concentrations in the sediments to identify the possible sources of organic pollutants.

RESULTS AND DISCUSSION

Spatial Variation of Sedimental Indicators

Due to the complex nature of organic matter in aquatic sediments, sedimental physio-chemical parameters, such as TOC, particle size fraction and C_{org}/N, are effective and widely used for determining the different signatures and transport routes of OM sources (e.g., Bröder et al., 2016; Koziorowska et al., 2016). Mud contents (silt–clay; <0.063 mm) showed a decreased trend from the nearshore to the offshore, with percentages ranging from 0.25 to 0.86% (**Figure 2E**). TOC contents ranged between 0.20 and 1.09% with a median value of 0.59%. High levels generally occurred in the mouths of the Huli River and Diyin River (**Figure 2A**). Higher levels of TOC and mud contents are generally detected in the mouths of rivers, indicating fresh terrigenous inputs linked to increased development (via land reclamation and construction activities) in this catchment. OMs exhibited an increased trend from the nearshore toward the offshore, with percentages ranging from 1.97 to 7.87% (**Figure 2B**). In contrast to OM, TC, TN and C_{org}/N of the sediments showed similar distribution trends, with high levels measured in the southeast of OB. The levels varied from 0.04 to 4.72% (with an average value of 1.27%), from 0.17 to 0.57% (with an average value of 0.28%), and from 0.90 to 5.57% (with an average value of 2.31%) for TC, TN, and C_{org}/N, respectively (**Figures 2C–F**). The C_{org}/N ratio generally provided valuable information concerning the origin of the OM. Typical Corg/N values derived from fresh marine biogenic OM range from 4 and

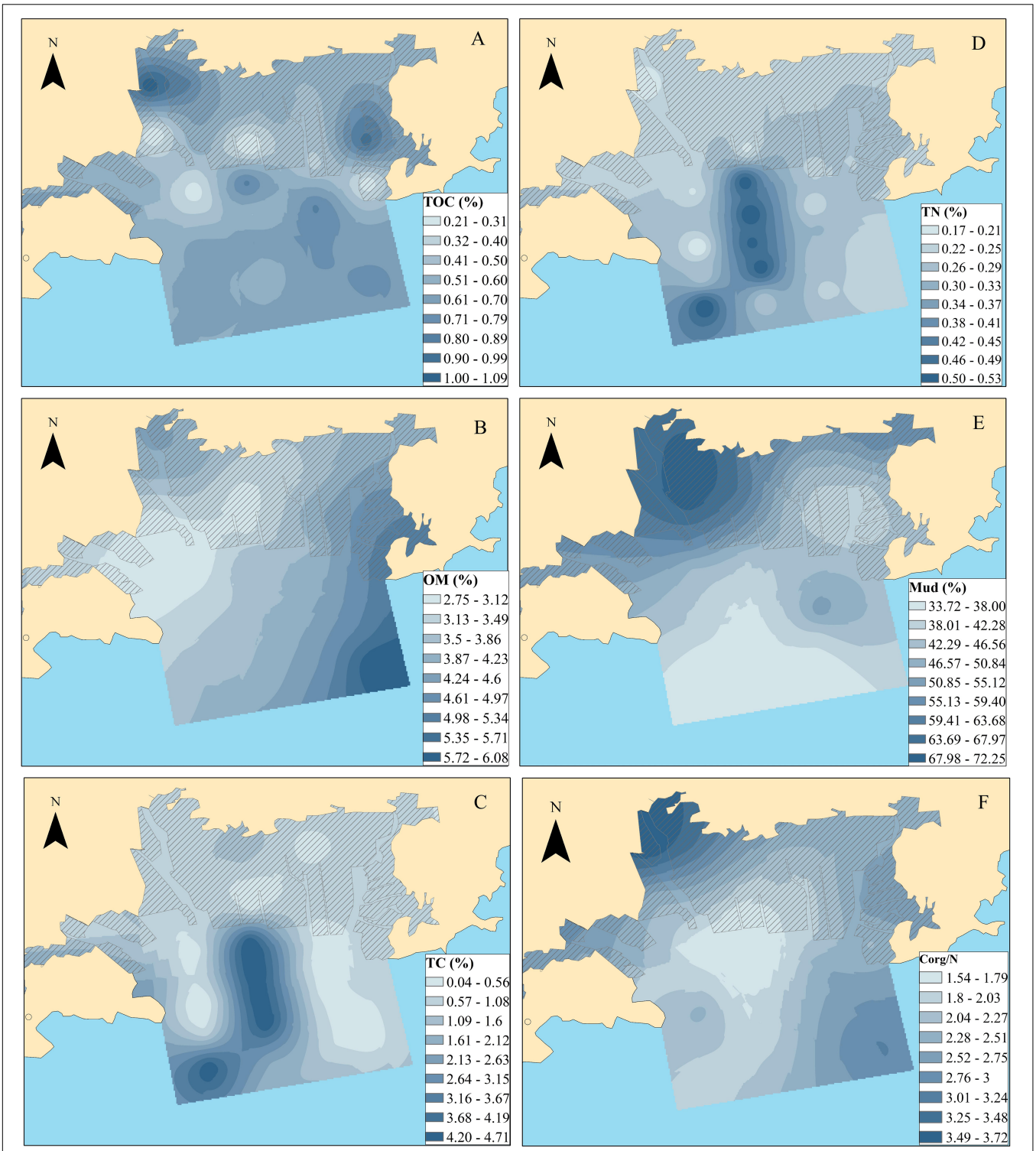


FIGURE 2 | Bulk physio-chemical parameters in the sediments of Qingduizi Bay [**(A)** TOC; **(B)** OM; **(C)** TC; **(D)** TN; **(E)** mud fractions (silt + clay); **(F)** C_{org}/N].

10, whereas OM from terrestrial sources has a ratio of higher than 20 due to the content of peptide material (Meyers, 1994). The low C_{org}/N values in the present study suggest a greater marine contribution than the terrestrial source. However, one should be cautious with such an interpretation since a preferential utilization of C and bacterial immobilization of N must be considered. Diagenetic processes and complicated transport routes should also be considered because of the special

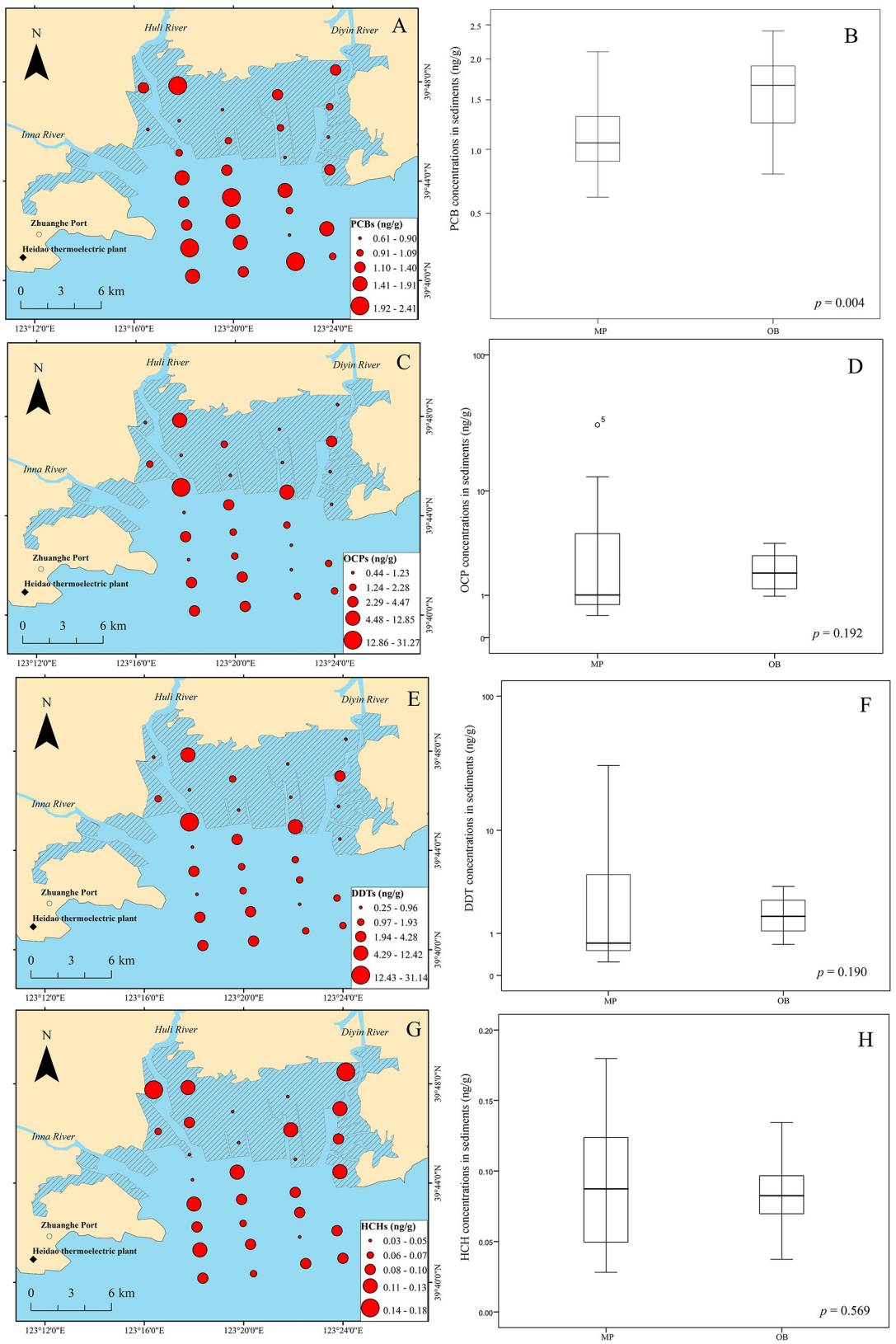


FIGURE 3 | Spatial distribution of PCBs **(A)**, OCPs **(C)**, DDTs **(E)** and HCHs **(G)**, and boxplots for PCBs **(B)**, OCPs **(D)**, DDTs **(F)** and HCHs **(H)** in the surface sediments of Qingduizi Bay.

geographical environment (riverine bay) and intense human activities (mariculture).

Occurrence of PCBs

The spatial distribution and levels of ΣPCBs (the sum of the 27 congeners of PCBs) in the sediments of Qingduizi Bay are shown in **Figure 3A**. PCBs were detected in all the sediment samples, suggesting ubiquitous contamination of these compounds in the aquatic environment of Qingduizi Bay. The residual values of Σ27PCBs ranged from 0.61 to 2.10 ng g^{-1} dw (mean: 1.11 ng g^{-1} dw) in MP and from 0.79 to 2.41 ng g^{-1} dw (mean: 1.57 ng g^{-1} dw) in OB (**Figure 3B**). Higher levels of PCBs were detected in OB ($p = 0.004$).

Previous studies have reported the PCB levels in surface sediments from other bays and estuaries around the world (**Table 1**). The PCB levels in Qingduizi Bay were generally far below those of more developed industrial estuaries and bays in China, such as Shantou Bay (Shi et al., 2016), three typical bays (Yueqing Bay, Xiangshan Bay, and Sanmen Bay) in the east China Sea (Yang et al., 2011), the Yangtze Estuary (Gao et al., 2013) and the Shuangtaizi Estuary (Yuan et al., 2015); and in other areas of the world, such as Casco Bay in the United States (Kennicutt et al., 1994), Rhone Prodelta in France (Tolosa et al., 1995) and Bizerte lagoon in Tunisia (Barhoumi et al., 2014), yet comparable to the PCB levels in the Daliao Estuary (China) (Men et al., 2014) and Guaratuba Bay (Brazil) (Combi et al., 2013).

The homolog profiles of PCB congeners in Qingduizi Bay are presented in **Figure 4**. Overall, MP and OB showed similar PCB congener compositions in the surface sediment. Light chlorinated congeners, such as tri-PCB and tetra-PCB, were the most abundant congeners in all sampling sites, accounting for 61.5–81.4% (mean: 71.6%) of the total PCBs. As semi-volatile persistent pollutants, the solubility and volatility of PCBs tend to decrease as the number of chlorine atoms increases. Light chlorinated congeners easily volatilize in air and accumulate in the sediment by atmospheric deposition, whereas highly chlorinated congeners are poorly diffused (Carroll et al., 1994). Consequently, high levels of light PCBs were detected in the sediments. The homolog profiles in the present study were consistent with those from other studies of the coastal regions (Liu et al., 2003; Lebeuf and Nunes, 2005; Barhoumi et al., 2014).

Occurrence of OCPs

The distribution of OCPs generally shows a different pattern to that of PCBs in Qingduizi Bay (**Figure 3C**). The total concentrations of the OCPs vary from 0.44 to 31.27 ng g^{-1} dw (mean: 3.38 ng g^{-1} dw) with an average detection frequency of 93.3%. No significant difference was found in the concentrations of OCPs between OB and MP ($p = 0.192$) (**Figure 3D**). The proportions of OCP compositions in sediment samples greatly varied: heptachlor, heptachlor epoxide, and γ-chlordane were absent; and α-chlordane, methoxychlor, endosulfan II, cyclodiene pesticides, and their metabolites, including aldrin, dieldrin, endrin and endrin aldehyde, were very low or negligible. Nevertheless, DDTs and their metabolites DDXs (e.g., *p,p′*-DDD, *p,p′*-DDE) collectively contributed 54.1–99.6%, and HCHs (α-HCH, β-HCH, γ-HCH, and δ-HCH) collectively contributed 0.1–25.0% of the total OCPs. As abundant and commonly used OCPs, DDTs, and HCHs were detected in all surface sediments of Qingduizi Bay.

The concentrations of DDTs varied from 0.25 to 31.14 ng g^{-1} dw (mean: 3.13 ng g^{-1} dw), and the highest level of DDTs was found at site P5 located in the western MP near the mouth of the Inna River (**Figure 3E**). Overall, the DDT levels showed no significant differences between MP and OB ($p = 0.190$) (**Figure 3F**). Among the DDT compounds, *p,p′*-DDT was the predominant isomer (0.12–29.56 ng g^{-1} dw), accounting for 33.0–94.6% of all DDTs, whereas *p,p′*-DDD

TABLE 1 | Levels of PCBs and OCPs in surface sediments from coastal area worldwide (ng g^{-1} dw).

Location	Year	PCBs	PCB congener numbers	DDTs	HCHs	References
Qingduizi Bay (China)	2012	0.61–2.41[a]	27	0.25–31.14	<0.03–0.18	Present study
Daliao Estuary (China)	2005 and 2007	0.83–7.29[b]	41	0.5–2.81	1.86–21.48	Wang et al., 2007; Men et al., 2014
Yueqing Bay, Xiangshan Bay and Sanmen Bay (China)	2006	9.33–19.60[c]	20	NA	NA	Yang et al., 2011
Yangtze Estuary (China)	2007 and 2010–2011	1.86–148.2[d]	28	n.d.–0.57	0.9–30.4	Liu et al., 2008; Gao et al., 2013
Shantou Bay (China)	2011	0.54–26.9[e]	26	0.14–6.46	0.35–4.54	Shi et al., 2016
Shuangtaizi Estuary (China)	2013	1.83–36.68[f]	28	0.02–0.47	0.07–7.25	Yuan et al., 2015
Rhone Prodelta (France)	1990	38–230[g]	9	73–704	NA	Tolosa et al., 1995
Casco Bay (United States)	1991	0.4–485[h]	20	<0.2–20	<0.07–0.48	Kennicutt et al., 1994
Guaratuba Bay (Brazil)	2010	n.d.–6.06[i]	46	n.d.–0.49	NA	Combi et al., 2013
Bizerte lagoon (Tunisia)	2011	0.8–14.6[j]	12	0.3–11.5	0.6–2.5	Barhoumi et al., 2014

[a]Sum of PCB congeners 8, 18, 28, 52, 44, 66, 101, 77, 81, 123, 118, 114, 153, 105, 138, 126, 187, 128, 167, 156, 169, 180, 157, 170, 189, 195, 206. [b]Sum of PCB congeners 17, 18, 28, 31, 33, 44, 49, 52, 70, 74, 82, 87, 95, 99, 101, 105, 110, 118, 128, 132, 138, 149, 151, 153, 158, 169, 170, 171, 177, 180, 183, 187, 191, 194, 195, 199/201, 205, 206, 208, 209. [c]Sum of PCB congeners 8, 18, 28, 44, 53, 66, 77, 101, 105, 118, 126, 128, 138, 153, 170, 180, 187, 195, 206, 209. [d]Sum of PCB congeners 8, 18, 28, 44, 52, 66, 77, 81, 101, 105, 114, 118, 123, 126, 128, 138, 153, 156, 157, 169, 170, 179, 180, 183, 187, 189, 206, 209. [e]Sum of PCB congeners 8, 18, 28, 29, 44, 50, 52, 66, 77, 87, 101, 104, 105, 118, 126, 128, 138, 153, 154, 170, 180, 187, 188, 195, 200, 206. [f]Sum of PCB congeners 8, 18, 28, 44, 52, 66, 77, 81, 101, 105, 114, 118, 123, 126, 128, 138, 153, 156, 157, 167, 169, 170, 180, 187, 189, 195, 206, 209. [g]Sum of PCB congeners 52, 101, 118, 128, 138, 153, 170, 187, 180. [h]Twenty PCB congeners without specific congeners. [i]Sum of PCB congeners 8, 18, 31, 28, 33, 52, 49, 44, 74, 70, 66/95, 56/60, 101, 99, 97, 81/87, 110/77, 151, 123/149, 118, 114, 153, 132, 105, 141, 138, 158, 126, 187, 183, 128, 167, 174, 177, 156, 157, 180, 169, 170, 201, 203, 189, 195, 194, 206, 209. [j]Sum of PCB congeners 28, 31, 52, 44, 101, 149, 118, 153, 138, 180, 194, 209.

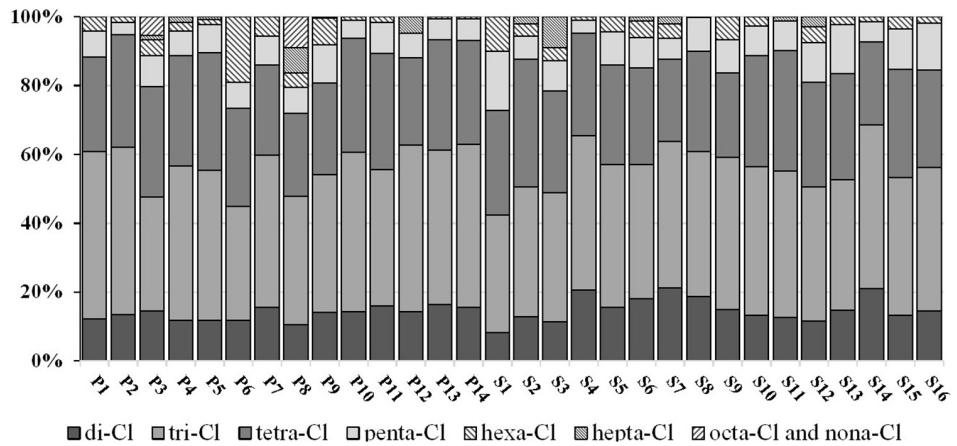


FIGURE 4 | PCB homolog compositions in the surface sediments of Qingduizi Bay.

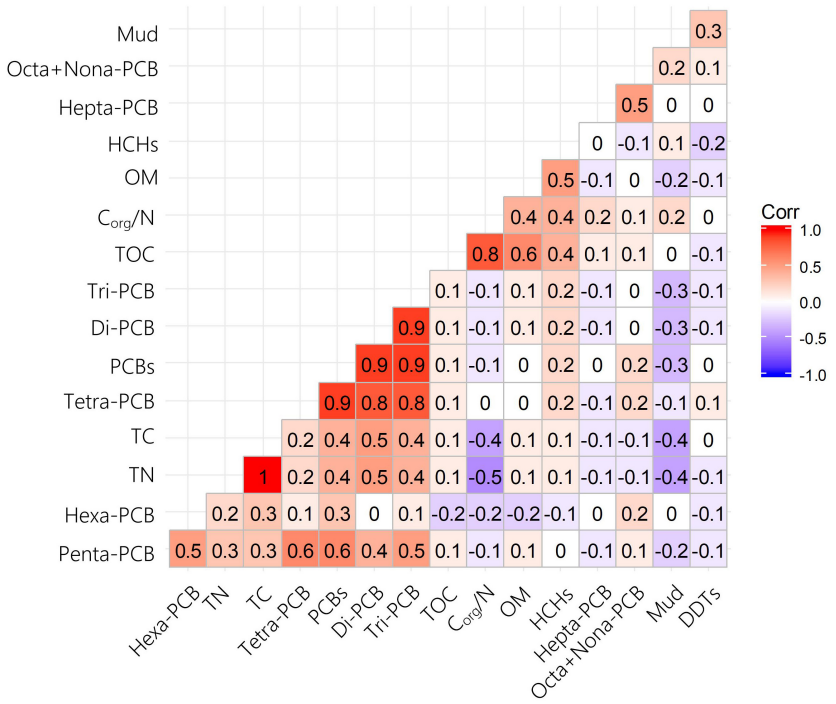


FIGURE 5 | Pearson correlations of PCB homolog compositions and physicochemical characteristics of sediments in Qingduizi Bay.

(0.06–1.91 ng g^{-1} dw) and *p,p′*-DDE (0.06–0.22 ng g^{-1} dw) were present in minimal amounts. When compared with other important bays and estuaries around the world, DDT concentrations in the surface sediments of Qingduizi Bay showed a range of variability. The DDT levels in this study were greater than those of most other Chinese bays and estuaries (Shantou Bay, Shuangtaizi Bay, the Daliao Estuary, the Yangtze Estuary, and other bays in the Eastern Sea) and Guaratuba Bay of Brazil, but were similar to those measured in Bizerte lagoon (Tunisia) and Casco Bay (United States). However, the ranges were more limited than those detected in developed coastal areas, such as the Rhone Prodelta in France (**Table 1**).

The levels of HCHs showed lower variability among sites (p = 0.569) (**Figure 3H**). Previous studies reported that 11,400 t of lindane (γ-HCH) were manufactured and used between 1991 and 2000, even after the prohibition on the usage and production of technical HCHs in 1983 (Li et al., 2001). The highest level of HCHs was found at site P1, located in the northwestern MP near the mouth of the Huli River with a concentration of 3.13 ng g^{-1} dw (**Figure 3G**). High levels of HCHs with elevated β-HCH fraction were observed in the mouths of the Huli River

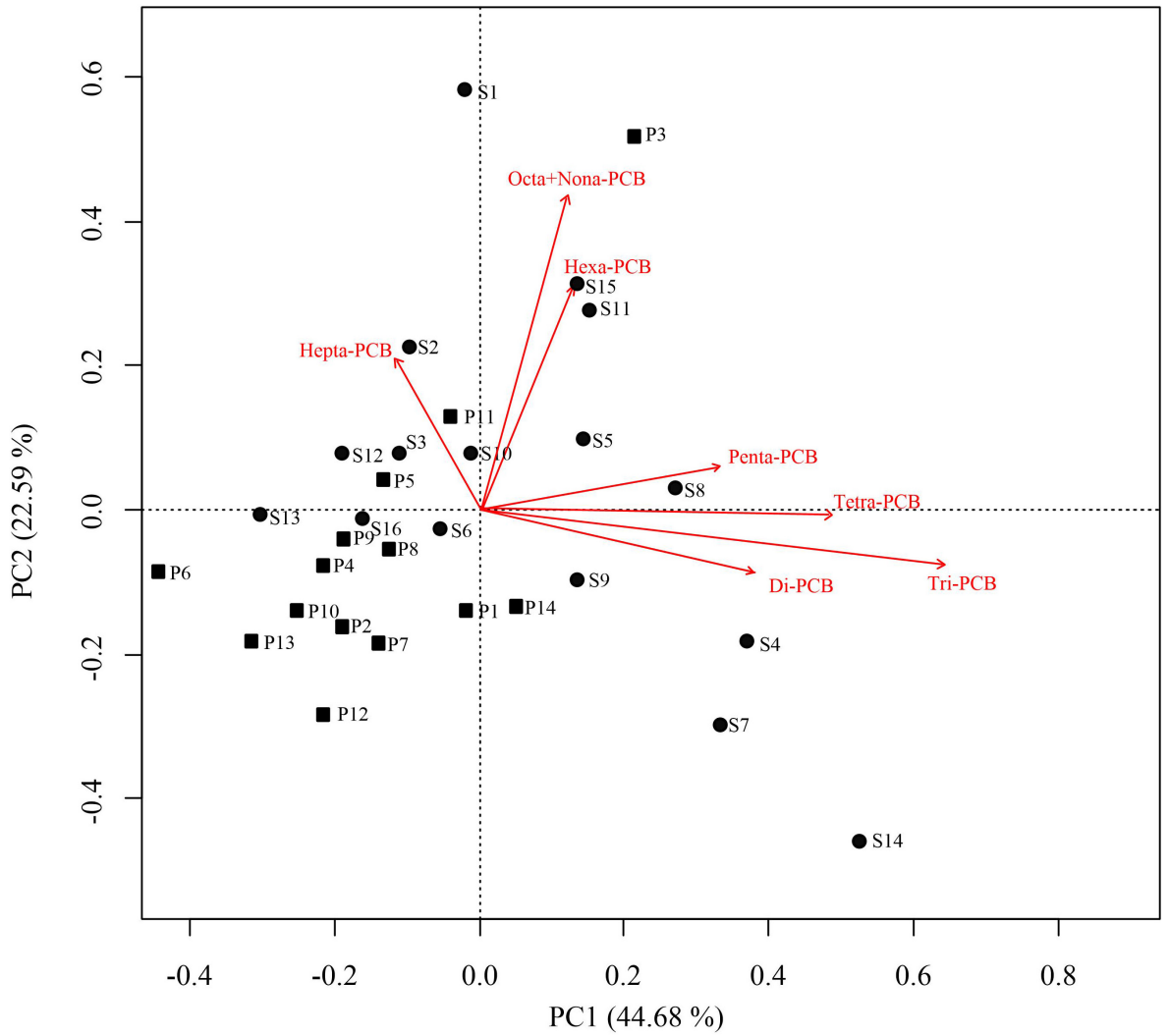


FIGURE 6 | PCA of PCB compositions in the surface sediments of Qingduizi Bay.

(P1) and Diyin River (P11 and P12), indicating the input of technical HCH residues to rivers from local agriculture runoff and aquaculture drug usage. The HCH levels in the surface sediment at Qingduizi Bay were similar to those at Casco Bay (United States) but were much lower than those in other estuaries and coastal sediments (**Table 1**).

Qualitative Analysis for PCBs Sources

The sections located in the western part of the bay showed higher levels of PCBs than that located in the eastern part of the bay, suggesting a possible presence of point sources in the western coast of Qingduizi Bay. The distribution patterns of PCBs could be based on various factors, such as distance from the source(s), hydrodynamic factors and the origin of the OM or clay swelling (Zhou et al., 2012). In the present study, the main point sources of the PCBs could be the Zhuanghe Port and Zhuanghe power plant, which are located on the westernmost shore of Qingduizi Bay. Inputs can probably be attributed to the exchange fluids in the transformers and capacitors from the power plant, as well as the paint additives of ships from the port. Nevertheless, the concentration of PCBs in P3 of MP (2.10 ng g^{-1} dw) was abnormally higher than those from other MP sites. P3 is speculated to be located near the mouth of Huli River, suggesting direct PCB inputs from the river runoff.

Theoretically, the distributions and levels of hydrophobic pollutants are influenced by the physico-chemical characteristics of sediments. Therefore, it is feasible to analyze the relationship between pollutants and sedimental characteristics to identify the potential sources and occurrences of pollution in estuaries or bays (Wu et al., 2015; Yang et al., 2015). PCBs are mainly derived from waste incineration, accidental fires, equipment/utility usage (e.g., old transformers and capacitors), and atmospheric deposition (Alonso-Hernandez et al., 2014). In the present study, Pearson's correlation coefficients were used to identify correlations among six sedimental physicochemical parameters and PCB homolog compositions (**Figure 5**). The results showed that all

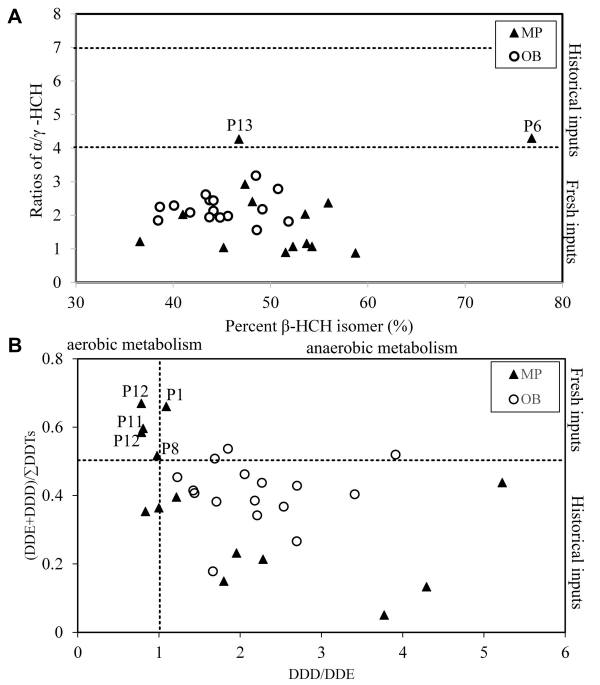


FIGURE 7 | Cross plots for the isomeric ratios of: **(A)** β-HCH percentage vs. **(B)** α/γ-HCH and DDD/DDE vs. (DDE + DDD)/ΣDDTs in different samples from Qingduizi Bay.

PCB compounds had weak correlations with physicochemical parameters, indicating that PCBs are more likely to come from atmospheric deposition than surface runoff.

Principal component analysis was also applied to analyze the potential sources of PCBs. Kaiser–Meyer–Olkin and Bartlett tests yielded 0.63 and 122.66 (df = 105, $p < 0.01$), respectively, indicating that this method is effective for source apportionment. Two principal components (PCs) were successfully extracted, which revealed the potential sources of PCBs in Qingduizi Bay and explained 67.27% of the total variance (**Figure 6**). PC1 accounted for 44.68% of the total variance and was mostly composed of light chlorinated congeners, including Di-PCB, Tri-PCB, Tetra-PCB and Penta-PCB, representing a non-point source deposition (e.g., atmospheric deposition and surface runoff). By contrast, PC2 accounted for 22.59%, and was composed of chlorinated congeners, such as Hepta-PCB, Octa-PCB, Nona-PCB and Hexa-PCB, indicating the point source deposition. The results were consistent with the conclusion of the Pearson's correlation analysis. Due to their characteristics of volatility, light chlorinated congeners volatilize easily to the atmosphere after being released. They are effectively adsorbed by suspended particulate matter and are finally deposited in sediments (Offenberg and Baker, 2002). Therefore, the conclusions of correlation analysis and PCA added credence to the view that light chlorinated congeners are derived from atmospheric deposition. As for highly chlorinated congeners, the existence of a single major source in the bay is possibly related to the point source (e.g., intense use of heat transfer and hydraulic fluids and solvent extenders in ship painting) (Hong et al., 2005; Erickson and Kaley, 2011).

Qualitative Analysis for DDT and HCH Sources

Dichlorodiphenyltrichloroethanes and technical HCH applications for agriculture purposes have been banned since 1983, whereas DDT continued to be used as a synthetic intermediate for the production of dicofol, which was not banned in China until 2018 (Guo et al., 2009; Grung et al., 2015; Yu et al., 2019). Several molecular ratios of selected OCP compounds, such as the abundance ratio of α-HCH/γ-HCH, (DDE + DDD)/DDT and DDE/DDD, were employed to understand their sources and fate in the environment.

Technical HCH contains 60–70% of α-HCH, 5–12% of β-HCH, 10–12% of γ-HCH and 6–10% of δ-HCH (Iwata et al., 1993), with a ratio of α/γ-HCH between 4 and 7 for technical compounds and nearly 0 for pure lindane, and the ratio would increase during the degradation process in the environment (Syed et al., 2014). The ratios of α/γ-HCH in the sediment of Qingduizi Bay varied from 0.88 to 4.29, while the ratios in 93% of sampling sites were below 4, implying that HCH residuals in sediments are mainly derived from the recent lindane usage and historical HCH residuals (**Figure 7A**). The percentage of β-HCH was used to identify the source of historical usage of HCHs, because the beta isomers are relatively resistant to microbial degradation (Law et al., 2001). The average compositions of β-HCH measured in the sediments ranged from 37 to 77% of the total HCHs, with an average of 48%, implying the mixture contained new and historical inputs of HCHs.

Generally, p,p'-DDT will be dechlorinated into p,p'-DDE under aerobic conditions and into p,p'-DDD under anaerobic conditions by microbial degradation (Hitch and Day, 1992). DDT sources in the sediments of Qingduizi Bay were identified by plotting p,p'-DDD/DDE against p,p'-(DDE + DDD)/ΣDDT (**Figure 7B**). Generally, the ratio of p,p'-(DDE + DDD)/ΣDDT < 0.5 indicates a fresh input because most DDTs have not started degrading yet (Hitch and Day, 1992). In the present study, 73% of the sampling sites were characterized by p,p'-(DDE + DDD)/ΣDDT ratios of <0.5, reflecting that new inputs of DDTs were predominant. Specifically, the ratios in several MP sites were less than 0.2, indicating a recent usage of DDTs in aquaculture. However, the ratios in five sites (P1, P4, P8, P9, and P11) were higher than 0.5, implying historical terrestrial usage of DDTs since these sites were located near the mouths of rivers. The ratios of p,p'-DDD/DDE in all sites of OB and most sites of MP were greater than 1, indicating the occurrence of a strong anaerobic transformation of DDT in the sediments of Qingduizi Bay.

Pesticide usage in agriculture and aquaculture is commonly known as one of the primary contributors to persistent halogenated compounds (PHCs) (Yu et al., 2011). Thus, PHCs that originate from mariculture should not be ignored, especially in intensive pond culture areas. The marine pollution derived from the rapid development of mariculture has elicited worldwide concern. Previous studies have frequently detected

TABLE 2 | Concentrations of PCB and OCP in surface sediments from Qingduizi Bay (ng g^{-1} dw) and toxicity guidelines.

Compound	Sediment quality guidelines				Range (mean)	[a]*n* of <ERL	[a]*n* of ERL–ERM	[a]*n* of >ERM	[a]*n* of <TEL	[a]*n* of TEL–PEL	[a]*n* of >PEL
	[b]ERL	[c]ERM	[d]TEL	[e]PEL							
ΣPCBs	50	400	21.5	189	0.61–2.41 (1.36)	30	0	0	30	0	0
ΣDDTs	1.58	46.1	3.89	51.7	0.25–31.14 (3.13)	18	12	0	26	4	0
p,p′-DDE	2.2	27	2.07	374	0.09–1.98 (0.22)	30	0	0	30	0	0
p,p′-DDD	2	20	1.22	7.81	0.06–1.91 (0.47)	30	0	0	28	2	0
p,p′-DDT	1	7	1.19	4.77	0.12–29.56 (2.45)	16	11	3	17	10	3

[a]*n, number of sites;* [b]*ERL, effects low range;* [c]*ERM, effects median range;* [d]*TEL, threshold effect level;* [e]*PEL, probable effect level.*

POPs, especially DDTs, in fishery feed (e.g., compound feed and trash fish) (Hites et al., 2004; Minh et al., 2006). The total areas of marine pond culture have been found to reach approximately 265,513 hectares, and more than 1.2×10^5 metric tons of unused feed were directly discharged into the sea without treatment (Cao et al., 2007). Therefore, pond culture activities could be a main contributor of DDTs in the coastal environment and organisms, as further confirmed by Yu et al. (2011).

Risk Assessment of PCBs and OCPs in the Sediment of Qingduizi Bay

Exogenic organic pollutants tend to accumulate in fatty tissue and are toxic to aquatic organisms because they are lipophilic (Zhu et al., 2015). The evaluation of potential ecotoxicological risks of POPs in estuarine and coastal areas has raised substantial concerns (Tolosa et al., 1995; Yuan et al., 2017). Ecological risk assessment is a useful tool to analyze and evaluate the adverse ecological effects caused by environmental contaminants. However, no specific guidelines are currently available for POPs in sediments in China. Based on the SQGs established by the United States Environmental Protection Agency (Usepa, 1998) and Canada (Ccme, 2002), the potential risks of PCBs and OCPs in the sediments from Qingduizi Bay were assessed (**Table 2**).

The present data and the guidelines were compared. The concentrations of PCBs and *p,p′*-DDE were all below the ERL and TEL values, suggesting that adverse biological effects would rarely occur for those compounds. As for *p,p′*-DDD, its concentrations in most sites (93.3% of total sites) were below TEL, indicating rather low ecotoxicity to aquatic organisms. Among all organic pollutants, *p,p′*-DDT and ΣDDTs were the most serious pollutants, and their levels in site 11 and site 12 respectively were above the ERL values. Even the levels of *p,p′*-DDT in three samples of MP were higher than ERM and PEL values, suggesting that adverse effects of *p,p′*-DDT and ΣDDTs were expected to occur in Qingduizi Bay.

CONCLUSION

Comprehensive surveys and experimental analyses of PCBs and OCPs in the sediments of Qingduizi Bay, a typical developing pond culture bay located in the northernmost part of the Yellow Sea of China, were conducted in this study. The results showed significant occurrences and levels of PCBs between MP and OB, whereas there were no significant differences of OCPs between MP and OB. Overall, PCBs and OCPs in the sediments have low to moderate levels without consideration of HCHs and DDTs. The inputs of PCBs could be attributed to the extensive historical application in adjacent areas, such as power plants and ship ports, whereas HCHs, which could be preserved in agricultural soils released via major river runoffs into the sea, could result mainly from historical applications. Nevertheless, DDTs showed recent usage and fresh inputs from aquaculture activities due to the high concentrations of *p,p′*-DDT in MP. Intensive mariculture activities could influence the levels and distributions of organic contaminants, especially for compounds contained in feed and pesticides. In addition, concerns should be raised regarding DDTs because the levels of several sites were above the SQGs and may cause ecotoxicological risks to marine organisms.

AUTHOR CONTRIBUTIONS

XlY, XtY, and WH conceptualized and executed the project. AZ, CG, and HZ collected the sediment samples. XL, XlY, MS, and LQ performed the experiments and conducted the laboratory analyses. XlY, LW, and GB performed the statistical analysis, interpretation of data, and the manuscript writing. XtY, WH, and GB edited and critically reviewed the final version of the manuscript which all the authors approved before submission.

FUNDING

This study was funded by the National Natural Science Foundation of China (Grant No. 41106115) and National Marine Public Welfare Research Projects (Grant Nos. 201305043 and 201105006).

ACKNOWLEDGMENTS

The authors express their gratitude to all the volunteers who participated in the field and laboratory work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2019.00602/full#supplementary-material

REFERENCES

Adeleye, A. O., Jin, H., Di, Y., Li, D., Chen, J., and Ye, Y. (2016). Distribution and ecological risk of organic pollutants in the sediments and seafood of Yangtze estuary and hangzhou bay. East China Sea. *Sci. Total Environ.* 541, 1540–1548. doi: 10.1016/j.scitotenv.2015.09.124

Alonso-Hernandez, C., Mesa-Albernas, M., and Tolosa, I. (2014). Organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) in sediments from the gulf of batabanó. *Cuba. Chemosphere* 94, 36–41. doi: 10.1016/j.marpolbul.2012.09.008

Bao, L.-J., Maruya, K. A., Snyder, S. A., and Zeng, E. Y. (2012). China's water pollution by persistent organic pollutants. *Environ. Pollut.* 163, 100–108. doi: 10.1016/j.envpol.2011.12.022

Barbier, E. B., Hacker, S. D., Kennedy, C., Koch, E. W., Stier, A. C., and Silliman, B. R. (2011). The value of estuarine and coastal ecosystem services. *Ecol. Monogr.* 81, 169–193.

Barhoumi, B., LeMenach, K., Dévier, M.-H., Hammami, B., Ameur, W. B., Hassine, S. B., et al. (2014). Distribution and ecological risk of polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) in surface sediments from the Bizerte lagoon, Tunisia. *Environ. Sci. Pollut. Res.* 21, 6290–6302. doi: 10.1007/s11356-013-1709-7

Bröder, L., Tesi, T., Salvadó, J. A., Semiletov, I. P., Dudarev, O. V., and Gustafsson, Ö (2016). Fate of terrigenous organic matter across the laptev sea from the mouth of the lena river to the deep sea of the arctic interior. *Biogeosciences* 13, 5003–5019. doi: 10.5194/bg-13-5003-2016

Cao, L., Naylor, R., Henriksson, P., Leadbitter, D., Metian, M., Troell, M., et al. (2015). China's aquaculture and the world's wild fisheries. *Science* 347, 133–135.

Cao, L., Wang, W., Yang, Y., Yang, C., Yuan, Z., Xiong, S., et al. (2007). Environmental impact of aquaculture and countermeasures to aquaculture pollution in China. *Environ. Sci. Pollut. Res.* 14, 452–462. doi: 10.1065/espr2007.05.426

Carroll, K. M., Harkness, M. R., Bracco, A. A., and Balcarcel, R. R. (1994). Application of a permeant/polymer diffusional model to the desorption of polychlorinated biphenyls from Hudson River sediments. *Environ. Sci. Technol.* 28, 253–258. doi: 10.1021/es00051a011

Ccme. (2002). *Canadian Sediment Quality Guidelines for the Protection of Aquatic Life*. Winnipeg: CCME.

Combi, T., Taniguchi, S., Figueira, R. C. L., Mahiques, M. M. D., and Martins, C. C. (2013). Spatial distribution and historical input of polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) in sediments from a subtropical estuary (Guaratuba Bay. SW Atlantic). *Mar. Pollut. Bull.* 70, 247–252. doi: 10.1016/j.marpolbul.2013.02.022

Craft, C., Seneca, E., and Broome, S. (1991). Loss on ignition and kjeldahl digestion for estimating organic carbon and total nitrogen in estuarine marsh soils: calibration with dry combustion. *Estuaries* 14, 175–179.

Editorial Board of China Bay Survey. (1991). *Survey of China Bays*. Beijing: China Ocean Press.

Erickson, M. D., and Kaley, R. G. (2011). Applications of polychlorinated biphenyls. *Environ. Sci. Pollut. Res.* 18, 135–151.

Gao, S., Chen, J., Shen, Z., Liu, H., and Chen, Y. (2013). Seasonal and spatial distributions and possible sources of polychlorinated biphenyls in surface sediments of Yangtze Estuary. *China. Chemosphere* 91, 809–816. doi: 10.1016/j.chemosphere.2013.01.085

Grung, M., Lin, Y., Zhang, H., Steen, A. O., Huang, J., Zhang, G., et al. (2015). Pesticide levels and environmental risk in aquatic environments in China—A review. *Environ. Int.* 81, 87–97. doi: 10.1016/j.envint.2015.04.013

Gui, D., Yu, R., He, X., Tu, Q., Chen, L., and Wu, Y. (2014). Bioaccumulation and biomagnification of persistent organic pollutants in indo-pacific humpback dolphins (Sousa chinensis) from the Pearl River Estuary. *China. Chemosphere* 114, 106–113. doi: 10.1016/j.chemosphere.2014.04.028

Guo, Y., Yu, H.-Y., and Zeng, E. Y. (2009). Occurrence, source diagnosis, and biological effect assessment of DDT and its metabolites in various environmental compartments of the Pearl River Delta. South China: a review. *Environ. Pollut.* 157, 1753–1763. doi: 10.1016/j.envpol.2008.12.026

Hitch, R. K., and Day, H. R. (1992). Unusual persistence of DDT in some western USA soils. *Bull. Environ. Contam. Toxicol.* 48, 259–264.

Hites, R. A., Foran, J. A., Carpenter, D. O., Hamilton, M. C., Knuth, B. A., and Schwager, S. J. (2004). Global assessment of organic contaminants in farmed salmon. *Science* 303, 226–229. doi: 10.1126/science.1091447

Hong, S. H., Yim, U. H., Shim, W. J., and Oh, J. R. (2005). Congener-specific survey for polychlorinated biphenlys in sediments of industrialized bays in Korea: regional characteristics and pollution sources. *Environ. Sci. Technol.* 39, 7380–7388. doi: 10.1021/es050397c

Ishikawa, Y., Noma, Y., Mori, Y., and Sakai, S. I (2007). Congener profiles of PCB and a proposed new set of indicator congeners. *Chemosphere* 67, 1838–1851. doi: 10.1016/j.chemosphere.2006.05.075

Islam, M. S., and Tanaka, M. (2004). Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. *Mar. Pollut. Bull.* 48, 624–649. doi: 10.1016/j.marpolbul.2003.12.004

Iwata, H., Tanabe, S., Sakai, N., and Tatsukawa, R. (1993). Distribution of persistent organochlorines in the oceanic air and surface seawater and the role of ocean on their global transport and fate. *Environ. Sci. Technol.* 27, 1080–1098. doi: 10.1021/es00043a007

Kennicutt, M. C., Wade, T. L., Presley, B. J., Requejo, A. G., Brooks, J. M., and Denoux, G. J. (1994). Sediment contaminants in casco bay, maine: inventories, sources, and potential for biological impact. *Environ. Sci. Technol.* 28, 1–15. doi: 10.1021/es00050a003

Koziorowska, K., Kuliński, K., and Pempkowiak, J. (2016). Sedimentary organic matter in two Spitsbergen fjords: terrestrial and marine contributions based on carbon and nitrogen contents and stable isotopes composition. *Cont. Shelf Res.* 113, 38–46. doi: 10.1016/j.csr.2015.11.010

Law, S. A., Diamond, M. L., Helm, P. A., Jantunen, L. M., and Alaee, M. (2001). Factors affecting the occurrence and enantiomeric degradation of hexachlorocyclohexane isomers in northern and temperate aquatic systems. *Environ. Toxicol. Chemis. Int. J.* 20, 2690–2698. doi: 10.1002/etc.5620201206

Lebeuf, M., and Nunes, T. (2005). PCBs and OCPs in sediment cores from the lower St. lawrence estuary, canada: evidence of fluvial inputs and time lag in delivery to coring sites. *Environ. Sci. Technol.* 39, 1470–1478. doi: 10.1021/es049051c

Li, Y., Cai, D., Shan, Z., and Zhu, Z. (2001). Gridded usage inventories of technical hexachlorocyclohexane and lindane for China with 1/6 latitude by 1/4 longitude resolution. *Arch. Environ. Contam. Toxicol.* 41, 261–266. doi: 10.1007/s002440010247

Li, Z., Lin, T., Li, Y., Jiang, Y., and Guo, Z. (2017). Atmospheric deposition and air-sea gas exchange fluxes of DDT and HCH in the Yangtze River Estuary, East China Sea. *J. Geophys. Res. Atmos.* 122, 7664–7677. doi: 10.1002/2016jd026330

Liu, G., Yuan, X., Guan, C., Luo, H., and Gu, Y. (2018). Influence of reclamation on off-shore macrobenthic community in the qingduizi bay. *Mar. Environ. Sci.* 663–669.

Liu, M., Cheng, S., Ou, D., Yang, Y., Liu, H., Hou, L., et al. (2008). Organochlorine pesticides in surface sediments and suspended particulate matters from the Yangtze Estuary. *China. Environ. Pollut.* 156, 168–173. doi: 10.1016/j.envpol.2007.12.015

Liu, M., Yang, Y., Hou, L.-J., Xu, S., Ou, D., Zhang, B., et al. (2003). Chlorinated organic contaminants in surface sediments from the Yangtze Estuary and nearby coastal areas. *China. Mar. Pollut. Bull.* 46, 672–676. doi: 10.1016/s0025-326x(03)00050-x

Long, E. R., Field, L. J., and MacDonald, D. D. (1998). Predicting toxicity in marine sediments with numerical sediment quality guidelines. *Environ. Toxicol. Chemis. Int. J.* 17, 714–727. doi: 10.1002/etc.5620170428

Long, E. R., Macdonald, D. D., Smith, S. L., and Calder, F. D. (1995). Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. *Environ. Manag.* 19, 81–97. doi: 10.1007/bf02472006

Men, B., He, M., Tan, L., and Lin, C. (2014). Distributions of polychlorinated biphenyls in the Daliao River estuary of Liaodong Bay. Bohai Sea (China). *Mar. Pollut. Bull.* 78, 77–84. doi: 10.1016/j.marpolbul.2013.11.005

Meyers, P. A. (1994). Preservation of elemental and isotopic source identification of sedimentary organic matter. *Chem. Geol.* 144, 289–302. doi: 10.1016/0009-2541(94)90059-0

Miao, L. J., Yu, Y. H., Guan, C. J., Lin, X., Kang, J., and Wei, Q. F. (2014). Application of opportunity cost method in the determination of marine ecological compensation standard - a case study of Zhuanghe Qingdui bay area. *Ocean Dev. Manag.* 5, 21–25.

Miglioranza, K. S., de Moreno, J. E., and Moreno, V. C. J. (2004). Organochlorine pesticides sequestered in the aquatic macrophyte *Schoenoplectus californicus* (CA Meyer) Sojak from a shallow lake in Argentina. *Water Res.* 38, 1765–1772. doi: 10.1016/j.watres.2004.01.017

Minh, N. H., Minh, T. B., Kajiwara, N., Kunisue, T., Iwata, H., Viet, P. H., et al. (2006). Contamination by polybrominated diphenyl ethers and persistent organochlorines in catfish and feed from Mekong River Delta, Vietnam. *Environ. Toxicol. Chem. Int. J.* 25, 2700–2708.

Montuori, P., Aurino, S., Garzonio, F., and Triassi, M. (2016). Polychlorinated biphenyls and organochlorine pesticides in Tiber River and Estuary: occurrence, distribution and ecological risk. *Sci. Total Environ.* 571, 1001–1016. doi: 10.1016/j.scitotenv.2016.07.089

Offenberg, J. H., and Baker, J. E. (2002). Precipitation scavenging of polychlorinated biphenyls and polycyclic aromatic hydrocarbons along an urban to over-water transect. *Environ. Sci. Technol.* 36, 3763–3771. doi: 10.1021/es025608h

Okay, O., Karacık, B., Başak, S., Henkelmann, B., Bernhöft, S., and Schramm, K.-W. (2009). PCB and PCDD/F in sediments and mussels of the Istanbul strait (Turkey). *Chemosphere* 76, 159–166. doi: 10.1016/j.chemosphere.2009.03.051

Primavera, J. (2006). Overcoming the impacts of aquaculture on the coastal zone. *Ocean Coastal Manag.* 49, 531–545. doi: 10.1016/j.ocecoaman.2006.06.018

R Core Team. (2016). *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing*. Vienna: R Core Team

Ricciardi, A., and Atkinson, S. K. (2004). Distinctiveness magnifies the impact of biological invaders in aquatic ecosystems. *Ecol. Lett.* 7, 781–784. doi: 10.1111/j.1461-0248.2004.00642.x

Richter, B. E., Jones, B. A., Ezzell, J. L., Porter, N. L., Avdalovic, N., and Pohl, C. (1996). Accelerated solvent extraction: a technique for sample preparation. *Anal. Chem.* 68, 1033–1039. doi: 10.1021/ac9508199

Sharma, B. M., Bharat, G. K., Tayal, S., Nizzetto, L., Čupr, P., and Larssen, T. (2014). Environment and human exposure to persistent organic pollutants (POPs) in India: a systematic review of recent and historical data. *Environ. Int.* 66, 48–64. doi: 10.1016/j.envint.2014.01.022

Shi, J., Li, P., Li, Y., Liu, W., Zheng, G. J., Xiang, L., et al. (2016). Polychlorinated biphenyls and organochlorine pesticides in surface sediments from Shantou Bay, China: sources, seasonal variations and inventories. *Mar. Pollut. Bull.* 113, 585–591. doi: 10.1016/j.marpolbul.2016.09.006

Soares, H., Boaventura, R., Machado, A., and Da Silva, J. E. (1999). Sediments as monitors of heavy metal contamination in the Ave river basin (Portugal): multivariate analysis of data. *Environ. Pollut.* 105, 311–323. doi: 10.1016/s0269-7491(99)00048-2

Syed, J. H., Malik, R. N., Li, J., Chaemfa, C., Zhang, G., and Jones, K. C. (2014). Status, distribution and ecological risk of organochlorines (OCs) in the surface sediments from the Ravi River. Pakistan. *Sci. Total Environ.* 472, 204–211. doi: 10.1016/j.scitotenv.2013.10.109

Tolosa, I., Bayona, J. M., and Albaiges, J. (1995). Spatial and temporal distribution, fluxes, and budgets of organochlorinated compounds in Northwest Mediterranean sediments. *Environ. Sci. Technol.* 29, 2519–2527. doi: 10.1021/es00010a010

Tsygankov, V. Y., Boyarova, M. D., and Lukyanova, O. N. (2015). Bioaccumulation of persistent organochlorine pesticides (OCPs) by gray whale and Pacific walrus from the western part of the Bering Sea. *Mar. Pollut. Bull.* 99, 235–239. doi: 10.1016/j.marpolbul.2015.07.020

Usepa. (1998). *Guidelines for Ecological Risk Assessment EPA/630/R-95/002F USEPA*. Washington, DC: USEPA.

Wang, H., He, M., Lin, C., Quan, X., Guo, W., and Yang, Z. (2007). Monitoring and assessment of persistent organochlorine residues in sediments from the Daliaohe River Watershed. Northeast of China. *Environ. Monit. Assess.* 133, 231–242. doi: 10.1007/s10661-006-9576-z

Wang, J., Tan, L., Zhang, W., and Lian, Z. (2010). Concentrations and distribution characteristic of PAHs, PCBs and OCPs in the surface sediments of Qingtao coastal area. *Environ. Sci.* 31, 2713–2722.

Wang, L., Yang, X., Zhang, A., Bidegain, G., Li, R., Na, G., et al. (2019). Distribution patterns and ecological risk of endocrine-disrupting chemicals at Qingduizi Bay (China): a preliminary survey in a developing maricultured bay. *Mar. Pollut. Bull.* 146, 915–920. doi: 10.1016/j.marpolbul.2019.07.026

Wang, T., Lu, Y., Zhang, H., and Shi, Y. (2005). Contamination of persistent organic pollutants (POPs) and relevant management in China. *Environ. Int.* 31, 813–821. doi: 10.1016/j.envint.2005.05.043

Wei, D., Kameya, T., and Urano, K. (2007). Environmental management of pesticidal POPs in China: past, present and future. *Environ. Int.* 33, 894–902. doi: 10.1016/j.envint.2007.04.006

Wu, J., Liu, G., Wang, N., and Li, A. (2011). Water quality and eutrophication in Qingduizi Bay from 2008 to 2009. *Fish. Sci.* 30, 708–710.

Wu, Q., Leung, J. Y., Yuan, X., Huang, X., Li, H., Huang, Z., et al. (2015). Biological risk, source and pollution history of organochlorine pesticides (OCPs) in the sediment in Nansha mangrove. *South China. Mar. Pollut. Bull.* 96, 57–64. doi: 10.1016/j.marpolbul.2015.05.047

Yang, H., Xue, B., Jin, L., Zhou, S., and Liu, W. (2011). Polychlorinated biphenyls in surface sediments of Yueqing Bay, Xiangshan Bay, and Sanmen Bay in East China Sea. *Chemosphere* 83, 137–143. doi: 10.1016/j.chemosphere.2010.12.070

Yang, X., Yuan, X., Zhang, A., Mao, Y., Li, Q., Zong, H., et al. (2015). Spatial distribution and sources of heavy metals and petroleum hydrocarbon in the sand flats of Shuangtaizi Estuary, Bohai Sea of China. *Mar. Pollut. Bull.* 95, 503–512. doi: 10.1016/j.marpolbul.2015.02.042

Yang, X., Zhang, P., Li, W., Hu, C., Zhang, X., and He, P. (2018). Evaluation of four seagrass species as early warning indicators for nitrogen overloading: Implications for eutrophic evaluation and ecosystem management. *Sci. Total Environ.* 635, 1132–1143. doi: 10.1016/j.scitotenv.2018.04.227

Yin, J. (2013). *The Causation Cognizance and Assessment of Marine Ecological Damage for Coastal Reclamation Aquaculture - a Case Study in Qingduizi Bay [M]*. Dalian: Dalian Ocean University.

Yu, H.-Y., Zhang, B.-Z., Giesy, J. P., and Zeng, E. Y. (2011). Persistent halogenated compounds in aquaculture environments of South China: implications for global consumers' health risk via fish consumption. *Environ. Int.* 37, 1190–1195. doi: 10.1016/j.envint.2011.04.012

Yu, S., Liu, W., Xu, Y., Zhao, Y., Cai, C., Liu, Y., et al. (2019). Organochlorine pesticides in ambient air from the littoral cities of northern China: spatial distribution, seasonal variation, source apportionment and cancer risk assessment. *Sci. Total Environ.* 652, 163–176. doi: 10.1016/j.scitotenv.2018.10.230

Yu, Y., Li, Y., Shen, Z., Yang, Z., Mo, L., Kong, Y., et al. (2014). Occurrence and possible sources of organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) along the Chao River. *China. Chemosphere* 114, 136–143. doi: 10.1016/j.chemosphere.2014.03.095

Yuan, X., Yang, X., Na, G., Zhang, A., Mao, Y., Liu, G., et al. (2015). Polychlorinated biphenyls and organochlorine pesticides in surface sediments from the sand flats of Shuangtaizi Estuary, China: levels, distribution, and possible sources. *Environ. Sci. Pollut. Res.* 22, 14337–14348. doi: 10.1007/s11356-015-4688-z

Yuan, X., Yang, X., Zhang, A., Ma, X., Gao, H., Na, G., et al. (2017). Distribution, potential sources and ecological risks of two persistent organic pollutants in the intertidal sediment at the Shuangtaizi Estuary. Bohai Sea of China. *Mar. Pollut. Bull.* 114, 419–427. doi: 10.1016/j.marpolbul.2016.09.058

Zhao, D., Zhang, P., Ge, L., Zheng, G. J., Wang, X., Liu, W., et al. (2018). The legacy of organochlorinated pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in Chinese coastal seawater monitored by semi-permeable membrane devices (SPMDs). *Mar. Pollut. Bull.* 137, 222–230. doi: 10.1016/j.marpolbul.2018.10.004

Zhou, S.-S., Shao, L.-Y., Yang, H.-Y., Wang, C., and Liu, W.-P. (2012). Residues and sources recognition of polychlorinated biphenyls in surface sediments of Jiaojiang Estuary. East China Sea. *Mar. Pollut. Bull.* 64, 539–545. doi: 10.1016/j.marpolbul.2011.12.023

Zhu, C., Wang, P., Li, Y., Chen, Z., Li, W., Ssebugere, P., et al. (2015). Bioconcentration and trophic transfer of polychlorinated biphenyls and polychlorinated dibenzo-p-dioxins and dibenzofurans in aquatic animals from an e-waste dismantling area in East China. *Environ. Sci. Process. Impacts* 17, 693–699. doi: 10.1039/c5em00028a

Zimmerman, J. R., Ghosh, U., Millward, R. N., Bridges, T. S., and Luthy, R. G. (2004). Addition of carbon sorbents to reduce PCB and PAH bioavailability in marine sediments: physicochemical tests. *Environ. Sci. Technol.* 38, 5458–5464. doi: 10.1021/es034992v

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

REVIEW
published: 15 October 2019
doi: 10.3389/fmars.2019.00633

Tributyltin: A Bottom–Up Regulator of the *Crangon crangon* Population?

*Koen F. V. Parmentier[1,2], Yves Verhaegen[1,3], Bavo P. De Witte[1], Stefan Hoffman[1], Daan H. R. Delbare[1], Patrick M. Roose[2], Ketil D. E. Hylland[4], Thierry Burgeot[5], Guy J. Smagghe[3] and Kris Cooreman[1]**

[1] Animal Sciences Unit, Flanders Research Institute for Agriculture, Fisheries and Food, Ostend, Belgium, [2] Operational Directorate Natural Environment, Royal Belgian Institute of Natural Sciences, Brussels, Belgium, [3] Laboratory of Agrozoology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium, [4] Department of Biosciences, University of Oslo, Oslo, Norway, [5] Unit of Biogeochemistry and Ecotoxicology, Institut Français de Recherche pour l'Exploitation de la Mer, Nantes, France

OPEN ACCESS

Edited by:
Julian Blasco, Spanish National Research Council (CSIC), Spain

Reviewed by:
Piero Giulio Giulianini, University of Trieste, Italy
Sumonto Mitra, Karolinska Institute (KI), Sweden

***Correspondence:**
Kris Cooreman
kris.cooreman@ilvo.vlaanderen.be

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *10 May 2019*
Accepted: *26 September 2019*
Published: *15 October 2019*

Citation:
Parmentier KFV, Verhaegen Y, De Witte BP, Hoffman S, Delbare DHR, Roose PM, Hylland KDE, Burgeot T, Smagghe GJ and Cooreman K (2019) Tributyltin: A Bottom–Up Regulator of the Crangon crangon Population? Front. Mar. Sci. 6:633. doi: 10.3389/fmars.2019.00633

The restrictions and the concerted action of the global ban on the use and presence of tributyltin (TBT) in marine applications to protect ecosystems in the marine environment in 2008 was mainly based on the economic impact on shellfish industries and the dramatic extinction of local mollusk populations in the past. In contrast to the vast datasets on effects on mollusks, the knowledge on impacts on species from other taxa remained in the uncertain until almost two decades ago. The assumption on a long-term TBT-mediated pernicious metabolic bottom–up regulation of the crustacean *Crangon crangon* population was provoked by the outcome of an EU-project 'Sources, Consumer Exposure and Risks of Organotin Contamination in Seafood.' This study reported high TBT body burdens in *C. crangon* in 2003, at the start of the transition period to the global ban. Experimental research on the TBT impact in *C. crangon* focused on agonistic interference with natural ecdysteroid hormones at the metabolic pathways regulating growth and reproduction and the biogeochemical distribution of the chemical. In this paper, metabolic, topical and population-relevant biological endpoints in *C. crangon* and other crustaceans are evaluated in relation to the temporal and spatial trends on TBT's occurrence and distribution in the field during and after the introduction of the tributyltin restrictions and endocrine-related incidents. Arguments are forwarded to relate the German Bight incident on growth and reproduction failure in the *C. crangon* population, despite the lack of direct evidence, to the pernicious impact of tributyltin in 1990/91 and previous years. The extreme occurrence of TBT in *C. crangon* from other parts of the southern North Sea and evidence on the high body burdens as dose metrics of exposure also feeds the suspicion on detrimental impacts in those areas. This paper further demonstrates the complexity of distinguishing and assessing the individual roles of unrelated stressors on a population in an integrated evaluation at the ecosystem level.

Keywords: tributyltin, *Crangon crangon*, endocrine disruption, ecosystem impact, ecdysteroids, ecdysteroid receptor

INTRODUCTION

The application of the broad-spectrum organotin biocide tributyltin as antifouling agent (**Figure 1**) since the mid-1900s was economically a success until, at the end of the 1970s, fertility and calcification impairments on oysters and induction of imposex and intersex in female snails linked TBT to the decline and local extinction of many mollusk species (Smith, 1981; Alzieu et al., 1982; Bryan et al., 1986, 1989; Gibbs et al., 1987; Waldock et al., 1988; Mensink et al., 1996a; Ide et al., 1997; Oehlmann et al., 1998, 2007). TBT-induced imposex and intersex in gastropod mollusks provided some of the strongest evidence on the occurrence of endocrine disruption in the field (Gibbs et al., 1988).

The economic damage on shellfish industries compelled countries to implement measures to assure the sustainable management of these resources. France was the first responding country by issuing a ban in 1982 on the application of organotin-based paints on ships < 25 m waterline and fish farms. Similar bans followed suit between 1987 and 1991 throughout the North Sea countries. In 1989, the EU imposed such measures to all Member States (EU Council Directive 89/677/EEC, European Commission, 1989) and the IMO acted accordingly on a global scale in 1990 (IMO resolution Mepc.29(25), 1990).

In 2001, the IMO adopted the 'International Convention on the Control of Harmful Antifouling Systems, 2001,' formalizing a global ban on the application of organotin antifouling agents on marine vessels after the deadline of 17 September, 2008 [AFS Convention, modified in 2010 (IMO resolution Mepc.195(61), 2010)] to include guidelines on survey and certification of antifouling systems on ships). The EU transposed the 2001 AFS Convention into Regulation (EC) 782/2003 (EU Regulation No. 782/2003, European Union, 2003) which banned the application of TBT on EU-flagged vessels starting on 1 January, 2003. That regulation further obliged all ships visiting EU ports from 1 January, 2008 onwards to be free of TBT or at least to bear a barrier coating.

In contrast to the vast datasets and assessments on endocrine TBT disruption in mollusks at population-relevant endpoints up to extinction, metabolic to apical effects on other taxa were seldom identified until during the last two decades. Research on effects in other taxa was initially based on lethal and sublethal endpoints. In crustaceans, acute 96 h-LC_{50} values of 28.5–41 μg TBT oxide (TBTO)/l did not draw up a particular sensitivity of adult brown shrimp (*Crangon crangon*; **Figure 2**; Champ, 1986; Verhaegen, 2012; Verhaegen et al., 2012). The larval stages of *C. crangon* showed more sensitive acute mortality at 1.5 μg TBTO/l and larval growth of the American lobster (*Homarus americanus*) nearly stopped at 1 μg TBTO/l (Laughlin and French, 1980; United States Environmental Protection Agency [EPA. US], 1985).

FIGURE 1 | Tributyltin treated **(left)** and untreated **(right)** marine exposed during 1 month.

FIGURE 2 | *Crangon crangon* (brown shrimp; © 2008 Hamal).

The outcome of the EU-project on Sources, consumer exposure and risks to organotins (OTs) accumulated in seafood (OT-SAFE; Willemsen et al., 2004) alerted serious concerns on the environmental risks related to the transfer of accumulated TBT in *C. crangon* to the human food chain as well as on the health of the population in its major habitat, the southern North Sea (Verhaegen et al., 2011, 2012; Verhaegen, 2012). The southern North Sea is the main fisheries area on *C. crangon*. This species was a target organism in the OT-SAFE study for its high economic value. The economic importance in Europe peaked since 2006 till 2014 at average annual catches of 30,000 to 38,000 tons and a third-place ranking in landing value of European fisheries products, exceeding 100 Million Euro in

Abbreviations: 9-*cis* RA, 9-*cis*-retinoic acid; AFS, Convention on the Control of Harmful Antifouling Systems of IMO; BCF, bioconcentration factor; BSAF, biota to sediment accumulation factor; cDNA, copy- or complement-DNA; CrcEcR, *C. crangon* ecdysteroid receptor; CrcRXR, *C. crangon* retinoid X-receptor; DBT, dibutyltin; Dw, dry weight; ECHA, European Chemicals Agency; EcR, ecdysteroid receptor; EFSA, European Food Safety Authority; EU, European Union; ICES, International Council for the Exploration of the Sea; IMO, International Maritime Organization; K_{ow}, Octanol-Water Partition Coefficient; LC_{50}, median lethal concentration; LOEC, Lowest Observed Effect Concentration; LPUE, Landings per Unit Effort; MBT, monobutyltin; MEPC, Marine Environment Protection Committee of IMO; NOEC, No Observed Effect Concentration; NR, nuclear receptor; OT, organotin; OT-SAFE, Sources, consumer exposure and risks to organotins (OTs) accumulated in seafood; pH_i, Intracellular pH; pK_a, Acid-dissociation constant; RXR, retinoid-X-receptor; SSD, Species Sensitive Distribution; TBT, tributyltin; TBTO, *Bis*(tributyl)tin oxide.

some years (ICES, 2011, 2017). Ecologically, its population can locally comprise up to 80% of the total epibenthic biomass in the southern North Sea (Bamber and Henderson, 1994; Cattrijsse et al., 1997).

This paper assesses different aspects of TBT pollution in *C. crangon* in its main distribution range, the southern North Sea and does not focus on an overall review on TBT data and effects in the marine environment, as the TBT levels vary by a high factor in different locations. For comprehensive reviews, we refer to, among other (Evans et al., 1996; Fent, 1996; Champ, 2000; Birchenough et al., 2002; Stronckhorst and van Hattum, 2003; Antizar-Ladislao, 2008; Morton, 2009; Oliveira et al., 2009; Rodríguez et al., 2009; Sousa et al., 2009; Bengtsson and Wernersson, 2012; Sunday et al., 2012; Matthiessen, 2013; Wetzel et al., 2013; OSPAR, 2014; Langston et al., 2015; Nicolaus and Barry, 2015; Wilson et al., 2015; Schøyen et al., 2018).

TBT LEVELS IN THE SOUTHERN NORTH SEA

It is undeniable that the several routes of bioaccumulation generated high TBT body burdens in many species from different taxa in charged areas. Thorough research on the impact of TBT in *C. crangon* followed on the OT-SAFE project (Willemsen et al., 2004). In 2003, at the start of the global TBT ban transition period, samples from the coastal sampling site BCS3 in Belgium in the southern North Sea contained high TBT levels in the edible tail tissue of the organism (average 326 μg TBT cation/kg tail dw) indicating a high accumulation potential, in comparison to, e.g., plaice (*Pleuronectes platessa*) muscle tissue, sampled at the same place and date at BCS3, contained TBT levels below the limit of detection (<4 μg TBT cation/kg ww; Willemsen et al., 2004). The levels of the other OTs monobutyltin, dibutyltin, monophenyltin, and diphenyltin were all not quantifiable (Verhaegen et al., 2012). The highest levels of TBT in *C. crangon* in 2003–2005, measured in the nearby Western Schelde estuary, ranged from 350 to 1700 μg TBT cation/kg dw upstream (Janssen et al., 2007). In 2009, these extreme TBT levels rapidly had decreased as a result of the 2003–2008 global ban. A large-scale geographical one-off survey along the southern North Sea coast in 2009 (**Figure 3**) revealed a rapid TBT elimination in *C. crangon* from open sea and the estuaries (Verhaegen, 2012; Verhaegen et al., 2012), compared to the data in Willemsen et al. (2004) and Janssen et al. (2007). In terms of consumer risks of TBT contamination in seafood, there is growing certainty that the current levels in *C. crangon* from open sea no longer present an intolerable human health risk (Verhaegen, 2012; Verhaegen et al., 2012). The authors calculated an allowed daily consumption of 5.22 ± 2.86 kg peeled *C. crangon* by a 60 kg weighing person in accordance with the maximum Tolerable Daily Intake (TDI) for TBT set by the EFSA. This renders brown shrimp again a healthy product, in comparison to its status a decade ago. In 2003, a daily consumption of 169 g peeled *C. crangon* would be sufficient to exceed the TDI (Willemsen et al., 2004). There is to our knowledge no information on risk to consumers available prior to 2001.

A baseline study of the TBT distribution in surface sediment of the Elbe estuary in 2011 ranged from 'undetectable' at the mouth to 100 μg TBT cation/kg sediment dw near the port of Hamburg (Wetzel et al., 2013; Sn to TBT cation conversion rate 2.44). At the 2003 and 2009 common sampling site BSC3, a 10-fold decrease in TBT levels was measured in the organism as well as in its habitat sediment.

Based on these TBT data, an average individual heavy metal-type biota to sediment accumulation factor 10 (BSAF ∼ 10) was derived from the sediment < 63 μm fraction data (Verhaegen, 2012; Verhaegen et al., 2012) using the method of Ankley et al. (1994) instead of using suspended solids data, as applied by Veltman et al. (2006). For an earlier year (1999), TBT contamination data in *C. crangon* were calculated from the 1999 Western Schelde sediment data and extrapolated 2003 BCS3 open sea sediment data using the BSAF: 900 and 650 μg TBT cation/kg dw *C. crangon* tail, respectively. These 1999 data suggest extreme contamination in the southern North Sea in the previous century prior and after the introduction of the first major TBT ban in 1989 on small ships.

In 1987, a high degree of imposex was observed in the vicinity of pleasure craft activity, fishing harbors and boat yards, reflecting localized inputs from these sources (Bailey and Davies, 1988). Another indication of the widespread TBT contamination in the southern North Sea are the data obtained from the mollusk *Buccinum undatum*. Data from 1995 on TBT, DBT, and MBT in samples from an offshore sampling station of Helgoland (German Bight, southern North Sea) ranged, respectively, from 2.73 to 38.2, 11.2 to 76.4, and 18.0 to 81.9 μg/kg dw (recalculated from the data of Ide et al., 1997 at a dw/ww conversion factor of 2.73). Similar TBT levels were found in this species from other areas in the southern North Sea: 2.5 to 28.8 μg TBT/kg dw along the coast of the Netherlands (ten Hallers-Tjabbes et al., 1996) and 11.5 to 11.8 μg TBT/kg dw in the Eastern Schelde (Mensink et al., 1996b).

The high distribution ratios of DBT/TBT and MBT/TBT in *Buccinum undatum* point to enhanced metabolic degradation of TBT which complicates the interpretation of body burden data in those metabolizing species. In snails, there is evidence that incorporated TBT undergoes biotransformation to DBT and MBT in the digestive gland (Bryan et al., 1993). Accumulation and catabolism of TBT is very species specific. Blue crab (*Callinectes sapidus*), fed contaminated prey grass shrimp (*Palaemonetes pugio*), significantly debutylated the accumulated TBT while this was not the case in the prey (Rice et al., 1989). In *C. crangon*, the unquantifiable DBT and MBT levels are a sign of reduced or no catabolism making the measured high TBT levels the actual body residues. Limited metabolic degradation was also observed in Caprellidae (Takahashi et al., 1999).

Tributyltin time trends following on the local and global TBT restrictions indicate sufficiently high elimination rates to drastically reduce the levels in a time span of a few years in recovering areas and have, unequally in time, led to a large-scale progressive recovery of the marine ecosystem (Evans et al., 1996; Birchenough et al., 2002; Morton, 2009; Oliveira et al., 2009; Rodríguez et al., 2009; Sousa et al., 2009; Verhaegen, 2012; Verhaegen et al., 2012; Matthiessen, 2013; OSPAR, 2014, 2017a,b;

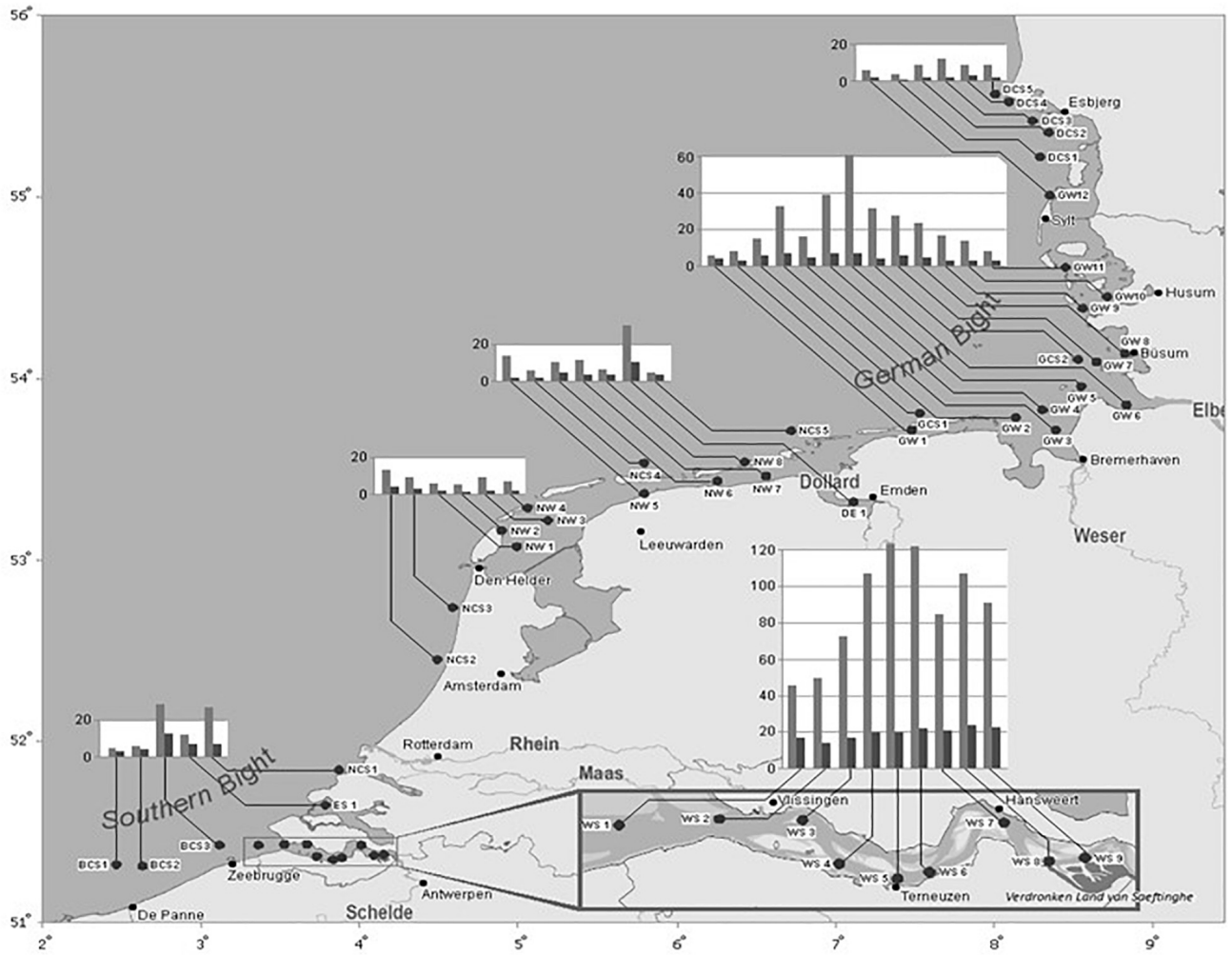


FIGURE 3 | Spatial distribution of TBT (light gray bars) and triphenyltin (dark gray bars) concentrations in *C. crangon* muscle (Y-axis, in μg/kg dw) along the south coast of the North Sea in 2009 (Verhaegen, 2012; Verhaegen et al., 2012). The X and Y axes represent the longitude Northern length (NL) and the latitude Eastern length (EL), respectively.

Langston et al., 2015; Nicolaus and Barry, 2015; Wilson et al., 2015; HELCOM, 2018; Schøyen et al., 2018). The unequal recovery in time is not surprising since the 1989 TBT restrictions on small ships had mainly a local impact in predominantly recreational and small ships areas. There is evidence from sites where commercial vessels were the only source of TBT (Birchenough et al., 2002). The TBT use in antifouling even peaked in 1996, despite the 1989 TBT-ban, when 85% of the larger ships were equipped with a TBT-based coating (OSPAR Commission, 2011). The rapid recovery of *C. crangon* between 2003/2005 and 2009 was, e.g., also seen in an earlier period in the Baltic Sea between 1998 and 2005 where the levels in mussel (*Mytilus edulis*) decreased from 250 to 300 μg TBT/kg dw in 1998 to a threshold value of 12 μg TBT cation/kg dw since 2005 (HELCOM, 2018). These threshold values seem long-lasting due to the negative influences of historical contamination in hot spots, repair docks, and harbor areas (Birchenough et al., 2002; Stronckhorst and van Hattum, 2003).

TBT: DISTRIBUTION, BIOAVAILABILITY, AND UPTAKE

The scientific debate on the biogeochemical behavior of TBT in the marine environment did not reach a commonly accepted consensus on its occurrence and distribution. Even though neutral TBT derivatives are known to partition to lipid and organic carbon (Meador, 2000; Brändli et al., 2009), the compound is readily ionizable depending on surrounding parameters, firstly pH and related confounding parameters electrochemical ligand binding sites, redox conditions, and salinity.

Partitioning equilibrium values such as the octanol-water partitioning coefficient K_{ow} are not very suitable to predict bioaccumulation of TBT since its derivatives are strongly pH-regulated. Many of the K_{ow} values were calculated or determined at unspecified pH values (Meador, 2011). The K_{ow} of TBT increased from 1600 to 12,000 in a pH range of 5.8 to 8.0

(Tsuda et al., 1990; Arnold et al., 1997). The usefulness of K_{ow} values on TBT in partitioning and bioaccumulation scenarios is therefore strictly conditional.

In aqueous solutions, paint released TBTO and TBT hydride hydrolyze with water forming cations (Eng et al., 1986; Arnold et al., 1997). At the acid-dissociation constant pK_a 6.25, half of the TBT is cationic and half is neutralized by anions (Arnold et al., 1997). In seawater at average pH 8 and ionic strength 0.5M 93% of the TBT in solution occurs as the hydroxide complex (Arnold et al., 1997), likely favored by the hydrolyzable nature of TBTO, TBT halides and TBT acetate (Eng et al., 1986). The halides were estimated to have a half-life of 60 days in aqueous media (Sunday et al., 2012) while biodegradation half-lives as low as 6 days have been reported in clean harbor water (Cooney, 1988; Adelman et al., 1990). The predominant occurrence of TBT hydroxide in marine systems was an important indication to link the partitioning behavior of TBT to that observed for neutral hydrophobic substances and predictions based on the K_{ow} equilibrium partitioning (Meador, 2000).

Equilibrium sorption experiments of TBT in estuarine sediments showed similarity of sorption and desorption coefficients indicating that TBT sorption is reversible (Unger et al., 1988). Later research revealed that the TBT sorption in sediment is characterized by a rapid reversible adsorption stage, where 80% of the final sediment concentration is adsorbed within 10 min (Langston and Pope, 1995; Champ and Seligman, 1996), and a slow, non-reversible stage of TBT diffusion into the porous microstructure of the organic material (Pignatello and Xing, 1996; Ma et al., 2000). A similar instant adsorption process was observed in soil while TBT was not easily leached off (Sunday et al., 2012). The sorption of the majority of the neutral TBT hydroxide form, in sediments from coastal waters at average pH 8 to the organic carbon in the sediment might be the principal process. However, a second seemingly less significant underestimated process may be the moored stable metal-type fixation of the remaining approximately 3% TBT cations to electronegative ligands in the sediment, thereby creating a quasi-continuous disequilibrium in the lipophilic partitioning of the neutral OH-form forcing into the direction of fixation on available ligands and steady state conditions. OT compounds are known to interact in the sediment by forming coordination complexes with electronegative oxygen and nitrogen, comparable with the metal-type accumulation in biota (Omae, 1989; Tanabe, 1999).

In biota, the metal-type fixation, accumulation, distribution and bioavailability to cellular processes of bioaccumulating TBT may substantially be activated and regulated by the acidic intracellular pH condition (pHi) which is in electrochemical equilibrium with the pH of the extracellular fluid. A study of Carter (1972) suggests four hypothetical intracellular compartments of different pH within large muscle cells of the crustacean giant barnacle (*Balanus nubilus*). The pHi of the largest compartment was 6.76 in a relative volume of 20 on 22 and a lowest mean pHi value of 6.12 ± 0.03 was measured in a relative volume of 0.5 on 22, closely in compliance with the mean calculated equilibrium pHi of 6.09 ± 0.03. According to Carter (1972) it is highly likely that the acidic compartment represents the aqueous phase of the cell cytoplasm. The two remaining compartments had a pHi of 7.0 and 7.5 in, respectively, relative volumes of 1.0 and 0.5 on 22. The pHi in all compartments and especially the acidic predominant part of the cells is thus much lower than the pH 8 in the surroundings in coastal waters. Carter (1972) supports the view that hydrogen protons are not distributed homogenously throughout the cytoplasm and that there exist intracellular compartments which have markedly different pHi, in contrast to the mean pHi measurements following, e.g., the homogenate technique of Pörtner et al. (1990) yielding higher integrated pH values. Integrated pHi values of 7.32 ± 0.04 in homogenized tail muscle of *C. crangon*, as reported by Abele-Oeschger et al. (1997) seem therefore not accurate enough to elucidate the actual TBT distribution and aggressive behavior in cells and tissues. The compartmentalized nature of the pHi and the predominant acidic conditions in the cells may provide new insights in the process of distribution and behavior. Acidic pHi values close to the TBT pK_a will increase the ratio of the cation, compared to the 'neutral' form (hydroxo-complex?) and increase the proportional interactions of the metal-type behavior of TBT by complex formation with ligands in phospholipids and proteins, as postulated by Arena et al. (1995), Tanabe (1999), Hunziker et al. (2001, 2002), and Strand and Jacobsen (2005). The neutral form appears to partition only to the lipid fraction of the biomembrane (Hunziker et al., 2001) while Doop et al. (2007) suggested that the toxicological potential depends on membrane permeability. In experimental conditions, the biomembrane-water distribution ratio of TBT exceeded the liposome-water distribution ratio at acidic pH 3, which is attributed to complex formation of the cationic species with ligands of the protein fraction. The distribution ratio of the TBT cation exceeded that of the neutral hydroxo-complex by a factor of 2. At higher pH 8.0 the biomembrane-water distribution ratio was found lower than the liposome-water distribution ratio (Hunziker et al., 2001).

The passive migration or electrochemical carrier-translocation of TBT cations or neutral forms through the plasma membrane into the cytoplasm is unclear and both pathways may operate simultaneously at different affinities in the prevailing pHi environment. Passive migration related to compound lipophilicity was demonstrated by Zucker et al. (1989) who measured decreased biomembrane potentials in murine erythroleukemic cells in the presence of TBT. On the other hand, in crustacean copepods, the TBT tissue residue as the dose metric associated with 50% mortality showed no correlation with the lipid content (Meador, 1997).

Conclusively, it is argued that the metal-type fixation in the acidic conditions in biota occurs at a higher ionic activity compared to the metal-type process in sediments at higher pH.

In experimental conditions, the TBT-exposed water pathway became standard in uptake and exposure studies since the toxic responses in mollusks, held in laboratories, showed good correlations with the direct TBT toxicity from bioconcentration. Some mollusks, e.g., *Mytilus edulis*, and especially predatory gastropods possess high TBT BCFs up to 10^5 compared to the moderate 10^3 in crustaceans (ECHA, 2008). A strong correlation between the LC_{50} and the BCF was also found in amphipod

species (Meador, 1997). An individual BCF for *C. crangon* is not known, instead, the TBT body residues are clearly related to the total TBT content of its habitat sediment (Verhaegen, 2012; Verhaegen et al., 2012).

A higher uptake in mollusks from water may be obvious due to the high BCF. Given the moderate BCF in crustaceans, the extremely high body burdens in *C. crangon* should be linked to biomagnification and the high average BSAF from contaminated sediment, rather than bioconcentration, confirming the findings of Veltman et al. (2006) on high accumulation rates in *C. crangon*. More studies describe both accumulation routes from diet and direct uptake from water (Strand and Jacobsen, 2005; Lee et al., 2006). Bryan et al. (1989) described a 50/50 accumulation ratio from diet and surroundings in the carnivorous gastropod *Nucella lapillus*.

MECHANISM OF ACTION OF TBT IN CRUSTACEANS

Several authors postulated in initial research that the site of the basic mechanism of action of TBT toxicity is the biomembrane (Massaro et al., 1989; Zucker et al., 1989; Gadd, 2000; Hunziker et al., 2001). It is in the meantime known that TBT-mediated endocrine disruption and imposex/intersex developments in the sensitive gastropods *Nucella lapillus*, *Nassarius reticulatus* (= *Hinia reticulata*) and other mollusks are related to TBT's agonistic interference with the growth- and reproduction NR retinoid-X and its ecdysteroid hormone triggers (Nishikawa et al., 2004; Sousa et al., 2010; Sternberg et al., 2010). It is this ligand-dependent nature that makes many NRs susceptible for exogenous chemicals at extremely low exposure doses. These adverse metabolic alterations at a range of cellular pathways in the mollusks rapidly translated into severe population-level changes in the past.

Similarly, ecdysteroid hormones trigger the specific regulation of several genes in different tissues and developmental stages of crustaceans, through interactions with the NRs EcR and/or RXR (Hopkins, 2009). In contrast to vertebrate RXR, invertebrate RXRs do not form active homodimers but serve as a heterodimerization partner for many other NRs, and as such are involved in the control of multiple endocrine pathways. EcR is the best known invertebrate partner protein for RXR. The name RXR refers to the natural 9-*cis*-RA, the putative ligand of vertebrate RXRs (Wolf, 2006), whereas the natural agonist(s) for invertebrate RXRs is still under debate. The putative endogenous ligand for crustacean RXR is the terpenoid methyl farnesoate, which has been confirmed in several crustacean species (Reddy et al., 2004; Wu et al., 2004; Laufer et al., 2005) but, e.g., not in the water flea *Daphnia magna* (Wang and LeBlanc, 2009).

Our current understanding of the principal endocrine mechanism of action of TBT in crustaceans is very similar to that in mollusks and refers to the interactions at the physical, functional and gene-expression levels of the ecdysteroid receptor – retinoid-X receptor dimer (CrcEcR-CrcRXR) complex (**Figure 4**; Verhaegen et al., 2010, 2011, 2012; Verhaegen, 2012). TBT caused a strong down-regulation of Ponasterone A-induced transactivation *in vitro* and strong down-regulated expression of CrcEcR/CrcRXR transcripts *in vivo* in the ovaries (**Figure 5**). The ovary-specific inhibition (**Figure 6**) is an initial indication of evolving reproductive impairment, similar to the impact on mollusks. *In silico* 3D-modeling confirmed a very good docking and blocking accommodation of TBT in the electrostatic and neutral areas of the full-length modeled CrcRXR–Ligand Binding Pocket (Verhaegen, 2012;

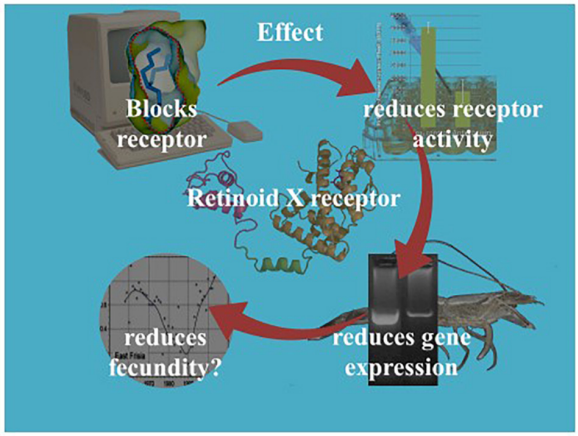


FIGURE 4 | Illustration of the retrospective/diagnostic ecotoxicological research on TBT's endocrine disrupting role in *C. crangon*, incorporating *in silico*, *in vitro,* and *in vivo* technologies.

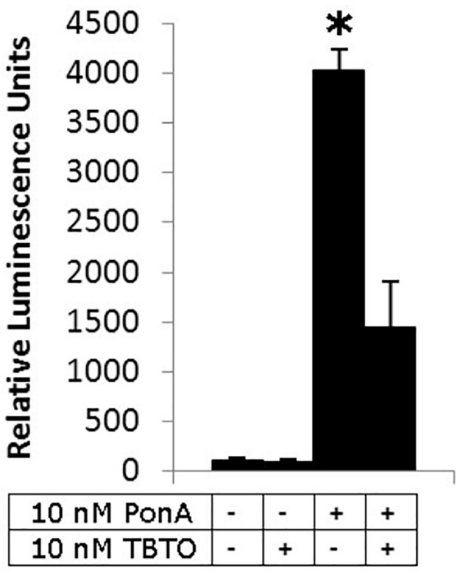


FIGURE 5 | Influence of 10 nM TBTO on reporter gene trans-activation by 10 nM PonA through CrcEcR–CrcRXR. Four different treatments were performed in quadruplicate (from left to right): a negative control, exposure to 10 nM TBTO, a positive control (10 nM PonA) and exposure to 10 nM TBTO in the presence of 10 nM PonA. Data are presented as the mean ± SEM ($n = 4$). An asterisk denotes a significant difference with the respective control (Student's *t*-test, two tailed, $p < 0.05$; Verhaegen et al., 2011; Verhaegen, 2012).

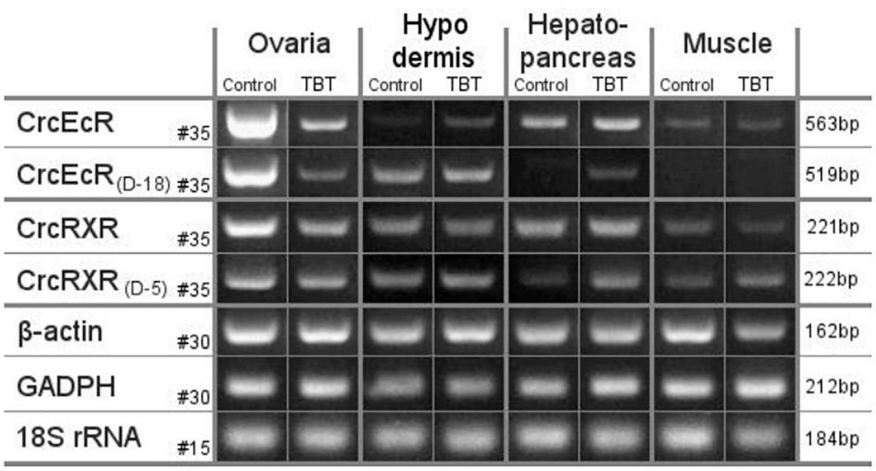


FIGURE 6 | mRNA levels in ovaries, hypodermis, hepatopancreas and muscle of *C. crangon* after exposure to TBT. Semiquantitative RT-PCR results of *C. crangon* exposed to 134 nM TBT for 96 h compared with mRNA expression in the control group for the non-truncated CrcEcR and CrcRXR isoforms. mRNA levels of the control genes b-actin, glyceraldehyde-3-phosphate (GADPH) and 18S rRNA are also represented. Amplified gene names and cycle number are given on the left, template tissue and experimental group are given at the top, amplicon size is given at the right. Truncated CrcEcR and CrcRXR isoforms are not shown as they were only detected in the ovaries at higher PCR cycle numbers. CrcEcRD-18 bands were clearly visible for hepatopancreas tissue of the control group and muscle tissue at 40 PCR cycles but not at 35 cycles (Verhaegen et al., 2011; Verhaegen, 2012).

Verhaegen et al., 2012). cDNA microarray analysis of 604 gender-related cDNA fragments, appended to CrcEcR and CrcRXR isoforms, revealed affected expression of 43 gene fragments and disruption of calcium homeostasis. Upregulation of vitellogenin and up- as well as downregulation of several cuticular proteins may cause a second endocrine disruption on the Y-organ – ecdysteroidal endocrine axis and evolving growth and reproduction impairment (Verhaegen, 2012). The crustacean endocrine axis comprises two major neuroendocrine glands situated in the eyestalks: the X-organ-sinus gland complex, and the Y-organ (Chang and Mykles, 2011).

In contrast to vertebrates and Lophothrochozoa (e.g., mollusks), arthropod vitellogenesis is regulated by ecdysteroid and juvenile hormones, and not by estrogens. In arthropods, vitellogenesis is negatively regulated by ecdysteroids (Hannas et al., 2010). Upregulation of vitellogenesis, as confirmed in Verhaegen's experiments (Verhaegen, 2012), would thus suggest an anti-ecdysteroidal effect of TBT (Verhaegen, 2012). Retarded molting, production of abnormalities in initial and final stages of limb regeneration and endocrine toxicity on ecdysis, vitellogenesis, and calcium resorption in a TBT environment were also proven in other studies on crustaceans (Nagabhushanam et al., 1990; Reddy et al., 1991, 1992; Wang et al., 2011; Hosamani et al., 2017). The interference of TBT in the calcium reabsorption inhibits the exoskeleton formation (Nagabhushanam et al., 1990).

The pathway of the TBT impact on metabolic stages of growth and reproduction in *C. crangon* seems to some extent identical to the deregulation in mollusks. This is not exceptional since the RXR, the fourth member of group B of the NR subfamily 2, is a highly conserved NR throughout the animal kingdom and mankind (Nishikawa et al., 2004). It is very likely that the mechanism of action of TBT on RXR is very similar in all examined taxa and occurs, presumably, in a thin line with natural ligands since Nishikawa et al. (2004) proved that the vertebrate natural RXR ligand 9-*cis*-RA, also induced imposex in rock shell *Thais clavigera*. This deterioration by natural ligands may be attributed to an important role of RXR in the differentiation and growth of male genital tracts in female gastropods (Nishikawa et al., 2004).

TBT TOXICITY AND TOPICAL AND POPULATION-RELEVANT ENDPOINTS

The highest sensitivity to TBT toxicity has long been assigned to gastropods. In the 80s and 90s, data on the TBT chemical status of the marine environment were commonly addressed via dose–response relationships in toxicity tests on mollusks in TBT-exposed water pathways. Lowest Observed Effect Concentrations as low as 1.0 to 2.0 ng TBT/l exposure water (Gibbs et al., 1987; Rainbow, 1995) brought imposex and intersex prevalence on the forefront as key indicators in monitoring programs in times of challenging developments of analytical techniques, sufficiently specific and sensitive enough to detect TBT concentrations at or below the ng/l (ppt) level in the water column for the reason that strong correlations were found between biological endpoints (e.g., LC_{50}) and the BCF (Meador, 1997) while other studies reported high correlations with whole-body TBT residues as well (King et al., 1989; Hagger et al., 2006).

Intersex is a phenomenon seen in mollusks at much higher TBT contamination. *E.g.,* intersex in the periwinkle *Littorina littorea* occurs at 100-fold higher TBT concentrations than imposex and is used as biomarker in mollusks in areas of high TBT exposure (Oehlmann et al., 1998). In laboratory tests on crustaceans TBT-induced macroscopic changes on the growth

and reproduction morphology have been reported for population relevant endpoints, such as intersex, fecundity, % ovigerous females, reproduction, larval development. LOEC and NOEC in long-term and full life-cycle bioassays on several species ranged, respectively, from 10 to 190 ng TBT/l and 6.0 to 224 ng TBT/l (Johansen and Mohlenberg, 1987; Bushong et al., 1988, 1990; Kusk and Petersen, 1997; Hall et al., 1998; Oberdörster et al., 1998; Baer and Owens, 1999; Ohji et al., 2002; Schmidt et al., 2005; Huang et al., 2006; Lagadic et al., 2017). One outlier NOEC of 1250 ng TBT/l was reported for adult *D. magna* in a 21 days test with offspring as biological endpoint (Oberdörster et al., 1998).

The development of SSD models, based on the lowest NOEC and LOEC data from water-exposure pathways, have shown that many aquatic species from different taxa, including crustaceans, are in the range of sensitivity of mollusks and some fish species showed higher sensitivity (Lagadic et al., 2017). The accumulation of TBT in organisms as well as surroundings in most charged areas in the past were therefore undeniably transcending the vulnerability of crustaceans and species from other taxa as well for population relevant endpoints.

It should be noted that the *in vivo* experiments of Verhaegen et al. (2011) on expression capacity of CrcEcR/CrcRXR transcripts in *C. crangon*'s tissues were performed at (sub)lethal TBT concentrations because of the necessity for using short-term exposure tests since attempts to culture *C. crangon* in full life-cycle conditions still remain unsuccessful. However, the *in vitro* inhibition of CrcEcR/CrcRXR transcripts and affected expression of genes, disruption of calcium homeostasis and the acute lethality of *C. crangon*'s and *H. americanus*' larvae demonstrated a higher toxicity at metabolic and topical endpoints (Laughlin and French, 1980; Verhaegen et al., 2011).

KNOWLEDGE GAPS

A critical gap in the toxicological research on TBT exposure is the need on information on the impact of the TBT body burdens on biological processes in affected organisms with the aim to more realistically assess the overall TBT impact in the field. It was postulated that the tissue residues reflect the bioavailability and effective target doses more accurately than the toxicity based on the water-exposure pathway (Meador, 2011) on standard laboratory-maintained organisms or their offspring. The variability in responses to toxicants in laboratory studies may occur over the natural, seasonal cycle of physiological variation that occurs in populations and organisms, held in laboratory, may become multi-fold more sensitive (Meador, 1993). Several studies have additionally found that when toxicity is expressed as a tissue residue, the variability between species, time periods and exposure conditions is greatly reduced (McCarty, 1991; van Wezel and Opperhuizen, 1995; Meador, 1997).

Unequal tissue distribution and TBT behavior may additionally influence the effective target dose to responsive tissue-specific biological endpoints. Several gastropod species concentrate up to 50% of the total TBT in the female gonads (Oehlmann et al., 1992; Stroben, 1994). The BCFs in the carp *Carassius carassius grandoculis*, obtained in exposure experiments, are also tissue-dependent and may vary almost 10-fold: 589 (muscle), 547 (vertebra), 5012 (liver), and 3162 (kidney) (Tsuda et al., 1986). Research on the chemical tissue partitioning of TBT in *C. crangon* was not a work package in Verhaegen (2012), however, in contrast to the absent responses in the hypodermis, hepatopancreas and muscle tissues, the exclusive and highly affected expression of CrcEcR/CrcRXR transcripts *in vivo* in the ovaries (**Figure 6**) may also indicate enhanced accumulation and disruption in that tissue.

TBT: A PUTATIVE BOTTOM–UP REGULATOR OF *C. crangon*'s POPULATION?

In contrast to the temporal and spatial trends in pernicious impacts on mollusks through female sterility associated with imposex and intersex, where the cause was rapidly attributed to TBT, and recovery following on the TBT restrictions, the assumption on TBT-mediated deregulation of *C. crangon* remained unclear and hypothetical since this species was commonly regarded as non-endangered during half a century. However, the underlying metabolic mechanism of action in both taxa was only unraveled until after 2000.

The question whether the TBT prevalence was an important indicator of the population health status of *C. crangon* is now no longer relevant as a result of the TBT bans. However, it remains a scientific necessity and societal obligation to be aware of the former threats on the population by the use of this chemical, in an ecosystem and economic perspective.

The most striking potential link between TBT and population-relevant endpoints in *C. crangon* may have happened before the TBT bans were introduced. Research on the seasonal spawning cycle and reproductive success in subareas of the German Bight in the North Sea in the period 1958–2005 linked reproductive impairment to low percentages of ovigerous females on the basis of morphological information (see review, Siegel et al., 2008). A decrease in the proportion of ovigerous females started in the western part of the German Bight (the Waddenzee) in the second half of the 1970s, where it dropped below 50% of the previous status. The absolute minimum was observed in the late 1980s with ovigerous female proportions below 10%. The subsequent low reproduction and recruitment caused dramatic drops in landings of consumption shrimp since the catches in the German Bight account for approximately 90% of the total European catch. Correlation analyses with common parameters, such as water temperature, river runoff, North Atlantic Oscillation climate index and the more obvious indicators predator and fishing mortality did not show any plausible proximate cause of this large-scale population impact (Siegel et al., 2008). Watermann and Dethlefsen (1983) postulated pollution-induced "dissolutions" of the shell and subsequent secondary infections. Unfortunately, a lack on data from other areas prevented larger geographical-scale comparisons and TBT-induced biological endpoints on growth and reproduction were at that time only extensively described in mollusks from TBT-charged areas. *C. crangon* is phylogeographically similar across its distribution

range (Luttikhuizen et al., 2008; Hoffman, unpublished results). The whole population for management purposes is considered as one stock (ICES, 2014) which may also indicate similarity in sensitivity to TBT in a large geographical area.

The nature of the observed disorders in the German Bight survey, percentage affected ovigerous females and low recruitment were later diagnosed in conventional full life-cycle exposure tests on several crustacean species, e.g., at a LOEC of 10 ng/l in the copepod *Schmackeria poplesia* (Ohji et al., 2002; Huang et al., 2006, 2010; Lagadic et al., 2017). The described 'dissolutions' of the shell may in turn refer to ecdysis and vitellogenin disruption in the process of the endocrine toxicity on expression of cuticular proteins and eventually vitellogenin, as postulated by Verhaegen (2012). Further, Reddy et al. (1992) reported significant effects after the third molt in the prawn *Caridina rajadhari*. Pollution-induced impacts by a range of other priority chemicals seem not an issue in this incident since downward trends were general (OSPAR, 2010) and the severity of the toxicities of many of these chemicals compared to the toxic potential of TBT may be arguable.

A second indication on the putative deterioration by TBT is the stock rebound in the German Bight in the course of the 1990s (Siegel et al., 2008), shortly after the European 1989 TBT ban was introduced. A rapid recovery was remarkable in yachting areas while industrialized maritime areas showed delays until the implementation of the global TBT-ban (Verhaegen, 2012; Verhaegen et al., 2012).

The breakdown of the catches in 1990/1991 was in the 2014 WGCRAN report (ICES, 2014) suggested to be caused by a mass invasion of 0-group whiting (*Merlangius merlangus*). It is obvious that a decrease in commercial landings is linked to rebuilding stocks of the main predators cod (*Gadus morhua*) and whiting. Both cod and whiting can have high shares of *C. crangon* in their stomach (Temming and Hufnagl, 2014) although both species seem to prefer the co-occurring gobies in their diet (Jansen, 2002) and growth rates are smaller on homogeneous *C. crangon* diets (Temming, 1995). Events of extremely abundant cod and/or whiting 0-groups occurred in the years 1959, 1961, 1970, 1977, 1983, 1990, and 1998 (Tiews, 1961; Berghahn, 1996; ICES, 1998; Siegel et al., 2005). Increased predator invasions matched to drops in commercial shrimp landings in several years (1977, 1983, 1990, and to a lesser extent 1998). Increased predator abundances are, however, in the event of the German Bight incident and the disastrous 1990/91 breakdown of the catches not a justifiable argument for the population-level occurrence of overall growth, reproduction and recruitment failure. The German Bight incident is, in our opinion, fully attributable to endocrine disruption by the most prevalent chemical TBT and predation mortality at that time may have played a secondary lesser impact on the stock.

A different scenario manifested post 2000 till now complicating the distinction between the proportional influences of the different established population drivers. The gradual increase in landings of consumption shrimp maximized after 2003 (>32,000t) to 37,000t in 2014 (ICES, 2011, 2017). The clear relationship between the stepwise temporal and spatial rebound of the stock, the implementation of the 1989 partial ban, the 2003–2008 global ban and the fading out of the TBT prevalence to threshold values may have restored sustainable shrimp fisheries making the other drivers such as predation mortality, climate change and fishing effort more visibly accountable. Data on stock assessments and landings statistics of cod and whiting confirmed downward trends by, respectively, 58 and 25% in numbers between 2001 and 2010. Updates with predator distribution maps and effects of climate change revealed a shift in range of distribution of both cod and whiting, resulting in a reduced overlap with *C. crangon* (Kirby et al., 2009; Temming and Hufnagl, 2014).

Estimates on predator influences on the population dynamics also fluctuated by the differences in trends of predator abundances. ICES (2015a,b) informed on abundances a downward trend on whiting and an upward trend on cod. Cod abundances in the North Sea have increased because cod fishing mortality rates have been reduced since 2000, in combination with a stronger spawning stock biomass in 1999, 2005, and 2009. The North Sea cod spawning biomass had tripled in the years after the lowest estimated level of 21,000t in 2006 (ICES, 2015a). However, these changes in predation levels had seemingly no profound impact on the commercial shrimp catches in the southernmost areas of the North Sea.

So far, the fluctuating influences of the predation mortality on *C. crangon*'s population have interfered with a putative impact of TBT preventing a clear signal on a population relevant endpoint. The far-reaching elimination of TBT from the marine environment has ensured that TBT's putative role on the health of the population has in the meantime minimized.

Recently, another stressor, fishing effort, which has never been regarded as a potential threat, has reached the highest efforts in 2013 and 2014 (ICES, 2015b). This impact resulted in an overall decrease in LPUE indicating an uncontrolled effort increase, overfishing and the need for a management plan to restore sustainable shrimp fisheries (ICES, 2013, 2014, 2016). 2015 was the turning year to again much lower commercial landings in 2016 (25,900t) and the lowest level since 1995 (ICES, 2017). In the years before 2000, Welleman and Daan (2001) quantified the yield of the total annual shrimp landings to only 5 to 10% of the total predation mortality. In 2012, an update of Welleman and Daan suggested fishing mortality beyond natural (predation) mortality (ICES, 2012). In 2015, the fishing pressure was estimated four times higher than natural mortality and the population was declared overfished (ICES, 2015b). Alarming is the U-turn to overfishing since, based on the findings of Welleman and Daan (2001), the yield of the landings could increase 10-fold or more, at zero predation mortality. It seems that the distinction between the influences of changes in fishing effort and predation are thin since the 2016 survey data again suggested a relationship between high abundances of whiting (*Merlangius merlangus*) and low shrimp abundances in the German areas (ICES, 2017). The suspected reason on the current overfishing can be found in the high shares of undersized shrimp compared to commercial sized shrimp in the catches, on average 0.85 L in the pulse gear and 0.68 L in the traditional gear per liter consumption shrimp (ICES, 2015b, 2016). These undersized shrimp are less discarded and landed nowadays

causing recruitment overfishing despite a survival range of discarded shrimp between 75 and 80% (ICES, 2016). The impact of this change in fisheries attitude seems enormous and would be better assessed when expressed in numbers instead of liters.

CONCLUSION

A potential population-level endocrine disruption by TBT may have interfered unnoticed during the past 60 years of *C. crangon*'s life history and almost the entire history of its recorded fisheries. Attention to (sub-)chronic TBT toxicity in crustaceans was long not an issue of particular interest due to the initial moderate lethal toxicity compared to mollusks, and *C. crangon*'s long-term non-endangered status.

Most assessments on TBT impact on organisms in the marine environment were based on water exposure toxicity correlations between effects and TBT levels in water. The development of SSD models were also based on water exposure toxicity data in often full life-cycle tests on several taxa. These SSD models demonstrate more similarity in sensitivity to TBT among taxa.

Despite the lack on full life-cycle toxicity information on topical endpoints in *C. crangon*, the available information on biological endpoints in *C. crangon* and other crustacean species, delivers in our view, sufficient arguments to strengthen the hypothesis on population-relevant endocrine disruption to clarify the German bight incident in before and during 1990/91.

The high TBT accumulation in charged areas, especially in organisms lacking TBT catabolism, suggests that biomagnification played a higher distinctive role than could be expected from bioconcentration. The extremely high body residues of TBT in the past and our predictions on enhanced intracellular partitioning allow to suggest that the TBT toxicity in the marine environment has been underestimated, compared to the water exposure pathway toxicity.

The common mechanism of action on RXR throughout the animal kingdom and mankind at the lower ppt level provoked the implementation of stringent environmental assessment criteria and quality standards in the marine environment to secure the ecosystem and human health (EFSA, 2004; European Union, 2005; United States Environmental Protection Agency [EPA.US], 2007; Cole et al., 2015; HELCOM, 2018).

The historical contamination in hotspots, harbors and estuaries in the marine environment and the continued on-land use (Cole et al., 2015) has brought the TBT levels to thresholds which may remain long-lasting, especially in anoxic sediments.

Up-to-now no safe alternative to TBT is available. The antifouling industry was forced to fall back on toxic booster biocides such as copper, triazines and not at least tralopyril, all having their respective and often unknown and severe impacts. It is therefore strongly recommended to scrutinize the potential environmental impact of all new antifoulants prior to their acceptance in antifouling applications.

AUTHOR CONTRIBUTIONS

GS and KC were the promotors and KP the daily mentor of co-author YV for his Ph.D. dissertation on Mechanism, Concentrations and Effects of Tributyltin in common shrimp *Crangon crangon*. The results of the research of YV and colleagues were frequently reviewed in the ICES Working Group on Biological Effects of Contaminants. This submitted manuscript (review) is a follow-up on the work of YV and colleagues on the potential detrimental role of tributyltin on the shrimp population in an integrated ecosystem assessment. All authors are or were actively involved in the tributyltin pollution research. ILVO was partner within the framework of the EU-project on 'Sources, Consumer Exposure and Risks of Organotin contamination in Seafood.'

REFERENCES

Abele-Oeschger, D., Sartoris, F. J., and Pörtner, H.-O. (1997). Hydrogen peroxide causes a decrease in aerobic metabolic rate and in intracellular pH in the Shrimp *Crangon crangon*. *Comp. Biochem. Physiol.* 117C, 123–129. doi: 10.1016/s0742-8413(97)00059-5

Adelman, D., Hinga, K. R., and Pilson, M. E. Q. (1990). Biogeochemistry of butyltins in an enclosed marine ecosystem. *Environ. Sci. Technol.* 24, 1027–1032. doi: 10.1021/es00077a012

Alzieu, C., Heral, M., Thibaud, Y., Dardignac, M., and Feuillet, M. (1982). Influence des peintures antisalissures a base d'organostanniques sur la calcification de la coquille de l'huitre *Crassostrea gigas*. *Revue des Travaux de l'Institut des pêches maritimes.* 45, 101–116.

Ankley, G. T., Leonard, E. N., and Mattson, V. R. (1994). Prediction of bioaccumulation of metals from contaminated sediments by the oligochaete *Lubriculus variegatus*. *Water Res.* 28, 1071–1076. doi: 10.1016/0043-1354(94)90192-9

Antizar-Ladislao, B. (2008). Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment. A review. *Environ. Int.* 34, 292–308. doi: 10.1016/j.envint.2007.09.005

Arena, G., Calì, R., Contino, A., Musumeci, A., Musumeci, S., and Purrello, R. (1995). Coordination properties of dialkyltin (IV) in aqueous solution. Thermodynamic study of dimethyltin (IV) complexes with l-amino acids. *Inorgan. Chim. Acta* 237, 187–191. doi: 10.1016/0020-1693(95)04655-S

Arnold, C. G., Weidenhaupt, A., David, M. M., Müller, S. R., Haderlein, S. B., and Schwarzenbach, R. P. (1997). Aqueous speciation and 1-octanol–water partitioning of tributyl- and triphenyltin: effect of pH and ion composition. *Environ. Sci. Technol.* 31, 2596–2602. doi: 10.1021/es970009%2B

Baer, K. N., and Owens, K. D. (1999). Evaluation of selected endocrine disrupting compounds on sex determination in *Daphnia magna* using reduced photoperiod and different feeding rates. *Environ. Contam. Toxicol.* 62, 214–221. doi: 10.1007/s001289900862

Bailey, S. K., and Davies, I. M. (1988). Tributyltin contamination in the Firth of Forth (1975 – 1987). *Sci. Total Environ.* 76, 185–192. doi: 10.1016/0048-9697(88)90106-4

Bamber, R. N., and Henderson, P. A. (1994). Seasonality of caridean decapod and mysid distribution and movements within the Severn Estuary and Bristol Channel. *Biol. J. Linnean Soc.* 51, 83–91. doi: 10.1006/bijl.1994.1009

Bengtsson, H., and Wernersson, A. (2012). *TBT, Koppar, Zink Och Irgarol I Dagvatten, Slam Och Mark I Småbåtshamnar*. Göteborg: Länsstyrelsen i Västra Götaland, 16.

Berghahn, R. (1996). Episodic mass invasions of juvenile gadoids into the Wadden Sea and their consequences for the population dynamics of brown shrimp (*Crangon crangon*). *Mar. Ecol.* 17, 251–260. doi: 10.1111/j.1439-0485.1996.tb00506.x

Birchenough, A. C., Evans, S. M., Moss, C., and Welch, R. (2002). Re-colonisation and recovery of populations of dogwhelks *Nucella lapillus* (L) on shores formerly subject to severe TBT contamination. *Mar. Pollut. Bull.* 44, 652–659. doi: 10.1016/s0025-326x(01)00308-3

Bryan, G. W., Bright, D. A., Hummerstone, L. G., and Burt, G. R. (1993). Uptake, tissue distnbution and metabolism of 14C-labelled tributyltin (TBT) in the dog whelk, *Nucella lapillus*. *J. Mar. Biol. Assoc. UK.* 73, 889–912. doi: 10.1017/s0025315400034792

Bryan, G. W., Gibbs, P. E., Hugett, R. J., Curtis, L. A., Bailey, D. S., and Dauer, D. M. (1989). Effects of tributyltin pollution on the mud snail, *Ilyanassa obsolete*, from the York river and Sarah creek, Chesapeake bay. *Mar. Pollut. Bull.* 20, 458–462. doi: 10.1016/0025-326x(89)90067-2

Bryan, G. W., Gibbs, P. E., Hummerstone, L. G., and Burt, G. R. (1986). The decline of the gastropod *Nucella lapillus* around south-west England; evidence for the effect of tributyltin from antifouling paints. *J. Mar. Biol. Assoc. UK.* 66, 611–640. doi: 10.1017/s0025315400042247

Brändli, R. C., Breedveld, G. D., and Cornelissen, G. (2009). Tributyltin sorption to marine sedimentary black carbon and to amended activated carbon. *Environ. Toxicol. Chem.* 28, 503–508. doi: 10.1897/08-236.1

Bushong, S. J., Hall, L. W. Jr., Hall, W. S., Johnson, W. E., and Herman, R. L. (1988). Acute toxicity of tributyltin to selected chesapeake bay fish and invertebrates. *Water Res.* 22, 1027–1032. doi: 10.1016/0043-1354(88)90150-9

Bushong, S. J., Ziegenfuss, M. C., Unger, M. A., and Hall, L. W. (1990). Chronic tributyltin toxicity experiments with the Chesapeake bay copepod, *Acartia tonsa*. *Environ. Toxicol. Chem.* 9, 359–366. doi: 10.1002/etc.5620090311

Carter, N. W. (1972). Intracellular pH. *Kidney Int.* 1, 341–346.

Cattrijsse, A., Dankwa, H. R., and Mees, J. (1997). Nursery function of an estuarine tidal marsh for the brown shrimp *Crangon crangon*. *J. Sea Res.* 38, 109–121. doi: 10.1016/s1385-1101(97)00036-1

Champ, M. A. (1986). "Organotin symposium: introduction and review," in *Proceedings of the Conference Oceans 86*, Vol. 4, ed. G. L. Maton, (Washington, DC: Marine Technology Society), 1093–1100.

Champ, M. A. and Seligman, P. F. (Eds) (1996). *Organotin: Environmental fate and effects*. Netherlands: Springer. 475–483.

Champ, M. A. (2000). A review of organotin regulatory strategies, pending actions, related costs and benefits. *Sci. Total Environ.* 258, 21–71. doi: 10.1016/s0048-9697(00)00506-4

Chang, E. S., and Mykles, D. L. (2011). Regulation of crustacean molting: a review and our perspectives. *Gen. Comp. Endocrinol.* 172, 323–330. doi: 10.1016/j.ygcen.2011.04.003

Cole, R. F., Mills, G. A., Parker, R., Bolam, T., Birchenough, A., Kröger, S., et al. (2015). Trends in the analysis and monitoring of organotins in the aquatic environment. *Trends Environ. Anal. Chem.* 8, 1–11. doi: 10.1016/j.teac.2015.05.001

Cooney, J. J. (1988). Microbial transformations of tin and tin compounds. *J. Indust. Microbiol.* 3, 195–204. doi: 10.1007/bf01569577

Doop, E., Hartmann, L. M., von Recklinghausen, U., Florea, A. M., Rabieh, S., Shokoushi, B., et al. (2007). The cyto- and genotoxicity of organotin compounds is dependent on the cellular uptake capability. *Toxicology* 232, 226–234. doi: 10.1016/j.tox.2007.01.014

ECHA (2008). *Member State Committee Support Document for Identification of Bis(tributyltin)Oxide as a Substance of Very High Concern*. Helsinki: European Chemicals Agency.

EFSA (2004). Opinion of the scientific panel on contaminants in the food chain on a request from the commission to assess the health risks to consumers associated with exposure to organotins in foodstuffs. *EFSA J.* 102, 1–119.

Eng, G., Bathersfield, O., and May, L. (1986). Mossbauer studies of the speciation of tributyltin compounds in seawater and sediment samples. *Water Air Soil Pollut.* 27, 191–197. doi: 10.1007/bf00464781

European Commission (1989). EU Council Directive 89/677/EEC of 21 December 1989 amending for the eighth time Directive 76/769/EEC on the approximation of the laws, regulations and administrative provisions of the member states relating to restrictions on the marketing and use of certain dangerous substances and preparations. *Off. J. Eur. Union* L398, 0019–0023.

European Union (2003). EU Regulation No 782/2003 of the European Parliament and of the Council of 14 April 2003 on the Prohibition of Organotin Compounds on Ships. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2003:115:0001:0011:EN:PDF (accessed October 11, 2019).

European Union (2005). *Common Implementation Strategy for the Water Framework Directive: Environmental Quality Standards (EQS), Substance Data Sheet Priority Substance no 30: Tributyltin Compounds (TBT ion). Final Version*. Brussels: European Union.

Evans, S. M., Evans, P. M., and Leksono, T. (1996). Widespread recovery of dogwhelks, *Nucella lapillus* (L) from tributyltin contamination in the North Sea and Clyde Sea. *Mar. Pollut. Bull.* 32, 263–269. doi: 10.1016/0025-326x(95)00127-9

Fent, K. (1996). Ecotoxicology of organotin compounds. *Crit. Rev. Toxicol.* 26, 1–117.

Gadd, G. M. (2000). Microbial interactions with tributyltin compounds: detoxification, accumulation, and environmental fate. *Sci. Total Environ.* 258, 119–127. doi: 10.1016/s0048-9697(00)00512-x

Gibbs, P. E., Bryan, G. W., Pascoe, P. L., and Burt, G. R. (1987). The use of the dogwhelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination. *J. Mar. Biol. Assoc. UK.* 67, 507–523. doi: 10.1016/j.marpolbul.2015.08.038

Gibbs, P. E., Pascoe, P. L., and Burt, G. R. (1988). Sex change in the female dogwhelk, *Nucella lapillus*, induced by tributyltin from antifouling paints. *J. Mar. Biol. Assoc. UK.* 68, 715–731. doi: 10.1017/S0025315400028824

Hagger, J. A., Depledge, M. H., Oehlmann, J., Jobling, S., and Galloway, T. S. (2006). Is there a causal association between genotoxicity and the imposex effect? *Environ. Health Perspect.* 114, 20–26. doi: 10.1289/ehp.8048

Hall, L. W. Jr., Bushong, S. J., Hall, W. S., and Johnson, W. E. (1998). Acute chronic effects of tributyltin on a Chesapeake Bay copepod. *Environ. Toxicol. Chem.* 7, 41–46. doi: 10.1002/etc.5620070107

Hannas, B. R., Wang, Y. H., Thomson, S., Kwon, G., Li, H., and LeBlanc, G. A. (2010). Regulation and dysregulation of vitellogenin mRNA accumulation in daphnids (*Daphnia magna*). *Aquat. Toxicol.* 101, 351–357. doi: 10.1016/j.aquatox.2010.11.006

HELCOM (2018). *TBT and Imposex. HELCOM Core Indicator Report*. Helsinki: HELCOM.

Hopkins, P. M. (2009). "Crustacean ecdysteroids and their receptors," in *Ecdysone: Structures and Functions*, ed. G. Smagghe, (Houten: Springer Verlag), 73–97. doi: 10.1007/978-1-4020-9112-4_3

Hosamani, N., Reddy, S. B., and Reddy, R. P. (2017). Crustacean molting: regulation and effects of environmental toxicants. *J. Mar. Sci. Res. Dev.* 7:5. doi: 10.4172/2155-9910.1000236

Huang, Y., Zhu, L. Y., and Liu, G. X. (2006). The effects of bis(tributyltin) oxide on the development, reproduction and sex ratio of calanoid copepod *Pseudodiaptomus marinus*. *Estuar. Coast. Shelf Sci.* 69, 147–152. doi: 10.1016/j.ecss.2006.04.010

Huang, Y., Zhu, L. Y., Qiu, X. C., and Zhang, T. W. (2010). Effect of bis(tributyltin) oxide on reproduction and population growth rate of calanoid copepod *Schmackeria poplesia*. *Chin. J. Oceanol. Limnol.* 28, 280–287. doi: 10.1007/soo343-010-9002-9

Hunziker, R. W., Escher, B. I., and Schwarzenbach, R. P. (2001). pH dependence of the partitioning of triphenyltin and tributyltin between phosphatidylcholine liposomes and water. *Environ. Sci. Toxicol.* 35, 3899–3904. doi: 10.1021/es010043v

Hunziker, R. W., Escher, B. I., and Schwarzenbach, R. P. (2002). Acute toxicity of triorganotin compounds: different specific effects on the energy metabolism and role of pH. *Environ. Toxicol. Chem.* 21, 1191–1197. doi: 10.1002/etc.5620210611

ICES (1998). *Report of the Working Group on Crangon Fisheries and Life History (WGCRAN) ICES CM 1998/G:8*, Chap. ICES. Port-en-Bessin, 99.

ICES (2011). *Report of the Working Group on Crangon Fisheries and Life History (WGCRAN) ICES CM 2011/SSGEF:11*. Ijmuiden: ICES, 38.

ICES (2012). *Report of the Working Group on Crangon Fisheries and Life History (WGCRAN) ICES CM 2012/SSGEF:09*. Porto: ICES, 75.

ICES (2013). *Report of the Working Group on Crangon Fisheries and Life History (WGCRAN) ICES CM 2013/SSGEF:12*. Copenhagen: ICES HQ, 25.

ICES (2014). *Report of the Working Group on Crangon Fisheries and Life History (WGCRAN) ICES CM 2014/SSGEF:08*. Hamburg: ICES, 40.

ICES (2015a). *Report of the Working Group for the Assessment of Demersal Stocks in the North Sea and Skagerrak (WGNSSK) ICES CM 2014/ACOM:13*. Copenhagen: ICES HQ, 1493.

ICES (2015b). *Report of the Working Group on Crangon Fisheries and Life History (WGCRAN) ICES CM 2015/SSGEPD:07*. Ijmuiden: ICES, 58.

ICES (2016). *Interim Report of the Working Group on Crangon Fisheries and Life History (WGCRAN) ICES CM 2016/SSGEPD:07*. Oostende: ICES, 33.

ICES (2017). *Interim Report of the Working Group on Crangon Fisheries and Life His-tory (WGCRAN) ICES CM 2017/SSGEPD:06*. Hamburg: ICES, 37.

Ide, I., Witten, E. P., Fisher, J., Kalbfus, W., Zellner, A., Stroben, E., et al. (1997). Accumulation of organotin compounds in the common whelk *Buccinum undatum* and the red whelk *Neptunea antiqua* in association with imposex. *Mar. Ecol. Prog. Ser.* 152, 197–203. doi: 10.3354/meps152197

IMO resolution Mepc.195(61) (2010). *Guidelines for Survey and Certification of Anti-fouling Systems on Ships*. Available at: http://www.imo.org/blast/blastDataHelper.asp?data_id=29924&filename=195(61).pdf (accessed October 11, 2019).

IMO resolution Mepc.29(25) (1990). *Amendments to the Annex of the Protocol of 1978 Relating to the International Convention for the Prevention of Pollution from Ships, 1973*. Available at: http://www.imo.org/blast/blastDataHelper.asp?data_id=15603&filename=29(25).pdf (accessed October 11, 2019).

Jansen, S. (2002). *Das Räuber-Beutesystem Juveniler Gadiden, Grundelnund Garnelen im Wattenmeer Nördlich Von Sylt*. PhD Dissertation. University of Hamburg: Hamburg. 155.

Janssen, C., Vinckx, M., De Brabander, H., Roose, P., and Vethaak, D. (2007). *Endocrine Disruption in the Scheldt Estuary Distribution. Exposure and effects (ENDIS-RISKS). D/2007/1191/45*. Brussels: Belgian Science Policy.

Johansen, K., and Mohlenberg, F. (1987). Impairment of egg production in *Acartia tonsa* exposed to tributyltin oxide. *Ophelia* 27, 137–141. doi: 10.1080/00785236.1987.10422017

King, N., Miller, M., and de Mora, S. (1989). Tributyl tin levels for seawater, sediment, and selected species incoastal Northland and Auckland, New Zealand. *N. Z. Mar. Fresh.* 23, 287–294. doi: 10.1080/00288330.1989.9516365

Kirby, R., Beaugrand, G., and Lindley, J. A. (2009). Synergistic effects of climate change and fishing in a marine ecosystem. *Ecosystems* 12, 548–561. doi: 10.1007/s10021-009-9241-9

Kusk, K. O., and Petersen, S. (1997). Acute and chronic toxicity of tributyltin and linear alkylbenzene sulfonate to the marine copepod *Acartia tonsa*. *Environ. Toxicol. Chem.* 16, 1629–1633. doi: 10.1002/etc.5620160810

Lagadic, L., Katsiadaki, I., Biever, R., Guiney, P. D., Karouna-Renier, N., Schwarz, T., et al. (2017). "Tributyltin: advancing the science on assessing endocrine disruption with an unconventional endocrine-disrupting compound," in *Reviews of Environmental Contamination and Toxicology*, Vol. 245, ed. P. De Voogt, (Cham: Springer), 65–127. doi: 10.1007/398_2017_8

Langston, W. J., and Pope, N. D. (1995). Determinants of TBT adsorption and desorption in estuarine sediments. *Mar. Pollut. Bull.* 31, 32–43. doi: 10.1016/0025-326x(95)91269-m

Langston, W. J., Pope, N. D., Davey, M., Langston, K. M., O'Hara, S. C. M., Gibbs, P. E., et al. (2015). Recovery from TBT pollution in english channel environments: a problem solved? *Mar. Pollut. Bull.* 95, 551–564. doi: 10.1016/j.marpolbul.2014.12.011

Laufer, H., Demir, N., Pan, X., Stuart, J. D., and Ahl, J. S. B. (2005). Methyl farnesoate controls adult male morphogenesis in the crayfish (*Procambarus clarkia*). *J. Insect Physiol.* 51, 379–384. doi: 10.1016/j.jinsphys.2005.02.007

Laughlin, R. B. Jr., and French, W. J. (1980). Comparative study of the acute toxicity of a homologous series of trialkyltins to larval shore crabs, *Hemigrapsus nudus*, and lobster, *Homarus americanus*. *Bull. Environ. Contam. Toxicol.* 25, 802–809. doi: 10.1007/bf01985611

Lee, C. C., Hsieh, C.-Y., and Tien, C.-J. (2006). Factors influencing organotin distribution in different marine environmental compartments, and their potential health risk. *Chemosphere* 65, 547–559. doi: 10.1016/j.chemosphere.2006.02.037

Luttikhuizen, P. C., Campos, J., van Bleijswijk, J., Peijnenburg, K. T. C. A., and van der Veer, H. W. (2008). Phylogeography of the common shrimp, *Crangon crangon* (L.) across its distribution range. *Mol. Phylogenet. Evol.* 46, 1015–1030. doi: 10.1016/j.ympev.2007.11.011

Ma, H. Z., Dai, S. G., and Huang, G. L. (2000). Distribution of tributyltin chloride in laboratory simulated estuarine microcosms. *Water Res.* 34, 2829–2841. doi: 10.1016/s0043-1354(00)00032-4

Massaro, E. J., Zucker, R. M., Elstein, K. H., Ting-Beall, H. P., and Easterling, R. E. (1989). Fixation of the plasma membrane/cytoplasm complex: a mechanism of toxic interaction of tributyltin. *Biol. Trace Elem. Res.* 21, 305–312. doi: 10.1007/bf02917268

Matthiessen, P. (2013). Detection, monitoring and control of tributyltin – An almost complete success story. *Environ. Toxicol. Chem.* 32, 487–489. doi: 10.1002/etc.2108

McCarty, L. S. (1991). "Toxicant body residues: implications for aquatic bioassays with some organic chemicals," in *Aquatic Toxicology and Risk Assessment. STP 1124*, Vol. 14, eds M. A. Mayes, and M. G. Barron, (Philadelphia, PA: American Society for Testing and Materials), 183–192.

Meador, J. P. (1993). The effect of laboratory holding on the toxicity response of marine infaunal amphipods to cadmium and tributyltin. *J. Exp. Mar. Biol. Ecol.* 174, 227–242. doi: 10.1016/0022-0981(93)90019-k

Meador, J. P. (1997). Comparative toxicokinetics of tributyltin in five marine species and its utility in predicting bioaccumulation and toxicity. *Aquat. Toxicol.* 37, 307–326. doi: 10.1016/s0166-445x(96)00827-2

Meador, J. P. (2000). Predicting the fate and effects of tributyltin in marine systems. *Rev. Environ. Contam. Toxicol.* 166, 1–48.

Meador, J. P. (2011). *Organotins in Aquatic Biota: Occurrence in Tissue and Toxicological Significance*. Washington, DC: U.S. Department of Commerce, 552.

Mensink, B. P., ten Hallers-Tjabbes, C. C., Kralt, J., Freriks, I. L., and Boon, J. P. (1996a). Assessment of imposex in the common whelk, *Buccinum undatum* (L.) from the Eastern Scheldt, the Netherlands. *Mar. Environ. Res.* 41, 315–325. doi: 10.1016/0141-1136(95)00022-4

Mensink, B. P., van Hattum, B., Vethaak, A. D., and Boon, J. P. (1996b). *The Development of Imposex in Relation to Organotin Contamination in the Common Whelk, Buccinum undatum. Field observations in the Eastern Sheldt and experimental exposure in a laboratory study*. den Burg: Netherlands Institute for Sea Research (NIOZ).

Morton, B. (2009). Recovery from imposex by a population of the dog whelk, *Nucella lapillus* (Gastropoda: Caenogastropoda), on the southeastern coast of England since May 2004: a 52-month study. *Mar. Pollut. Bull.* 58, 1530–1538. doi: 10.1016/j.marpolbul.2009.05.012

Nagabhushanam, R., Reddy, P. S., and Sarojini, R. (1990). Tributyltin oxide induced alterations in exuvial weight and calcium content of the prawn, *Caridina rajadhari*. *Proc. Anim. Sci.* 99, 397–400. doi: 10.1007/bf03191873

Nicolaus, E. E. M., and Barry, J. (2015). Imposex in the dogwhelk (*Nucella lapillus*): 22 years monitoring around England and Wales. *Environ. Monit. Assess.* 187:736. doi: 10.1007/s10661-015-4961-0

Nishikawa, J., Mamiya, S., Kanayama, T., Nishikawa, T., Shiraishi, F., and Horiguchi, T. (2004). Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. *Environ. Sci. Technol.* 38, 6271–6276. doi: 10.1021/es049593u

Oberdörster, E., Rittschof, D., and LeBlanc, G. A. (1998). Alteration of [14C]-testosterone metabolism after chronic exposure of *Daphnia magna* to tributyltin. *Arch. Environ. Contam. Toxicol.* 34, 21–25. doi: 10.1007/s002449900281

Oehlmann, J., Bauer, B., Minchin, D., Schulte-Oehlmann, U., Fioroni, P., and Markert, B. (1998). Imposex in *Nucella lapillus* and intersex in *Littorina littorea*: interspecific comparison of two TBT-induced effects and their geographical uniformity. *Hydrobiologia* 378, 199–213. doi: 10.1007/978-94-011-5336-2_22

Oehlmann, J., Di Benedetto, P., Tilmann, M., Duft, M., Oetken, M., and Schulte-Oehlmann, U. (2007). Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. *Ecotoxicology* 16, 29–43. doi: 10.1007/s10646-006-0109-x

Oehlmann, J., Stroben, E., and Fioroni, P. (1992). The rough tingle *Ocenebra erinace* (Gastropoda Murcidae): an exhibitor of imposex in comparison to *Nucella lapillus*. *Helgoländer Meeresunters* 46, 311–328. doi: 10.1007/bf02367102

Ohji, M., Arai, T., and Miyazaki, N. (2002). Effects of tributyltin exposure in the embryonic stage on sex ratio and survival rate in the caprellid amphipod *Caprella danilevskii*. *MEPS* 235, 171–176. doi: 10.3354/meps235171

Oliveira, I. B., Richardson, C. A., Sousa, A. C. A., Takahashi, S., Tanabe, S., and Barroso, C. M. (2009). Spatial and temporal evolution of imposex in dog whelk *Nucella lapillus* (L.) populations from North Wales, UK. *J. Environ. Monit.* 11, 1462–1468. doi: 10.1039/b906766c

Omae, I. (1989). Organotin chemistry. 355.

OSPAR (2010). *Quality Status Report 2010*. London: OSPAR Commission, 2010.

OSPAR (2014). *Imposex and TBT: Status, Trends and Effects in Marine Molluscs: an Improving Situation?* London: OSPAR Commission.

OSPAR (2017a). Available at: https://oap.ospar.org/en/ospar-assessments/intermediate-assessment-2017/pressures-human-activities/contaminants/organotin-sediment/ (accessed October 11, 2019).

OSPAR (2017b). Available at: https://oap.ospar.org/en/ospar-assessments/intermediate-assessment-2017/pressures-human-activities/contaminants/imposex-gastropods/ (accessed October 11, 2019).

OSPAR Commission (2011). *Background Document on Organic tin Compounds*. London: OSPAR Commission, 33.

Pignatello, J. J., and Xing, B. (1996). Mechanisms of slow sorption of organic chemicals to natural particles. *Environ. Sci. Technol.* 30, 1–11. doi: 10.1021/es940683g

Pörtner, H. O., Boutilier, R. G., Tang, Y., and Toews, D. P. (1990). Determination of intracellular pH and PCO2 after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol.* 81, 255–273. doi: 10.1016/0034-5687(90)90050-9

Rainbow, P. S. (1995). Biomonitoring of heavy metal availability in the marine environment. *Mar. Pollut. Bull.* 31, 183–192. doi: 10.1016/0025-326X(95)00116-5

Reddy, P. R., Nagaraju, G. P. C., and Reddy, P. S. (2004). Involvement of methyl farnesoate in the regulation of molting and reproduction in the freshwater crab *Oziotelphusa senex* senex. *J. Crust. Biol.* 24, 511–515. doi: 10.1651/C-2478

Reddy, P. S., Nagabhushanam, R., and Sarojini, R. (1992). Retardation of moulting in the prawn, *Caridina rajadhari*, exposed to tributyltin oxide (TBTO). *Proc. Nat. Acad. Sci. India* 62, 353–356.

Reddy, P. S., Sarojini, R., and Nagabhushanam, R. (1991). Impact of tributyltin oxide (TBTO) on limb regeneration of the prawn, *Caridina rajadhari*, after exposure to different time intervals of amputation. *J. Tiss. Res.* 1, 35–39.

Rice, S. D., Short, J. W., and Stickle, W. B. (1989). Uptake and catabolism of tributyltin by blue crabs fed TBT contaminated prey. *Mar. Environ. Res.* 27, 137–145. doi: 10.1016/0141-1136(89)90005-6

Rodríguez, J. G., Tueros, I., Borja, A., Franco, J., García Alonso, J. I., Garmendia, J. M., et al. (2009). Butyltin compounds, sterility and imposex assessment in *Nassarius reticulates* (Linneaus, 1758), prior to the 2008 European ban on TBT antifouling paints, within Basque ports and coastal areas. *Cont. Shelf Res.* 29, 1165–1173. doi: 10.1016/j.csr.2009.01.005

Schmidt, K., Steinberg, C. E. W., Staaks, G., and Pflugmacher, S. (2005). Influence of a xenobiotic mixture (PCB and TBT) compared to single substances on swimming behavior or reproduction of *Daphnia magna*. *Acta Hydrochim. Hydrobiol.* 33, 287–300. doi: 10.1002/aheh.200400579

Schøyen, M., Green, N. W., Hjermann, D. Ø, Tveiten, L., Beylich, B., Øxnevad, S., et al. (2018). Levels and trends of tributyltin (TBT) and imposex in dogwhelk (*Nucella lapillus*) along the Norwegian coastline from 1991 to 2017. *Mar. Environ. Res.* 144, 1–8. doi: 10.1016/j.marenvres.2018.11.011

Siegel, V., Damm, U., and Neudecker, T. (2008). Sex-ratio, seasonality and long-term variation in maturation and spawning of the brown shrimp *Crangon crangon* (L.) in the German Bight (North Sea). *Helgoland Mar. Res.* 62, 339–349. doi: 10.1007/s10152-008-0121-z

Siegel, V., Gröger, J., Neudecker, T., Damm, U., and Jansen, S. (2005). Longterm variation in the abundance of the brown shrimp *Crangon crangon* (L.) population of the German Bight and possible causes for its interannual variability. *Fish Oceanogr.* 14, 1–16. doi: 10.1111/j.1365-2419.2004.00301.x

Smith, B. S. (1981). Tributyltin compounds induce male characteristics on female mud snails *Nassarius obsoletus* = *Ilyanassa obsolete*. *J. Appl. Toxicol.* 1, 141–144. doi: 10.1002/jat.2550010302

Sousa, A. C. A., Barroso, C. M., Tanabe, S., and Horiguchi, T. (2010). "Involvement of retinoid X receptor in imposex development in *Nucella lapillus* and *Nassarius reticulatus* - Preliminary results," in *Interdisciplinary Studies on Environmental Chemistry - Biological Responses to Contaminants*, eds N. Hamamura, S. Suzuki, S. Mendo, C. M. Barroso, H. Iwata, and S. Tanabe, (Tokyo: Terrapub), 189–196.

Sousa, A. C. A., Laranjeiro, F., Takahashi, S., Tanabe, S., and Barroso, C. M. (2009). Imposex and organotin prevalence in a European post-legislative scenario: temporal trends from 2003 to 2008. *Chemosphere* 77, 566–573. doi: 10.1016/j.chemosphere.2009.06.049

Sternberg, R. M., Gooding, M. P., Hotchkiss, A. K., and LeBlanc, G. A. (2010). Environmental-endocrine control of reproductive maturation in gastropods: implications for the mechanism of tributyltin induced imposex in prosobranchs. *Ecotoxicology* 19, 4–23. doi: 10.1007/s10646-009-0397-z

Strand, J., and Jacobsen, J. A. (2005). Accumulation and trophic transfer of organotins in a marine food web from the Danish coastal waters. *Sci. Total Environ.* 350, 72–85. doi: 10.1016/j.scitotenv.2005.02.039

Stroben, E. (1994). *Imposex Und Weitere Effekte Von Chronischer TBT-Intoxikation bei Einigen Mesogastropoden und Bucciniden (Gastropoda, Prosobranchia)*. Göttingen: Cuvillier.

Stronckhorst, J., and van Hattum, B. (2003). Contaminants of concern in Dutch marine harbor sediments. *Arch. Environ. Contam. Toxicol.* 45, 306–316.

Sunday, A. O., Alafara, B. A., and Oladele, O. G. (2012). Toxicity and speciation analysis of organotin compounds. *Chem. Spec. Bioavailab.* 24, 216–226. doi: 10.3184/095422912X13491962881734

Takahashi, S., Tanabe, S., Takeuchi, I., and Miyazaki, N. (1999). Distribution and specific bioaccumulation of butyltin compounds in a marine ecosystem. *Arch. Environ. Contam. Toxicol.* 37, 50–61. doi: 10.1007/s002449900489

Tanabe, S. (1999). Butyltin contamination in marine mammals, a review. *Mar. Pollut. Bull.* 39, 62–72. doi: 10.1016/s0025-326x(99)00064-8

Temming, A. (1995). *Die quantitative Bestimmung der Konsumtion von Fischen. Experimentelle, Methodische und theoretische Aspekte*. Hamburg: Universität Hamburg.

Temming, A., and Hufnagl, M. (2014). Decreasing predation levels and increasing landings challenge the paradigm of non-management of North Sea brown shrimp (*Crangon crangon*). *ICES J. Mar. Sci.* 72, 804–823. doi: 10.1093/icesjms/fsu194

ten Hallers-Tjabbes, C. C., Mensink, B. P., van Hattum, B., and Boon, J. P. (1996). "Impact of traditional antifouling in open sea, risk and ecological consequences," in *Proceedings of the International Sysmposium on Directorate-General for Shipping and Marine Affairs and the Organotin Environmental Programme Association*, (The Hague), 36–42.

Tiews, K. (1961). The role of whiting as an undesirable guest in German coastal waters. *ICES Near Northern Seas Committee* 28, 137–143.

Tsuda, T., Nakanishi, H., Aoki, S., and Takebayashi, J. (1986). Bioconcentration of butyltin compounds by round crucian carp. *Toxicol. Environ. Chem.* 12, 137–143. doi: 10.1080/02772248609357154

Tsuda, T. S., Aoki, S., Kojima, M., and Harada, H. (1990). The influence of pH on the accumulation of tri-n-butyltin chloride and triphenyltin chloride in carp. *Comp. Biochem. Physiol.* 95C, 151–153. doi: 10.1016/0742-8413(90)90096-r

Unger, M. A., MacIntyre, W. G., and Huggett, R. J. (1988). Sorption behaviour of tributyltin on estuarine and freshwater sediments. *Environ. Toxicol. Chem.* 7, 907–915. doi: 10.1002/etc.5620071107

United States Environmental Protection Agency [EPA. US], (1985). *Office of Pesticide Programs. (1985). Tributyltin Support Document*. Washington, D.C: United States Environmental Protection Agency.

United States Environmental Protection Agency [EPA.US], (2007). *Ambient Aquatic Life Water Quality Criteria for Tributyltin (TBT)*. Washington, D.C: United States Environmental Protection Agency.

van Wezel, A. P., and Opperhuizen, A. (1995). Narcosis due to environmental pollutants in aquatic organisms: residue-based toxicity, mechanisms, and membrane burdens. *Crit. Rev. Toxicol.* 25, 255–279. doi: 10.3109/10408449509089890

Veltman, K., Huijbregts, M. A. J., van den Heuvel-Greve, M. J., Vethaak, A. D., and Hendriks, A. J. (2006). Organotin accumulation in an estuarine food chain: comparing field measurements with model estimations. *Mar. Environ. Res.* 61, 511–530. doi: 10.1016/j.marenvres.2006.02.005

Verhaegen, Y. (2012). *Mechanism, Concentrations and Effects of Tributyltin in Common Shrimp Crangon crangon*. PhD. Dissertation. Ghent University: Ghent.

Verhaegen, Y., Monteyne, E., Neudecker, T., Tulp, I., Smagghe, G., Cooreman, K., et al. (2012). Organotins in North Sea brown shrimp (*Crangon crangon* L.)

after implementation of the TBT ban. *Chemosphere* 86, 979–984. doi: 10.1016/j.chemosphere.2011.11.028

Verhaegen, Y., Parmentier, K., Swevers, L., Renders, E., Rougé, P., De Coen, W., et al. (2011). The heterodimeric ecdysteroid receptor complex in the brown shrimp *Crangon crangon*: EcR and RXR isoform characteristics and sensitivity towards the marine pollutant tributyltin. *Gen. Comp. Endocrinol.* 172, 158–169. doi: 10.1016/j.ygcen.2011.02.019

Verhaegen, Y., Parmentier, K., Swevers, L., Rougé, P., Soin, T., De Coen, W., et al. (2010). The brown shrimp (*Crangon crangon* L.) ecdysteroid receptor complex: cloning, structural modeling of the ligand-binding domain and functional expression in an EcR-deficient Drosophila cell line. *Gen. Comp. Endocrinol.* 168, 415–423. doi: 10.1016/j.ygcen.2010.05.007

Waldock, M. J., Waite, M. E., and Thain, J. E. (1988). Inputs of TBT to the marine environment from shipping activity in the UK. *Environ. Technol. Lett.* 9, 999–1010. doi: 10.1080/09593338809384662

Wang, Y. H., Kwon, G., Li, H., and LeBlanc, G. A. (2011). Tributyltin synergizes with 20-hydroxyecdysone to produce endocrine toxicity. *Toxicol. Sci.* 123, 71–79. doi: 10.1093/toxsci/kfr154

Wang, Y. H., and LeBlanc, G. A. (2009). Interactions of methyl farnesoate and related compounds with a crustacean retinoid X receptor. *Mol. Cell. Endocrinol.* 309, 109–116. doi: 10.1016/j.mce.2009.05.016

Watermann, B., and Dethlefsen, V. (1983). Black spot disease of shrimps - an environmental problem, a disease or wounds. *Fischwirtsch* 30, 24–29.

Welleman, H. C., and Daan, N. (2001). Is the Dutch Shrimp Fishery Sustainable? *Senckenbergiana Maritima* 31, 321–328. doi: 10.1007/bf03043040

Wetzel, M. A., Winterscheid, A., and Wahrendorf, D.-S. (2013). Baseline of the butyltin distribution in surface sediments (0 – 20cm) of the Elbe estuary (Germany, 2011). *Mar. Pollut. Bull.* 77, 418–423. doi: 10.1016/j.marpolbul.2013.09.021

Willemsen, F., Wegener, J.-W., Morabito, R., and Pannier, F. (2004). *Sources, Consumer Exposure and Risks of Organotin Contamination in Seafood. Final Report of the European Commission, 2004. Research Project OT-SAFE (QLK1-2001-01437).*

Wilson, J. G., Minchin, D., McHugh, B., McGovern, E., Tanner, C. J., and Giltrap, M. (2015). Declines in TBT contamination in Irish coastal waters 1987 – 2011, using the dogwhelk (*Nucella lapillus*) as a biological indicator. *Mar. Pollut. Bull.* 100, 289–296. doi: 10.1016/j.marpolbul.2015.08.038

Wolf, G. (2006). Is 9-cis-retinoic acid the endogenous ligand for the retinoic acid-X receptor? *Nutr. Rev.* 64, 532–538. doi: 10.1111/j.1753-4887.2006.tb00186.x

Wu, X., Hopkins, P. M., Palli, S. R., and Durica, D. S. (2004). Crustacean retinoid-X receptor isoforms: distinctive DNA binding and receptor-receptor interaction with a cognate ecdysteroid receptor. *Mol. Cell. Endocrinol.* 218, 21–38. doi: 10.1016/j.mce.2003.12.013

Zucker, R. M., Elstein, K. H., Easterling, R. E., and Massaro, E. J. (1989). Flow cytometric comparison of the effects of trialkyltins on the murine erythroleukemic cell. *Toxicol.* 58, 107–119. doi: 10.1016/0300-483x(89)90001-2

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

ORIGINAL RESEARCH
published: 15 November 2019
doi: 10.3389/fmars.2019.00688

Mussel Caging and the Weight of Evidence Approach in the Assessment of Chemical Contamination in Coastal Waters of Finland (Baltic Sea)

Kari K. Lehtonen[1], Giuseppe d'Errico[2], Samuli Korpinen[1], Francesco Regoli[2], Heidi Ahkola[3], Tanja Kinnunen[1] and Anu Lastumäki[1]*

[1] Marine Research Centre, Finnish Environment Institute, Helsinki, Finland, [2] Dipartimento Di Scienze Della Vita E Dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy, [3] Finnish Environment Institute, Jyväskylä Office, Jyväskylä, Finland

OPEN ACCESS

Edited by:
Francois Galgani, Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), France

Reviewed by:
Periyadan K. Krishnakumar, King Fahd University of Petroleum and Minerals, Saudi Arabia
Halldór Pálmar Halldórsson, University of Iceland, Iceland

***Correspondence:**
Kari K. Lehtonen
kari.lehtonen@ymparisto.fi

Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *28 February 2019*
Accepted: *25 October 2019*
Published: *15 November 2019*

Citation:
Lehtonen KK, d'Errico G, Korpinen S, Regoli F, Ahkola H, Kinnunen T and Lastumäki A (2019) Mussel Caging and the Weight of Evidence Approach in the Assessment of Chemical Contamination in Coastal Waters of Finland (Baltic Sea). Front. Mar. Sci. 6:688. doi: 10.3389/fmars.2019.00688

Contamination status of coastal areas of Finland (northern Baltic Sea) markedly affected by anthropogenic activities (harbors, shipyards and maritime activity, industry, municipal and agricultural inputs, legacy contamination) was assessed for the first time using the weight of evidence (WOE) approach. The key element of the study was the caging (transplantation) of Baltic mussels (*Mytilus trossulus*) for the measurement of tissue accumulation of polycyclic aromatic hydrocarbons (PAHs) and applying a suite of biomarkers of biological effects of contaminants. Additional variables included in the assessment were trace metals in seawater, macrozoobenthos, near-bottom oxygen levels and eutrophication indicators. The chemical parameters were supported by passive sampling of PAHs and organotins at the study sites. The integrated approach combining all the line of evidence (LOE) variables into the WOE showed separation of some sites as more affected by hazardous substances than others, with the most contaminated areas found around harbor and ship yard areas. The contaminant levels measured in the different matrices were not alarmingly high at none of the areas compared to many other areas within or outside the Baltic Sea under more heavy anthropogenic impact, rarely exceeding any given threshold values for Good Environmental Status of the EU Marine Strategy Framework Directive. However, significant biological effects were recorded in mussels in the most contaminated sites, signifying that the combined effects caused by the contaminants and other environmental factors are disturbing the health of marine organisms in the area. The results of this successful combined application based on the mussel transplantation method and the WOE approach are highly encouraging for further trials in developing the monitoring of chemical contamination in the Baltic Sea.

Keywords: Baltic Sea, biomonitoring, marine pollution, mussel caging, biomarkers, weight of evidence, integrated assessment

INTRODUCTION

Mussels belonging to the genus *Mytilus* are globally used bioindicator organisms in marine pollution monitoring (see e.g., recent reviews by Beyer et al., 2017; Krishnakumar et al., 2018, and references within). By efficiently filtering large amounts of water and using waterborne particles as food they become exposed to extensive amounts of various types of hazardous substances in the dissolved form or as bound to particles (Viarengo and Canesi, 1991). In the low-diversity brackish-water ecosystem of the Baltic Sea the Baltic mussel (*Mytilus trossulus*) is an ecological key species (Koivisto and Westerbom, 2010; Väinölä and Strelkov, 2011), which has been used in biomonitoring and case studies concerning chemical contamination (e.g., Baršienė et al., 2006; Kopecka et al., 2006; Lehtonen et al., 2006a; Schiedek et al., 2006). By applying the caging approach, i.e., transplantation of bioindicator organisms, it is possible to expose them in sites of interest along a pollution gradient or hot spots; this approach has been successfully undertaken in various sea areas (e.g., Andral et al., 2004, 2011; Regoli et al., 2004; Smolders et al., 2004; Damiens et al., 2007; Tsangaris et al., 2010; Serafim et al., 2011; Marigómez et al., 2013; Lekube et al., 2014; Moschino et al., 2016), and also in the Baltic Sea (Rank et al., 2007; Dabrowska et al., 2013; Turja et al., 2013, 2014, 2015; Lehtonen et al., 2016).

In the current study, mussels were transplanted in 2016 and 2017 in cages along the Baltic Sea coast of Finland outside 10 main cities with significant ports to assess the contamination status of the areas. The cages were deployed for ca. two months and the mussels were examined for selected biomarker responses [antioxidative defense system (ADS), biotransformation, and neurotoxicity], and for the accumulation of polycyclic aromatic hydrocarbons (PAH) and organotin compounds. In addition, passive samplers were deployed in the cages for the detection of the aforementioned groups of compounds. As biomarkers of the ADS response and damage caused by oxidative stress enzymatic activities of catalase (CAT) and glutathione reductase (GR), and the level of lipid peroxidation (LPO) were measured in the digestive gland of mussels. Phase II biotransformation enzyme glutathione *S*-transferase (GST), also involved in ADS, was determined in the same tissue. Activity of acetylcholinesterase (AChE), an indicator of neurotoxic effects, was measured in the gill tissue. A morphometric body condition index (CI) was determined as a background parameter for the nutritional status of the mussels.

Polymer based passive samplers are increasingly used in studying harmful substances in different environmental matrices (Smedes et al., 2009; Rusina et al., 2010; Tucca et al., 2014; Pintado-Herrera et al., 2016). They enable the determination of low concentrations of hydrophobic substances which in grab water samples remain below the detection limit. The samplers measure only freely dissolved chemical concentration which can penetrate through cell walls and be harmful to organisms (Mayer et al., 2003; Alvarez et al., 2004; Vrana et al., 2005). In this study, passive samplers were deployed at half of the mussel caging sites for the additional detection of PAH and organotin compounds.

A classical weight of evidence (WOE) elaboration was applied to integrate all the data for a holistic assessment of the health status of the study sites. The WOE approach combines data from different typologies of investigations, or lines of evidence (LOEs), and typically integrates chemical results with assessment of various biological effects (Piva et al., 2011; Benedetti et al., 2012). Individual LOEs are independently elaborated to summarize specific Hazard Quotients (HQs) before their final integration when a different weight is given according to their ecological relevance. Among the advantages of a similar approach is the possibility to discriminate differences between evaluations from various typologies of studies. Despite the choice of the most appropriate LOEs depends on local objectives and specificities, WOE studies have been increasingly adopted for environmental quality characterization and as a fundamental component within Ecological Risk Assessment (ERA) strategies and recommended within recent European directives for evaluation of ecological status through multiple quality indicators (Benedetti et al., 2012).

In the present work, a WOE investigation was applied integrating chemical characterization of water (LOE1), bioaccumulation (LOE2), and biomarkers (LOE3) in caged mussels, benthic community status (LOE4). Data on oxygen level and eutrophication conditions were further considered and integrated as additional LOEs (LOE5 and LOE6) in the final WOE elaboration.

MATERIALS AND METHODS

Study Sites, Collection of Mussels, and the Caging Set-Up

Mussels for the caging experiment were collected by scuba diving in Hanko, Finland (**Figure 1**) in May in 2016 and 2017 and transported in ambient seawater in thermo-insulated boxes to the Marine Research Laboratory of the Finnish Environment Institute (SYKE) in Helsinki. Mussels with shell length of 25–30 mm were selected and epibionts were removed from the shell surfaces. The mussels were kept at 10°C for 2 days in aerated water collected from the sampling site. In 2016, the cagings were done at five sites outside the cities of Kotka (site depth: 21 m), Helsinki (21 m), Parainen (38 m), Pori (23 m), and Vaasa (24 m) (five sites), and in 2017 at five sites at Porvoo (17 m), Hanko (28 m), Naantali (15 m), Uusikaupunki (18 m), and Rauma (13 m) (**Table 1**). The deployment sites covered the whole area of natural occurrence of the species in Finnish coastal waters with variability in some environmental factors. Salinity ranged from around 4.5 (Kotka) to 6.3 (Hanko) at the Gulf of Finland sites, from 4.7 to 5.2 at the Archipelago Sea sites, and from 5.8 (Uusikaupunki) to 5.0 (Vaasa) at the Bothnian Sea sites.

Mussels were placed inside the cages for ca. 8–9 weeks until recovery in late July. In this sea area the major spawning period of mussels is early summer (Benito et al., 2019). Although the general seasonal reproduction pattern is quite clear, the exact timing is dependent on various environmental factors (e.g., nutrition and temperature) that differ between locations, even on a small scale. In addition, even within a population it is common to observe individuals in a different spawning stage. Thus, by the end of the exposure period the mussels at all the study sites had most likely recovered from spawning stress.

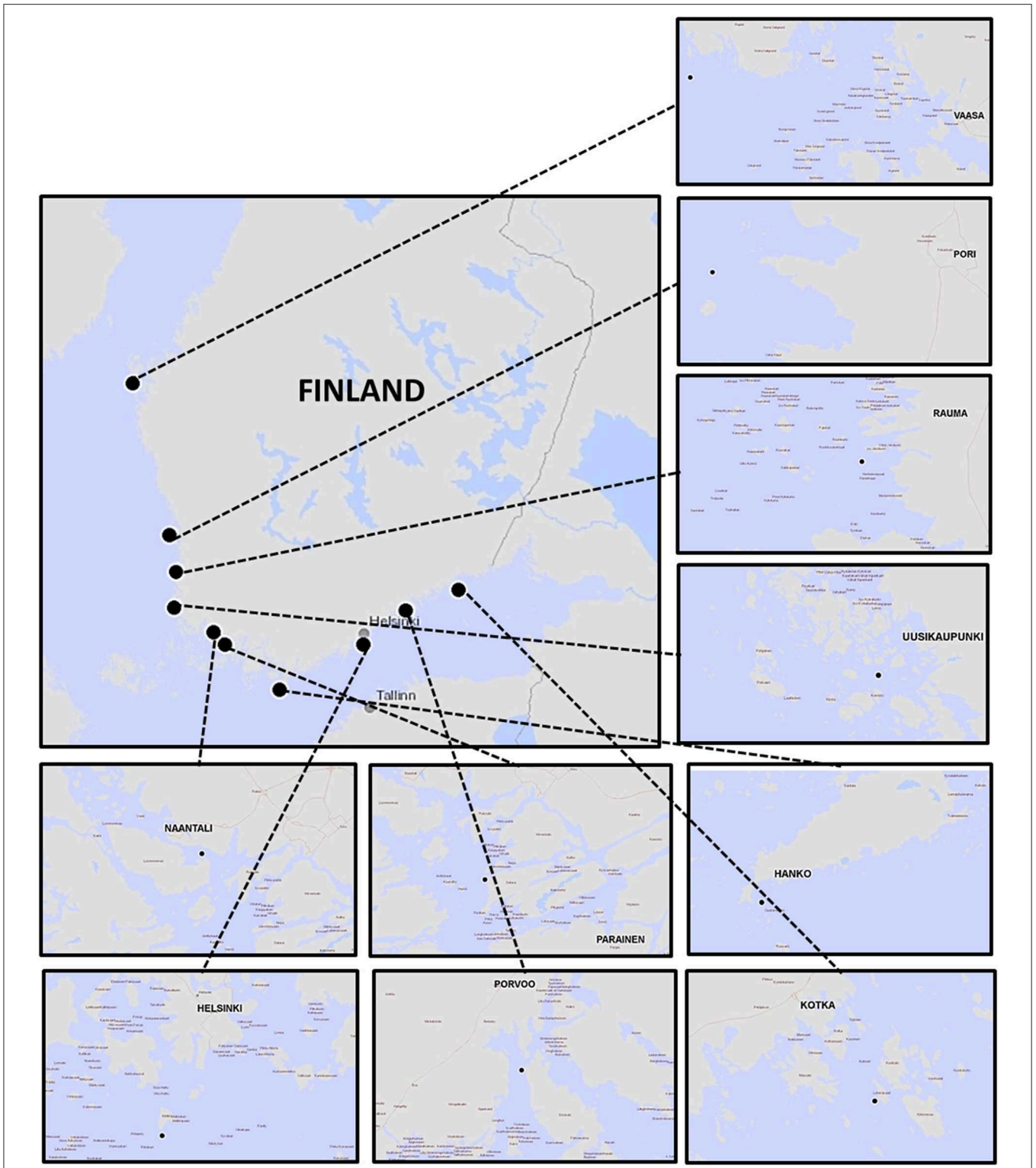


FIGURE 1 | Study sites along the coast of Finland. The black dot in each site map represent the exact site of the mussel cage.

The mussel cages were constructed of stainless steel and had a rectangular shape (height 60 cm, width 40 × 40 cm). Within the frame structure there were five easily removable steel net boxes (mesh size of 10 × 10 mm) in which the mussels (ca. 200 individuals per cage) were randomly distributed. The cages were deployed at the water depth of 7–8 m, anchored to the bottom

TABLE 1 | Some key characteristics of the study sites.

City	Inhabitants	Main pollution sources	Location of cage
Kotka	54,000	Old paper mill industry, harbor, shipyard, maritime traffic, river input	Open archipelago
Porvoo	51,000	Major oil refinery and terminal, small boat harbor, river input	Dense archipelago
Helsinki	650,000 (1.100,000)*	Maritime traffic, major WWTP, river input	Open sea
Hanko	8,500	Maritime traffic, harbor, small boat harbor	Open archipelago
Parainen	16,000 (190,000)**	Agriculture, mining	Very dense archipelago
Naantali	20,000 (190,000)**	Shipyard, major oil refinery and terminal, agriculture, major river input	Very dense archipelago
Uusikaupunki	16,000	Industry, harbor, agriculture	Dense archipelago
Rauma	40,000	Shipyard, industry, agriculture	Dense archipelago
Pori	85,000	Industry, harbor, agriculture, river input	Open sea
Vaasa	68,000	Agriculture, maritime traffic, river input	Open archipelago

Helsinki-Espoo-Vantaa metropolitan area.
**City of Turku nearby up the River Aura.*

with a rope attached to an object weighing ca. 70 kg, and held in a stable vertical position by submerged buoys. For maritime safety, each cage was marked with a 4 m ODAS buoy equipped with radar reflectors and a light blinking at 20 s intervals.

After the recovery of the cages the mussels were immediately dissected aboard the vessel for biomarker and chemical analyses. Digestive gland and gill tissues were taken for the various enzymatic biomarker analyses ($n = 15$ individuals), snap-frozen in liquid nitrogen and later stored at −80°C until analysis. Shell length of individual mussels was measured for the morphometric condition index (CI) determination ($n = 15$). For chemical contamination measurements the whole soft tissue was dissected from 60 to 120 individuals after a depuration period of 4–5 h. These samples were pooled (2–4 pools) and stored in glass vials in a −20°C freezer.

Biomarker Measurements

ADS, Biotransformation Phase II Activity, and Neurotoxicity

The ADS response was assessed by measuring the enzymatic activity of CAT, GR, and SOD as well as the level of lipid peroxidation (LPO). GST was measured for biotransformation Phase II activity, and acetylcholinesterase activity (AChE) was measured to assess neurotoxicity. For the enzymatic assays, digestive glands of mussels ($n = 15$) were homogenized in potassium phosphate buffer (100 mM, pH 7.4) and gills ($n = 15$) in sodium phosphate buffer (200 mM, pH 7.0) containing 0.1% Triton-X.

GST activity was estimated by measuring the formation rate of the conjugated substrate [chlorodinitrobenzene (CNDB)-glutathione (GSH)] at 340 nm (Habig et al., 1974). Final concentrations of 1 mM CNDB (Sigma 237329) and 1 mM GSH (Sigma G6529) in potassium phosphate buffer (100 mM, pH 7.0) were used in the reaction. CAT activity was measured as CAT mediated degradation of hydrogen peroxide (H_2O_2) at 240 nm (Claiborne, 1985). The reaction mixture contained 4.3 μM H_2O_2 (Fluka 95302) in potassium phosphate buffer (100 mM, pH 7.0). GR activity was measured indirectly as consumption of NADPH in the reduction of oxidized glutathione (GSSG) (Carlberg and Mannervik, 1975). The reaction mixture contained 2 mM EDTA (Sigma E5134), 0.5 mM GSSG (Sigma G4376), and 0.1 mM NADPH (Sigma N7505) in potassium phosphate buffer (100 mM, pH 7.5). Levels of LPO were measured as the generation of thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979). The reaction mixture contained 0.24 M trichloroacetic acid (Riedel de Haën 33731), 60 mM Tris-HCl with 0.1 mM DTPA and 16 mM 2-thiobarbituric acid (Sigma T5500). The amount of TBARS was measured by reading absorbance at 535 nm. Analyses of AChE activity were performed from gill samples as described in Bocquené and Galgani (1998) with modifications as in Leiniö and Lehtonen (2005).

All the enzymatic assays, LPO, and protein content (Bradford, 1976) used for the calculation of specific enzymatic activities were measured in 96-well microplates using the TECAN Infinite 200 (TECAN) spectrophotometer with Magellan software.

Morphometric Condition Index

The CI determined in mussels ($n = 15$) using the formula CI = (soft tissue dry weight [mg]/shell length $[mm]^2$) * 100.

Contaminant Measurements in Soft Tissues of Mussels

PAH Compounds

Pooled samples of mussel soft tissue were homogenized and 5–10 g was taken for the extraction. Internal (Fluoro-PAHs, Chiron) and yield PAH standards were added to the homogenate. Five milliliter of water and 10 ml of ethylacetate were added and the samples were shaken for 1 min. A salt mixture consisting of 4 g $MgSO_4$ and 2 g NaCl was added. The samples were again shaken for 1 min and then centrifuged for 10 min. Five milliliters of the ethylacetate extract supernatant was taken and 200 μl of isooctane added to the extract. Ethylacetate was evaporated under a nitrogen flow and 1 ml of hexane was added. The

extract was purified in a column containing glass wool, Na_2SO_4, and silica. The PAH compounds, now in hexane, were eluted with hexane/dichloromethane (3:1, v/v). After elution 0.5 ml isooctane was added as solvent keeper and the solvent was evaporated under a gentle flow of nitrogen until a final volume of 0.5 ml was reached. Ten microliters of injection standards (deuterated PAHs, Dr. Ehrenstorfer) were added and the sample was analyzed with a Thermo GC-MS/MS (Trace 1310 GC Ultra gas chromatograph and TSQ Quantum XLS ultra mass spectrometer). Measurements were done with selected reaction monitoring (SRM) mode. Identification of the PAH compounds was done by selecting two typical fragment ions.

Organotins

From samples collected in 2017, organotins were extracted with acetic acid from freeze-dried homogenized mussel tissue samples to ether-hexane mixture tropolone for complexation. The tissues were broken down with tetramethylammoniumhydroxide prior to extraction. Ethyl derivatives of the compounds were formed using Na-tetraethylborate. The derivatized extracts were cleaned in columns containing activated aluminum oxide using ether-hexane solution as the eluent. The compounds determined were monobutyltin (MTB), DBT, TBT, monophenyltin (MPhT), DThT, triphenyltin (TPhT), and dioctyltin (DOT). For quantitation, the perdeuterated analog of each compound was used as an internal standard, and added to the samples at the beginning of the procedure except for DOT for which perdeuterated DPhT was used. Calibration samples, two zero samples and two control samples treated similarly to the actual samples were analyzed for each sample series. The compounds were determined by using gas chromatography (Hewlett Packard 6890) with high resolution mass spectrometer (Autospec Ultima) using HP-1 capillary capillary column (J&W Scientific: 12 m, i.d. 0.20 mm, 0.33 μm). The detection limits of the method are 0.1–1.1 ng g wet wt.$^{-1}$ depending on the compound. The method is accredited.

Contaminant Measurements Using Passive Samplers

Again only in 2017, polydimethylsiloxane (PDMS) passive samplers (SSP-M823-010, area 90 × 55 mm, thickness 250 μm) were attached to the cages placed at five sites to assess the concentrations of waterborne PAHs and organotins. Two samplers were applied at each caging site. At the time of retrieval the samplers were rinsed with ultrapure water, wrapped in aluminum foil and transferred to the laboratory in a tightly closed container. The samplers were stored in a freezer (−20°C) until extraction. After deployment the organotins were extracted from the samplers with acetic acid to ether-hexane which contained tropolone. The compounds were ethylated with sodium tetraethyl borate, cleaned with activated aluminum oxide column and eluted with ether-hexane. Organotins were analyzed with GC-MS/MS (Agilent 7010, column J&W Scientific, length 12 m, i.d. 0.20 mm, film thickness 0.33 μm) using multiple reaction monitoring (MRM).

For PAH analyses the samplers were shaken in methanol for 24 h and the extract was evaporated to a smaller volume. The extract was cleaned with EPH cartridge (Biotage) and the PAH compounds were eluted with dichloromethane, which was again evaporated to a smaller volume and exchanged to cyclohexane and analyzed with GC/MS. The PDMS samplers are deployed until the equilibrium between the sampler and the surrounding medium has been reached (Smedes et al., 2009). The average concentration of studied chemical in seawater during the deployment can be calculated if the sampler-water partition ($K_{s,w}$) coefficient is known. $K_{s,w}$ between PMDS passive sampler and TBT, diphenyltin (DPhT) and dibutyltin (DBT) has been determined in a laboratory experiment (Ahkola et al., 2018).

Determination of Trace Metals in Water

The seawater samples were preserved with concentrated nitric acid (super purity grade) as 150 μl per 30 ml sample. The samples were diluted 5x before the measurement. The internal standard elements (Rh, Ir) were added on-line using the ICP-MS peristaltic pump. To remove spectral interferences ICP-MS measurement was performed in KED (Kinetic Energy Discrimination) mode using helium as collision gas. Concentrations of the following trace metals are reported here: arsene (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn).

Additional Data

The HERTTA open environmental database of SYKE containing the national monitoring data was utilized to acquire data on macrozoobenthos in areas in the vicinity of the mussel caging sites. Data from sampling stations in these areas (1–12, depending on site) were elaborated to the brackish water benthic index (BBI; Perus et al., 2007) which offers a salinity-corrected tool for classification of the soft-bottom zoobenthos under the demands of the European Union Water Framework Directive (EU WFD). Near-bottom (1 m above the seafloor) oxygen concentrations were also collected from the HERTTA database. Status classifications of nitrogen (N), phosphorus (P) and chlorophyll *a* (chl*a*) in the water column were taken from the EU WFD assessments to represent the eutrophication status of the caging areas. As the status classification ("Bad," "Poor," "Moderate," "Good," "High") is calculated for a limited coastal waterbody area from several stations and based on 6-year means of concentrations in the upper 5-m surface waters in the summer months, the obtained assessment status is only a robust estimate over these highly dynamic variables.

Statistical Analysis

The SYSTAT™ software were used to calculate the statistics. All data were tested for the normality and homogeneity of variance with Bartlett's test. One-way ANOVA followed by Tukey's *post hoc* HSD multiple comparisons. Principal Component Analysis (PCA) was used for exploratory analysis of interactions between individual PAH compounds measured in the tissues of mussels and the biomarkers applied.

The WOE Approach

Data on water chemistry, bioaccumulation and variations of biomarkers in caged mussels as well as benthic community status were elaborated within the WOE model, which summarizes

hazard indices for various LOEs in specific modules before their overall integration. The computation rules of the WOE model have been successfully applied to classify environmental hazards in different conditions of origin, typology, and intensity of pollution, including highly and moderately contaminated sites from industrial areas, harbors, brackish environments, and the Costa Concordia shipwreck on the Italian coast (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al., 2014; Bebianno et al., 2015), and most recently in the assessment of environmental effects of offshore oil platforms in the Adriatic Sea (Regoli et al., 2019) In the latter paper, full and updated descriptions of the LOE/WOE calculation methodologies are presented.

Briefly, the evaluation of chemical hazard in LOE1 is initially based on the calculation for each pollutant of Ratio to Reference (RTR), i.e., the ratio between concentration measured in seawater and relative threshold values which included lower EAC and EC from OSPAR (2004) and Danish EPA 1182/2007 levels. From the calculated ratio to reference, a RTRw is obtained by the application of a correction factor (w) which, depending to the typology of chemicals, ranges from 1 to 1.3 for "non priority" ($w = 1$), "priority" ($w = 1.1$) or "priority and hazardous" pollutants ($w = 1.3$) according to EC Directive 2008/105 (EC, 2008). In the calculation of the specific HQ for chemistry (HQC), an average RTRw is obtained for all of the parameters with RTR <1 (i.e., values below the SQG), while for those with RTR >1, the RTRw are individually added into the summation Σ:

$$HQ_C = \frac{\sum_{j=1}^{N} RTR_W(j)_{RTR(j)\leq 1}}{N} + \sum_{k=1}^{M} RTR_W(k)_{RTR(k)>1}$$

The values of HQC are assigned to one class of chemical hazard (absent or negligible, slight, moderate, major, severe) depending on the number, typology and magnitude of exceeding chemicals (see Piva et al., 2011 for detailed thresholds levels).

For the elaboration of chemical results in caged mussels (LOE2), the RTRw is calculated for each parameter as the increase of concentration compared to control specimens, corrected for the typology of pollutant and the statistical significance of the difference, and assigned to one of five classes of effect. The cumulative HQ for bioavailability (HQBA) does not consider parameters with RTRw <1.3 (concentrations ≤ control value for a priority and hazardous pollutants), calculates the average for those with RTRw ranging between 1.3 and 2.6 (i.e., up to 2-fold increase compared to controls for a priority and hazardous pollutant), and adds the summation (Σ) of all those with RTRw ≥2.6):

$$HQ_{BA} = \frac{\sum_{n=1}^{j} RTR_W(n)_{1.3\leq RTR_w<2.6}}{j} + \sum_{n=1}^{K} RTR_W(n)_{RTR_W\geq 2.6}$$

The level of cumulative HQBA is summarized in one class of hazard for bioavailability, from "Absent" to "Severe," depending on the distribution of analyzed chemicals within the different classes of effect (Regoli et al., 2014).

Results on biomarkers are elaborated (LOE3) considering a "weight" based on the toxicological relevance of each biological endpoint, and a "threshold" indicating the minimum variation considered of biological significance for various responses. For every analyzed biomarker, the measured variation is compared to the specific threshold, then corrected for the weight of the response and the statistical significance of the difference compared to controls. Each biomarker response is assigned by the model to 1 of 5 classes of effect (from "Absent" to "Severe"); the calculation of the HQ for biomarkers (HQBM) does not consider the contribution of responses with an effect lower or equal to threshold ("Absent" or "Slight"), calculates the average for those with an effect up to 2-fold compared to the threshold ("Moderate") and adds the summation (Σ) for the responses more than 2-fold greater than the respective threshold, i.e., "Major" or "Severe" (Regoli et al., 2014):

$$HQ_{BM} = \left(\frac{\sum_{j=1}^{N} Effect_W(j)_{1<Effect(j)\leq 2}}{num\ biomark_{1<Effect(j)\leq 2}} + \sum_{k=1}^{M} Effect_W(k)_{Effect(j)>2} \right)$$

According to variations measured for various biomarkers the model summarizes the level of cumulative HQBM in one of five classes of hazard for biomarkers, from "Absent" to "Severe" (Regoli et al., 2014).

For benthic communities (LOE4), using the approach of Perus et al. (2007) for the classification of BBI, the interval of values between 0 and the threshold "Not-good status/Good status" has been divided in two equal classes, respectively assigned to level of hazard "Major" and "Severe." Similarly, the interval of values ranging between the threshold "Not-good status/Good status" and one has been divided in three classes corresponding to a level of hazard "Moderate" (with BBI ranging within the first 30% of the interval threshold-to-1), "Slight" (in the following 35% of the interval threshold-to-1) and "Absent" (in the last 35% of the interval threshold-to-1). The samples of each site were initially processed individually to calculate the HQ and level of hazard: the HQ of single samples were then averaged to obtain the mean hazard level for each site.

For the LOE on oxygen levels (LOE5), considering the values indicated as the threshold "Good"-"Moderate" (4 mg l^{-1}), threshold "Moderate"-"Bad" (2 mg l^{-1}), the minimum oxygen level (0 mg l^{-1}) and typically higher level of oxygen in investigated sites (10 mg l^{-1}), the following classes of hazard have been considered for hypoxic conditions: 0–1 mg/L, HQ "Severe"; 1–2 mg l^{-1}, HQ "Major"; 2–4 mg l^{-1}, HQ "Moderate"; 4–7 mg l^{-1}, HQ "Slight"; 7– ≥10 mg l^{-1}, HQ "Absent".

A "eutrophication index" (LOE6) was calculated combining and differently weighting the evaluations of "chl*a* status" (weight 40%), "P status" (30%) and "N status" (30%).

The elaborations of results from individual water chemistry, bioaccumulation, and biomarkers in caged mussels, benthic communities, oxygen levels, and eutrophication status were

TABLE 2 | Trace metals in water (μg l^{-1}, average values 1–10 m depth).

Site	As	Cd	Co	Cr	Cu	Ni	Pb	Zn
Kotka	0.72	0.01	0.025	0.01	1.05	0.77	0.04	1.0
Porvoo	0.92	0.01	0.100	0.30	1.10	0.86	0.04	1.0
Helsinki	0.85	0.01	0.060	0.01	1.00	0.77	0.04	**2.3**
Hanko	1.00	0.01	0.025	0.01	0.90	0.75	0.01	1.0
Parainen	0.98	0.01	0.135	0.15	1.40	1.02	0.06	**1.6**
Naantali	1.10	0.01	0.325	0.75	1.35	1.35	0.20	**2.8**
Uusikaupunki	1.00	0.01	0.145	0.25	0.95	1.20	0.08	1.5
Rauma	1.15	0.02	0.410	1.20	**1.60**	1.95	0.37	**5.3**
Pori	0.96	0.01	0.025	0.01	1.00	1.04	0.03	1.0
Vaasa	0.84	0.01	0.080	0.01	1.10	1.20	0.02	**2.2**
Thresholds	3.50	0.25	NA	3.4	1.45	9.60	7.20	1.1

Values exceeding the thresholds are shown in bold. NA, not available.

finally integrated in the WOE assessment, giving a different weight to various lines of evidence, to summarize an overall WOE index, finally assigned to one of five classes of risk from "Absent" to "Severe" (Piva et al., 2011).

RESULTS

Trace Metals in Seawater

Concentrations of As, Cd, Co, Cr, Cu, Ni, Pb, and Zn at the caging sites are presented in **Table 2**. Only values of Zn (five stations) and Cu (1) exceeded the water quality threshold values derived from literature (**Table 2**). However, below these threshold levels significant variability was recorded, between the sites, especially in regard to Co (up to ca. 20 times differences), Cr (up to 100 times) and Pb (up to ca. 40 times). When these data were elaborated according to weighted criteria, the summarized level of hazard was "Absent" for Kotka, Porvoo, Hanko and Pori, "Slight" in Helsinki, Parainen, Uusikaupunki and Vaasa, "Moderate" in Naantali and Rauma. At all these stations the parameter contributing most to the calculated HQ was Zn (**Table 3**).

PAHs and Organotins in Mussel Tissues

Uusikaupunki, Pori and Vaasa emerged as the sites with clearly lower total PAH tissue levels compared to the others (9.5–29.7 μg kg^{-1}, **Figure 2**). Mussels in Kotka showed the highest total tissue concentrations of PAHs (96.6 μg kg^{-1}) with Hanko and Naantali coming up next (88.5 and 86.0 μg kg^{-1}, respectively). Mussels in Kotka and Parainen showed a quite different PAH compound profile compared to the others with proportionately higher share of benzo compounds (61 and 53% of total, respectively) while these were not recorded at all at stations Porvoo, Hanko, Naantali, Uusikaupunki, and Pori. Another notable distinction was the presence of methylated naphthalenes, which were very prominent at the sites characterized by low PAH tissue levels, namely Pori (100% of total) and Vaasa (49%) but completely absent at Porvoo, Naantali, Uusikaupunki, and Rauma.

The levels of organotins were measured only in mussels caged in 2017 at five stations, being clearly highest in mussels caged in Naantali with 223 μg kg dry $wt.^{-1}$ of total organotins, which of 80% consisting of TBT (**Figure 3**). For the rest of the sites the organotin levels were between 18.2 (Hanko) and 54.3 μg kg dry $wt.^{-1}$ (Porvoo). TPhT was recorded at every site with the levels ranging from 4.3 (Rauma) to 18.3 μg kg dry $wt.^{-1}$ (Porvoo). DBT was measured only in mussels caged at the Porvoo site (16.6 μg kg dry $wt.^{-1}$).

The weighted elaboration of bioaccumulation data provided a clear discrimination between the sampling areas with a level of hazard ranging from "Absent"/"Slight" in Pori, Vaasa, Uusikaupunki, and Helsinki to "Moderate" in Hanko and "Major" for mussels caged in Kotka, Porvoo, Parainen, Naantali, and Rauma (**Table 3**). Perylene, methylnaphatalenes, benzo[a]pyrene (B[a]P), and organotins were the compounds contributing most to the "Major" bioavailability hazard.

PAHs and Organotins in Passive Samplers

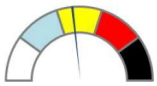
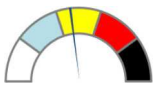
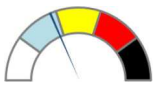
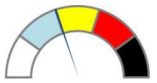
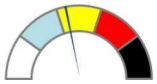
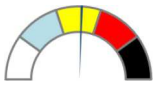
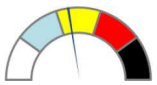
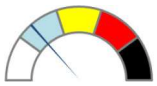
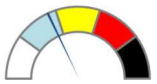
Concentrations of PAHs and organotins in PDMS passive samplers were measured only in 2017 at five caging sites. The total concentration patterns of both groups of compounds coincided generally well with those measured in mussel tissues (data not shown). The μg kg $sampler^{-1}$ concentrations can be converted to ng l^{-1} or pg l^{-1} when the sampler-water partition coefficient $K_{s,w}$ is known for the used chemical and the PDMS sampler type. The partition coefficients for certain PAHs (anthracene, acenaphthene, B[a]P, chrysene, fluoranthene, fluorene, naphthalene, phenanthrene, pyrene), and organotins (TBT, TPhT, DBT) are available from the literature (Smedes et al., 2009; Ahkola et al., 2018). As the partition coefficient depends on the characteristics of the studied compound the average concentrations during the sampling period differ from the μg kg $sampler^{-1}$ ones. With PAHs the partition factors differ between 3.10 and 5.92 (naphthalene–B[a]P), which means that naphthalene prefers to water phase more than B[a]P. High concentrations of naphthalene were observed at all study sites despite it did not stand out when the μg kg $sampler^{-1}$ amounts were studied. The highest concentrations of PAHs were found at Porvoo and Uusikaupunki but due to the lack of the $K_{s,w}$ all the detected compounds are not included in this calculation (**Figure 4**). The results suggest that the dissolved fraction collected by passive samplers differs markedly from the one bioaccumulated by mussels.

The profile of total organotin concentrations was quite alike to that measured in mussels, implying that the bioaccumulation of organotins to mussels and PDMS samplers is more similar than in case of PAHs. Similar to the mussel tissue samples the peak concentrations of total organotins in passive samplers were measured in Naantali and the lowest ones in Hanko. TBT was discovered at all five sampling sites and TPhT and DBT from four sites except in Hanko, which also showed a "Moderate" bioavailability hazard level (**Table 3**). TBT was again clearly the prominent organotin compound.

Biomarker Responses

Marked differences were observed in the measured biomarkers (**Figure 5**), some coinciding with higher concentrations of the measured compounds in mussel tissues and passive samplers. ANOVA and Tukey *post hoc* statistics on differences between biomarker responses between the study stations are presented in **Table 4**, showing significant differences between the sites.

TABLE 3 | Elaborations with levels of hazard assigned to the different LOEs and the final WOE.

Site	Chemical characterization	Bioavailability	Biomarkers	Benthic communities	Near-bottom oxygen	Eutrophication	Weight of Evidence integration	
Kotka	HQ: 0.284 Absent -	HQ: 63.417 Major BaP–DBahA–BkF; PER	HQ: 4.229 Moderate CAT–GST	HQ: 67.174 Major	Absent	Major	MODERATE	
Porvoo	HQ: 0.311 Absent -	HQ: 63.030 Major ANT–FLU; PER	HQ: 4.642 Moderate GST-LPO-CAT; GR	HQ: 46.078 Moderate	Slight	Major	MODERATE	
Helsinki	HQ: 2.271 Slight 100% Zn	HQ: 14.842 Slight -;-	HQ: 2.517 Moderate -; GST	HQ: 31.326 Slight	Absent	Major	SLIGHT	
Hanko	HQ: 0.28 Absent -	HQ: 29.925 Moderate -; 1-MetNAPH	HQ: 2.714 Moderate GR-GST; -	HQ: 46.377 Moderate	Absent	Moderate	SLIGHT	
Parainen	HQ: 1.7 Slight 100% Zn	HQ: 59.329 Major BbF–BaP; PER	HQ: 2.008 Slight LPO;-	HQ: 48.291 Moderate	Absent	Major	MODERATE	
Naantali	HQ: 2.829 Moderate 100% Zn	HQ: 80.710 Major FLU; PER–OSn	HQ: 2.402 Moderate GST-CAT; -	HQ: 49.020 Moderate	Absent	Major	MODERATE	
Uusikaupunki	HQ: 1.566 Slight 100% Zn	HQ: 1.985 Slight -;-	HQ:2.42 Moderate CAT-GST; -	HQ: 9.520 Absent	Slight	Moderate	SLIGHT	
Rauma	HQ: 6.18 Moderate 81.5% Zn	HQ: 64.589 Major -; PER–BaP	HQ: 2.125 Slight CAT; -	HQ: 33.676 Slight	Absent	Moderate	MODERATE	
Pori	HQ: 0.293 Absent -	HQ: 0 Absent -;-	HQ: 0 Absent -;-	HQ: 50.986 Moderate	Absent	Slight	SLIGHT	
Vaasa	HQ: 2.199 Slight 100% Zn	HQ: 4.296 Slight -;-	HQ: 1.0 Slight -;-	HQ: 59.938 Moderate	Absent	Slight	SLIGHT	

Hazard Quotient (HQ) is provided for chemical characterization of seawater (showing the percentage of the parameter contributing most to the HQ), bioavailability (parameters showing major or severe effects), biomarkers (parameters showing moderate or major effects), and benthic communities.

An exploratory PCA examining the connections between the bioaccumulation of the different PAHs and biomarker responses are presented in **Figure 6**. The results show clear connections between the tissue levels of certain groups of PAH compound types (methylated naphthalanes, benzo compounds, and "classic" parent PAHs), and the biomarker responses.

According to the toxicological relevance and the magnitude of variations observed for each biomarker, the overall significance of observed variations was summarized in a HQ "Absent" or "Slight" for Pori, Vaasa, Rauma and Parainen, and "Moderate" for all the other stations (**Table 3**).

Supporting Data: Macrozoobenthos, Near-Bottom Oxygen, and Eutrophication Status

From the obtained BBI values the level of hazard elaborated for benthic communities was "Absent" or "Slight" for Uusikaupunki, Rauma, Helsinki, "Moderate" for Porvoo, Hanko, Parainen, Naantali, and "Major" for Kotka (**Table 3**). The oxygen levels corresponded to an hypoxic hazard level summarized as "Absent" for all the stations except for Porvoo and Uusikaupunki ("Slight"). Eutrophication hazard obtained from the results on P, N, and chl*a* ranged from "Slight" (Pori and Vaasa), "Moderate" (Uusikaupunki, Rauma, and Hanko) to "Major" (all the other sites, **Table 3**).

Application of the WOE Approach

In the conclusive WOE elaboration, the HQs obtained from various LOEs were normalized to a common scale and integrated giving them a different weight according to the ecological relevance of each typology of analyses. The weights assigned in this study to various LOEs were 0.9 for trace metal analyses of seawater, 1.2 for bioavailability in transplanted mussels, 1.0 for biomarkers, 1.3 for benthic communities, 0.5 for oxygen, and 0.5 for eutrophication. A large number of heterogeneous analytical results were summarized in a WOE evaluation, which discriminated various sites increasing from "Slight" (Pori, Uusikaupunki, Vaasa, Helsinki, Hanko) to "Moderate" (Parainen, Rauma, Porvoo, Naantali, and Kotka) (**Table 3**). The overall class derived from a combination of specific HQs ranging

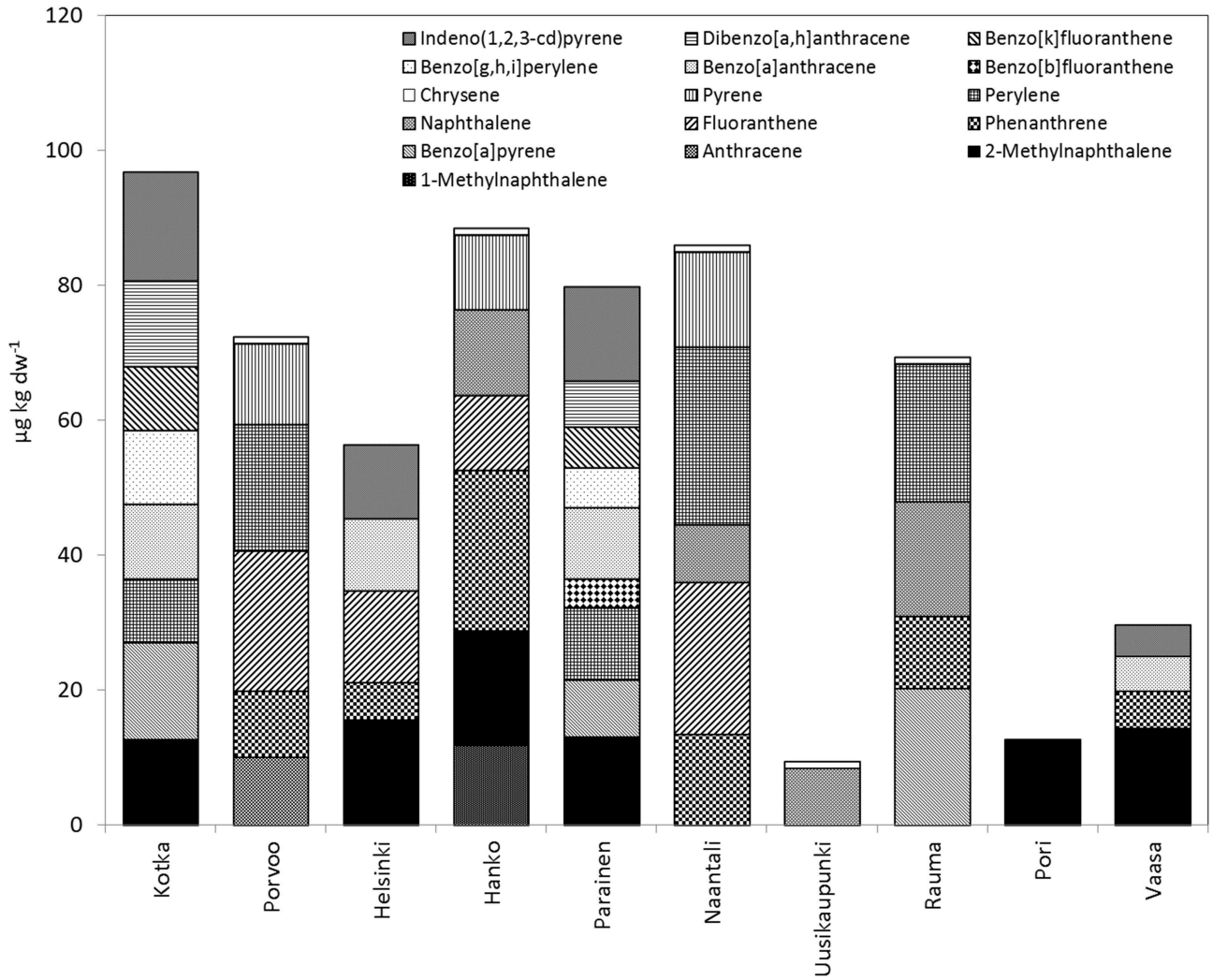


FIGURE 2 | PAH compounds (µg kg dry wt.$^{-1}$) in soft tissues of caged mussels from the study sites. Fluorene, acenaphthene, acenaphthylene, and benzo[e]pyrene were not detected in any of the samples.

from "Absent"/"Slight" to "Moderate" (WOE "Slight") or from "Absent"/ "Slight" to "Major" (WOE "Moderate").

DISCUSSION

Holistic interpretation of data on contaminant concentrations, biomarker responses, and various environmental conditions is typically highly complex. This study shows that the WOE approach provides robust support for the understanding and communicating the results. In combination with the presenting of the actual results it also provides transparency of the basis of the interpretation.

Site-Specific Differences in Chemical Hazard and Bioaccumulation

Examining the map, different types of coastal environments where the cagings were performed can be seen, showing open sea sites with less coastal anthropogenic impacts, sites deep inside a dense archipelago close to major harbors, maritime and near-shore industrial activities, and something between these. The first category includes Hanko, Uusikaupunki, Pori and Vaasa, while the rest belong to mainly to the second one. It should also be understood that the exact placing of the cages in the study areas has an obvious effect on the results since there are specific point sources and various environmental gradients in each area. For a comprehensive and detailed study, the number of caging units per area should be manifold to be able to characterize the area in a smaller scale. In addition, in some cases the deployment of the cages exactly to the locations foreseen as optimal in regard to the assessment of local pollution was not possible due to factors such as the proximity of major ship routes, shallowness, and the presence of underwater rocks dangerous to surface navigation of the research vessel. However, since mussels filter seawater very effectively and the it is moving along horizontal and vertical

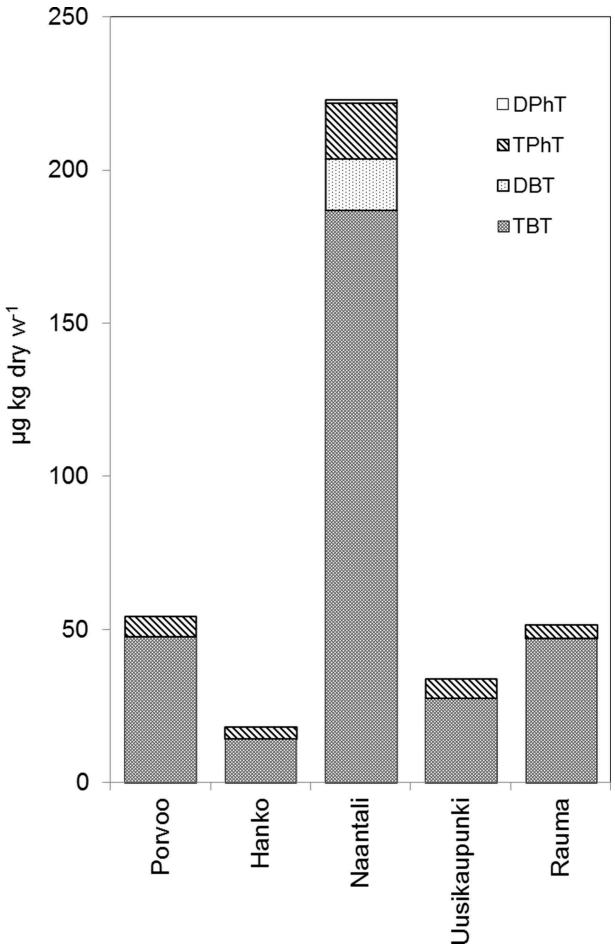


FIGURE 3 | Organotins (µg kg dry wt.$^{-1}$) in soft tissues of caged mussels from five study sites in 2017. MBT, MPhT, and DOT were not detected in any of the samples.

currents (considering also strong resuspension in shallow coastal areas especially impacted by intensive ship traffic) the mussels in a caging unit actually represent the average conditions within a quite large area. Nevertheless, the results from this study should not be taken as exact indicators of local conditions especially in the more open areas that are obviously less impacted by near-coast activities.

Chemical characterization using the concentrations of trace metals in seawater did not show any extreme situations in any of the study areas. Regarding Zn, values exceeding the given threshold of 1.1 µg l^{-1} were observed at half (5) of the sites. The highest values above the threshold by five times was recorded at Rauma and is probably related to high resuspension of sediments mainly due to ongoing dredging operations in the harbor area during the time of caging; this may also be reason for the occurrence of some other contaminants in passive samplers and in mussel tissues. Rauma was also the only site where the threshold for Cu was exceeded. For Zn, the second highest value (2.8 µg l^{-1}) recorded in Naantali could be associated also to a constantly high sediment resuspension due to the extensive maritime traffic in the area, consisting of oil tankers to the large oil terminal as well as daily passenger ferries between Finland and Sweden. The nearby Parainen, also within the same dense achipelago with limited water exchange, is also strongly affected by ferry traffic and the resulting sediment resuspension; these two sites are characterized by higher concentrations of most of the measured trace metals in seawater which are likely derived from the resuspended sediments. Earlier studies on mussels, both native (Lehtonen et al., 2006a) and caged mussels (Lehtonen et al., 2016) in the area have shown a contamination gradient in the region with the inner part being more polluted. Obviously, there is a large number of other groups of contaminants that are also released from sediments during resuspension; if the trace metals indeed originate from this matrix they act as a proxy for the overall exposure to other contaminants as well. One of the groups is organotins (mainly TBT), which in Naantali showed by far the highest concentrations both in mussel tissues (>4-fold higher levels compared to Rauma and Porvoo) as well as in passive samplers (3-fold). In this study, sediment particles introduced to the water phase by resuspension appear to be the most likely source of organotins detected both in caged mussels and passive samplers.

In general, the total PAH levels measured in this study, ranging from 9.5 to 96.9 ng g^{-1} dry wt., coincide very well with the earlier studies in coastal areas of Finland (Turja et al., 2013, 2014, 2015; Lehtonen et al., 2016). However, they are significantly lower than those measured e.g., in the Northern Atlantic ($\sum PAH_{16}$ 72–2217 ng g^{-1} dry wt; EU H2020 GRACE project, unpublished data). Examining the bioaccumulation of PAH compounds in the caged mussels, the cities of Vaasa, Pori and Uusikaupunki, all along the Bothnia Sea coast, stand out as the least polluted by these compounds. Between Pori and Uusikaupunki there is Rauma, where the resuspension related to the dredging activities mentioned above may have caused the elevated tissue levels not observed at the other Bothnian Sea sites, with high-molecular weight compounds B[a]P and perylene comprising an unusually high share of 59% of total PAHs. Mussels caged in Rauma contained no detectable levels of methylated naphthalenes, which were in turn commonly observed at the other sites and at relatively marked shares of total PAHs, e.g., in the nearby Pori 2-methylnaphthalenes formed 100% of the measured PAHs.

Notably, mussels caged near the large oil terminals in Porvoo and Naantali did not contain methylated naphthalenes, which are often connected to oil. Instead, phenanthrene, fluoranthene, perylene, and pyrene formed 61 and 76% of total PAHs at these sites (respectively), much more than in the other areas. With the exception of anthracene (in Porvoo) and naphthalene (in Naantali) the PAH profiles were identical and this is quite likely connected to the nearby main environmental stressors, identified as oil industry and transport, and maritime traffic.

The PAH profiles at Kotka and Parainen are quite different compared to other sites. The total levels are similar to some other sites but the share of benzo compounds reaches 61 and 53%, respectively. In comparison, no benzo compounds were detected in mussels at half (5) of the study sites. Compared to the three other sites in the Gulf of Finland (Porvoo, Helsinki and

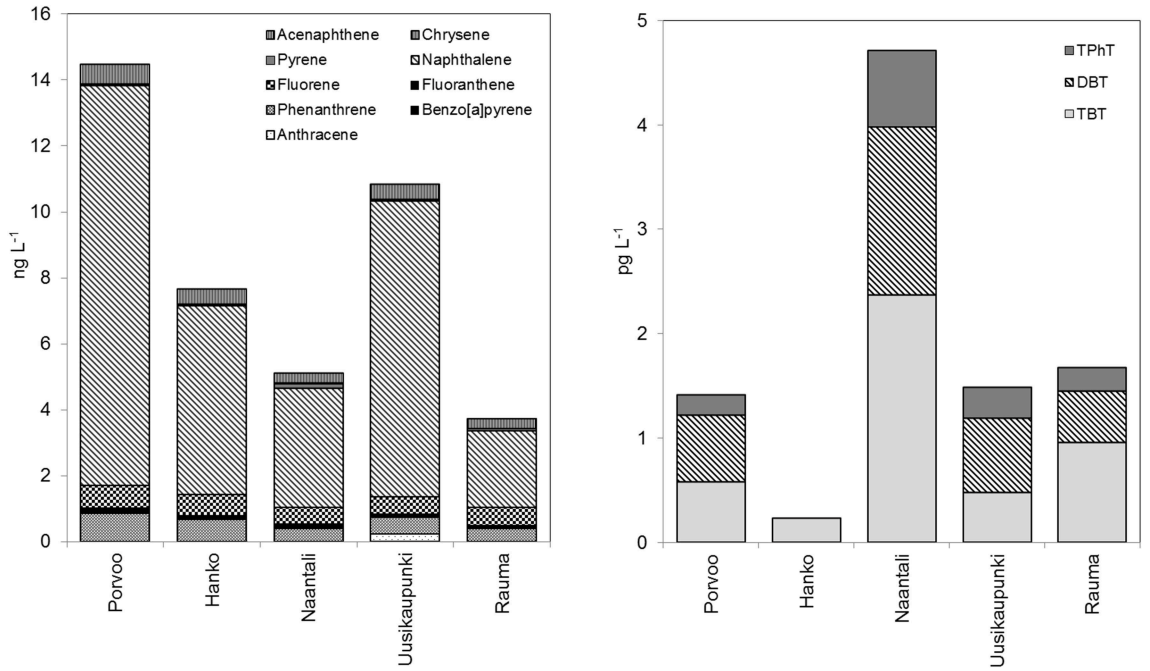


FIGURE 4 | Seawater concentrations of PAHs (ng L^{-1}) and organotins (pg L^{-1}) calculated from accumulated amounts in passive samplers depolyed at five mussel caging sites in 2017.

Hanko) no bioaccumlation of phenanthrene and fluoranthene, both relatively abundant at all these sites, could be detected in mussels. The Kotka region under the influence of the large Kymijoki is notoriously known for its high contamination by the abundant pulp mill industry and one of the rivers is one of the hotspots for dioxins (HELCOM, 2010). The riverborne contaminants have greatly polluted also the coastal sediments with various types of substances, including PAHs. The highly different PAH profile especially compared to the other Gulf of Finland caging sites suggest a different exposure scheme in the Kotka sea region, possibly related to the high industrial activities of the past.

The caging site at Hanko is next to the open sea and affected mainly by maritime traffic. Methylated naphthalenes consisted a significant share (33%) of the bioaccumulated PAH compounds in mussels. Mussels caged near Helsinki, another open-sea site greatly affected by traffic but also by other sources related to the metropolitan area, contained 28% of these compounds. Taking in account that in Pori and Vaasa, the sites with the lowest PAH contamination, the shares of methylated naphthalenes was largest (up to 100%) it may be considered that these sites are significantly less affected by other types of PAHs, e.g., those released to the water column via intensive resuspension of highly contaminated sediments such as found in Naantali, Parainen, and Kotka.

High tissue concentrations (on a regional scale) of phenanthrene in mussels collected from the Hanko region have been recorded also in other studies (Turja et al., 2014; Lehtonen et al., 2016; Turja et al. submitted manuscript). Also, elevated concentrations of methyl phenanthrenes (not measured here) have been observed (Turja et al., submitted manuscript), possibly connected to the anomalous concentrations of methyl naphthalenes measured in the present study. Thus, although the overall assessment of the Hanko site does not indicate any great environmental concern, these two single indicators show that further studies are needed in this area, especially since the observations already cover a period of over 10 years.

The PDMS passive samplers measure only freely dissolved chemical fraction so the concentrations determined with field deployed passive sampler are lower than the total ones. The average concentrations could be determined only to certain PAHs and organotins for which sampler water partition coefficients exist. The environmental quality standard (EQS) TBT in surface water is very low (0.2 ng L^{-1}) and in most cases near the laboratory detection limit (EC, 2008). With PDMS passive sampler the presence of TBT in aquatic environment can be detected in much lower concentrations than before, which further enables a more reliable risk assessment.

Site-Specific Differences in Biomarkers

When examining the results on biomarker responses it is of key importance to take into account and understand the effects of factors not related to chemical contamination such as salinity and food availability (eutrophication status) that vary greatly between the study areas. Both the Gulf of Finland and the Archipelago Sea are considered highly eutrophicated areas while the Bothnian Sea suffers much less from these problems (HELCOM, 2018).

In this part of the Baltic Sea mussels live at the absolute limits of salinity tolerance (e.g., Westerbom et al., 2002); this has crucial implications on their growth and general physiology,

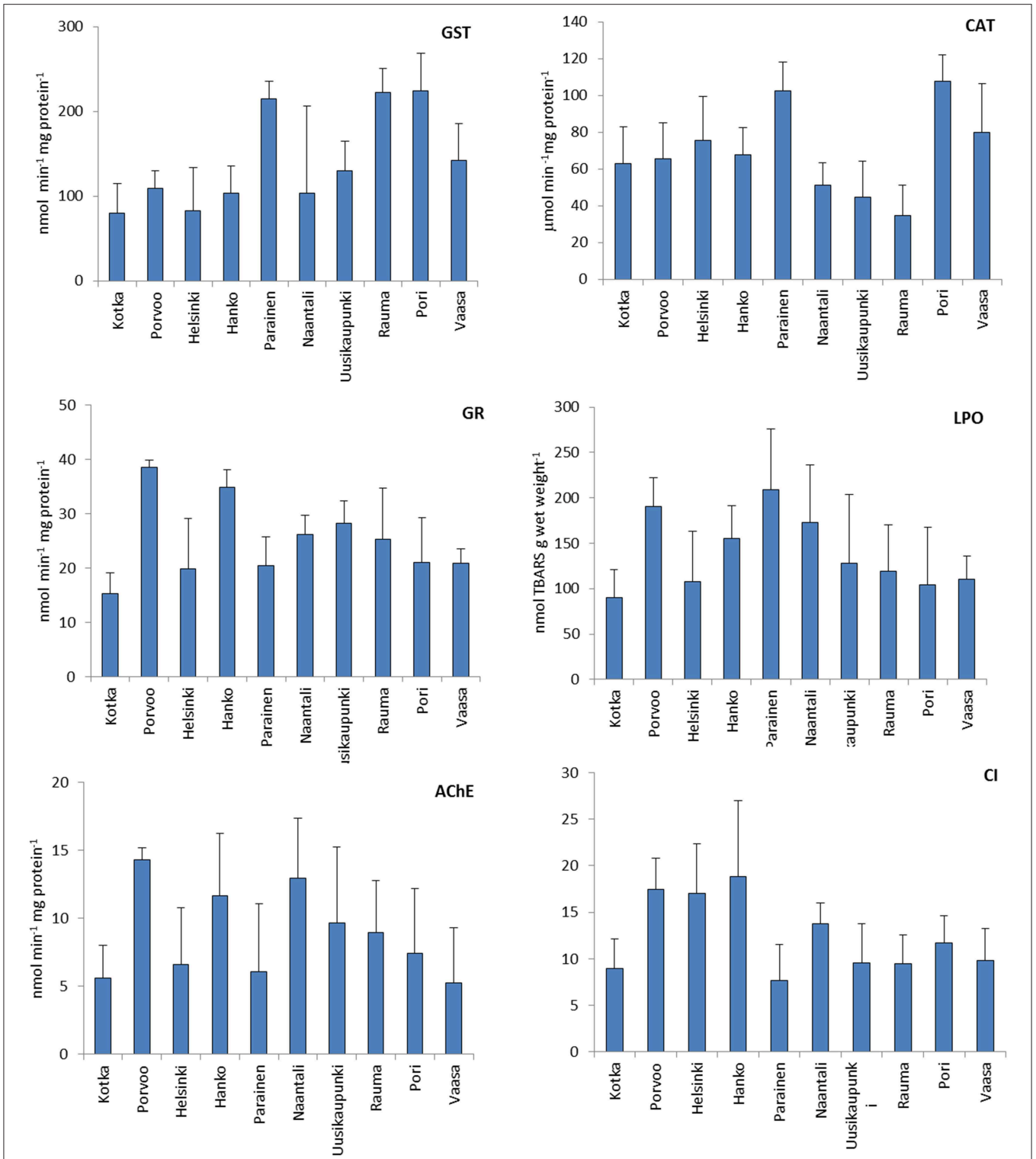


FIGURE 5 | Biomarkers (mean ± SD) measured in caged mussels from the study sites. GST, glutathione *S*-transferase; CAT, catalase (CAT); GR, glutathione reductase; LPO, lipid peroxidation; AChE, acetylcholinesterase; CI, condition index. Statistical significances: see **Table 3**.

which in turn may, at some point, lead to increased susceptibility to pollution insults or the ability to respond to pollution with protective cellular mechanisms. Although the salinity differences might appear small (2–3 per mille) compared to full-marine areas, they are crucially important at this critical range. In addition, salinity conditions in the Baltic Sea are

TABLE 4 | ANOVA performed on each biomarker at the different study locations with *post hoc* Tukey's HSD multiple comparisons.

		Hanko	Helsinki	Kotka	Naantali	Parainen	Pori	Porvoo	Rauma	Uusikaupunki
GST	Helsinki	0.967								
	Kotka	0.923	1.000							
	Naantali	1.000	0.969	0.926						
	Parainen	**0.000*** **	**0.000*** **	**0.000*** **	**0.000*** **					
	Pori	**0.000*** **	**0.000*** **	**0.000*** **	**0.000*** **	1.000				
	Porvoo	1.000	0.858	0.759	1.000	**0.000*** **	**0.000*** **			
	Rauma	**0.000*** **	**0.000*** **	**0.000*** **	**0.000*** **	1.000	1.000	**0.000*** **		
	Uusikaupunki	0.872	0.143	0.089	0.868	**0.000*** **	**0.000*** **	0.972	**0.000*** **	
	Vaasa	0.380	**0.014***	**0.007****	0.374	**0.001*** **	**0.000*** **	0.623	**0.000*** **	0.999
CAT	Helsinki	0.996								
	Kotka	1.000	0.902							
	Naantali	0.641	0.124	0.932						
	Parainen	**0.002****	**0.044***	**0.000*** **	**0.000*** **					
	Pori	**0.000*** **	**0.005****	**0.000*** **	**0.000*** **	1.000				
	Porvoo	1.000	0.979	1.000	0.792	**0.001*** **	**0.000*** **			
	Rauma	**0.003****	**0.000*** **	**0.025***	0.644	**0.000*** **	**0.000*** **	**0.008****		
	Uusikaupunki	0.168	**0.011***	0.492	0.999	**0.000*** **	**0.000*** **	0.282	0.967	
	Vaasa	0.903	1.000	0.572	**0.024***	0.196	**0.036***	0.789	**0.000*** **	**0.001*** **
GR	Helsinki	**0.000*** **								
	Kotka	**0.000*** **	0.699							
	Naantali	**0.013****	0.215	**0.000*** **						
	Parainen	**0.000*** **	1.000	0.642	0.483					
	Pori	**0.000*** **	1.000	0.376	0.499	1.000				
	Porvoo	0.903	**0.000*** **	**0.000*** **	**0.000*** **	**0.000*** **	**0.000*** **			
	Rauma	**0.004****	0.463	**0.002****	1.000	0.741	0.776	**0.000*** **		
	Uusikaupunki	0.179	**0.025***	**0.000*** **	0.998	0.107	0.097	**0.002****	0.976	
	Vaasa	**0.000*** **	1.000	0.448	0.478	1.000	1.000	**0.000*** **	0.754	0.093
LPO	Helsinki	0.453								
	Kotka	0.078	0.999							
	Naantali	0.998	0.074	**0.005****						
	Parainen	0.252	**0.000*** **	**0.000*** **	0.792					
	Pori	0.352	1.000	1.000	**0.047**	**0.000*** **				
	Porvoo	0.809	**0.004****	**0.000*** **	0.998	0.998	**0.002****			
	Rauma	0.836	1.000	0.946	0.305	**0.002****	1.000	**0.036***		
	Uusikaupunki	0.965	0.994	0.755	0.550	**0.006****	0.984	0.100	1.000	
	Vaasa	0.553	1.000	0.995	0.109	**0.000*** **	1.000	**0.007****	1.000	0.998
AChE	Helsinki	**0.028***								
	Kotka	**0.002****	1.000							
	Naantali	0.997	**0.001*** **	**0.000*** **						
	Parainen	**0.006****	1.000	1.000	**0.000*** **					
	Pori	0.124	1.000	0.960	**0.007****	0.996				
	Porvoo	0.717	**0.000*** **	**0.000*** **	0.996	**0.000*** **	**0.000*** **			
	Rauma	0.753	0.870	0.413	0.192	0.649	0.991	**0.013***		
	Uusikaupunki	0.940	0.585	0.154	0.418	0.320	0.900	**0.047***	1.000	
	Vaasa	**0.001*** **	0.996	1.000	**0.000*** **	1.000	0.890	**0.000*** **	0.271	0.084
CI	Helsinki	0.986								
	Kotka	**0.000*** **	**0.000*** **							
	Naantali	**0.033***	0.692	0.051						
	Parainen	**0.000*** **	**0.000*** **	0.998	**0.003****					
	Pori	**0.000*** **	0.062	0.743	0.937	0.206				
	Porvoo	0.996	1.000	**0.000*** **	0.345	**0.000*** **	**0.007****			
	Rauma	**0.000*** **	**0.000*** **	1.000	0.131	0.976	0.912	**0.000*** **		
	Uusikaupunki	**0.000*** **	**0.001*** **	1.000	0.163	0.961	0.939	**0.000*** **	1.000	
	Vaasa	**0.000*** **	**0.001*** **	1.000	0.223	0.927	0.969	**0.000*** **	1.000	1.000

*Statistically significant differences are indicated in bold. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.*

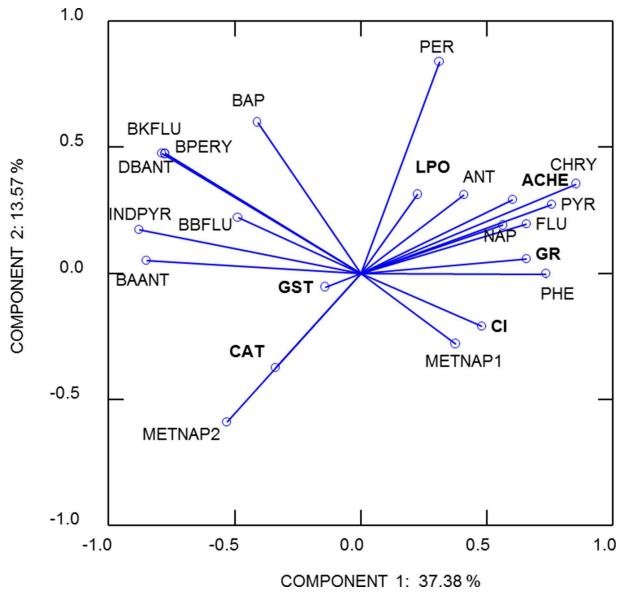


FIGURE 6 | Principal Component Analysis (PCA) on biomarkers and PAH compounds. GST, glutathione *S*-transferase; CAT, catalase; GR, glutathione reductase; LPO, lipid peroxidation; AChE, acetylcholinesterase; CI, condition index; METNAP1, 1-methylnaphthalene; METNAP2, 2-methylnaphthalene; ANT, anthracene; BAP, benzo[a]pyrene; PHE, phenanthrene; FLU, fluoranthene; NAP, naphthalene; PER, perylene; PYR, pyrene; CHRY, chrysene; BBFLU, benzo[b]fluoranthene; BAANT, benzo[a]anthracene; BPERY, benzo[g,h,i]perylene; BKFLU, benzo[k]fluoranthene; DBANT, dibenzo[a,h]anthracene; INDPYR, indeno(1,2,3-cd)pyrene.

stable, e.g., estuarine areas are naturally lower in salinity and devoid of any marine-originated brackish water adapted biota, and seasonal variations through ice melt and river discharge are low. In case of mussels transplanted in the most low-saline sites in the eastern Gulf of Finland (Kotka, Porvoo, Helsinki) and Bothnian Sea (Pori, Vaasa) the effects of extreme salinity conditions and energy-demanding osmotic stress can affect some of the biomarkers.

While high eutrophication typically is a serious environmental problem, in the case of caged mussels in this short-term exposure it can be considered as a positive growth-enhancing factor. In the present study the difference in eutrophication status is reflected in the significantly higher CI values of mussels transplanted in the Gulf of Finland (Porvoo, Helsinki, Hanko) compared to the Bothnian Sea (axis Uusikaupunki-Vaasa), indicating poorer growth opportunities in the latter area. The observation that most of the Bothnian Sea sites were less contaminated with PAHs (with the exception of Rauma discussed earlier) compared with the whole Gulf of Finland the low CI is indeed caused by nutrition or salinity stress. The abundant food conditions in the Gulf of Finland "balance out" the higher contaminant stress in the region so that the mussels are able to grow and attain energy needed for the defense mechanisms; however, in the case of Kotka it appears that not even the good food conditions are sufficient to counteract the combined effects of elevated contamination levels and low salinity. At Parainen and Naantali in the inner Archipelago Sea food is abundant and also salinity is more tolerable, and the markedly lower CI values compared to Porvoo, Helsinki and Hanko are likely due to contaminant stress.

Despite of the use of AChE activity as a specific indicator of exposure to some pesticicides (e.g., Bocquené and Galgani, 1998) it has been recently used also as an indicator of non-specific stress since it responds to a variety of chemicals and other factors such as trace metals, hydrocarbons, detergents, and algal toxins (Zinkl et al., 1991; Payne et al., 1996; Guilhermino et al., 1998; Lehtonen et al., 2003, respectively). The lowest values are measured at the low-salinity ends, Kotka and Vaasa, the former considered polluted and the latter among the cleanest ones of the study sites. Thus, salinity obviously affects mussels in these low-salinity areas. The PCA carried out on PAHs and biomarkers shows clear separation of groups of bioaccumulated PAH compounds and their interrelationships with selected biomarker responses. An interesting result is that at the sites where the mussels were characterized by zero bioaccumulation of benzo compounds (Porvoo, Hanko, and Naantali) they also had a significantly higher AChE activity. The most relevant pairwise comparison can be made with Naantali and Parainen, relatively close by and similar environmental conditions, with mussels containing no benzo compounds at the former site having a significantly higher AChE activity compared to the those at the latter having a share of 42% of their PAHs as these types. The total tissue PAH concentrations were similar at both sites, 79.9 and 86 μg kg dry wt.$^{-1}$, respectively for Parainen and Naantali. An important observation is also that at the low-salinity Kotka site the mussels showed high levels of benzo compounds and a very low AChE activity.

Still focusing on the sites with high benzo compound bioaccumulation and especially the Archipelago Sea site pair Naantali and Parainen, the activity of GST is 2-fold higher at the latter site characterized by the high content and share of benzo compounds. This explicitly indicates an elevated detoxification and phase II biotransformation of these compounds. In Kotka the bioaccumulation of benzo compounds was also high but as can be depicted from a very low AChE activity the mussels are apparently unable to respond to the challenge since GST activity is also very low. Conclusively, the contaminant load jointly with threshold-level salinity presents the mussels caged at the Kotka site a too challenging situation to cope with. The results obtained here obviously call for more detailed investigations on the relationships of the bioaccumulation of different PAH compounds and biomarkers measured in mussels under field exposure conditions.

WOE Elaboration

A typical challenge in multidisciplinary biomonitoring is the difficulty to summarize the overall significance of heterogeneous results to better evaluate and quantify environmental impacts and risks on both geographical and temporal scales. This work corroborates previous findings that the criteria of a quantitative WOE approach reflect an important advancement in such risk assessment procedures. Various typologies of results (i.e., the

LOEs) are independently elaborated through logical flowcharts and mathematical algorithms, which provide synthetic indices of hazard for each of the considered LOEs, before their final integration in a quantitative WOE evaluation (Piva et al., 2011). The hazards for water chemistry and bioavailability in caged mussels were based on the number, magnitude and potential toxicity of chemicals exceeding accepted quality guidelines or natural concentrations in reference organisms, while the evaluation of the biomarker considered, for each parameter, is based on the toxicological relevance of the measured endpoint and the magnitude of variations compared to specific thresholds. Benthic community data were elaborated to provide a commonly accepted ecological descriptor, and additional indices on oxygen and eutrophication were included in the overall integration. Among the advantages of a similar WOE approach is the possibility to discriminate differences between evaluations from various typologies of studies: elevated chemical loads in environmental matrices are not necessarily bioavailable and, at the same time, unexpected biological effects are caused by low levels of pollutants acting through synergistic mechanisms. Another relevant feature of this WOE approach is the weighted elaboration of data, allowing to abandon the "pass-to-fail" approach where even a single parameter slightly below or above a threshold would determine the chemical classification. Similarly, biological evaluations are not based on variations of individual responses, thus providing a more integrated assessment of the ecotoxicological or ecological hazards. As recently shown in a complex monitoring scenario of off-shore platforms (Regoli et al., 2019), this study confirmed the practical importance of a transparent procedure combining a scientifically sound approach with the possibility to synthesize the overall significance of the results obtained. Concerning the different typologies of the results obtained in the present study on the coastline of Finland the WOE integration summarized a "Slight" environmental impact in half of the study areas (Vaasa, Pori, Uusikaupunki, Hanko, and Helsinki) and "Moderate" in the other ones, none of the sites showing "Major" or "Severe" impacts. Conclusively, the approach provides elaborated hazard indices in a user-friendly format useful to support a more comprehensive process of risk assessment and "site-oriented" management decisions.

CONCLUSIONS

The key message of this study is that the use of mussel caging and WOE is an effective and practical method for evaluating the contamination status of marine coastal areas in the Baltic Sea. Both are novel methods in this region; caging of bivalves has so far been tested in research projects only (e.g., Rank et al., 2007; Dabrowska et al., 2013; Turja et al., 2013, 2014, 2015; Lehtonen et al., 2016; Kholodkevich et al., 2017) but it has not been included in regular monitoring programmes in any of the surrounding countries. The current study was targeted at developing the caging methodology and assessing its efficiency in the coastal waters of Finland and the results show convincingly that a fully integrated field sampling methodology including simultaneous chemical and biological effects parameters (biomarkers) is a cost-efficient and effective methodology. The application of biomarkers and other biological effects methods has gained more ground in the monitoring of marine pollution (Cajaraville et al., 2000; Hagger et al., 2009; Lam, 2009) and has been recommended based on the results of large research programmes carried out also in the Baltic Sea (Lehtonen et al., 2006b, 2014). Combined with the WOE approach an integrated assessment on the status of chemical contamination can be obtained and also linked with other environmental indicators (community analyses, eutrophication, hydrography) a more holistic assessment can be achieved, showing more clearly the linkages between contamination between hazardous substances and other stressors, both natural and anthropogenic. An integrated chemical-biological monitoring yields significantly more information on the contamination status of aquatic environments compared to the chemistry-only based approach, presenting early warning signals of biological effects and also mixture/multistressor effects that would remain undiscovered without using biomarkers. However, due to the complex biological and physico-chemical interactions biomarker results measured in field populations (also caged organisms) should always be carefully examined to ensure correct data interpretation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

KL, HA, TK, and AL contributed conception and design of the study. All authors contributed to the organization of the database, wrote sections of the manuscript, and contributed to manuscript revision, read and approved the submitted version. KL, GE, and FR performed the statistical analysis. KL wrote the first draft of the manuscript.

ACKNOWLEDGMENTS

The authors are grateful to the crew of r/v Aranda and technical staff of SYKE for highly professional work performances during the deployment and recovery of the mussel cages. Jari Nuutinen and Timo Sara-Aho (SYKE) performed the PAH and trace metal analyses, respectively. Thanks are also directed to Jaakko Mannio, Katri Siimes, and Emmi Vähä for management, help, and expertize during the projects leading to this paper and its preparation. The projects MerPAH and UuPri during which the data was collected were funded by the Ministry of the Environment of Finland.

REFERENCES

Ahkola, H., Siimes, K., and Rantakokko, P. (2018). "Suitability of silicone passive sampler for monitoring TBT concentrations in surface water," in *2nd Water JPI Conference, Emerging Pollutants in Freshwater Ecosystems* (Helsinki: Poster).

Alvarez, D. A., Petty, J. D., Huckins, J. N., Jones-Lepp, T. L., Getting, D. T., and Goddard, J. P. (2004). Development of a passive, *in situ*, integrative sampler for hydrophilic organic contaminants in aquatic environments. *Environ. Toxicol. Chem.* 23, 1640–1648. doi: 10.1897/03-603

Andral, B., Galgani, F., Tomasino, C., Bouchoucha, M., Blottiere, C., Scarpato, A., et al. (2011). Chemical contamination baseline in the Western Basin of the Mediterranean Sea based on transplanted mussels. *Arch. Environ. Contam. Toxicol.* 61, 261–271. doi: 10.1007/s00244-010-9599-x

Andral, B., Stanisiere, J. Y., Sauzade, D., Damier, E., Thebault, H., Galgani, F., et al. (2004). Monitoring chemical contamination levels in the Mediterranean based on the use of mussel caging. *Mar. Pollut. Bull.* 49, 704–712. doi: 10.1016/j.marpolbul.2004.05.008

Baršienė, J., Lehtonen, K. K., Koehler, A., Broeg, K., Vuorinen, P. J., Lang, T., et al. (2006). Biomarker responses in flounder (*Platichthys flesus*) and mussel (*Mytilus edulis*) in the Klaipeda-Butinge area (Baltic Sea). *Mar. Pollut. Bull.* 53, 422–436. doi: 10.1016/j.marpolbul.2006.03.009

Bebianno, M. J., Pereira, C. G., Rey, F., Cravo, A., Duarte, D., D'Errico, G., et al. (2015). Integrated approach to assess ecosystem health in harbor areas. *Sci. Total Env.* 514, 92–107. doi: 10.1016/j.scitotenv.2015.01.050

Benedetti, M., Ciaprini, F., Piva, F., Onorati, F., Fattorini, D., Notti, A., et al. (2012). A multidisciplinary weight of evidence approach toward polluted sediments: integrating sediment chemistry, bioavailability, biomarkers responses and bioassays. *Environ. Int.* 38, 17–28. doi: 10.1016/j.envint.2011.08.003

Benedetti, M., Gorbi, S., Fattorini, D., Pacitti, D., d'Errico, G., Piva, F., et al. (2014). Environmental hazards from natural seepage: integrated classification of risk from sediment In review chemistry, bioavailability and biomarkers responses 512 in sentinel species. *Environ. Pollut.* 185, 116–126. doi: 10.1016/j.envpol.2013.10.023

Benito, D., Ahvo, A., Nuutinen, J., Bilbao, D., Saenz, J., Etxebarria, N., et al. (2019). Influence of season-depeding ecological variables on biomarker baseline levels in mussels *Mytilus trossulus* from two Baltic Sea subregions. *Sci. Total Env.* 689, 1087–1103. doi: 10.1016/j.scitotenv.2019.06.412

Beyer, J., Green, N. W., Brooks, S., Allan, I. J., Ruus, A., Gomes, T., et al. (2017). Blue mussels (*Mytilus edulis* spp.) as sentinel organisms in coastal pollution monitoring: a review. *Mar. Environ. Res.* 130, 338–365. doi: 10.1016/j.marenvres.2017.07.024

Bocquené, G., and Galgani, F. (1998). Biological effects of contaminants: cholinesterase inhibition by organophosphate and carbamate compounds. *ICES Tech. Mar. Environ. Sci.* 22:1–12.

Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1006/abio.1976.9999

Cajaraville, M. P., Bebianno, M. J., Blasco, J., Porte, C., Sarasquete, C., and Viarengo, A. (2000). The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *Sci. Total Environ.* 247, 295–311. doi: 10.1016/S0048-9697(99)00499-4

Carlberg, I., and Mannervik, B. (1975). Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J. Biol. Chem.* 250, 5475–5480.

Claiborne, A. (1985). "Catalase activity," in *Handbook of Methods for Oxygen Radical Research.* ed R. A. Greenwald (Boca Raton, FL: CRC Press), 283–284.

Dabrowska, H., Kopko, O., Turja, R., Lehtonen, K. K., Gora, A., and Polak-Juszczak, L. (2013). Sediment contaminants and contaminant levels and biomarkers in caged mussels (*Mytilus trossulus*) in the southern Baltic Sea. *Mar. Environ. Res.* 84, 1–9. doi: 10.1016/j.marenvres.2012.11.001

Damiens, G., Gnassia-Barelli, M., Loques, F., Romeo, M., and Salbert, V. (2007). Integrated biomarker response index as a useful tool for environmental assessment evaluated using transplanted mussels. *Chemosphere* 66, 574–583. doi: 10.1016/j.chemosphere.2006.05.032

EC (2008). Directive 2008/105/EC of the European Parliament and the Council of 16 December 2008 on environmental quality standards in the field of water policy. *Off. J. Euro. Union* 348, 84–97. Available online at: http://data.europa.eu/eli/dir/2008/105/oj

Guilhermino, L., Barros, B., Silva, M. C., and Soares, A. M. V. M. (1998). Should the use of inhibition of cholinesterase as a specific biomarker for organophosphate and carbamate pesticides be questioned? *Biomarkers* 3, 157–163. doi: 10.1080/135475098231318

Habig, W. H., Pabst, M. J., and Jakoby, B. (1974). Glutathione-*S*-transferase. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130–7139.

Hagger, J. A., Galloway, T. S., Langston, W. J., and Jones, M. B. (2009). Application of biomarkers to assess the condition of European marine sites. *Environ. Pollut.* 157, 2003–2010. doi: 10.1016/j.envpol.2009.02.038

HELCOM (2010). "Hazardous substances in the Baltic Sea–an integrated thematic assessment of hazardous substances in the Baltic Sea," in *Baltic Sea Environment Proceedings* (Helsinki), 120B.

HELCOM (2018). "State of the Baltic Sea–second HELCOM holistic assessment 2011-2016," in *Baltic Sea Environment Proceedings* (Helsinki), 155.

Kholodkevich, S. V., Kuznetsova, T. V., Sharov, A. N., Kurakin, A. S., Lips, U., Kolesova, N., et al. (2017). Applicability of a bioelectronic cardiac monitoring system for the determination of biological effects of pollution in bioindicator species in the Gulf of Finland. *J. Mar. Sys.* 171, 8151–8158. doi: 10.1016/j.jmarsys.2016.12.005

Koivisto, M. E., and Westerbom, M. (2010). Habitat structure and complexity as determinants of biodiversity in blue mussel beds on sublittoral rocky shores. *Mar. Biol.* 157, 1463–1474. doi: 10.1007/s00227-010-1421-9

Kopecka, J., Lehtonen, K. K., Baršienė, J., Broeg, K., Vuorinen, P. J., Gercken, J., et al. (2006). Measurements of biomarker levels in flounder (*Platichthys flesus*) and blue mussel (*Mytilus trossulus*) from the Gulf of Gdansk (southern Baltic). *Mar. Pollut. Bull.* 53, 406–421. doi: 10.1016/j.marpolbul.2006.03.008

Krishnakumar, P., Ali Qurban, M., and Sasikumar, G. (2018). "Biomonitoring of trace metals in the coastal waters using bivalve molluscs," in *Trace Elements–Human Health and Environment.* ed H. Saled (InTech Open),153–176.

Lam, P. K. S. (2009). Use of biomarkers in environmental monitoring. *Ocean Coast. Manag.* 52, 348–354. doi: 10.1016/j.ocecoaman.2009.04.010

Lehtonen, K. K., Kankaanpä, H., Leiniö, S., Sipiä, V. O., Pflugmacher, S., and Sandberg-Kilpi, E. (2003). Accumulation of nodularin-like compounds from the cyanobacterium *Nodularia spumigena* and changes in acetylcholinesterase activity in the clam *Macoma balthica* during short-term laboratory exposure. *Aquat. Toxicol.* 64, 461–476. doi: 10.1016/S0166-445X(03)00101-2

Lehtonen, K. K., Leiniö, S., Schneider, R., and Leivuori, M. (2006a). Biomarkers of pollution effects in the bivalves *Mytilus edulis* and *Macoma balthica* collected from the southern coast of Finland (Baltic Sea). *Mar. Ecol. Prog. Ser.* 322, 155–168. doi: 10.3354/meps322155

Lehtonen, K. K., Schiedek, D., Koehler, A., Lang, T., Vuorinen, P. J., Förlin, L., et al. (2006b). The BEEP project in the Baltic Sea: overview of results and outline for a regional biological effects monitoring strategy. *Mar. Pollut. Bull.* 53, 523–537. doi: 10.1016/j.marpolbul.2006.02.008

Lehtonen, K. K., Sundelin, B., Lang, T., and Strand, J. (2014). Development of tools for integrated monitoring and assessment of hazardous substances and their biological effects in the Baltic Sea. *Ambio* 43, 69–81. doi: 10.1007/s13280-013-0478-3

Lehtonen, K. K., Turja, R., Budzinski, H., and Devier, M.-H. (2016). An integrated chemical-biological study using caged mussels (*Mytilus trossulus*) along a pollution gradient in the Archipelago Sea (SW Finland, Baltic Sea). *Mar. Environ. Res.*119, 207–221. doi: 10.1016/j.marenvres.2016.06.003

Leiniö, S., and Lehtonen, K. K. (2005). Seasonal variability in biomarkers in the bivalves *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea. *Comp. Biochem. Physiol. C* 140, 408–421. doi: 10.1016/j.cca.2005.04.005

Lekube, X., Izagirre, U., Soto, M., and Marigomez, I. (2014). Lysosomal and tissue-level biomarkers in mussels cross-transplanted among four estuaries with different pollution levels. *Sci. Total Environ.* 472, 36–48. doi: 10.1016/j.scitotenv.2013.10.075

Marigómez, I., Zorita, I., Izagirre, U., Ortiz-Zarragoitia, M., Navarro, P., Etxebarria, N., et al. (2013). Combined use of native and caged mussels to assess biological effects of pollution through the integrative biomarker approach. *Aquat. Toxicol.* 136, 32–48. doi: 10.1016/j.aquatox.2013.03.008

Mayer, P., Tolls, J., Hermens, L., and Mackay, D. (2003). Equilibrium sampling devices. *Environ. Sci. Technol.* 37, 184–191. doi: 10.1021/es032433i

Moschino, V., Del Negro, P., De Vittor, C., and Da Ros, L. (2016). Biomonitoring of a polluted coastal area (Bay of Muggia, Northern Adriatic Sea): a five-year study using transplanted mussels. *Ecotoxicol. Environ. Saf.* 128, 1–10. doi: 10.1016/j.ecoenv.2016.02.006

Ohkawa, H., Ohishi, N., and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95, 351–358. doi: 10.1016/0003-2697(79)90738-3

Payne, J. F., Mathieu, A., Melvin, W., and Fancey, L. L. (1996). Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. *Mar. Pollut. Bull.* 32, 225–231 doi: 10.1016/0025-326X(95)00112-Z

Perus, J., Bonsdorff, E., Bäck, S., Lax, H. G., Villnäs, A., and Westberg, V. (2007). Zoobenthos as indicators of ecological status in coastal brackish waters: a comparative study from the Baltic Sea. *Ambio* 36, 250–256. doi: 10.1579/0044-7447(2007)36[250:ZAIOES]2.0.CO;2

Pintado-Herrera, M. G., Lara-Martin, P. A., Gonzalez-Mazo, E., and Allan, I. J. (2016). Determination of silicone rubber and low-density polyethylene diffusion and polymer/water partition coefficients for emerging contaminants. *Environ. Toxicol. Chem.* 35, 2162–2172. doi: 10.1002/etc.3390

Piva, F., Ciaprini, F., Onorati, F., Benedetti, M., Fattorini, D., Ausili, A., et al. (2011). Assessing sediment hazard through a Weight of Evidence approach with bioindicator organisms: a practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays. *Chemosphere* 83, 475–485 doi: 10.1016/j.chemosphere.2010.12.064

Rank, J., Lehtonen, K. K., Strand, J., and Laursen, M. (2007). DNA damage, acetylcholinesterase activity and lysosomal stability in native and transplanted mussels (*Mytilus edulis*) in areas close to coastal chemical dumping sites in Denmark. *Aquat. Toxicol.* 84, 50–61 doi: 10.1016/j.aquatox.2007.05.013

Regoli, F., d'Errico, G., Nardi, A., Mezzelani, M., Fattorini, D., Benedetti, M., et al. (2019). Application of a weight of evidence approach for monitoring complex environmental scenarios: the case-study of off-Shore platforms. *Front. Mar. Sci.* 6:377. doi: 10.3389/fmars.2019.00377

Regoli, F., Frenzilli, G., Bocchetti, R., Annarumma, F., Scarcelli, V., Fattorini, D., et al. (2004). Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. *Aquat. Toxicol.* 68, 167–178. doi: 10.1016/j.aquatox.2004.03.011

Regoli, F., Pellegrini, D., Cicero, A. M., Nigro, N., Benedetti, M., Gorbi, S., et al. (2014). A multidisciplinary weight of evidence approach for environmental risk assessment at the Costa Concordia wreck: integrative indices from Mussel Watch. *Mar. Environ. Res.* 96, 92–104. doi: 10.1016/j.marenvres.2013.09.016

Rusina, T. P., Smedes, F., and Klanova, J. (2010). Diffusion coefficients of polychlorinated biphenyls and polycyclic aromatic hydrocarbons in polydimethylsiloxane and low-density polylethylene polymers. *J. Appl. Polym. Sci.* 116, 1803–1810. doi: 10.1002/app.31704

Schiedek, D., Broeg, K., Baršienė, J., Lehtonen, K. K., Gercken, J., Pfeifer, S., et al. (2006). Biomarker responses and indication of contaminant effects in blue mussel (*Mytilus edulis*) and female eelpout (*Zoarces viviparus*) from the western Baltic Sea. *Mar. Pollut. Bull.* 53, 387–405. doi: 10.1016/j.marpolbul.2005.11.013

Serafim, A., Lopes, B., Company, R., Cravo, A., Gomes, T., Sousa, V., et al. (2011). A multi-biomarker approach in cross-transplanted mussels *Mytilus galloprovincialis*. *Ecotoxicology* 20, 1959–1974. doi: 10.1007/s10646-011-0737-7

Smedes, F., Geertsma, R. W., Zande, T. V. D., and Booij, K. (2009). Polymer-water partition coefficients of hydrophobic compounds for passive sampling: application of cosolvent models for validation. *Environ. Sci. Technol.* 43, 7047–7054. doi: 10.1021/es9009376

Smolders, R., Bervoets, L., De Coen, W., and Blust, R. (2004). Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. *Environ. Pollut.* 129, 99–112. doi: 10.1016/j.envpol.2003.09.027

Tsangaris, C., Kormas, K., Strogyloudi, E., Hatzianestis, I., Neofitou, C., Andral, B., et al. (2010). Multiple biomarkers of pollution effects in caged mussels on the Greek coastline. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 151, 369–378. doi: 10.1016/j.cbpc.2009.12.009

Tucca, F., Moya, H., and Barra, R. (2014). Ethylene vinyl acetate polymer as a tool for passive sampling monitoring of hydrophobic chemicals in the salmon farm industry. *Mar. Poll. Bull.* 88, 174–179. doi: 10.1016/j.marpolbul.2014.09.009

Turja, R., Höher, N., Snoeijs, P., Baršienė, J., Butrimavičienė, L., Kuznetsova, T., et al. (2014). A multibiomarker approach for the assessment of pollution impacts in two areas in the Swedish Baltic Sea coast using caged mussels (*Mytilus trossulus*). *Sci. Total Environ.* 473–474, 398–409. doi: 10.1016/j.scitotenv.2013.12.038

Turja, R., Lehtonen, K. K., Meierjohann, A., Brozinski, J., Vahtera, E., Soirinsuo, A., et al. (2015). The mussel caging approach in assessing biological effects of wastewater treatment plant discharges in the Gulf of Finland (Baltic Sea). *Mar. Pollut. Bull.* 97, 135–149. doi: 10.1016/j.marpolbul.2015.06.024

Turja, R., Soirinsuo, A., Budzinski, H., Devier, M.-H., and Lehtonen, K. K. (2013). Biomarker responses and accumulation of hazardous substances in mussels (*Mytilus trossulus*) transplanted along a pollution gradient close to an oil terminal in the Gulf of Finland (Baltic Sea). *Comp. Biochem. Physiol. C* 157, 80–92. doi: 10.1016/j.cbpc.2012.09.006

Väinölä, R., and Strelkov, P. (2011). *Mytilus trossulus* in Northern Europe. *Mar. Biol.* 158, 817–833. doi: 10.1007/s00227-010-1609-z

Viarengo, A., and Canesi, L. (1991). Mussels as biological indicators of pollution. *Aquaculture* 94, 225–243. doi: 10.1016/0044-8486(91)90120-V

Vrana, B., Mills, G. A., Allan, I. J., Dominiak, E., Svensson, K., Knutsson, J., et al. (2005). Passive sampling techniques for monitoring pollutants in water. *Trends Anal. Chem.* 24, 845–868. doi: 10.1016/j.trac.2005.06.006

Westerbom, M., Kilpi, M., and Mustonen, O. (2002). Blue mussels, *Mytilus edulis*, at the edge of the range: population structure, growth and biomass along a salinity gradient in the north-eastern Baltic Sea. *Mar. Biol.* 140, 991–999. doi: 10.1007/s00227-001-0765-6

Zinkl, J. G., Lockhart, W. L., Kenny, S. A., and Ward, F. J. (1991). "The effects of cholinesterase inhibiting insecticides on fish," in *Cholinesterase Inhibiting Insecticides,* ed P. Mineau (Amsterdam: Elsevier), 233–254.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership