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The Use of the Marine Microalga *Tisochrysis lutea* (*T-iso*) in Standard Toxicity Tests; Comparative Sensitivity With Other Test Species

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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

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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

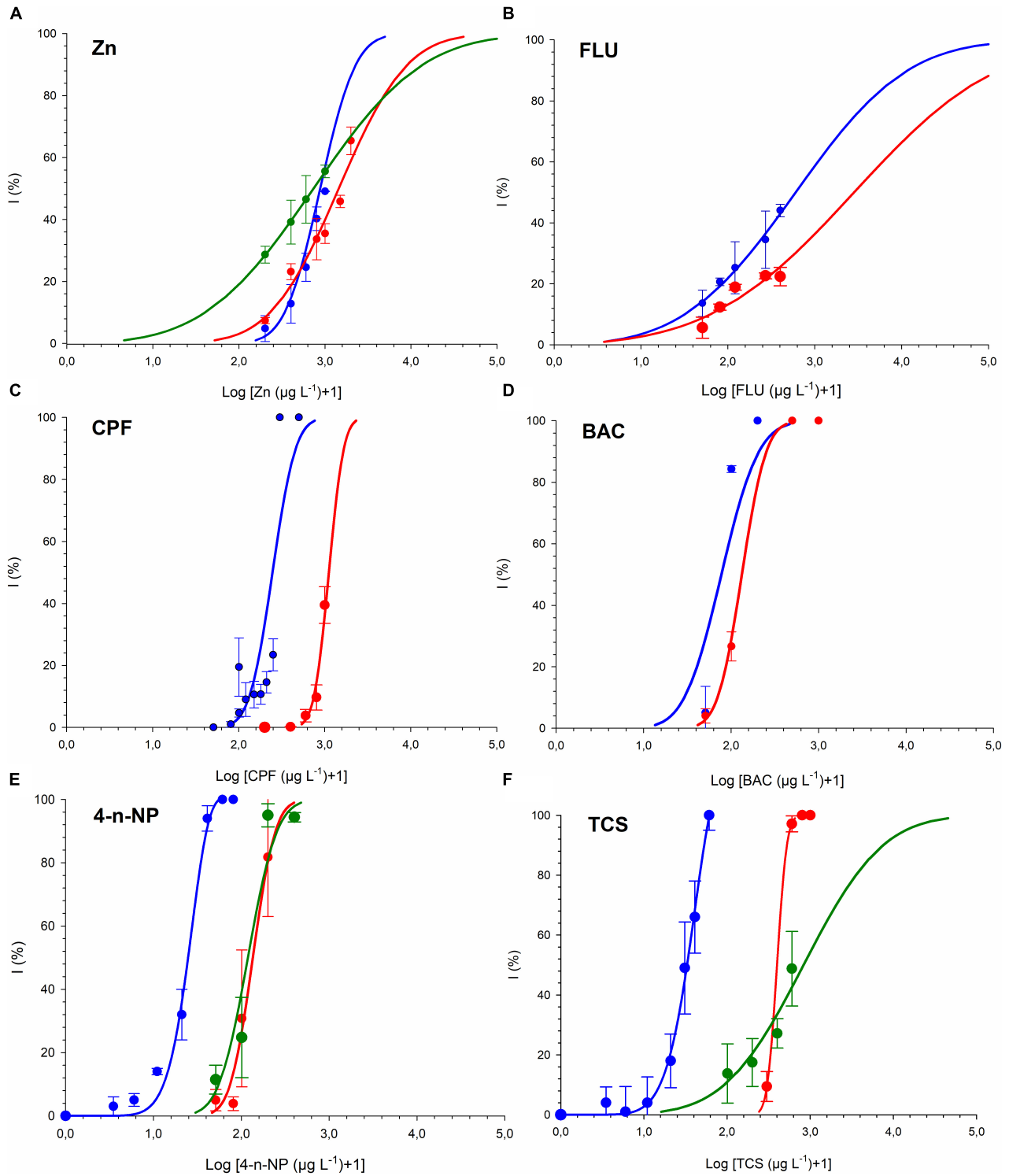

FIGURE 1 | Growth inhibition of *T-iso* (blue), *P. tricornutum* (red) and *R. subcapitata* (green) exposed to **(A)** Zinc **(B)** Fluoranthene **(C)** Chlorpyrifos **(D)** Benzalkonium chloride **(E)** 4-n-Nonylphenol. **(F)** Triclosan. Blue curve from panel **(E,F)** published in Tato et al. (2018).

(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.

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