Monitoring Non-indigenous Species in Port Habitats: First Application of a Standardized North American Protocol in the Mediterranean Sea

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Baseline port monitoring for fouling communities is an essential tool to assess non-indigenous species (NIS) introduction and spread, but a standardized and coordinated method among Mediterranean and European countries has not yet been adopted. In this context, it is important to test monitoring protocols that allow for the collection of standardized and directly comparable data, replicated across time and space. Here, for the first time in the Mediterranean Sea, we tested a standardized protocol developed by the Smithsonian Environmental Research Center (SERC) and used now in several countries. The 3-year monitoring survey (2018–2020) was conducted in the Gulf of La Spezia (Ligurian Sea, Italy), with the deployment of a total of 50 PVC panels per year in five different sites (a commercial harbor, three marinas and a site in the proximity of a shellfish farm). A total of 79 taxa were identified, including 11 NIS, ranging from zero to seven NIS for each panel. In comparison with previous surveys, new NIS arrivals were observed in the Gulf of La Spezia: *Botrylloides* cf. *niger*, *Branchiomma* sp., *Branchiomma luctuosum*, *Paraleucilla magna,* and *Watersipora arcuata*. At the end of the 3-year monitoring, mean richness? and percent cover of NIS were measured, and both measures differed across the monitoring sites, with higher values in two marinas and in the commercial harbor. Among years, richness of NIS was relatively stable at each monitoring site. The structure of the fouling was influenced more by native and cryptogenic species than by NIS. Moreover, among the monitoring sites, the density of artificial structures was not a reliable predictor or proxy for local NIS abundance. This first application of the SERC method in the Mediterranean Sea, demonstrates both pros and cons, including the detection of new NIS reported here. Further direct comparisons with other NIS monitoring tools are recommended, and additional tests to assess its effectiveness in this biogeographical area are encouraged. A broader application of this and other standard methods across temporal and spatial scales in the Mediterranean basin should be implemented, providing critical data needed to assess changes in the structure of fouling communities.

Keywords: macrozoobenthos, sessile fauna, settlement panels, marine bioinvasions, Ligurian Sea, fouling

INTRODUCTION

The introduction of non-indigenous species (NIS) in marine environments is considered one of the most important factors contributing to global change (Ruiz et al., 1997; Occhipinti-Ambrogi, 2007; Galil et al., 2018a), including both ecological and economic impacts (Occhipinti-Ambrogi, 2007; Katsanevakis et al., 2014a; Ojaveer et al., 2015). The Mediterranean Sea is among the most invaded marine provinces (Bailey et al., 2020), with almost 800 multicellular NIS recorded (Galil et al., 2018b), and the main vectors of introduction include: the opening and enlargement of the Suez Canal, aquaculture and shipping (Ruiz et al., 2000; Galil et al., 2014, 2015; Katsanevakis et al., 2014b; Tsiamis et al., 2020). While most species introduced through the Suez Canal and by aquaculture vectors are generally confined in relatively restricted areas, vessels can further spread NIS throughout the whole Mediterranean Basin (Galil et al., 2018a). The maritime traffic involved in the introduction and spread of NIS includes both large commercial and small recreational vessels, which can carry species inside the ballast waters or as biofouling on vessel hulls (Ruiz et al., 2000; Clarke Murray et al., 2011; Canning-Clode et al., 2013; Zabin, 2014; Ferrario et al., 2017; Galil et al., 2018b). As defined by the International Maritime Organization, biofouling is "the accumulation of aquatic organisms such as microorganisms, plants and animals, on surfaces and structures immersed in or exposed to the aquatic environment" (IMO, 2012). The macrozoobenthic component of the fouling communities is usually composed of both sessile epibionts—sponges, serpulids, bivalves, barnacles, bryozoans, tunicates—and mobile marine invertebrates, like but not limited to flatworms, amphipods, isopods, sea spiders and brittle stars (Connell and Glasby, 1999; Glasby and Connell, 1999; Connell, 2001; Lord et al., 2015; Gavira-O'Neill et al., 2018; Culver et al., 2021).

Maritime traffic plays a key role in the introduction of fouling NIS, favoring their first settlement and establishment in ports and then creating hotspot areas for NIS. It has long been considered that large commercial ports act as primary hubs for fouling NIS, due to the transit of transoceanic cargo ships, while small boats are involved in their secondary spread in marinas and neighboring areas (Minchin et al., 2006; Floerl et al., 2009; Hulme, 2009; Ros et al., 2013; Zabin, 2014; Marchini et al., 2015). On the other hand, recent studies have suggested the importance of recreational boating in the primary introduction and spread of NIS (Ashton et al., 2014; Ferrario et al., 2017; Ulman et al., 2019a,b). After introduction to a port environment, the settlement of NIS can be facilitated both by the large abundance of available artificial substrates (Glasby et al., 2007) and the tolerance of these species to different environmental conditions, including polluted waters (Piola and Johnston, 2008; Piola et al., 2009).

For decades, fouling assemblages have served as a model to study bioinvasion processes (Ruiz et al., 2009; Canning-Clode et al., 2011; Marraffini et al., 2017; Leclerc et al., 2018; Marasinghe et al., 2018; Vieira et al., 2018; Ferrario et al., 2020). Furthermore, ports and marinas can be considered ideal sites for the early detection and monitoring of NIS presence

and abundance, due to their importance as hotspot areas of NIS (Olenin et al., 2011; Ojaveer et al., 2014; Marraffini et al., 2017). However, no formal protocol has yet been adopted for the standardized assessment of fouling NIS in the European and Mediterranean marine regions. Monitoring of marine fouling communities is fundamental to track the introduction and spread of NIS, as well as to evaluate the power of legislation designed for limiting further introductions (Ruiz and Hewitt, 2002; Lehtiniemi et al., 2015; Marraffini et al., 2017). The implementation of long-term monitoring programs and a preventive approach can contribute to the assessment of the potential risks of NIS that have been demonstrated to negatively affect native communities—e.g., contributing to the decrease of vulnerable species (Marraffini et al., 2017; Occhipinti-Ambrogi, 2021). For these reasons, a standardized monitoring and a coordination among countries is highly recommended, in order to obtain comparable data replicated across time and space (Hewitt and Martin, 2001; Olenin et al., 2010, 2011; Latombe et al., 2017; Bailey et al., 2020). This is also in accordance with the Marine Strategy Framework Directive (MSFD 2008/56/EC), suggesting a development and application of standardized methods to detect NIS—a descriptor included in the achievement of the Good Environmental Status in European marine waters.

Several methodologies for sampling fouling communities in port habitats have been developed over the years. Most common techniques include (i) Rapid Assessment Surveys—RAS (e.g., Cohen et al., 2005; Campbell et al., 2007; Olenin et al., 2007; Minchin, 2012; Lehtiniemi et al., 2015; Marchini et al., 2015; Gewing and Shenkar, 2017; Ulman et al., 2017); (ii) net-assisted scraping (e.g., Ferrario et al., 2017; Ulman et al., 2017; Tempesti et al., 2020), including quadrat scraping during snorkeling or scuba diving (e.g., Neves et al., 2007; Awad et al., 2014; Ulman et al., 2019b); and artificial substrate units (ASU), such as (iii) two-dimensional settlement panels (e.g., Relini, 1977; Canning-Clode et al., 2013; Dos Santos Schwan et al., 2016; Lezzi et al., 2017; Leclerc and Viard, 2018; Marasinghe et al., 2018; Carlton, 2019; Leclerc et al., 2019); and (iv) three-dimensional artificial habitat collectors (e.g., Fowler et al., 2013; Gestoso et al., 2019; Outinen et al., 2019; Holmes and Callaway, 2020; Obst et al., 2020; Ros et al., 2020). Any of the above methods involves both advantages and disadvantages, and their success or suitability depend on the aim of the study. For example, quantitative methods (e.g., quadrats, settlement panels) are useful to thoroughly assess the entire fouling community but require a lot of fieldwork and laboratory effort. Conversely, qualitative methods like RAS allow for the collection of a lot of data on species distribution in a short timeframe, but the focus is typically only on target NIS already known and easily recognizable in the field. This method can limit the early detection of new arrivals, particularly of inconspicuous NIS. Net-assisted scraping probably ensures the most comprehensive picture of the fouling community, yet it requires long laboratory analyses and does not provide quantitative outputs (although semi-quantitative abundance estimates are possible; Ferrario et al., 2017). In addition, some methods are more suitable for sessile taxa (e.g., settlement panels), while other ones are better for mobile taxa

(e.g., scrapes, 3-D ASU). Light exposure and orientation of the ASU also affects the ability of collecting the photosynthetic component of fouling communities. Therefore, the best practice for comprehensive sampling fouling communities should be a simultaneous multi-method approach in the same study area, as suggested by Tait et al. (2018) and Kakkonen et al. (2019).

In recent years, several sampling efforts focused along European and Mediterranean ports were conducted to monitor NIS presence. Using RAS, Ashton et al. (2006) and Nall et al. (2015) sampled in Scotland, while Bishop et al. (2015) sampled with the same method in the English Channel. In the Normandy region, Verlaque and Breton (2019) found macroalgal NIS through a RAS by scuba diving, involving citizen scientists after a multi-year field survey. In the ports of Gdynia and Gdańsk (Baltic Sea), Brzana et al. (2019) found a non-indigenous tanaid species using both settlement panels and artificial habitat collectors. In the subtropical island of Madeira (Atlantic Ocean), Canning-Clode et al. (2013) monitored NIS presence and abundance with a 6-year fouling survey through settlement panels. Regarding the Mediterranean Sea, Ferrario et al. (2017) sampled ports in the Western sector (Sardinian Sea, Ligurian Sea, and North Tyrrhenian Sea) by scraping the artificial hard substrates with a rigid hand-held net and Tempesti et al. (2020) sampled in the same way in the port area of Leghorn. In the Taranto Sea (Ionian Sea), Lezzi et al. (2017) carried out a 2-year macrofouling monitoring program using PVC settlement panels, while along the Slovenian coasts Fortič et al. (2019) combined different methods (RAS, scraping net and scuba dives) in their monitoring. In the Aegean Sea, Bariche et al. (2020) reported for the first time a non-indigenous nudibranch, using Autonomous Reef Monitoring Structures (ARMS) protocol, proposed by Obst et al. (2020). In the Southern Mediterranean Sea, Chebaane et al. (2019) sampled through RAS fishing ports and marinas in Monastir Bay, Tunisia. Lastly, the broadest investigation of non-indigenous invertebrates in Mediterranean marinas was conducted by Ulman et al. (2017) with both modified RAS and scrapes by hand-held rigid net. During the last few years, the metabarcoding approach has also started to be employed in NIS surveys with satisfying results (see also Borrell et al., 2017; Miralles et al., 2021; Pearman et al., 2021). Metabarcoding can be considered as an efficient tool to detect cryptic species or early life stages, but one of the main disadvantages is the incompleteness of genetic sequence reference libraries (Zaiko et al., 2018; Duarte et al., 2021). For this reason, an integrative approach should be considered as the gold standard (Padial et al., 2010; Cahill et al., 2018; Obst et al., 2020; Duarte et al., 2021).

In this study we applied for the first time in the Mediterranean Sea—namely in the Gulf of La Spezia (Ligurian Sea. Italy)—an international standard protocol for sessile fouling NIS monitoring in port habitats, with the aim to assess: (i) the fouling communities and NIS colonization in different harbor sites; (ii) the development of NIS colonization in the fouling communities over time; (iii) the effectiveness of this protocol for the early detection of newly introduced NIS in the Mediterranean; and (iv) the relationship between abundance of NIS and density of artificial structures in port habitats.

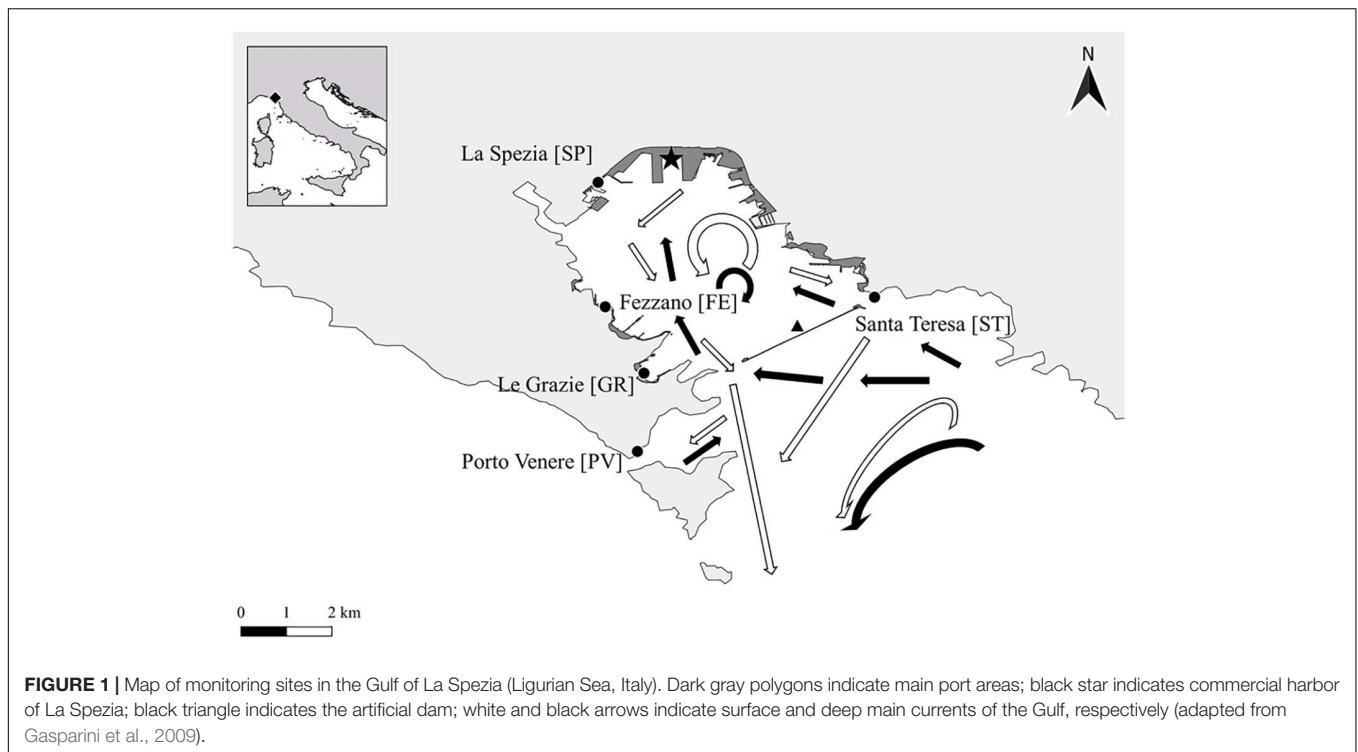# MATERIALS AND METHODS

Study Area

The Gulf of La Spezia (44.09°N, 9.85°E) is one of the largest bays of the Ligurian Sea (Italy), characterized by the presence of several anthropogenic activities, including a commercial harbor and several marinas, and featuring intense anthropogenic influences such as urban runoff, naval industries, a military base, an electric power-plant and aquaculture facilities (Gasparini et al., 2009). The bay is about 5 km wide and 10 km long, and is separated in two parts by an artificial offshore dam about 2 km long (Gasparini et al., 2009).

The fouling monitoring program included five sites inside the gulf (**Figure 1**): in the marinas of Fezzano, Le Grazie and Porto Venere (FE, GR, and PV, respectively), near the commercial harbor of La Spezia (SP), and in a small marina near a mussel culture facility in Santa Teresa (ST). The trend of sea surface temperature (−0.915 m) from August 2018 to August 2020 was obtained by extrapolating data from loggers (ONSET HOBO WaterTemp Pro v2) deployed in the gulf for other experimental purposes by ENEA Marine Environment Research Centre (La Spezia).

Monitoring Protocol

The monitoring was carried out for three consecutive years (2018–2020) by applying a standardized protocol developed by the Smithsonian Environmental Research Center (Edgewater, MD, United States) that is being used to detect and track NIS in the U.S as well as Canada, Ecuador, and Panama (see also de Rivera et al., 2005; Gartner et al., 2016; Simkanin et al., 2016; Marraffini et al., 2017; Tracy et al., 2017; Chang et al., 2018; Jurgens et al., 2018; Newcomer et al., 2018, 2019; Carlton, 2019; Torchin et al., 2021). A total of 50 PVC settlement panels (14 × 14 cm) were deployed in the summer of each year in the Gulf of La Spezia, allocating 10 panels in each site. Specifically, the passive collector (substrate) is composed of a settlement panel attached to a brick, with a sanded surface facing downward to minimize algal growth and facilitate invertebrate recruitment (Crooks et al., 2011; Marraffini et al., 2017; Jimenez et al., 2018). The experimental units were secured to docks or floating pontoons using rope, suspended 1 m below the sea surface, and immersed for 3 months during the summer season, in order to maximize the larval recruitment (Freestone et al., 2011; Marraffini et al., 2017). In 2018, the panels were immersed from July to October (87 days of deployment), in 2019 from April to July (89 days), and in 2020 from May to August (101 days). Differences in community assembly time across years were due to logistical constraints, in addition to mobility restrictions related to the health emergency of Coronavirus disease (COVID-19), and were considered during data analysis. At the end of the immersion period, all panels were retrieved, photographed and observed under dissecting microscopes for taxonomic identification of sessile invertebrates.

The percent cover of the whole fouling assemblage was assessed by point count method (n = 50 points; Chang et al., 2018), using a plastic grid (7 × 7 points, with an additional 50th

FIGURE 1 | Map of monitoring sites in the Gulf of La Spezia (Ligurian Sea, Italy). Dark gray polygons indicate main port areas; black star indicates commercial harbor of La Spezia; black triangle indicates the artificial dam; white and black arrows indicate surface and deep main currents of the Gulf, respectively (adapted from Gasparini et al., 2009).

point chosen randomly) positioned upon the plates observed under a dissecting microscope. With this method it is possible to observe more than one taxon in the same counting point, therefore percent cover for the whole community may be exceed 100%. Fouling communities on panels were retrieved and observed *in vivo* in 2018, while in 2019 and 2020 the panels were preserved in 70% ethanol upon retrieval and stored for later analysis.

Data Analysis

Species richness (S), NIS/native ratio and percent cover of each taxon were calculated for each panel ($N = 10$ per site). S and percent cover were assessed separately for all taxa, together with the assignment of biogeographic status *sensu* Chapman and Carlton (1991): NIS, native (including cryptogenic species—i.e., species that are not discernibly native or introduced, as defined by Carlton, 1996) and unresolved status (due to insufficient taxonomic resolution e.g., damaged specimens). Differences in mean values richness and percent cover of native and cryptogenic species and NIS were assessed by two-way ANOVA (Type III tests with two orthogonal fixed factors: "year," three levels: 2018, 2019, 2020; and "site," five levels: FE, GR, PV, SP, ST; unequal sample size). When pooling across sampling years, differences in mean richness and percent cover of NIS were assessed by one-way ANOVA (fixed factor "site," five levels: FE, GR, PV, SP, ST; unequal sample size). In case of non-homogeneity of the variances, data were properly transformed. A more conservative critical value ($\alpha < 0.01$) was chosen if the data transformation was not successful. Tukey's tests were used for *post hoc* comparisons of means.

Percent cover data were analyzed using principal coordinate analysis (PCO) in order to visualize the pattern of clustering among fouling communities from each site during the years of monitoring. The ordination technique was carried out on centroids (year × site), based on Bray-Curtis similarity on fourth root transformed taxa percent cover data. PCO both for the whole community, and for NIS and native species component were applied separately. Distance-based test for homogeneity of multivariate dispersions (PERMDISP) and permutational multivariate analysis of variance (PERMANOVA) were carried out to test differences in NIS percent cover values of communities among sites (Anderson et al., 2008).

Correlation between NIS percent cover and density of immersed artificial structures (e.g., docks, pontoons) of each monitoring site was tested, as suggested by Susick et al. (2020). Density of each site was measured through satellite images as the linear length of mooring structures within a 200 m diameter circle of the potential marine habitat, centered on the spatial centroid of deployed panels. Length of artificial structure visible above water was measured using the path ruler tool in Google Earth Pro v. 7.3.3.7699 (Susick et al., 2020).

Analyses were carried out with the software Microsoft Excel, R (R Core Team., 2017), QGIS (QGIS Development Team., 2020) and PRIMER 6 with PERMANOVA + add-on package (Clarke and Gorley, 2006; Anderson et al., 2008).

RESULTS

From August 2018 to August 2020, the range of surface temperature varied from 12 to 27°C (**Supplementary Figure 1**).

Mean temperatures during the monitoring periods were $25 \pm 1°C$ in 2018, $19 \pm 3°C$ in 2019 and $21 \pm 2°C$ in 2020.

Overall, we collected 126 out of the 150 panels initially deployed in the 3 years; the remaining panels (nine in 2018, four in 2019, and 11 in 2020) were lost due to external causes (e.g., coastal storms or vandalism).

After the 3-year monitoring, a total of 79 sessile taxa representing seven phyla were documented from the Gulf of La Spezia (**Figure 2**), including 11 NIS: four Bryozoa, four Annelida, one Porifera, and two Tunicata (**Table 1**). Among all the NIS observed, one has not been identified at species level (i.e., *Branchiomma* sp.). This species has been firstly assigned to *Branchiomma boholense* (Grube, 1878), but an accurate analysis of the specimens revealed divergent taxonomic characters, that cannot be attributed to the other *Branchiomma* spp. known to occur in the Mediterranean Sea, including the non-indigenous *Branchiomma bairdi* (McIntosh, 1885), *B. boholense* and *Branchiomma luctuosum* (Grube, 1870). Thus, further analyses are needed to confirm its identity, which could also be assigned to a fourth NIS belonging to the genus *Branchiomma*. The majority of the NIS were detected during all three monitoring campaigns, with the exception of *Botrylloides* cf. *niger* Herdman, 1886—found only in 2018—and *B. luctuosum*, found in 2018

and 2020. NIS were detected on all panels sampled over 3 years, except for a single panel at PV in 2019. Furthermore, nine panels exhibited only one NIS, seven of which were collected in PV from 2018 to 2020. The highest values of NIS richness ($S_{NIS} = 7$) found on single panels were from FE 2018, GR 2020, and ST 2020.

For species richness, the lowest value of mean S at each site, i.e., S_{tot} ($\pm$ SD), was observed in SP in 2018 (11 ± 4), while the highest in ST in the same year (22 ± 2) (**Figure 3**). Regarding the mean S_{NIS}, the lowest value was recorded in PV in 2019 (1 ± 1) and the highest in FE, GR and ST in 2018, as well as in FE and GR in 2020 (about five species on average). S of native and cryptogenic species (S_{native}) was the lowest in SP 2018 (7 ± 3) and the highest in PV 2020 (18 ± 3) (**Figure 3**). The mean NIS/(native + cryptogenic species) ratio showed a similar pattern, registering the lowest values in PV 2019 and 2020 (0.1 ± 0.04) and the highest in SP 2018 (0.6 ± 0.2). S_{native} resulted significantly different among sites, but not across years ($F = 11.3172$, $p = 9.73 \times 10^{-8}$; $F = 1.6857$, $p = 0.19003$, respectively). The site $\times$ year interaction resulted significant ($F = 3.9884$, $p = 3.462 \times 10^{-4}$) and *post hoc* test revealed that a single site (SP) contributed to this interaction (2018 vs. 2019 adjusted $p = 2.913 \times 10^{-4}$; 2018 vs. 2020 adjusted $p = 7.745 \times 10^{-4}$). Similarly, S_{NIS} showed significative differences
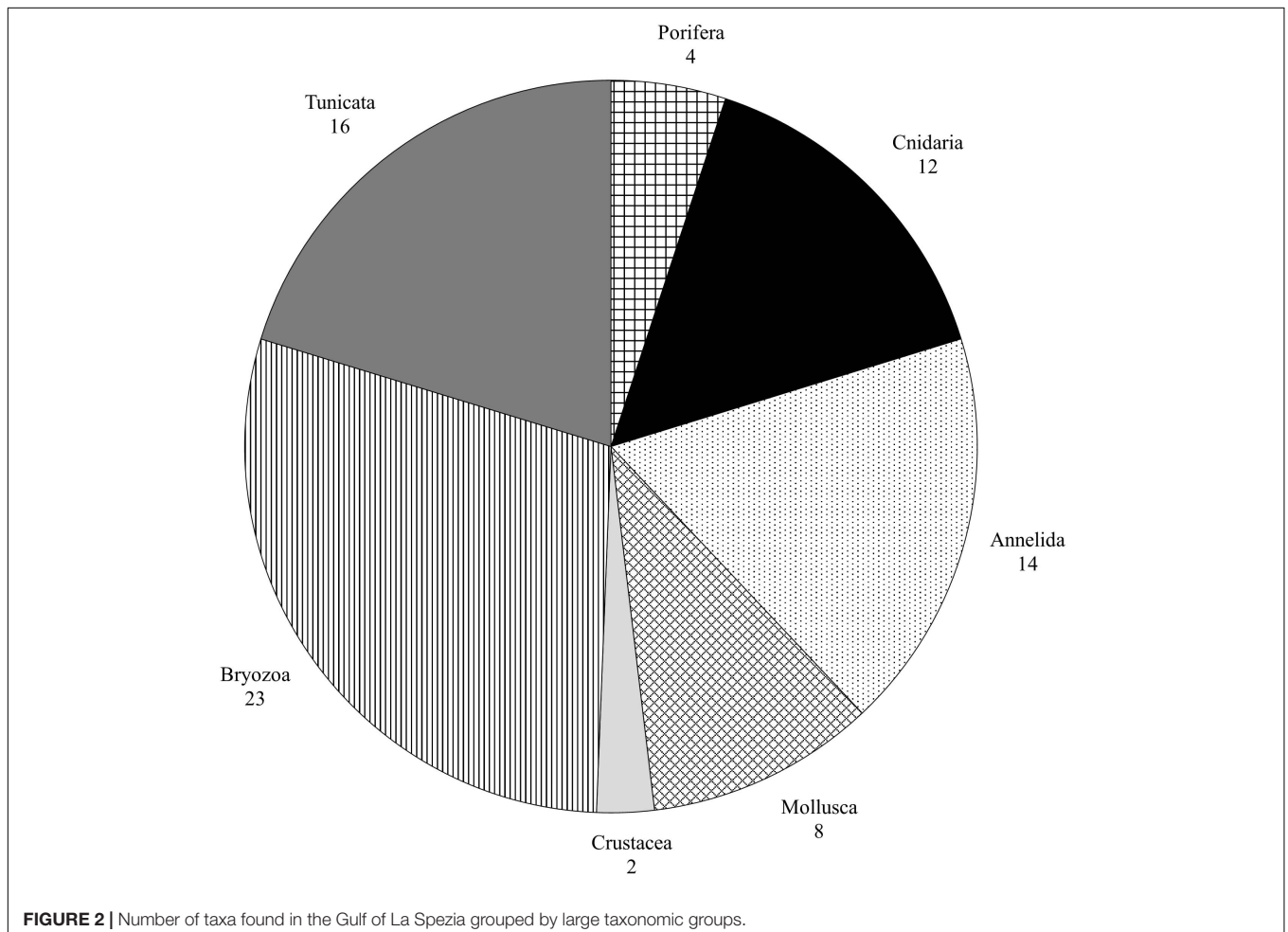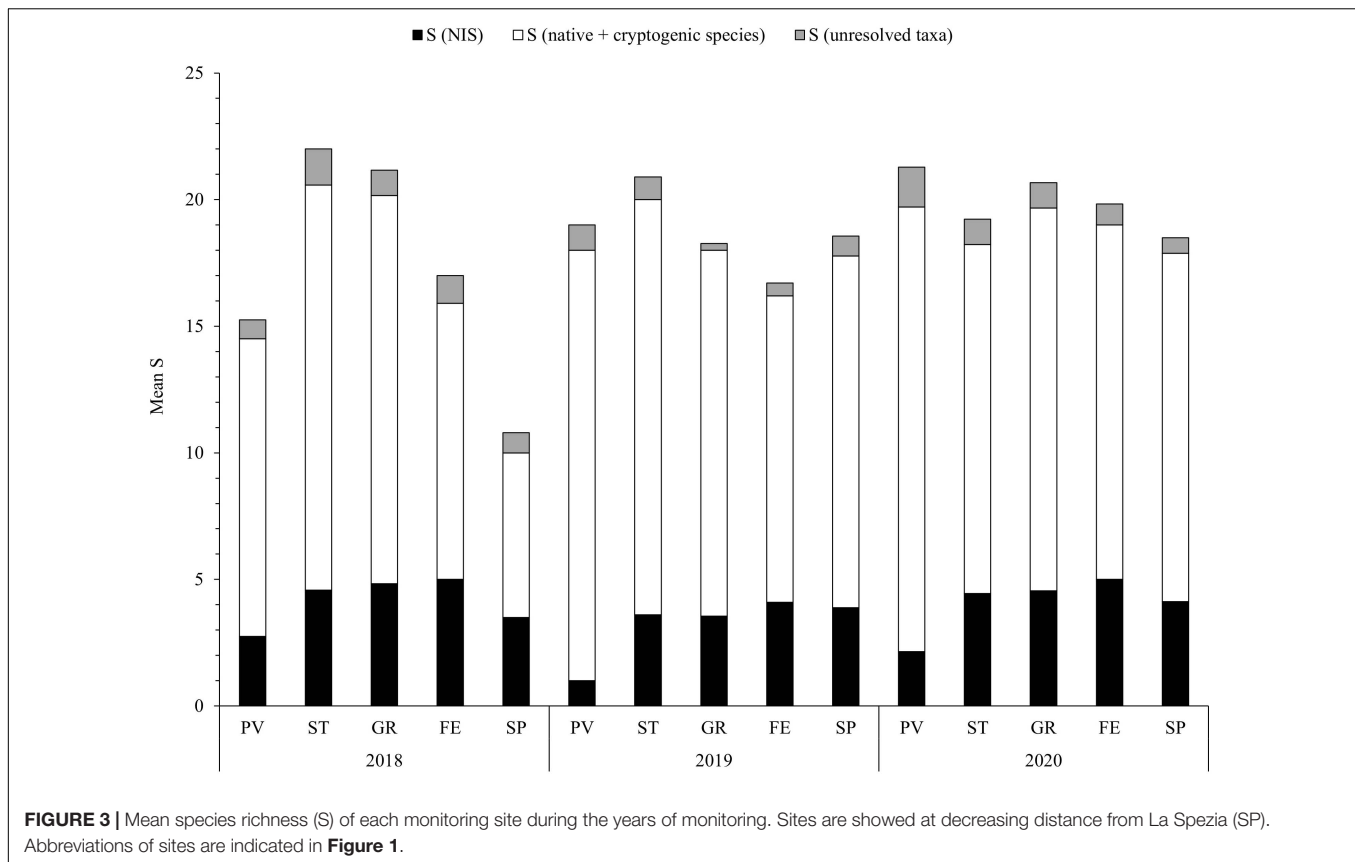

FIGURE 2 | Number of taxa found in the Gulf of La Spezia grouped by large taxonomic groups.

TABLE 1 | List of the NIS found in the investigated localities.

Phylum	Species	Fezzano			Le Grazie			Porto Venere			La Spezia			Santa Teresa		
		2018	2019	2020	2018	2019	2020	2018	2019	2020	2018	2019	2020	2018	2019	2020
Porifera	*Paraleucilla magna Klautau, Monteiro and Borojevic, 2004	■	■	■	■	■	■			■	■		■	■	■	■
Bryozoa	Amathia verticillata (Delle Chiaje, 1822)				■		■	■	■	■	■		■			
	Celleporaria brunnea (Hincks, 1884)	■	■	■	■	■	■	■		■	■	■	■		■	■
	Tricellaria inopinata d'Hondt and Occhipinti Ambrogi, 1985	■		■	■	■	■	■		■	■		■	■	■	■
	*Watersipora arcuata Banta, 1969	■		■		■		■		■	■	■		■	■	■
Annelida	*Branchiomma sp.	■		■		■	■		■	■		■	■	■	■	■
	*Branchiomma luctuosum (Grube, 1870)		■	■	■		■	■		■		■	■		■	■
	Hydroides dirampha Mörch, 1863	■		■	■		■	■		■	■		■		■	■
	Hydroides elegans (Haswell, 1883)	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Tunicata	*Botrylloides cf. niger Herdman, 1886											■		■	■	■
	Styela plicata (Lesueur, 1823)	■	■	■	■	■	■			■	■	■	■	■	■	■

Asterisk indicates NIS found for the first time in the Gulf of La Spezia.

FIGURE 3 | Mean species richness (S) of each monitoring site during the years of monitoring. Sites are showed at decreasing distance from La Spezia (SP). Abbreviations of sites are indicated in **Figure 1**.

only among sites ($F = 5.5382$, $p = 4.21 \times 10^{-4}$) and not across years ($F = 1.7775$, $p = 0.1738433$), and no interaction was detected ($F = 0.988$, $p = 0.4493411$). Among the sites, PV showed significant differences with all the other four sites (adjusted $p < 0.001$).

Merging the data of the 3 years of monitoring, the sites with the highest and the lowest values of mean S_{tot} were ST (21 ± 3) and SP (16 ± 5), respectively; while considering S_{NIS}, the highest value was found in FE (5 ± 1) and the lowest in PV (2 ± 1). Most of the sites displayed higher values of mean S_{native}, namely GR (15 ± 4), PV (15 ± 4), and ST (15 ± 3); conversely, SP (11 ± 4) showed a slightly lower value (**Table 2**). During the 3 years of

TABLE 2 | Mean values of S ($\pm$ SD) per site for the entire 3-year period of monitoring.

Site	S (total species)	S (NIS)	S (native + cryptogenic species)
FE	17.5 ± 4.4	4.7 ± 1.2	12.1 ± 3.7
GR	19.8 ± 4.9	4.2 ± 1.3	14.9 ± 4.1
PV	18.3 ± 4.4	2.0 ± 1.2	15.2 ± 4.0
SP	15.7 ± 4.7	3.8 ± 1.2	11.1 ± 4.2
ST	20.6 ± 3.0	4.2 ± 1.3	15.4 ± 2.9

S (total species, including unresolved taxa), mean number per plate of taxa in each site; S (NIS), mean number per plate of NIS in each site; S (native + cryptogenic species), mean number per plate of native and cryptogenic species in each site. Abbreviations of sites are explained in **Figure 1**.
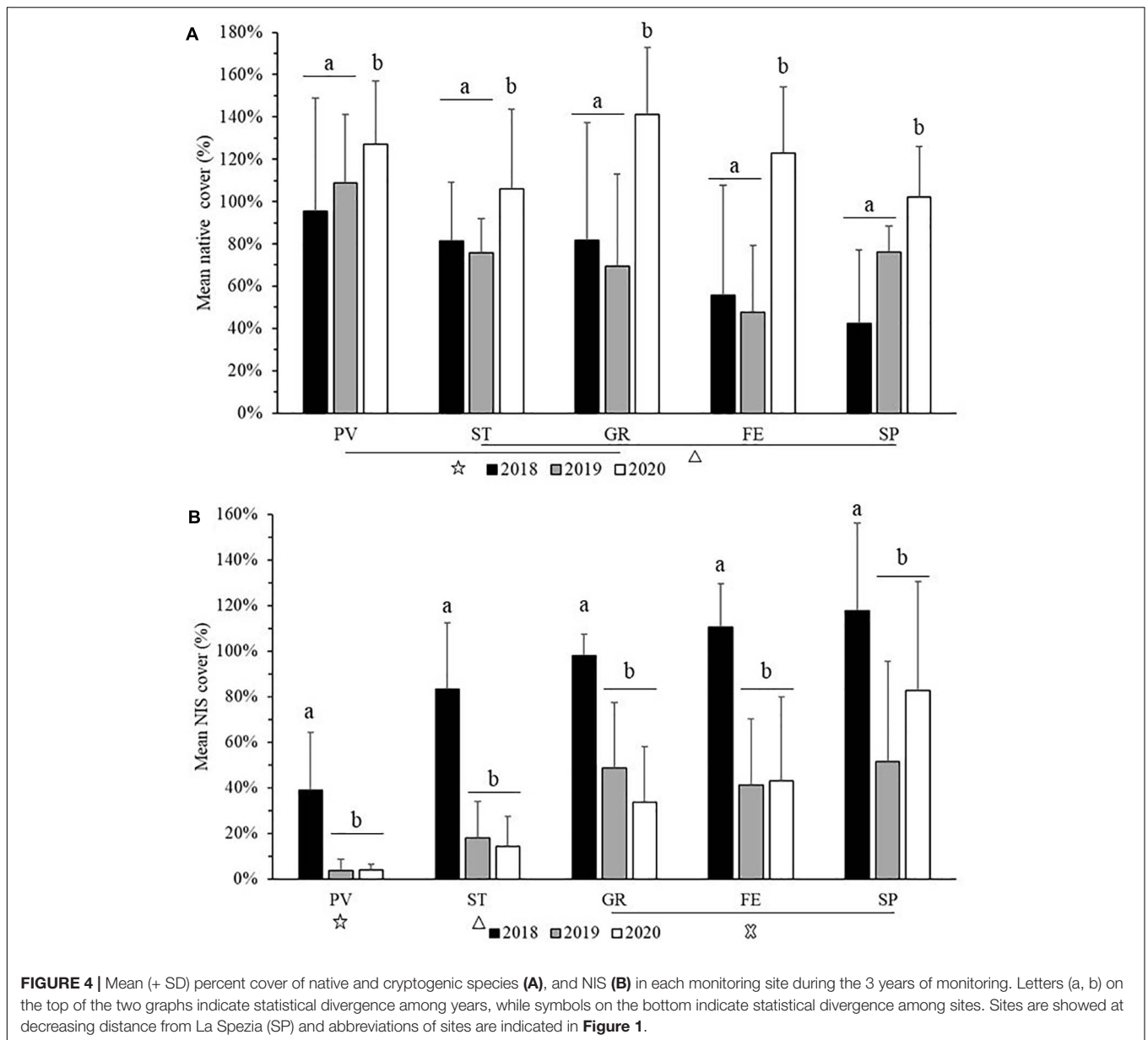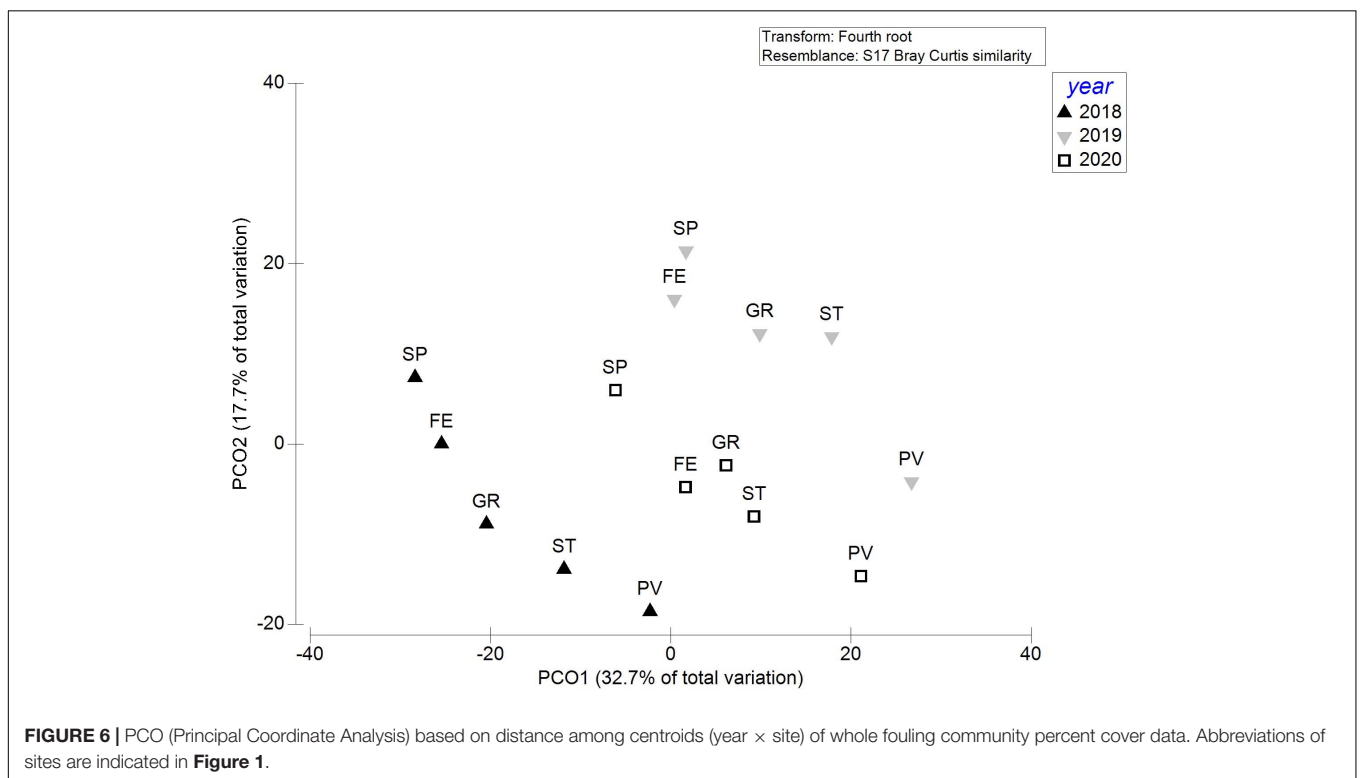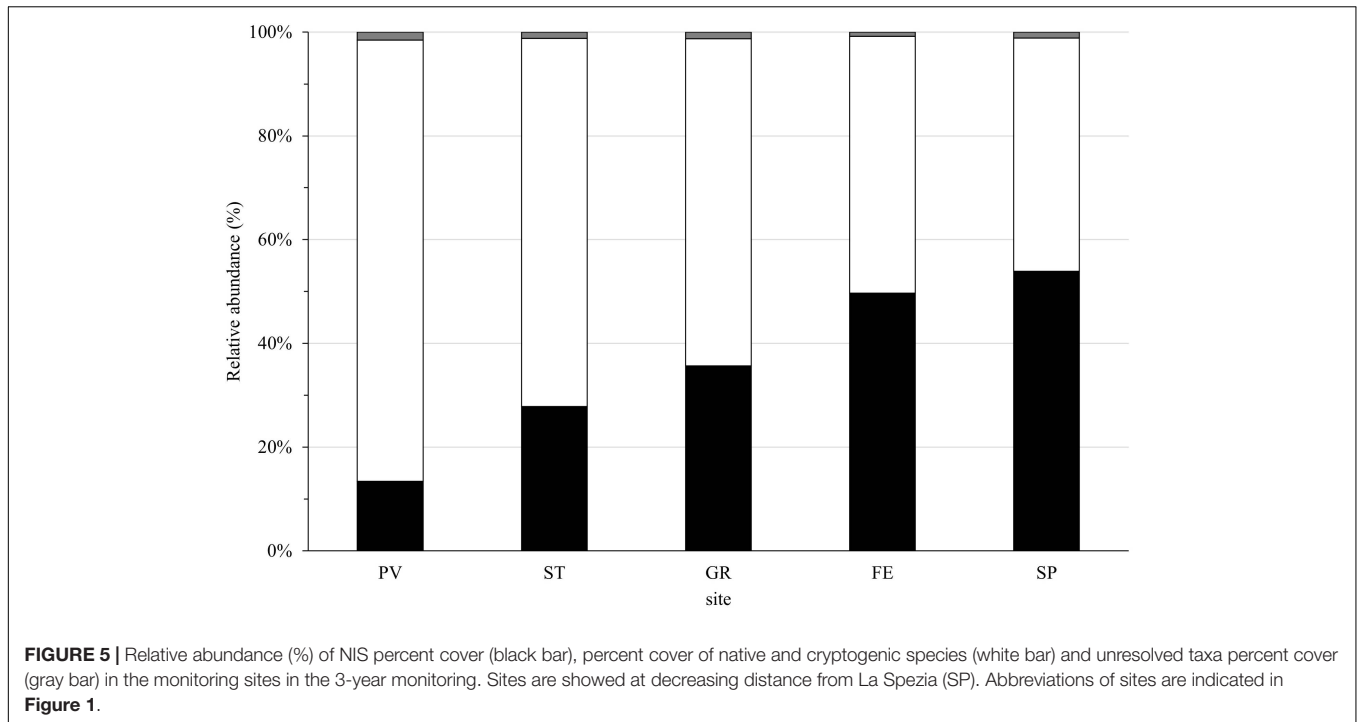
monitoring, mean percent cover of NIS was the lowest in PV ($17 \pm 23\%$) and the highest in SP ($85 \pm 50\%$).

Mean percent cover of native and cryptogenic species (**Figure 4A**) was highest value in GR 2020 ($141 \pm 32\%$) and lowest in SP 2018 ($42 \pm 35\%$). For NIS, mean percent cover (**Figure 4B**) was highest in SP 2018 ($118 \pm 38\%$) and lowest in PV 2019 ($4 \pm 5\%$). Percent cover of native species was significantly different across years ($F = 9.0727$, $p = 2.241 \times 10^{-4}$) and sites ($F = 3.1586$, $p = 0.0168232$), with no significant interaction ($F = 1.5418$, $p = 0.1508879$). Across years, the values observed in 2020 showed significant differences with 2018 and 2019 (adjusted $p < 0.001$); among sites, PV resulted significantly different from FE and SP (adjusted $p < 0.01$) (**Figure 4A**). Significant main effects were detected for NIS cover among sites ($F = 7.5473$, $p = 2.05 \times 10^{-05}$) and across years ($F = 14.5618$, $p = 2.42\text{E-}06$), but no interaction effect was detected ($F = 1.2237$, $p = 0.2918$). *Post hoc* tests revealed that percent cover of NIS was different between 2018 and the other 2 years ($p < 0.001$); between PV and the other four sites ($p < 0.01$); between ST and the other sites ($p < 0.01$) (**Figure 4B**).

The investigated sites were significantly different in terms of mean richness and percent cover of NIS, considering the 3 years of monitoring ($F = 14.691$, $p = 8.257 \times 10^{-10}$; $F = 13.494$, $p = 4.00 \times 10^{-9}$, respectively). Tukey's tests showed a significant difference in richness between PV and all the other sites ($p < 0.001$) when considering S_{NIS}. On the basis of NIS cover a significant difference came out between the sites PV-FE,

FIGURE 4 | Mean (+ SD) percent cover of native and cryptogenic species **(A)**, and NIS **(B)** in each monitoring site during the 3 years of monitoring. Letters (a, b) on the top of the two graphs indicate statistical divergence among years, while symbols on the bottom indicate statistical divergence among sites. Sites are showed at decreasing distance from La Spezia (SP) and abbreviations of sites are indicated in **Figure 1**.

PV-GR, and PV-SP ($p < 0.001$); ST-FE ($p < 0.05$) and ST-SP ($p < 0.001$). Relative abundance—i.e., percent cover data standardized to 100%—of unresolved taxa resulted very low in all sites (1–2%); while values for NIS and native species were similar in FE and SP (about 50% each); and in GR, PV, and ST the relative abundances were higher for the native species (63, 85, and 71%, respectively) in comparison with the NIS (36, 13, and 28%, respectively; **Figure 5**).

Regarding the structure of fouling communities, PCO showed slightly separated clusters per sampling year and the same distribution of sites within each year, with SP and PV being the most diversified sites (**Figure 6**). This pattern probably follows the percent cover of native species rather than NIS, which did not show a clear arrangement (**Supplementary Figure 2**). Furthermore, the values on percent cover of the

non-indigenous component were more clumped than the native one (**Supplementary Figure 2**). PERMANOVA main test on NIS percent cover data of communities among monitoring sites (years merged) showed significant dissimilarity among community composition [pseudo-F = 13.138, p(perm) = 0.0001, unique permutations: 9915], while PERMDISP highlighted significant deviations of samples from centroid [$F = 3.7702$, p(perm) = 0.0116], rejecting the hypothesis of no difference in dispersion among the groups, i.e., "non-homogeneity" of samples dispersion. Moreover, pairwise PERMANOVA tests showed significant differences among sites, except for FE and GR [$t = 1.3337$, p(perm) = 0.1371, unique permutations: 9956], which therefore showed not-dissimilar NIS communities.

Density of artificial structures was the highest in SP (36.28 km $\times$ km^{-2}) and the lowest in PV (13.74 km $\times$ km^{-2}), with

FIGURE 5 | Relative abundance (%) of NIS percent cover (black bar), percent cover of native and cryptogenic species (white bar) and unresolved taxa percent cover (gray bar) in the monitoring sites in the 3-year monitoring. Sites are showed at decreasing distance from La Spezia (SP). Abbreviations of sites are indicated in **Figure 1**.

FIGURE 6 | PCO (Principal Coordinate Analysis) based on distance among centroids (year × site) of whole fouling community percent cover data. Abbreviations of sites are indicated in **Figure 1**.

intermediate values in GR (29.04 km × km^{-2}), FE (22.91 km × km^{-2}), and ST (14.51 km × km^{-2}). Correlation between NIS percent cover of samples (126) and these calculated densities resulted low (adjusted R^2 = 0.2144, p = 2.876 × 10^{-8}; **Supplementary Figure 3**).

DISCUSSION

The 3-year monitoring on fouling communities in the Gulf of La Spezia allowed us to collect 79 taxa, half of which belong to Bryozoa and Tunicata, and to detect a total of 11 NIS. Seven

NIS were already reported in the Gulf in 2010–2013 (Lodola et al., 2012, 2015; Ferrario et al., 2017; Katsanevakis et al., 2020), while five are new records, namely the sponge *Paraleucilla magna* Klautau, Monteiro and Borojevic, 2004, the sabellid polychaetes *Branchiomma* sp. and *B. luctuosum*, the bryozoan *Watersipora arcuata* Banta, 1969 and the ascidian *Botrylloides* cf. *niger*. Interestingly, in this study three NIS from Ferrario et al. (2017), observed in the harbor of La Spezia or in the marina of Lerici (near Santa Teresa site), were not recorded during the 3 years of monitoring, namely *Amphibalanus improvisus* (Darwin, 1854), *Arcuatula senhousia* (Benson, 1842), and *Ficopomatus enigmaticus* (Fauvel, 1923).

For the NIS observed in the present study, *Paraleucilla magna* is the only sponge reported as non-indigenous in the Mediterranean Sea (Longo et al., 2007). It was first described in Brazil and its putative native origin is the Indo-Pacific region and Australia, while its current distribution in the Mediterranean includes Italy, Spain, Malta, Croatia, Greece, Turkey, and Cyprus (Ulman et al., 2017). Although *P. magna* was already reported in the Ligurian Sea, namely along the Portofino coasts (Bertolino et al., 2013; Longobardi et al., 2017), this study provides a new record for the Gulf of La Spezia. The hypothesis expressed by Bertolino et al. (2013) and Longobardi et al. (2017) on the potential expansion of this species from the mussel farms of the Gulf of La Spezia to Portofino area, is strengthened by this last finding. The highest mean cover of *P. magna* was indeed found in Santa Teresa, the monitoring site near aquaculture facilities.

Regarding *Branchiomma* sp., we think this taxon must be further investigated, as this genus showed intraspecific variability that can lead to species misidentification (Capa et al., 2013). For example, a recent taxonomic study suggested that most of the Mediterranean specimens previously identified as *B. bairdi* should be probably be considered as *B. boholense*, with the exception of data reported by Ulman et al. (2019a) in different Mediterranean countries (Del Pasqua et al., 2018; Langeneck et al., 2020).

Branchiomma luctuosum, originally described from the Red Sea (Grube, 1870), was found for the first time in the Mediterranean Sea in Italy in 1978 (Knight-Jones et al., 1991). Then, *B. luctuosum* was recorded in several localities in the whole Mediterranean Sea, where it is considered established and able to colonize both artificial and natural substrates (Giangrande et al., 2012; Fernández-Romero et al., 2021). In the Western Mediterranean, *B. luctuosum* was found along the Spanish coasts since 2004 (El Haddad et al., 2012; López and Richter, 2017), in the Gulf of Genoa in 2011–2015 (Bianchi et al., 2018), in Sardinia in 2014 (Ferrario et al., 2017; Langeneck et al., 2020), and more recently in Leghorn in 2016 (Langeneck et al., 2020; Tempesti et al., 2020); the latter locality being about 37 nautical miles away from La Spezia.

The bryozoan *Watersipora arcuata* was reported for the first time in the Mediterranean Sea in 2013 in the Ligurian Sea (Ferrario et al., 2015). This species, likely originating from the Tropical Eastern Pacific, has now been recorded in most Mediterranean regions, namely Spain, Italy, Malta, and Turkey (Ulman et al., 2017; Ferrario et al., 2018). Furthermore, a recent revision of historical samples revealed its presence along the South Atlantic coast of Spain since 1990, suggesting its pathway

of introduction in the Mediterranean Sea through the Gibraltar Strait (Reverter-Gil and Souto, 2019).

Finally, *Botrylloides niger*, a colonial ascidian native from the tropical Western Atlantic, is reported in several tropical and warm water regions across the world (Sheets et al., 2016), including Madeira island (Ramalhosa et al., 2021). In the Mediterranean Sea, it was first found in 2006 in Israel (Rubinstein et al., 2013) and erroneously identified as *Botrylloides leachii* (Savigny, 1816; Griggio et al., 2014). In general, species in the genus *Botrylloides* are easily confused (Brunetti, 2009; Viard et al., 2019), and in absence of molecular analyses, we use a conservative approach by considering the identification as tentative, pending further verification.

Our monitoring showed that mean NIS richness away varied from 1 to 5 species among sites and years, with similar values across the years of monitoring. For this reason, we did not find a clear increasing pattern in new NIS detections in the different sites, but continuation of this monitoring is required to identify more robust temporal patterns. On the other hand, native species richness significantly varied only in La Spezia among years, highlighting a stable number of native species during the monitoring. It should be highlighted that *B.* cf. *niger* was recorded only in 2018 in ST, but in the following years it did not disappear from the marina. Despite not being found on panel communities, it was still observed, even if in very low abundances, on other artificial substrates (e.g., on the supporting structure of the panels and on floating pontoons; authors personal observation). For this reason, *B.* cf. *niger* may be considered as an established NIS in the study area. After 3 years of monitoring, Porto Venere was found to be the least impacted site in terms of both richness and percent cover of NIS, while the other sites showed a higher impact by NIS, with values of mean NIS percent cover ranging from 34% (Santa Teresa) to 85% (La Spezia).

In general, the different submersion periods—mid-summer in 2018 and early spring in the following years—affected the development of fouling assemblages, mainly in terms of differences in taxa percent cover. NIS richness were indeed similar across the years of monitoring, while in 2018 definitely higher mean values of NIS cover were observed in all sites, compared to the other years of monitoring. The latter result is probably a consequence of the later submersion period of 2018, which led to the dominance on the panels of one NIS, namely *Hydroides elegans* (Haswell, 1883). A similar phenomenon with the dominance of one single species in fouling communities was also observed in marinas of South-eastern Brazil (Dos Santos Schwan et al., 2016), and could be related to the seasonal reproductive peaks (Reinhardt et al., 2013; Lezzi and Giangrande, 2018; Fortiè et al., 2021). These preliminary results suggest that maintaining the monitoring program between May and July is advisable. In fact, the number of detected NIS resulted independent from the submersion period, but during the period May-July it is possible to better capture the entire spectrum of potential colonizers, avoiding the dominance of a single species.

Considering the whole data set on NIS percent cover of the 3-year monitoring, higher values were found in La Spezia, with Fezzano, Le Grazie, Santa Teresa, and Porto Venere gradually showing lower values. The same gradient from La Spezia to Porto Venere was also observed when considering the structure

of the entire community, and the gradient was probably more influenced by the composition of the native species than to the non-indigenous one. These patterns may reflect the different geographic features of the monitoring sites. In fact, the sites are located at an increasing distance from the main harbor of La Spezia, one of the largest commercial ports in Italy (accessed 1 March 2021)[1]. The harbor of La Spezia hosts international cargo ships and passengers' vessels, as well as local ferries.

The distance of sites from the main harbor appears to be proportional to the mean NIS percent cover, while the proximity of Santa Teresa to the mussel farm showed higher abundances of *P. magna*, a NIS associated to aquaculture (Bertolino et al., 2013), as well as the exclusive presence of *B.* cf. *niger* in comparison with other sites (see figshare repository)[2]. In addition, the water circulation inside the Gulf is different in Porto Venere compared to the other sites, due to both the presence of the dam and the distance from the open sea (**Figure 1**; Gasparini et al., 2009). This circulation could advantage NIS larval dispersal and settlement inside the dam, where water exchange is surely lower than in Porto Venere (Gasparini et al., 2009). On the other hand, the density of artificial structures seems not to be a factor influencing NIS distribution in the area. The observed variability of NIS percent cover in the dataset can also be related to other features of sites, like the presence of floating pontoons, or the proximity to aquaculture facilities or to commercial harbors (Nall et al., 2015; Ulman et al., 2019a). All these aspects seem to affect the distribution of NIS and the composition of fouling communities in the Gulf of La Spezia. A regular data collection in the Gulf will allow us to examine in depth these results and better clarify the observed dynamics, but this first attempt should be considered in future management measures to prevent NIS introduction in the area, by implementing more rigid controls in sites closer to the harbor of La Spezia. Moreover, a longer data collection could also allow for obtaining limited statistical dispersion of samples within sites, in order to assess if difference in NIS percent cover in monitoring sites is purely due to the dissimilarity among sites and not to dispersion of samples (Anderson et al., 2008).

In conclusion, this monitoring allowed us to implement, for the first time in the Mediterranean Sea, an international standard protocol developed by the SERC to assess fouling communities and detect NIS. The number of detected NIS was in line with previous monitoring carried out in the Gulf of La Spezia and in other Ligurian port localities, but with additional new records for the area and three NIS no longer observed (Ferrario et al., 2017; Ulman et al., 2019a; Katsanevakis et al., 2020). These results are consistent with the role of commercial harbors and recreational marinas as hot-spot sites for the introduction of NIS (Clarke Murray et al., 2011; Ros et al., 2013; Ferrario et al., 2017; Verlaque and Breton, 2019), and the importance of continuous monitoring to detect changes in marine communities (Olenin et al., 2011; Ojaveer et al., 2014; Lehtiniemi et al., 2015). This monitoring protocol seems to be a strong and effective tool to control the presence and abundance of fouling NIS in the Mediterranean ports. Its main advantages are cost-effectiveness, ease of application and the capability to obtain quantitative

results in a relatively short time. On the other hand, the effort for sampling and analyzing the panels is considerable. Finally, this protocol is predominantly focusing on the assessment of the sessile hard-bottom fauna, not providing complete quantitative data on the mobile component living on the panels (e.g., see Vicente et al., 2021), as well as on organisms dwelling in soft bottoms (e.g., see Chatzinikolaou et al., 2018), that should ideally be also considered for future monitoring programs on NIS in port habitats.

While this methodology provides a reliable option for sampling and monitoring fouling communities in the Mediterranean area, further analyses to test its effectiveness should be encouraged in other regional localities, together with a comparison to other fouling monitoring tools, including the use of genetic approaches (e.g., Ardura and Planes, 2017; Borrell et al., 2017; Marraffini et al., 2017; Cahill et al., 2018; Kakkonen et al., 2019; Obst et al., 2020 Duarte et al., 2021). Similarly successful implementation in other locales could lead to the proposal of this method as an adopted standard for the assessment of marine bioinvasions in the Mediterranean. In fact, the application of the SERC method across the Mediterranean Sea could provide a simple option to gain standardized and quantitative data in a key invasion-prone habitat, allowing rapid assessment and one approach to evaluate changes in the structure of fouling communities, as it is being carried out along multiple coasts and countries in the Americas.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JF, EK, GMR, and AO-A conceived the study. MT, JF, EK, AM, and MFR performed the field work and laboratory analyses. MT carried out the statistics and wrote the first draft of the manuscript, with the contribution of JF. AO-A and GMR acquired funds. All the authors contributed to the manuscript revision, read and approved the submitted version.

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[1]https://www.assoporti.it/en/home/

[2]https://doi.org/10.6084/m9.figshare.14758383.v2

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.700730/full#supplementary-material

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