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Anthropogenic stressors that favour nuisance species. A study from environmental DNA in marine plankton samples

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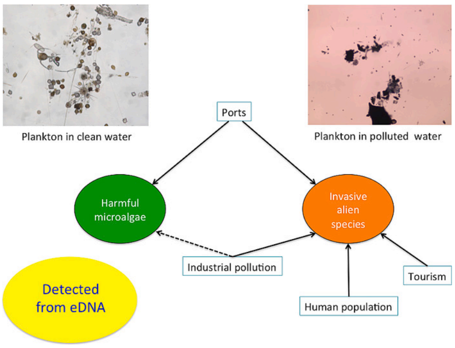
HIGHLIGHTS

- Invasive species and toxic algae are species of biosecurity concern.
- Environmental DNA was extracted from water samples in Bay of Biscay.
- Metabarcoding revealed invasive metazoans and harmful algae species.
- Ports, pollution and tourism were associated with nuisance species.
- Preventing pollution and promoting awareness about invasions are priority for ocean's health.

GRAPHICAL ABSTRACT



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ABSTRACT

Anthropogenic stressors reduce marine biodiversity. Tolerant species may develop in altered areas where they occupy niches that native species are unable to use. Species of biosecurity concern, like invasive aliens or harmful microalgae, are especially successful in disturbed areas. Here, we employed multiple regression approach to investigate the relationship between the proportion of planktonic stages of invasives and anthropogenic stressors. Planktonic species were inventoried from environmental DNA on water samples from the Bay of Biscay. Key findings were that the proportion of invasives was associated with port and touristic activities that are vectors for biological invaders, and with industrial pollution, likely for their tolerance to disturbed environments. The proportion of toxic algae was correlated with port activity, reinforcing the role of maritime traffic as a vector of harmful microalgae. An increase of nuisance species is expected under the current growth of pollution in the ocean. Promoting awareness of biological invasions among maritime sectors and tourists, and controlling pollution, seem priorities for environmental conservation in this region and elsewhere.

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1. Introduction

Aquatic biosecurity has been defined as the national, regional, and international efforts to prevent, reduce, and manage the introduction of pests, diseases, or unwanted organisms, as well as short-term response and long-term control of established pests (Dahlstrom et al., 2011). Many of those unwanted organisms are species tolerant to environmental disturbances and can flourish in circumstances that are adverse for the majority of biota (Byers, 2002; Hellmann et al., 2008). Among them, invasive alien species (IAS) – introduced by humans into new environments, deliberately or inadvertently- are considered biopollutants (Pourang Eslami et al., 2016). The costs of biological invasions are increasing worldwide in the last decades, especially damage compensation costs. They cause enormous economic losses to marine resources (Cuthbert et al., 2021) because they induce significant ecosystem changes (Olenin et al., 2007), altering the trophic web (Ardura et al., 2021) and causing finally the extinction of many native species (Bell et al., 2021; Clavero and García-Berthou, 2005).

Main vectors of marine IAS are maritime transport (Costello et al., 2022), with floating litter as secondary dispersal vectors (Fernandez et al., 2022); aquaculture (Naylor et al., 2001; Crego-Prieto et al., 2015); or aquarium species trade (Padilla and Williams, 2004). Tourism is another vector; in highly touristic coastal areas invasive species tend to increase, sometimes propagated for ornamental purposes (Nunes et al., 2020), and in the case of aquatic species for a higher dispersal via yachts and other maritime activities (Anderson et al., 2015). Ports are heavily transformed ecosystems considered IAS hubs (Floerl et al., 2009; Hulme, 2021). They receive constantly new organisms via trade routes, and their biological communities are exposed to diverse pollutants; the term "biological portuarization" has been coined to define the evolution of marine species in human-altered ports (Touchard et al., 2023). This happens because IAS are typically tolerant to environmental stressors, being associated with disturbed areas where they can occupy degraded niches that are empty when local, less tolerant species cannot (Lenz et al., 2011). Accordingly, biopollutants correlate negatively with spatial protection, which limits stressors in protected areas (Ardura et al., 2016). The clear relationship between anthropic disturbances and biopollutants is reflected in the fact that human population size is the best predictor for bioinvasion rate at a large geographical scale (Pyšek et al., 2010). IAS are also concentrated in highly disturbed areas, for example in industrialized zones (Salomidi et al., 2013); aquatic pollution has been reported to increase the rate of successful biological invasions (Crooks et al., 2011). Some zones subjected to salinity variations, like estuaries, are naturally stressed and tend to be especially sensitive to species invasions (e.g., Cognetti and Maltagliatià, 2000; Occhipinti-Ambrogi, 2007; Pejovic et al., 2016).

Other species of biosecurity concern are harmful algae (HABs, for harmful algae blooms; Lundholm et al., 2009 onwards): unicellular toxic algae that can experience outbreaks (red tides). Maritime traffic is also a vector of HABs, which can be transported by ships (Ardura et al., 2020; Wang et al., 2022) and/or other human-mediated vectors (Zingone et al., 2021). Toxic microalgae are favoured by high nutrient inputs derived from human activities like agriculture, aquaculture and sewage (e.g., Streicher et al., 2021; Tsikoti and Genitsaris, 2021). They have been associated with varied anthropogenic stressors, defined as disturbances or pressures caused by human activities (Vitousek et al., 1997), including tourism, industry and others (Ardura et al., 2024). The role of tourism in this case would be an augmentation of nutrient concentration due to increased sewage in touristic seasons when population size increases exceeding wastewater treatment plants (WWTP) capacity, although physical conditions (wind speed) and the local hydrodynamics can dilute or disperse phytoplankton biomass (Davidson et al., 2014).

One of the knowledge gaps in biosecurity is the relative weight of different environmental features in the allowance of IAS settlement. Audrézet et al. (2021) found differences between invertebrate IAS species for their capacity to attach to different materials, perhaps mediated by the larval ecology. For this, Audrézet et al. (2022) recommended to increase research efforts to understand the mechanisms of recruitment of marine pests, especially through the analysis of concomitant factors. In this study we explored possible relations between species of biosecurity concern and different stressors that may be synergistic with each other, like port activity, pollution or tourism. As Zaiko et al. (2018) proposed, we used environmental DNA (eDNA hereafter) for the identification of risk species. The methodology of DNA extraction from water samples, high throughput sequencing –or PCR amplification with species-specific primers- and species assignment of the sequences from comparison with reference databases has been validated in many studies (Ardura et al., 2021; Nagarajan et al., 2022). Here we analysed surface seawater, for which the completeness of reference species DNA sequences in public databases is quite good – at least in comparison with deep-water organisms (Duhamet et al., 2023). The study site (Asturias coast, southwest Bay of Biscay) was chosen for featuring significant variation at a relatively short scale for biopollution (Miralles et al., 2016), including planktonic species (Borrell et al., 2017).

From the tolerance of nuisance species to environmental disturbances (Byers, 2002; Hellmann et al., 2008), we forecasted the associations presented in the following departure hypotheses:

i) The proportion of IAS and HABs will be associated with the ports. Zones with lower port activity will exhibit fewer invasive species than zones with more maritime traffic, probably moderated or enhanced by other concomitant factors.
ii) The association between nuisance species and ports may change depending on concomitant disturbances. Tourism and aquaculture are expected to enhance the two types of nuisance species. Environmental pollution and salinity alterations due to rivers and human population size will likely increase IAS, while WWTPs would contribute to HABs enhancement.

2. Material and methods

2.1. Study region

The case study is Asturias in southwest Bay of Biscay (Fig. 1; map

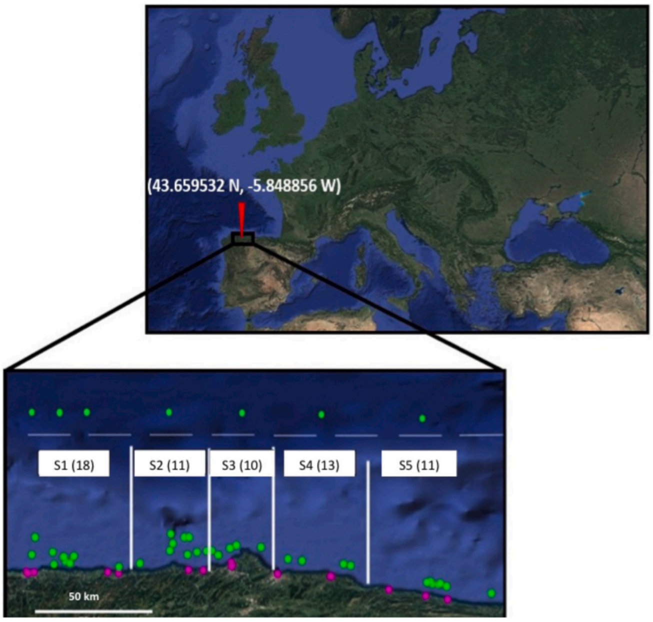

Fig. 1. Map showing Asturias coast within South Bay of Biscay waters, indicating the sampling points analysed in each of the five sectors considered (S1 to S5) as coloured dots. Green dots: sampling points from open waters; pink dots: inshore water samples.

elaborated using the Free and Open Source QGIS® free software v. 3.16). The region is characterized by a temperate Atlantic climate. Main oceanographic features are the dominant eastward North Atlantic current parallel to the coast and the Iberian Poleward current off northern Spain, called Navidad current –also eastwards- during the winter (Frouin et al., 1990; DeCastro et al., 2011; Le Hénaff et al., 2011). There are numerous bathymetric irregularities that make currents and wind to fluctuate in nearby zones (Domínguez-Cuesta et al., 2019). The coast morphology is determined by cliffs, rocky and some sandy beaches, with a dense fluvial network of five main basins and many small, short rivers (Cotilla Rodríguez et al., 2005). Thus, the coast is patchy, alternating sheltered coves with exposed beaches where the effect of tides is more intense. The organisms inhabiting the coast are, consequently, exposed differentially to large environmental disturbances, even at a very short scale of a few kilometres (Muñoz-Colmenero et al., 2015).

The region contains two commercial international ports (Gijon and Aviles) and many small fishing ports and marinas. Aquaculture is concentrated in the estuaries (*rías*) of Eo and Villaviciosa for molluscs (the latter was closed for pollution the last years), and fish farms in the rivers, principally of rainbow trout. Tourism, concentrated in the central and eastern part of the region during the summer, is increasing in the last decades (Valdes Pelaez et al., 2011). Industry and mining have left a legacy of coastal pollution by heavy metals and other contaminants (e.g., Garcia-Ordiales et al., 2019; Navarro-Murillo et al., 2024), and the central part of the regional coast has been reported as a heavily polluted area (e.g., Sierra et al., 2014).

For this study, the area was divided in five sectors (Fig. 1) that are exposed to different anthropogenic stressors. From west to east, Sector 1 is characterized by forestry and agriculture in the land, small fishing ports and oyster culture in Ria del Eo. Sector 2 has principally agriculture and receives water from rivers crossing the main valleys with coal mining in the region. Sector 3 has a high population density, industry, and Aviles international commercial port. Sector 4 has the international commercial port of Gijón which is the largest and most polluted city of Asturias, and mollusc aquaculture in Ria de Villaviciosa at the east. Finally, Sector 5 is the most touristic in the region for its scenic beaches, has small to medium fishing ports, and transformation of agriculture and husbandry products (cheese, flours, meat) at industrial scale.

2.2. Stressors related with IAS and HABs

Environmental stressors that may contribute to the appearance of nuisance species were considered and measured in each sector. Details about the information sources of these stressors are given in Table 1 and commented next.

The human population (e.g., Pyšek et al., 2010; Spear et al., 2013) was measured from total population and population density of the coastal counties in each sector, taken from the Spanish National Institute of Statistics for 2019.

For maritime ports (e.g., Hulme, 2021), in each sector we considered proxies for the activity of local and international ports. Annual regional catch in 2020 landed in the fishing ports of each sector was taken as a proxy of port activity in each sector (Accessed in February 2022). Cargo tons arriving in the ports of the region (only in the two international ports) was employed a proxy of commercial maritime transport.

For aquaculture (e.g., Naylor et al., 2001) we considered the aquaculture facilities operating in each sector. The majority are rainbow trout farms that were scored as 0.5 point per farm. Shellfish production facilities, concentrated in two *rias* as explained above, were weighted by cultured surface.

Industrial pollution is important because it facilitates the establishment of tolerant invasive species (e.g., Salomidi et al., 2013). Here we have taken as a proxy the number of industries in each sector, ranking the sectors in a Likert scale.

Tourism is important because it increases the local population, thus the anthropogenic stressors related with mobility and displacement –more inadvertent movements of IAS- and higher volume of wastewater with nutrient inputs –more opportunities for HABs development. The proxy for this stressor was the number of tourist beds in the coastal counties of each sector (see Table 1 for the link to the official statistics of the region provided by SADEI, Asturias Society of Economic and Industrial Studies).

Table 1
Stressors considered in this study that may enhance nuisance species. Source of the information and estimation measurements are presented, and references supporting the relationship of each stressor with invasive alien species. The webpages provided were accessed on February 2025.

Stressor	Measure or proxy - source	References
Human population size	Number of inhabitants and population density in coastal counties - https://www.ine.es/dyngs/INEbase/categoria.htm?c=Estadistica_P&cid=1254734710990.	Pyšek et al. (2010); Spear et al. (2013)
Port activity	Proxy for fishing ports activity: annual catch - https://tematico.asturias.es/dgpesca/fich/992020_Z.pdf Proxy for commercial ports activity: annual cargo tons received - https://www.puertos.es/es-es/estadisticas/Paginas/default.aspx	Costello et al. (2022)
Aquaculture	Trout farms - http://www.serida.org/publicacionesdetalle.php?id=1481 Shellfish farming - https://www.boe.es/diario_boe/txt.php?id=BOE-A-2019-6958	Naylor et al. (2001); Crego-Prieto et al. (2015)
Industrial pollution	1–5 Likert scale from the lowest to the highest number of pollutant industries	Crooks et al. (2011); Salomidi et al. (2013)
Tourism	Number of tourist beds in coastal counties - https://www.sadei.es/sadei/informacion-estadistica/banco-de-datos_263_1_ap.html?f=11_02_02$$11020202.px	Anderson et al. (2015); Ardura et al. (2024)
Freshwater discharge	River order - 1 to 5 from small streams with no tributaries to large rivers with five branching levels	Occhipinti-Ambrogi (2007); Keller et al. (2011)
WWTP	Number of WWTPs in each sector - https://consorcioaa.com/infraestructuras/saneamiento/	Passy et al. (2016); Preisner et al. (2021)

Since estuaries are zones prone to biological invasions –IAS may settle in regions with salinity fluctuations (Occhipinti-Ambrogi, 2007; Pejovic et al., 2016), the freshwater discharge in each sector was estimated also from a proxy because there are no registers of discharge for small rivers in the region. The river order (Strahler, 1957) was considered as a proxy, assuming that the discharge is bigger in larger, more complex rivers (Table 1).

Finally, the number of wastewater treatment plants (WWTP) in each coastal region was considered because they are a possible source of eutrophication and HABs (Passy et al., 2016; Preisner et al., 2021). The WWTPs of the region can be found in the official site of the Water Consortium of Asturias accessed in February 2023 (Table 1).

2.3. Sampling protocol and samples processing

Surface water samples were collected during spring and summer in front of the coast using sterile bottles previously rinsed with distilled water. Water samples were taken at different distances from the shore to open waters (Supplementary Table 1), with a fair coverage of the five sectors in water volume and number of sampling points (Fig. 1). Fisher volunteers collected most samples from open waters from fishing vessels ($N = 45$), thanks to the collaboration of the Federation of Fishing Guilds of Asturias. From each sampling point a sample of 5 L was taken for DNA analysis (225 L in total). The samples closest to the shore ($N = 8$, 3 L per sample, corresponding to a total of 24 L) were directly taken from beaches or piers trying to match more or less open water samples locations in each sector. An additional 6 L sample (PS116-C4, Supplementary Table 1) was taken from RV Polarstern in autumn, as explained in Garcia-Vazquez et al. (2021). Thus, in total 231 L were collected from open waters. It should be noted that these water samples were not collected in the same season while the growth of species differs among seasons. This will be taken into account when analysing the results.

The water samples were stored cold in portable coolers until arriving in the laboratory, where they were immediately filtered through PES Supor 200 Membrane Filters (Pall Corporation, Life Sciences) of 0.2 μm pore for retention of eDNA. Filtration was done using a vacuum pump and cleaned sterilized materials inside a laminar flow cabinet to prevent contamination. Membranes for DNA analysis were immediately stored in 100 % ethanol.

Further contamination control measures were taken. Equipment and materials were cleaned with 10 % bleach and triple-rinsed with distilled water, filtered through 0.45 μm pore membranes.

2.4. Environmental DNA analysis

Environmental DNA was employed to detect species present in water samples, using Next Generation Sequencing (NGS) as explained in Garcia-Vazquez et al. (2021). In summary, DNA was extracted from membranes and ethanol pellets using PowerWater DNA Isolation Kit (Qiagen). A fragment of the cytochrome oxidase I gene (COI thereafter) was amplified on eDNA samples using the primers mlCOIintF43 (Leray et al., 2013) and jgHCO219844 (Geller et al., 2013) modified with a PGM sequencing adaptor, sample barcodes and a "GAT" spacer. PCR was conducted in a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA) employing the mix and conditions described in detail in Garcia-Vazquez et al. (2021).

Negative controls of field sampling (a sterile water bottle carried along samples transport), DNA extraction and PCR were employed, as well as in house positive controls with known concentrations of DNA of different species.

The sequences obtained from NGS were quality-filtered using R wrapper JAMP v0.67 (https://github.com/VascoElbrecht/JAMP). The sequences were filtered by length (amplicon size 313 ± 10 bp reads were retained) using Cutadapt v1.15 (Martin, 2011). Expected errors probabilities were calculated to filter reads qualities (Max ee = 0.5; −fastq_qmax 60) using Usearch v11.0.667_i86 (Edgar, 2010). For taxonomic classification, filtered sequences were compared against a public COI reference Nt database (NCBI, accessed on 22/09/2021) and stored locally. The database was downloaded using the esearch query "COI NOT Bacteria NOT environmental NOT viruses NOT unclassified" and constructed with the respective taxonomic information using the script "Entrez_qiime.py" by (Baker, 2017). Finally, "qiime feature-classifier" command within QIIME™ 2 pipeline v2020.2 (Bolyen et al., 2019) was employed to assign the taxonomy, using a 97 % as identity percentage threshold and an e-value of 10^{-50}.

The resulting tables of molecular operational taxonomic units (MOTUs) were expert-checked to adapt taxonomy to WORMS (2022). Terrestrial and miscellaneous species like marine birds or marine mammals, clearly not belonging to marine plankton in any of their life stages, were excluded from downstream analysis. Organisms inhabiting brackish waters were allowed because they could theoretically be present in near-shore locations with river discharges.

In addition to NGS, specific primers designed to detect toxic *Alexandrium* species (Galluzzi et al., 2009) were employed for PCR amplification on eDNA samples. PCR was performed using an Applied Biosystems 2720 Thermal Cycler in a final volume of 20 μL containing 10 μL of template DNA and 10 μL of PCR mix. The PCR mix composition was: 4 μL of 5× Green Go Taq® Flexi Buffer, 2 μL of MgCl2 (25 mM), 1 μL of dNTP mix (0.025 μL of each dNTP (from EURX 100 mM Ultrapure dNTPs Set) plus 0.9 μL of distilled water (Laboratorios Serra Pamies), 0.4 μL of both Primers, 0.25 μL of Go Taq® G2 Flexi DNA Polymerase (5 u/μL), 1 μL of BSA (Bovine Serum Albumin; 50 mg/mL; from EURX) and 1.05 μL of distilled water).

The PCR conditions were as follows: 7 min at 95 °C, 40 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension of at 72 °C for 7 min. Negative controls (no-template) were included on each PCR reaction.

2.5. Downstream analysis of the eDNA dataset

The MOTU list obtained from the water samples collected in this study was completed with organisms identified from previous water eDNA sampling in the same region during 2016 (Borrell et al., 2017), in the understanding that a species is present in a location for some time –a few years– while local extinction events are relatively rare. The original table with the list of those species inferred from eDNA in Borrell et al. (2017) is published in open access at doi:https://doi.org/10.1371/journal.pone.0183347.s001. It was taxonomically checked to retain only aquatic organisms.

Four biotic indices were calculated. Biodiversity analysis in each sector was conducted following Ardura et al. (2021). Briefly, the organisms of the MOTU list were grouped by phylum. Taxonomic diversity was estimated from the MOTU list. The number of species per phylum was taken as unit (n) to calculate Shannon diversity index, as:

$$H = -\Sigma_i (n_i/n)\ ln(n_i/n)$$

The geographic origin and distribution of the species were checked in WORMS (2022) and Algaebase (Guiry et al., 2014). Harmful algae were identified from the IOC-UNESCO list (Moestrup, 2009), and invasive species from IUCN Global Invasive Species Database (http://www.iucngisd.org/gisd/, accessed July 2022). The proportion of IAS over the total number of identified species and the proportion of potential HABs over the number of phytoplankton species (an indicator of possible eutrophication) were calculated.

Finally, environmental changes derived from anthropogenic stressors may benefit some species that are rarely subjects of studies (Willmott et al., 2022). The proportion of commercial species (any species exploited by the fishing fleet in the region) was calculated here for comparison with unwanted species.

2.6. Statistics

Contingency Chi-square was used to test for possible differences between sectors in the distribution of eDNA samples by month. Seasonal differences for the proportion of nuisance species were tested using one-way ANOVA with seasons as variation factor (three levels being winter, spring and summer). Alternative Kruskal-Wallis tests were employed when ANOVA conditions were not met.

A principal component analysis (PCA) was run to identify biotic and abiotic variables that characterize the five coastal sectors considered, and to visualize relationships among them in a scatter plot, like for example between industrial pollution and the proportion of IAS MOTU. PCA was also employed to visualize associations between the richness of different planktonic taxa and MP pollution in these sectors. Correlation option was employed, and variable loadings calculated for each PC. In scatter plots, diagonal lengths were built proportional to the relative contribution of each variable to the PCs represented.

Exploratory analysis of the dataset continued with pairwise correlations (Pearson's r) between the considered variables: independent, which are all the abiotic factors, and dependent variables, considering the proportion of nuisance species (IAS and HABs) and the proportion of reads of those species. Normality of the variables was checked using Shapiro-Wilk tests. Multiple regression analysis was employed to identify significant associations between independent (abiotic factors) and dependent variables (proportion of nuisance species) after controlling the rest, i.e. to know the independent variables associated significantly with the variation of the dependent variables. Regression requirements were checked using Durbin-Watson statistic for residual autocorrelations, and Breusch-Pagan statistic for homoscedasticity. With five sectors ($N = 5$), the maximum number of independent variables that can be tested in each multiple regression analysis is three, thus we chose those that were closer to the dependent variable in the PCA, exhibiting higher correlation values in exploratory pairwise tests. Multiple regressions with significant adjusted R^2 as determined from significant F were considered significant.

Statistics was performed using the free software PAST v.3 (Hammer et al., 2001).

3. Results

3.1. Species detected from eDNA

NGS datasets are available on NCBI's Sequence Read Archive repository with the BioProject ID numbers PRJNA821035 (Biosample number SAMN27035741) and PRJNA675458 (BioSample number SAMN16708468). The number of reads and taxonomic status of the molecular operational taxonomic units (MOTUs) i.e., putative species are in the Supplementary Table 2.

To check for a possible bias in plankton diversity due to different sampling seasons (Supplementary Table 1), the number of eDNA samples per month were compared among sectors. The difference was not significant ($\chi^2 = 27.8$, 20 d.f., $p = 0.15 > 0.05$, n.s.), thus differences between sectors may not be principally attributed to different sampling seasons.

In total 108 putative species were identified from eDNA (Supplementary Table 2). Several nuisance species were identified from the MOTU list: four alien species that are potentially invasive in Europe, and three potential HABs (Table 2). We also detected eDNA of three fish species of commercial interest that represent in total >2058.3 landing tons in the region (Table 2). The four IAS were the polychaetes *Ficopomatus enigmaticus* and *Polydora triglanda*, and the Ascidian *Cnemidocarpa*, in the sectors at the east of Cape Peñas; the acorn barnacle *Austrominius modestus* was in Sector 3 in the centre of the region (Table 2). The three potential HABs (genera *Alexandrium*, *Gymnodinium* and *Gyrodinium*) were distributed across the region.

Table 2
Species of interest found from the five sectors considered. Presence/absence: 1/0. The number of reads (in parenthesis) cannot be given for *Alexandrium* because it was detected from final time PCR with specific primers.

Species	Reference	Coastal sector				
		1	2	3	4	5
Potential IAS in Europe						
Ficopomatus enigmaticus	Miralles et al. (2016)	0	0	0	1 (1)	1 (6)
Polydora triglanda	Miralles et al. (2016)	0	0	0	0	1 (8)
Austrominius modestus	Miralles et al. (2016)	0	0	1 (20)	0	0
Cnemidocarpa sp.	Gaber & Elghazaly (2021)	0	0	0	1 (811)	1 (4)
Harmful algal bloom species						
Alexandrium spp.	Lundholm et al. (2009 onwards)	1	1	0	0	0
Gymnodinium spp.	Lundholm et al. (2009 onwards)	0	0	0	0	1 (30)
Gyrodinium spp.	Lundholm et al. (2009 onwards)	1 (3)	0	1 (88)	1 (1)	1 (134)

Commercial fish	Catch tonnes	Coastal sector				
		1	2	3	4	5
Dicentrarchus labrax	57,969.8 kg catch	0	0	0	0	1 (135)
Sarda sarda	10,936.3 kg catch	1 (2)	0	0	0	0
Thunnus alalunga	1,989,426.7 kg catch	1 (21)	1 (2)	0	0	1 (1)

The proportion of HABs, IAS and commercial fish found from water eDNA was different across the region, as it was the general community profile (Table 3). Sector 1 contained a higher proportion of zooplankton (versus phytoplankton) species, especially in comparison to sectors 2 and 3 that had <30 % of animal species identified from eDNA. The taxonomic diversity was higher in sectors 4 and 5 at the east of Cape Peñas than in those located at the west (Table 3). The proportion of HABs over the total number of phytoplankton species was higher in the sectors 1 and 2 at the west, while invasive species were concentrated in the central and eastern sectors 3 to 5 as seen above. DNA of commercial fish was found in the sectors 1 and 2 at the west and 5 at the east; not in the more polluted and populated sectors 3 and 4. Finally, taxonomic diversity was higher at the east than at the west (Table 3).

The plankton community changed seasonally in this study, with phytoplankton representing 56.6 % of the detected planktonic species in spring, 22.2 % in summer and 33.3 % in autumn samples ($\chi^2 = 6.7$, 2 d.f., $p = 0.035$). However, the proportion of nuisance species did not change significantly among seasons, being 0.05 (SD = 0.02), 0.01 (SD 0.01) and 0.08 (SD 0.05) in winter, spring and summer respectively (Kruskal-Wallis tie-corrected Hc = 2.829, $p = 0.243$). This, together with no differences in sampling seasonality among sectors seen above, would further support no biased results due to seasonality.

3.2. Overview of the biotic pollution and abiotic stressors in the marine sectors considered

The abiotic stressors in each sector are summarized in Table 4. As explained in 2.2, human population is concentrated in Sectors 3 and 4, where the two industrial ports of the region are located and there is more industry. Surprisingly, the number of WWTPs is lower in these two sectors (Table 4). Sector 4 contains globally larger river discharges. The total population size is clearly larger in Sector 4, and the population density in Sector 3. Sector 5 is the first in WWTPs, the second with Sector 1 in aquaculture, the third in fishing ports and the fourth in human population, river discharges and industrial pollution.

In the PCA, PC 1 and PC 2 explained 81.9 % of the variance (Supplementary Table 3B). The fishing activity in ports, WWTP number and population density had the highest loading in PC 1; population size, aquaculture and cargo in PC2; and the proportion of IAS, diversity, and rivers in PC3. The scatter plot of PC 2 on PC 1 showed differences between the spatial sectors considered (Fig. 2). Sectors 1 and 2 were located in the same quadrant with the variables commercial fish and WWTP. Ports (fishing activity), and population density were together

Table 3
Biotic variables in the five sectors considered within Bay of Biscay. Proportion of invasive species, commercial fish species, zooplankton fraction over the total number of species detected, harmful algae over the total number of phytoplankton species detected (HABs), and taxonomic diversity measured as Shannon index with the number of species per class as unit. For the proportion of invasive species, commercial fish and zooplankton taxa, calculations made from the number of reads are given in parenthesis.

	Sector 1	Sector 2	Sector 3	Sector 4	Sector 5
% Invasive species	0	0	3.57 % (0.4 %)	5.13 % (7.2 %)	5.08 % (0.3 %)
% Commercial fish	3.3 % (0.28 %)	4.8 % (0.12 %)	0	0	3.4 % (2.6 %)
% HABs	7.7 %	8.3 %	5.6 %	4.8 %	6.1 %
% zooplankton taxa	47.5 % (12.8 %)	28.6 % (29.1 %)	25 % (6.5 %)	35.9 % (17.1 %)	37.3 % (30.8 %)
Taxonomic diversity	2.03	1.92	2.19	2.31	2.57

Table 4
Measures of environmental stressors in the five coastal sectors analysed in Asturias (southwest Bay of Biscay). Population density in inhabitants per km^2. The intensity of the impact of ports is measured from the fishing activity as a proxy: ktons of landings in fishing ports of each sector. Industrial pollution and tourism are presented in 1–5 (min–max) Likert scale. Cargo as number of Mtons arriving in the commercial ports of Avilés (Sector 3) and Gijón (Sector 4). Tourism was measured from the number of tourist beds in each sector. WWTP, wastewater treatment plants.

Stressor	Sector 1	Sector 2	Sector 3	Sector 4	Sector 5
Population	34,183	6858	100,558	306,889	24,804
Population density	45.44	62.99	1224.08	506.13	50.15
Aquaculture	2	0.5	0	2.4	2
Port fishing activity	0.95	0.23	12.49	5.2	2.17
Cargo	0	0	5.5	18.01	0
Rivers	61	59	20	76	56
Industrial pollution	1	3	4	5	2
Tourism	3	2	1	4	5
WWTP number	4	4	1	2	5

with HABS in the quadrant containing Sector 3. Sector 4 was closer to the diagonals of IAS, cargo, population and industrial pollution. Finally, Sector 5 (at the east of Cape Peñas like Sector 4) was near the diagonals of the proportion of zooplankton species, tourism, rivers and aquaculture. Overall, this scatter plot suggested positive associations between HABs and port fishing activity and IAS and commercial ports; invasive species would be also associated with population size and industrial pollution, close to diversity and opposite to commercial fish, that would be not far from WWTPs. Diversity was positively correlated with the proportion of invasive species ($r = 0.898$ with $p = 0.038$).

3.3. *Association of nuisance species with environmental stressors*

The results of multiple regression analysis (Table 5) were quite consistent with the associations visualized in the PCA of Fig. 2. The proportion of IAS detected from eDNA in this study was significantly associated with tourism, fishing ports activity, and with industrial pollution. HABs were significantly and positively associated with fishing ports, while negatively with aquaculture. Industrial pollution was also associated with HABs but the correlation did not reach statistical significance (Table 5).

Considering the number of reads instead of presence/absence of nuisance species, the number of IAS reads was significantly correlated with cargo tons ($r = 0.962$, $p = 0.009$) and with the population size ($r = 0.968$, $p = 0.007$). The same calculation cannot be made for HABs because *Alexandrium* was detected from conventional PCR.

Since both IAS and HABs were positively associated with port activity we explored further the relationship between this factor and biota in this region. The differences in diversity between sectors can be visualized in Fig. 3. The taxonomic catalogue was not significantly different comparing all the sectors individually ($\chi^2 = 65.38$, 72 d.f., $p = 0.69$); however, grouping sectors 1 and 2 (with lower port activity) with the other group of sectors for the taxonomic catalogue, the difference was significant ($\chi^2 = 33.5$, 21 d.f., $p = 0.04$). The three sectors with higher port activity were enriched in phytoplankton in comparison with the other two (57.1 % versus 43.6 % of the species), while having fewer Arthropoda and Mollusca (herbivores) (Supplementary Table 2). Nematoda DNA appeared only in the Sector 5. The more polluted sectors with higher port activity (3, 4 and 5) exhibited a higher Shannon taxonomic diversity, although the ANOVA comparing the sectors 1 and 2 with the sectors 3, 4 and 5 did not reach significance ($F_{1,3} = 6.56$, $p = 0.083$).

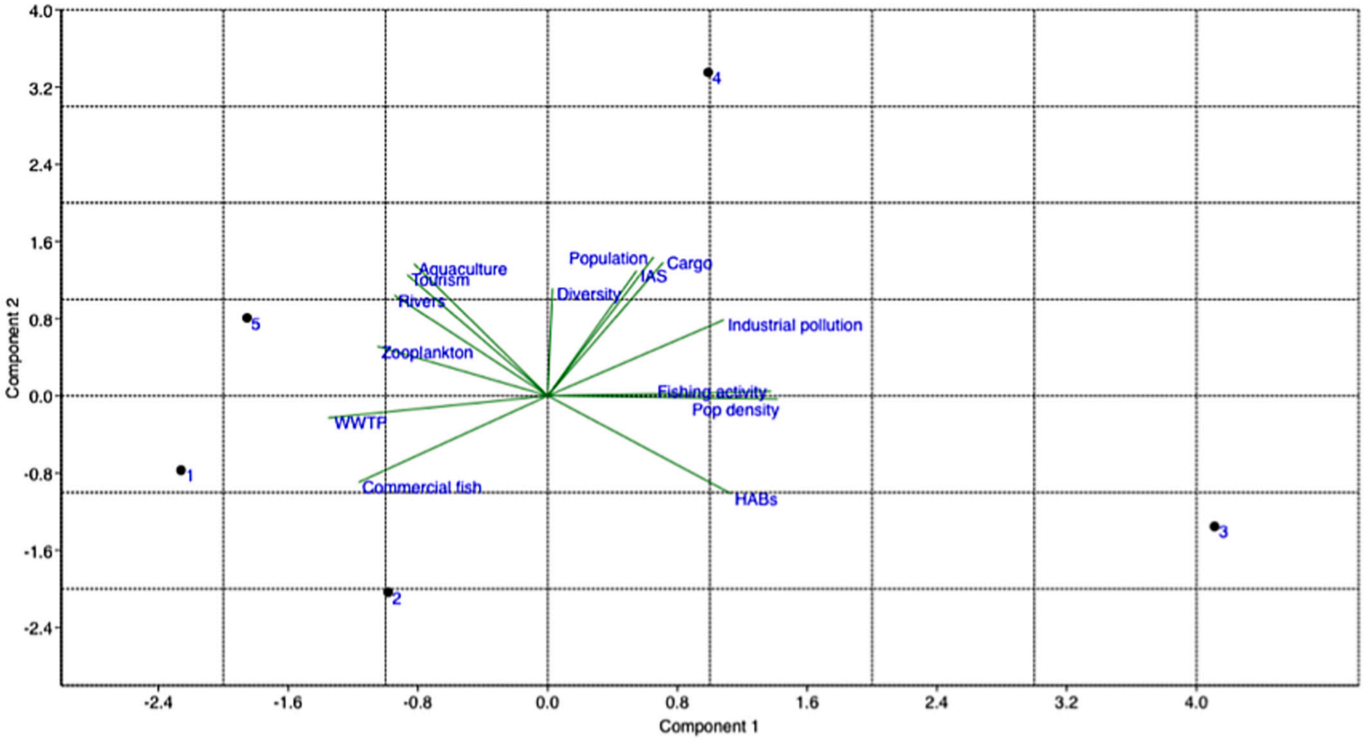

Fig. 2. Scatter plot of the Principal Components 1 and 2 showing the biotic and abiotic variables analysed as diagonals, their length being proportional to their relative weight. HABs, harmful algae; IAS, invasive alien species; WWTP, wastewater treatment plants.

Table 5
Multiple regression analysis showing significant multiple regressions for dependent biotic variables and independent environmental stressors. SE, standard error.

Dependent variable: proportion of IAS

Multiple R = 1		Coefficient	SE	t	p	R^2
Multiple R^2 = 1	Constant	−0.013	0.0008	16.3	0.039	
Adjusted R^2 = 0.999	Tourism	0.019	0.0003	60.397	0.011	0.88
F(3,1) = 1788.5	Ports	0.002	8.89×10^{-5}	22.687	0.028	0.22
p = 0.017	Industrial pollution	0.0008	0.0004	22.707	0.027	0.28

Dependent variable: proportion of HABs

Multiple R = 0.9998		Coefficient	SE	t	p	R^2
Multiple R^2 = 0.9995	Constant	0.191	0.0018	103.53	0.006	
Adjusted R^2 = 0.998	Ports	0.004	0.0002	21.87	0.029	0.64
F(3,1) = 727.52	Aquaculture	−0.018	0.0007	24.66	0.026	0.77
p = 0.027	Industrial pollution	0.005	0.0005	9.71	0.07	0.06

4. Discussion

4.1. Relationships between stressors and biopollution

The two departure hypotheses were accomplished in this study. First, we found an association between port activity and metazoan biopollutants, and also between port activity and HABs, confirming Hypothesis i and supporting previous studies on these species of concern for biosecurity (e.g., Seebens et al., 2013; Ardura et al., 2020; Costello et al., 2022). Areas with lower port activity contained fewer eukaryotic IAS and HABs than sectors with more fishing activity, and in the case of IAS also with commercial ports. Since our study was based on eDNA we have not seen physically the organisms. Thus we cannot be sure the statistical association is due to small propagules (eggs, early stage larvae) of metazoan IAS or to established populations.

For Hypothesis ii, it was generally supported because, as expected, other factors were also significantly associated with nuisance species. Industrial pollution was significantly associated with invasive species, that for their higher tolerance (Karatayev et al., 2008; Frü et al., 2012; Havel et al., 2015) resist pollution better than natives (Crooks et al., 2011; Salomidi et al., 2013). Interestingly, industrial pollution was also associated with HABs; this is one of the disturbances related with toxic microalgae in Mediterranean lagoons (Ardura et al., 2024). Tourism was positively associated with invasive species, as found by Anderson et al. (2015). Likely the higher movement of leisure vessels and other small and large recreational boats in highly touristic areas helps these species to spread.

However, not all the expectations of Hypothesis ii were met in our results. For example, both IAS and HABs were expected to be positively associated with aquaculture, for different reasons: IAS from escapes or as accompanying species, and HABs for the nutrient input of aquaculture activities. However, aquaculture was not positively associated with nuisance species in this study; moreover, it was negatively associated with HABs, which is quite unexpected. Davidson et al. (2014) found physical factors like winds and currents to influence strongly HABs distribution. In the study region dominant currents go eastwards (DeCastro et al., 2011; Domínguez-Cuesta et al., 2019). Perhaps HABs are associated with nutrient inputs from aquaculture, but the currents displace them to the sectors at east. Here aquaculture is concentrated in the Sector 1 at the west and in sectors 4 and 5 at the east, being almost negligible in the central part (sectors 2 and 3, see Table 4). If currents displace HABs eastwards, they would appear in the central sectors and at the east of sectors 4 and 5 outside the sampling region.

We found also interesting results using the number of reads instead of presence/absence of the nuisance species. It has to be taken into account that the number of reads does not represent the abundance of a species in number of individuals, being in the best case a semi-quantitative indicator of the biomass (e.g., Ershova et al., 2021; Blabolil et al., 2022). Even with this handicap, we found a positive correlation between the proportion of IAS reads and factors related with ports and pollution (like cargo and human population size), suggesting an association of invasive species with the population size (Pyšek et al., 2010).

4.2. Associations of stressors and biopollution with diversity

Although the negative impact of chemical contaminants on diversity is clear and has been sufficiently demonstrated (Johnston and Roberts, 2009), in our results, planktonic diversity was not depressed in more polluted sectors. A possible explanation could be due to the proportional increase of IAS in those sectors, that are simultaneously exposed to maritime traffic and pollution. Moreover, diversity and IAS were significantly correlated in our study. These tolerant invasive species would contribute to maintain or even increase the total taxonomic diversity, explaining the paradoxical increase of diversity with pollution. Elliott and Quintino (2007) found a similar effect in highly stressed estuarine areas, where biota is well-adapted to cope with that stress.

Another interesting result of this study was the significant difference in the taxonomic catalogue between the sectors with invasive species and those where IAS were not detected. With more phytoplankton and less herbivores like arthropods and gastropods, we could speculate an increase of primary producers in zones with IAS. An increase of primary producers following IAS introductions was found in very distant regions like for example Azores or Polynesia (Ardura et al., 2021; Faria et al., 2022). In Azores, the increase of primary producers following invasion by the brown macroalga *Rugulopteryx okamurae* is accompanied by a reduction of diversity (Faria et al., 2022), an effect that we have not seen in our study. It must be noted that the IAS found here (two Polychaeta, one Ascidian, one acorn barnacle) were all sessile animals, suspension feeders; thus their introduction is unlikely related with an increase of primary producers. A more probable explanation for this apparent change could be the well-known interchange of algae promoted by ships (Micael et al., 2014; Seebens et al., 2016), including indeed native algae.

4.3. Limitations of the study

In this study we employed relatively small water volumes per sampling point to perform eDNA metabarcoding. Such small water volumes may be sufficient to identify species of interest from eDNA (Borrell et al., 2017; Garcia-Vazquez et al., 2021).

Another possible limitation was that species were identified only from their DNA traces, not observed visually. However, most of the species detected here from eDNA, especially IAS, have been confirmed from conventional sampling and morphological identification in the region (Miralles et al., 2021, 2016), in some cases after a first detection from eDNA as an early alert (Borrell et al., 2017; Muñoz-Colmenero et al., 2018). The validity of eDNA metabarcoding for species detection in diversity surveys has been confirmed in numerous studies (Garcia-Vazquez et al., 2021; Jeunen et al., 2019; Valentini et al., 2016). Thus, even if the data here presented are based on eDNA solely, we could consider them sufficiently robust.

Here the samples were taken in different seasons, from spring to autumn. Seasonal fluctuations of plankton are indeed expected in

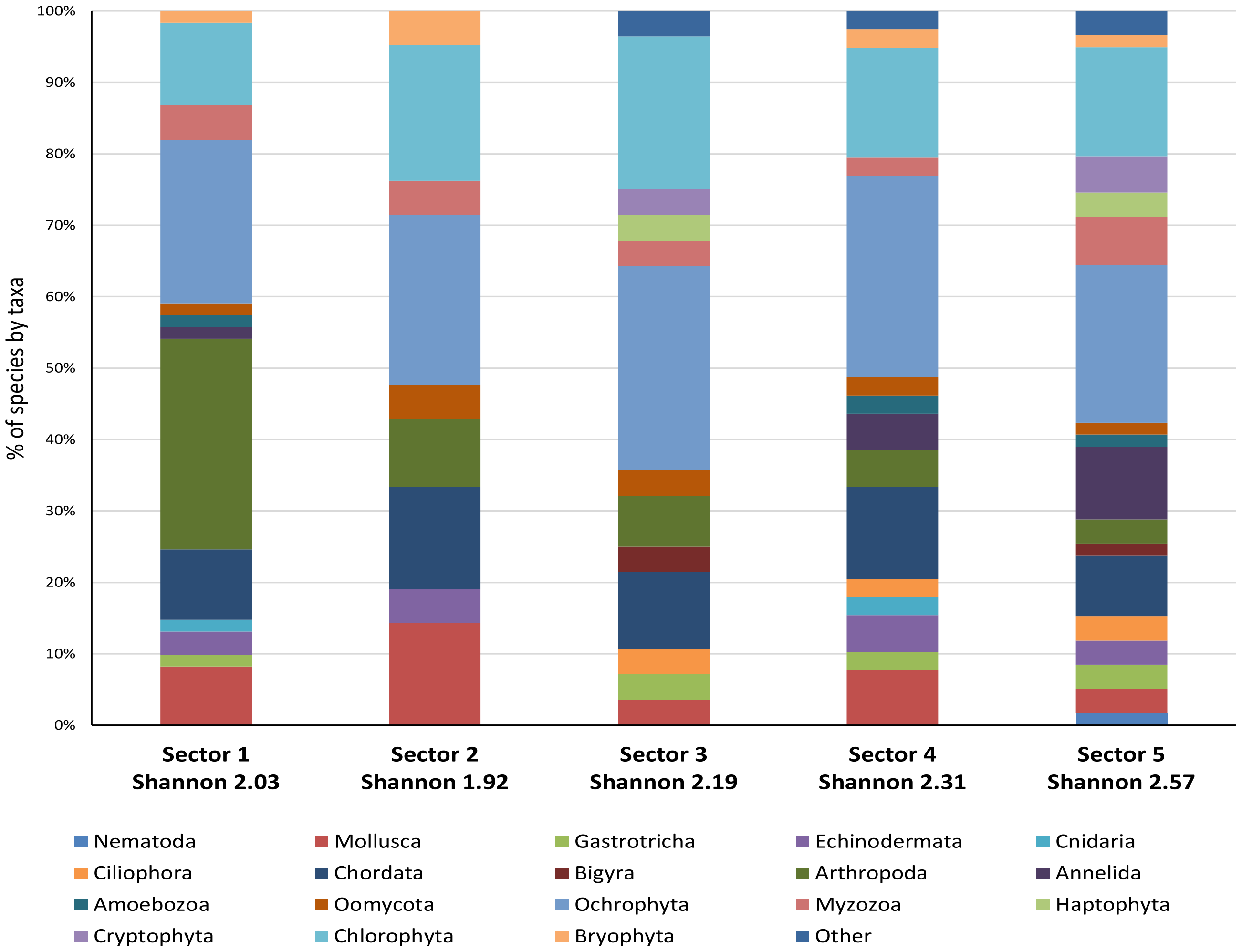

Fig. 3. Taxonomic composition of the communities detected from environmental DNA in the five sectors analysed within South Bay of Biscay. Shannon MOTU taxonomic diversity is given for each sector.

temperate regions like the southern Bay of Biscay (Huskin et al., 2006; Muñiz et al., 2018), thus the results should be taken with caution. However, the analysis of sampling seasonality suggests there is not a big bias due to this factor in our dataset. The proportion of samples taken in spring, summer and autumn was not different between sectors.

Indeed the composition of plankton community varied seasonally in the region in our eDNA data, with a higher proportion of phytoplankton species in spring and autumn, when phytoplankton blooms occur in the North Atlantic (e.g., Martinez et al., 2011). Diatoms like *Thalassiosira* and *Skeletonema* that are involved in those phytoplankton blooms in the Northeast Atlantic (Carstensen et al., 2015) occurred in our dataset (see Supplementary Table 2), thus this seasonal variation fits the expectations. However, despite the normal community variation the proportion of nuisance species DNA did not vary significantly among seasons in our dataset, which seems to be normal if the invasive species are already established. This, together with the similar sampling seasonality in the five sectors considered, would support the analysis by sectors is not too much biased by seasonal plankton variations. Repeating the sampling from the same locations in different seasons would be recommended to capture the real plankton diversity and MP pollution in these waters.

Finally, water quality parameters such as dissolved oxygen, nitrates, salinity, pH and others affect the distribution of planktonic species including IAS (e.g., Purmalis et al., 2021). They should be considered in futures studies to understand the spread of eukaryote nuisance species. Notwithstanding it, the region is affected by pervasive pollution derived from industrial activities and mining, as published by other authors (Garcia-Ordiales et al., 2019; Navarro-Murillo et al., 2024), and differences in pollution between different parts of the coast have been reported (Sanz-Prada et al., 2022), thus the analysis comparing differentially industrialized sectors seems to be opportune for the region.

5. Management implications

Port activity significantly explained the level of IAS in our study, as in many other regions. Working together with maritime sectors to improve the control of biopollution is a recommendation derived from this study.

Indeed, the control of pollution is strongly encouraged; it will benefit not only human health but also a healthy ecosystem and native biodiversity. Industrial companies should work together with the regional government to implement measures for reducing emissions and industrial sewage.

Tourism appeared here associated with invasive species. From the type of species, all sessile with no interest for aquariums or as pets, they were likely inadvertently dispersed in small ports and marinas by leisure vessels; recreational boats are an important factor of IAS dispersal (e.g., Kelly et al., 2013). Contacting tourists in marinas, and campaigning in the touristic sector for encouraging control and awareness about invasive species would be also recommended.

6. Conclusions

Working on water samples for biota inventory through eDNA, a significant association between pollution, maritime traffic and biopollution was found in southwest Bay of Biscay. The proportion of IAS seems to be favoured by port activity, tourism (likely leisure boating) and industrial pollution. Moreover, IAS concentration was positively correlated with plankton diversity. Port activity was also associated with the proportion of potential HAB-causing species. Despite some limitations, the present study could be considered robust enough to show positive, significant associations between pollution, biopollution and plankton diversity. The control of pollution and approaching maritime sectors for increasing awareness about biological invasions should be priorities for the sake of marine biodiversity conservation.

CRediT authorship contribution statement

Sara Fernandez: Writing – review & editing, Software, Methodology, Investigation, Formal analysis, Data curation. **Susana Acle:** Writing – review & editing, Investigation. **Eduardo Dopico:** Writing – review & editing, Resources, Investigation. **Paula Masiá:** Writing – review & editing, Methodology, Investigation. **Daniel Menéndez:** Writing – review & editing, Investigation. **Johannes Rick:** Writing – review & editing, Investigation. **Alba Ardura:** Writing – review & editing, Project administration, Investigation, Funding acquisition. **Eva Garcia-Vazquez:** Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.179194.

Data availability

Data will be made available on request.

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