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Building on gAMBI in ports for a challenging biological invasions scenario: Blue-gNIS as a proof of concept.

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ABSTRACT

The status of aquatic ecosystems has historically been monitored by the use of biotic indices. However, few biotic measures consider the presence of non-indigenous species as a sign of anthropogenic pollution and habitat disturbance even when this may seriously affect the metric scores and ecological status classifications of an environment. Today, biological invasions are currently one of the greatest threats to biodiversity and sustainable blue economies around the world. In this work, environmental assessments were conducted in the Port of Gijon, Northern Spain, using eDNA metabarcoding, and the gAMBI (genetics based AZTI Marine Biotic Index) was estimated. Results indicate a high/good ecological status within the port. However, nine non-indigenous species and five invasive species were found, and a modification of the gAMBI that includes species

invasiveness was proposed: Blue-gNIS. The index was preliminary tested against existing validated indices such as gAMBI, BENTIX (based on the ecology of macroinvertebrates) and ALEX (based on the invasiveness of the species). Blue-gNIS classified the port in a good ecological status and showed its potential usefulness to achieve more complete water quality assessments of ports.

KEY WORDS

Metabarcoding, invasive species, AMBI, gAMBI, biotic index, Blue-gNIS, Blueports.

INTRODUCTION

Marine ecosystems and their biodiversity are fundamental resources for society because economic activities, such as fishing, tourism, aquaculture and shipping depend on them (Baine et al., 2007; FAO 2012; Gössling et al., 2018). Currently, global warming, pollution and overexploitation are some of the anthropogenic stressors that have led to drastic changes in marine ecosystems, reducing their biodiversity and altering ecosystem functions and services (Halpern et al., 2008; McCauley et al., 2015; Halpern et al., 2015).

Biological invasions are also an important threat to biodiversity (Molnar et al., 2008). Marine ecosystems are facing constant introductions of new species, mostly in ports that are the main entry gates for non-indigenous species (NIS), occurring principally through biofouling and ballast water (Katsanevakis et al., 2013; Nunes et al., 2014). When non-indigenous species manage to establish reproductively viable populations in new areas and begin to disperse and proliferate uncontrollably, they can outnumber native species, dominate the ecosystem and generate serious environmental impacts, becoming invasive alien species (IAS). Since eradication is more difficult in late than early invasion stages, new strategies are needed for the effective prevention and early detection of nuisance organisms (Gherardi & Angiolini, 2009; Ujiyama et al., 2018).

The current situation revealed that despite all the available knowledge about NIS detection and prevention, appropriate strategies are far from being effectively implemented within the ports. To address this problem, several policies and directives have been developed to protect marine ecosystems, such as the EU Water Framework Directive (WFD, Directive 2000/60/EC) and Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC). Their main objective is to improve the ecological status of European aquatic ecosystems and to reach an overall “good ecological status”. To do this, the member states are required to perform periodic evaluations of their water bodies (De Jonge et al., 2006). To date, different aquatic ecosystems, including rivers, lakes, transitional and coastal waters, have been analyzed in European monitoring programs (Zacharias et al., 2020). However, less than 3% of the reported information is from coastal waters (EEA, 2012), which contain modified habitats such as ports where metal pollution, oil spills, garbage, antifouling paints, ballast waters and greenhouse gas emissions can affect the local biodiversity (UNCTAD, 2015; Yu et al., 2017).

Through the execution of periodic monitoring, it is possible to assess the environmental status of these aquatic ecosystems, and in this way, action strategies can be developed to prevent further deterioration and biodiversity loss (Birk et al., 2012; Borja et al., 2010). In this context, the use of biotic indices has become very relevant at the time of communicating and presenting the results from monitoring networks or environmental impact studies to managers, stakeholders or policy makers, as these indices constitute an easy method to transmit the results in a simple and understandable way (Borja et al., 2019). However, most of the biotic indices employed for environmental assessments are solely based on ecological traits from specific taxa such as macroinvertebrates and do not consider other important aspects such as the species invasiveness. Biological invasions are currently considered the second cause of biodiversity loss (Bellard et al., 2016) so that there is an urgent need to use biotic indices that combine both, ecology and invasiveness, when performing environmental evaluations.

Aquatic pollution triggers the decline in pollution-sensitive species and leaves free ecological niches that can be occupied by pollution tolerant species which in many cases can be non-indigenous or invasive species (Crooks et al., 2011). In this way, biodiversity losses triggered by pollution may lead to a reduction in the resilience of ecosystems to invasion (Shea and Chesson, 2002; Miralles et al., 2016), indicating that there is a need to periodically monitor local environmental conditions to avoid the deterioration of the ecosystem and increase the resistance to invasion events. The prevention of the introduction of invasive alien species (IAS) is one of the lines of action that have been stated by the European Commission for the EU Blue Growth strategy (European Commission, 2017; Eikeset et al., 2018). The introduction of IAS into ports, coastal areas and watersheds is damaging aquatic ecosystems around the world, with estimated direct costs of many millions of dollars spent on monitoring, prevention of spread and remediation of the ecosystems (Walsh et al., 2016; Interwies and Khuchua, 2017). Thus, biological invasions are one of the greatest threats to biodiversity and sustainable blue economies that can also affect human health (Bayliss et al., 2017) and therefore must be included in any environmental quality status evaluations.

The AZTI Marine Biotic Index (AMBI) (Borja et al., 2000) is currently one of the most used biotic indices (Borja et al., 2015; Abaza et al., 2018; Belhaouari et al., 2019; Yan et al., 2020) and it is useful for environmental quality assessments of marine ecosystems. It is currently included in both the WFD and MSFD directives for the purpose of improving the quality and preventing the further deterioration of aquatic environments (Borja et al., 2009). The AMBI is based on macroinvertebrate species that are classified into five ecological groups, depending on their sensitivity/tolerance to disturbance, and it is known to be useful for detecting anthropogenic changes in an environment (Borja et al., 2000). New biological indices have been developed, such as the gAMBI (genetics-based AMBI), which is a modification of the AMBI that works with genetic data (Aylagas et al., 2014) that can be based on presence/absence information but also on relative abundance data, including the number of reads obtained for each species (Aylagas et al., 2018). This methodology allows faster and cheaper marine monitoring and health status assessment compared to other methods. However, these indices do not take into

account the invasiveness of non-indigenous species, which should be considered for a more complete quality assessment of an area. In fact, it has been demonstrated that the presence of invasive species may affect the metric scores and ecological status classifications of an environment (MacNeil et al., 2013; Mathers et al., 2016).

DNA metabarcoding has become popular in recent years as a useful tool for evaluating the ecological and environmental status of aquatic ecosystems (Baird and Sweeney 2011; Baird and Hajibabaei 2012; Taberlet et al., 2012; Keck et al., 2017; Hering et al., 2018; Pawlowski et al. 2018). It is a technique based on gene markers used to identify taxa-specific sequences from the released organism's DNA, allowing the simultaneous identification of multiple taxa from bulk or environmental samples. Although there are multiple issues that are still unresolved when using metabarcoding techniques (e.g. incomplete reference databases, primer biases, and unstandardized bioinformatic processes), in contrast with classical methods that employ species identifications based on morphological traits, DNA metabarcoding is more time and cost-effective and increases the taxonomic resolution, species detectability (specially for earlier life stages or fragmented/destroyed samples) and comparability across geographic regions (Pawlowski et al. 2018). It is a technique that provides better taxonomic characterizations, with the potential of revealing hidden diversity (Lindeque et al., 2013), and has also become successful in terms of efficient monitoring of endemic, endangered and invasive alien species (Ficetola et al., 2008; Dejean et al., 2012; Valentini et al., 2016; Blackman 2017; Borrell et al., 2017; Hering et al., 2018).

In this research, we targeted the industrial Port of Gijon, (central Cantabrian Coast, northern Spain), which receives large international and national cargo vessels, and used DNA metabarcoding as a tool to evaluate the ecological status of the port. We also propose a slight modification of the gAMBI to obtain a new exploratory multihabitat index called Blue-gNIS based not only on the ecology but also on the invasiveness of the detected species. The aim is to obtain better characterizations of coastal waters where non-indigenous species can seriously affect local biodiversity. Blue-gNIS could become a useful tool for biomonitoring programs in ports where intense marine traffic can act as a vector for the introduction and spread of harmful species.

MATERIALS AND METHODS

The Port of Gijon (Bay of Biscay) characterization and sampling

The Port of Gijon is located on the Cantabrian coast (5°41'W and 43°34'N) and is one of the main seaports in the Atlantic Arc and the leading port for bulk solid movement in Spain (https://www.puertogijon.es/en/). The Port of Gijon occupies 415 hectares and has more than 7,000 linear meters of docks, and it includes areas divided by the type of traffic (solid and liquid bulk and container terminals and multipurpose facilities for various types of traffic). The port also has a small marina with recreational boats located outside the main docks.

The traffic data from the Port of Gijón (period 2004-2017) was obtained from Gijon Port Authority (2017) and it was measured by employing the gross tonnage arrived from each biogeographical zone (measured in GTs), which is related to the capacity of ships and the surface of the ship's hull on which species can be transported (Davidson et al., 2009).

The sampling was conducted in July 2017. The port was divided into 5 sites following the dock distribution within the port, namely, sites A, B, C, D and the marina (E), and within each site, two points were chosen (Figure 1). Four samples were taken at each point, with two replicates in the water column and another two in the sediment. Water samples were taken using Niskin bottles between the surface and 1 m depth. For the sediment, a Van Veen grab was used to collect a total surface of 90cm^2 in each replicate. Samples were stored in 50 mL vials and introduced in ice-cold bags for the transportation to the University of Oviedo, where they were stored at -20 °C.

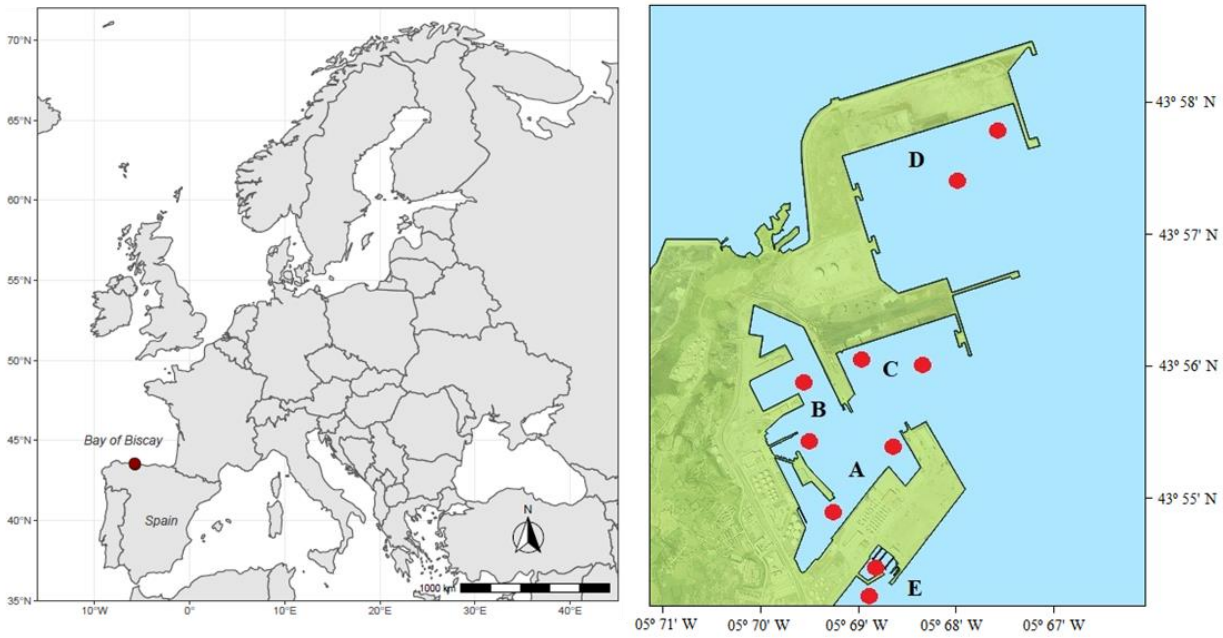

Figure 1. Sampling locations within the Port of Gijon in the southern central area of the Bay of Biscay (5°41'W , 43°34'N).

Environmental DNA extractions

The eDNA extractions were conducted under sterile conditions inside a laminar airflow chamber previously disinfected with UV light and 10% bleach solution. Negative controls were used in all filtration and extraction processes. The negative control for the filtration was 1L of milliQ water and all filtrations took place under sterile conditions in the laboratory of eDNA in the Genetics Department from the University of Oviedo. All samples were carefully preserved in cold (under 5ºC) before filtered. Filtrations were done immediately after collected (a time scale of hours). Finally, we used pumps from Labbox Labware (Spain). The water samples (1 L per sample) were filtered through 0.22 µm sterile nitrocellulose membranes (Prat Dumas, France), and then, the DNA was extracted using a PowerWater® DNA Isolation Kit (Qiagen Laboratories, USA). For the

sediments, 10 g per sample was vortexed for initial homogenization, and then the DNeasy PowerMax Soil® DNA Isolation Kit (Qiagen Laboratories, USA) was used following the manufacturer 's instructions. The correct extraction of the DNA was visually assessed on 1.5% agarose gel (by checking the presence of bands of the expected size), and the samples were quantified using the Picogreen method and Victor-3 fluorometry (Invitrogen, cat. #P7589). A positive DNA control was used during the whole sequencing process and employing the same conditions as for eDNA samples. It contained equimolarly pooled DNA (50ng µL-1) belonging to 9 different macroinvertebrate species and another 10 additional species (Supplementary Table 1).

PCR amplification, next-generation sequencing and bioinformatics analyses

PCR amplifications of the mitochondrial cytochrome oxidase subunit I gene (COI) were undertaken on an Eppendorf Mastercycler (Eppendorf, Germany) in a total volume of 53 µl using 25 µl of MyTaq™Red Mix which includes KAPA HiFi HotStart DNA Polymerase (Bioline, USA), 2 µl of each primer and 3 µl of template DNA using the universal primers mlCOIintF (5'- GGWACWGGWTGAACWGTWTAYCCYCC-3') and jgHCO2198 (5'-TAIACYTCIGGRTGICCRAARAAYCA -3') (Leray et al. 2013). For the index PCR, 5 µl of indexes were used from the Nextera XT index kit (FC-131-1001 or FC-131-1002) following the protocols described in the 16S Metagenomic Sequencing Library Preparation Manual from Macrogen Korea (Illumina, 2011). After multiple trials, for the best amplification success, the PCR conditions were adjusted as follows: for the first PCR, 1x: 95 °C for 3 min; 25x: 95 °C for 30 sec, 44.7 °C for 30 sec and 72 °C for 30 sec; and finally, 1x: 72 °C for 5 min followed by a 4 °C hold. Conditions for the index PCR were: 1x: 95 °C for 3 min; 8x: 95 °C for 30 sec, 44.7 °C for 30 sec and 72 °C for 30 sec; and finally, 1x: 72 °C for 5 min followed by a 4 °C hold. Library construction included quality controls for the size (Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip) and quantity (Roche's Rapid library-standard quantification solution and calculator). The bands of the expected size (313 bp) were sequenced by 300bp paired ends in the Illumina Miseq system (Macrogen, Korea) and the BCL (base calls) binary was converted into FASTQ utilizing illumina package bcl2fastq2-v2.20.0 conversion software. Scythe (v0.994) (Buffalo, 2011) and Sickle (Joshi & Fass, 2011) programs were used to remove adapter sequences. After adapter trimming, reads shorter than 36bp were dropped in order to produce clean data

Bioinformatics analyses were performed using QIIME2 (Bolyen et al., 2018). An initial quality filter was performed by cutting forward and reverse reads to a specified length when the nucleotide assignment qualities showed Phred scores lower than 20 (at 260bp for forward reads and 210bp for reverse reads). Then, paired end reads were merged, and chimeras were removed using the consensus method, which performs de novo identification for each sample and removes all amplicon sequence variants identified as chimeras. To finish the filtering step, the remaining sequences were dereplicated.

An updated COI sequence database was generated by downloading data from the current NCBI webpage (September 2020). Only nonenvironmental DNA belonging to voucher specimens was considered, and all eukaryotic organisms were included. To do this, the following key words were employed in the NCBI browser: Mitochondrial, Cytochrome

Oxidase 1 (and corresponding abbreviations CO1, COI and cox1), voucher, and nonenvironmental. This generated a dataset composed of more than 455,000 fasta-formatted entries for the COI gene belonging to 123,439 different species. The script entrez_qiime.py (Baker, 2016) was then used to generate the taxonomy file associated with the generated database. Taxonomic assignments were done by using the qiime feature-classifier plugin from QIIME2 (version 2019.4.0) with a 90% of minimum identity and E-value of 1e-50 following Fernandez et al. (2018). Sequences were rearranged and clustered using the vsearch cluster-features-denovo plugin, version 2019.7.0 (Rognes et al., 2016). A similarity threshold of 97% was employed because it is considered the level at which species differ in the case of the COI gene (Hebert et al., 2003). Sequences with a higher similarity percentage were clustered into the same operational taxonomic unit (OTU). Once the OTU table was created, a final filtering step was performed, and only marine OTUs were retained for further analyses. The assigned marine OTUs were individually revised and named using WoRMS taxonomy (Horton et al., 2019) as a model to avoid discrepancies or outdated nomenclature.

Biotic indices (gAMBI and Blue-gNIS) and ecological status evaluations

gAMBI is a biological index that classifies species into five different ecological groups, depending on their tolerance to pollution: group 1, contains the most pollution-sensitive species that cannot survive in polluted areas, followed by groups 2 and 3 which contain species that show more tolerance to pollution. Groups 4 and 5 contain the most pollution-tolerant species that usually inhabit disturbed areas. The percentage of species belonging to each group defines the final index value, which can range from 0 (unpolluted areas) to 6 (heavily polluted areas). Each index score has an associated ecological status that can be high, good, moderate, poor or bad (Supplementary Table 2). The index is calculated by the following formula:

$$gAMBI = \frac{[(0x\%G1) + (1{,}5x\%G2) + (3x\%G3) + (4{,}5x\%G4) + (6x\%G5)]}{100}$$

The environmental assessment of the port of Gijon was carried out by estimating the gAMBI value for each station. The index values were calculated using the AMBI software downloadable at https://ambi.azti.es/es/descarga-de-ambi/, which contains a list of macroinvertebrates classified into the five ecological groups.

In this work, we propose an adaptation of gAMBI, namely, Blue-gNIS (named after the EU Blue Growth strategy), which not only considers species ecology or tolerance level to anthropogenic stressors, but also takes into account the invasiveness of species inhabiting the area under assessment. The Blue-gNIS uses the same formula as the gAMBI, and has the same range, from 0 (undisturbed areas) to 6 (extremely disturbed areas). The only difference is in the classification of species into ecological groups, which is done by combining ecological traits with the invasion history (Figure 2). First, in order to

determine the group to which each species belongs, the invasiveness is analyzed by searching for the species distributional data. In this research, the following databases were consulted: AquaNIS (AquaNIS, 2015), DAISIE (Roy et al., 2019) ISSG (http://www.issg.org/database), GRIIS (http://www.griis.org), CABI (CABI, 2019), Algaebase (Guiry & Guiry, 2019) and Marine Planktonic Copepods (Razouls et al., 2019). Species introduction events were analyzed using the AquaNIS webpage as the main source of information. With this information, species were classified as native, NIS (exotic species without reports of producing environmental impacts in the area under study) or IAS (exotic species that produce environmental impacts in the area under study). Cryptogenic species were not included in the analysis. Finally, a search for previous reports of the presence of the assigned NIS and IAS was done to check their current status in the area under study (Figure 2).

Once the invasiveness is assessed, the ecological information is added. In the case of native species, those species belonging to gAMBI (macroinvertebrate species) were classified based on the existing values (that are based on the ecology of the species). For NIS and IAS, the initial ecological group was determined following the categories from the ALEX (ALien Biotic IndEX), which is an index that considers species invasiveness (Çinar and Bakir, 2014). In the case of NIS, they were initially classified into ecological group 3, as these species show more tolerance than native species to anthropic environments and pollution, along with the ability to survive in extreme conditions, such as in ballast tanks, where many of these species are transported to recipient regions (Piola and Johnston, 2009). This value can be increased, depending on the ecological traits of the species. If the NIS has an existing AMBI value, and if it is higher than 3, the species will be classified into that group, but if, on the contrary, the value is lower than 3, the species will remain in group 3. If there is no previous AMBI ecological group for the species, a bibliographical search is conducted to determine its ecological traits (such as tolerance to hypoxic conditions, heavy metals, eutrophication, high temperatures, etc). This way all the detected non-indigenous species are considered in the Blue-gNIS index calculation. In the case in which reports reveal the species presence in disturbed conditions, the ecological group is increased from 3 to 4. The same criteria were employed for IAS, but in this case, following the categories from Çinar and Bakir (2014), these species were initially classified into group 4, because aquatic pollution increases the relative success of invasive species (Crooks et al., 2011). This value can also be increased to group 5 if they meet the previously specified conditions or if they have specific ecological traits related to disturbed habitats (Figure 2).

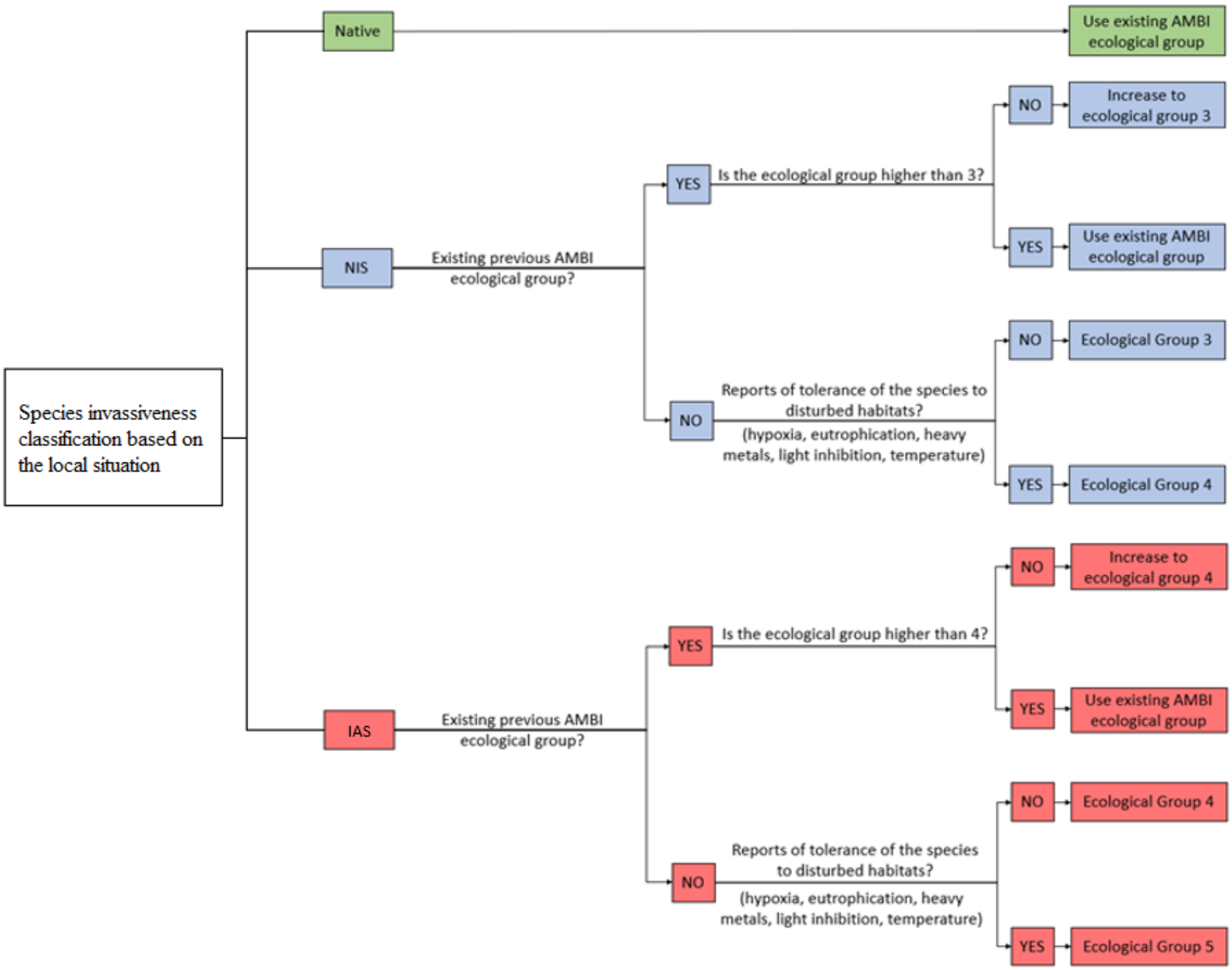

Figure 2. Criteria for the classification of species into ecological groups for Blue-gNIS estimations.

Apart from these criteria for species classification into ecological groups, the Blue-gNIS has another difference relative to the gAMBI in the taxa that are considered for the environmental assessment. In the case of the gAMBI, only macroinvertebrate taxa are employed for the index calculation. On the other hand, regarding the invasiveness of the species, the presence of any NIS is considered informative for the Blue-gNIS, whether macroinvertebrate or not. For example, if non-indigenous macrophytes are detected during the environmental assessment, they are included in the analysis. In this way, a better representation of the real status of the port is obtained because all the NIS present in the area under study are taken into account. Moreover, in order to classify these species into ecological groups, not only their invasiveness but also their ecological traits are considered. For instance, macrophytes are classified depending on their ecological group

defined by the Ecological Evaluation Index (EEI) that uses only macrophytes as bioindicator species (Orfanidis et al., 2011).

Testing the performance of Blue-gNIS

The performance of the Blue-gNIS was calibrated by comparing the obtained results with validated biotic indices reported by Aylagas et al., (2014) (gAMBI) and Simboura and Zenetos, (2002) (Bentix), which are based on species ecology, and the one from Çinar and Bakir (2014) (ALEX), which is based on species invasiveness. This way, Blue-gNIS was preliminary calibrated considering both, the ecological component (against gAMBI and Bentix) and the component related to biological invasions (against ALEX). Both presence/absence and quantitative data were used in the comparisons. The index scores and the associated ecological status were calculated following the formulas from the authors mentioned above. These scores were normalized to the Ecological Quality Ratio (EQR) and compared using Spearman's rank correlations due to the lack of linearity among them. Data used to perform these comparisons were not only those obtained in this study for the port of Gijon, but also additional data belonging to previous studies conducted in the Cantabrian Sea, such as those of Borrell et al. (2017) (metabarcoding in Ports), Borrell et al. (2018) (metabarcoding in estuaries) and Miralles et al. (2019) (specific eDNA detection of *Crepidula fornicata* in different locations of the Bay of Biscay and unpublished metabarcoding data) were used in order to obtain a better calibration of the Blue-gNIS (Supplementary Table 3). Rarefaction plots were generated for all these data, and only samples reaching the plateau and thus representing an adequate sampling depth and species richness within these studies were selected for the analysis (Supplementary Figure 1).

Statistical analyses

Statistical analyses were conducted using the PAST program (Hammer et al. 2001) on both, presence/absence and quantitative metabarcoding data. Normality was checked in the dataset, and then diversity permutation tests and diversity t-tests were performed to compare differences in biodiversity levels among different sampling methods and port stations. The Shannon index was chosen for these comparisons (Herrera et al., 2007; Ransome et al., 2017; Lacoursière-Roussel et al., 2018; Wangesteen et al., 2018). ANOVA tests were conducted for samples obtained in the same substrate (water or sediment) within each station. Similarities between stations and sampling techniques were determined using Bray-Curtis distances and a nonmetric multidimensional scaling (nmMDS) analysis after checking the stress and r2 values in Shepard plots. Tolerable stress levels were considered those below 0.2 (Oksanen et al., 2016). A PERMANOVA test was conducted using 9999 permutations and Bray-Curtis similarity index to compare sediment and water samples. Regarding the calibration of Blue-gNIS, the normality of the parameters was checked performing Shapiro Wilk tests and Bonferroni correction was applied to the performed correlations.

RESULTS

Metabarcoding analyses

The quantity of DNA obtained from water and sediment samples that was used for High-throughput sequencing (HTS) ranged between 0.01 ng µL-1 and 80.55 ng µL-1 (Supplementary Table 4) and provided a total of 3,342,049 reads. For bioinformatics analyses, the quality-filtering step removed too-short, low-quality and chimeric reads, resulting in 1,896,906 sequences. A total of 241,756 sequences, with an average length of 365 bp, were successfully assigned against the COI database and classified into 452 OTUs belonging to different taxonomic levels. Some terrestrial taxa were identified, which were mainly insects (193 OTUs) and terrestrial mammals (96 OTUs). After filtering these taxa, 141 marine OTUs were obtained, which were classified into 25 classes mainly composed of Florideophyceae, Hexanauplia and Polychaeta. From this dataset macroinvertebrates, NIS and IAS were employed for biotic index calculations (Supplementary Table 5).

Samples obtained from the same substrate (water or sediment) within each station were combined for posterior analyses as they did not show statistically significant differences. The nonmetric multidimensional scaling (nmMDS) analysis based on Bray Curtis distances, showed a stress value of 0.12 and r^2 values of 0.58 and 0.30 for axes 1 and 2, respectively, in the Shepard plot (Supplementary Figure 2). This plot indicates how well the Multidimensional Scaling reflects the actual proximities. Results indicate a good correlation between the original distances (target rank) and the transformed ones (obtained rank). A clear differentiation between samples taken from water and those taken from sediment was observed (Figure 3). In both cases (sediment and water), station E (the recreational marina) was the most dissimilar compared to stations A, B, C and D, which were much more similar to each other. The PERMANOVA analysis showed statistically significant differences between stations' beta diversities (p=0.006) when comparing water and sediment samples (Supplementary table 6).

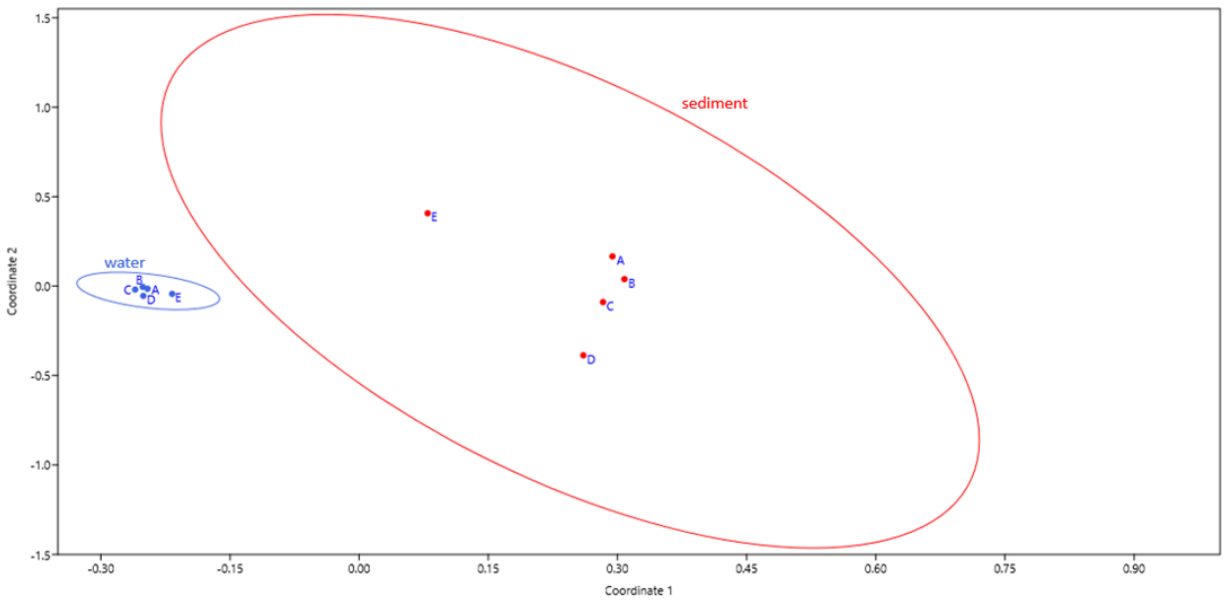

Figure 3. Non-metric Multidimensional Scaling of the metabarcodes found in each station for water (blue dots) and sediment (red dots) samplings in the Port of Gijon, Bay of Biscay. Circles indicate 95% confidence ellipses.

Some taxa showed a greater abundance in sediment than in water, as in the case of annelids, for which 15 OTUs were detected in sediments and only three in water. A greater diversity of annelids was detected in the sediment samples than in the water samples (diversity t test for the Shannon index: t= -4.27, df= 4.84, P= 0.0086). Taxa such as Nematoda, Nemertea and Phaeophyceae could only be detected in sediment samples (Figure 4). More OTUs were detected in water than in sediment in the cases of Arthropoda (17 in water and 10 in sediment), Mollusca (11 in water and 7 in sediment) and Chordata (composed only of Actinopterygians), which were only detected in water samples. For species level, only a 4% was found in both water and sediment samples.

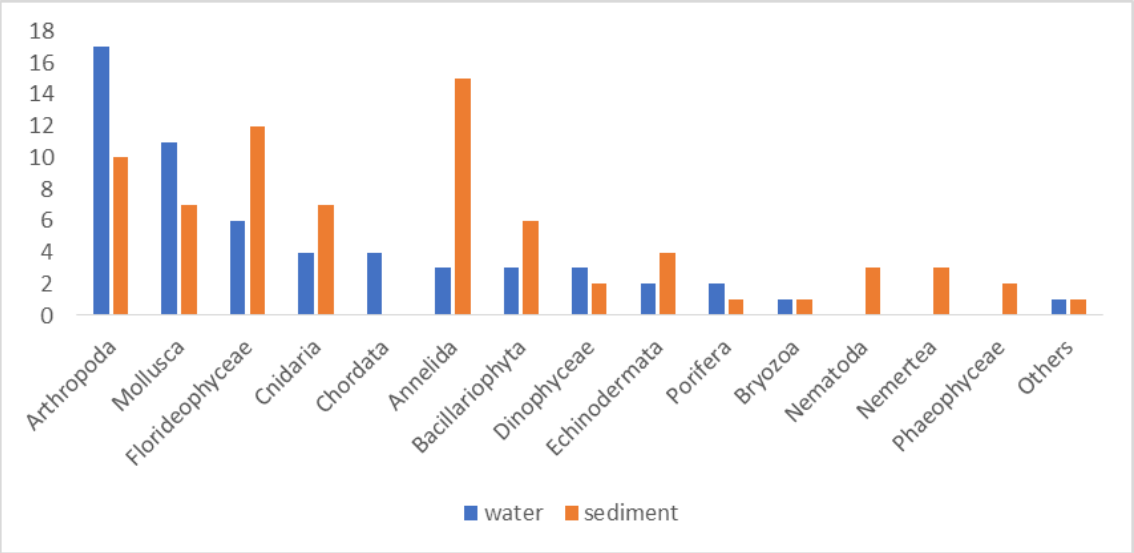

Figure 4. Number of OTUs detected using metabarcoding for major taxa in sediment and water samples from the Port of Gijon, Bay of Biscay.

Nine NIS (*Paracalanus quasimodo, Oncaea waldemari, Clytia gregaria, Grateloupia imbricata, Neogastroclonium subarticulatum,* and *Hymeniacidon gracilis*) and five IAS (*Asparagopsis armata, Bonnemaisonia hamifera, Dasysiphonia japonica, Bugula neritina* and *Botryocladia wrightii*) were detected in the Port of Gijon. These IAS were all Florideophyceae, except *Bugula neritina*, which is a bryozoan. Almost all species were detected several times and at different stations of the port, except four species that only appeared in a single station (Table 1). All of the IAS that were detected in this study had been previously reported in the port of Gijon or in the Cantabrian Sea. Regarding NIS, although five species have no reports to date, the other four have already been reported in the study area (Supplementary Table 7).

Table 1. NIS (non-indigenous species) and IAS (invasive alien species) found in the Port of Gijon, Bay of Biscay, using metabarcoding on eDNA from different stations and substrates. Their invasion status in the area under study and introduction events in different biogeographic regions suggesting most probable vectors and pathways: NEA (North East Atlantic), MED (Mediterranean), NP (Northern Pacific), SP (Southern Pacific), WA (Western Atlantic), SWA (South West Atlantic), NWA (North West Atlantic); HF (Hull Fouling), WC (Water Currents), AQ (Aquaculture), BW (Ballast Water).

Class	Species	Native range	Introduction events (AquaNIS)	Most probable pathway	Current status in Gijon	Substrate		Station				
						Water	Sediment	A	B	C	D	E
Hexanauplia	*Paracalanus quasimodo*	Western Atlantic, North East Pacific, Baltic Sea	-	-	NIS	X		X	X	X	X	
Hexanauplia	*Oncaea waldemari*	English Channel to Baltic Sea	-	-	NIS	X		X			X	X
Hydrozoa	*Clytia gregaria*	North Pacific and New Zealand	-	-	NIS		X			X		
Florideophyceae	*Asparagopsis armata*	Australia and New Zealand	NEA, MED, NP, WA	HF, WC, AQ	IAS	X	X	X	X	X		X
Florideophyceae	*Bonnemaisonia hamifera*	North West Pacific	NEA, MED, SP	HF, WC, AQ	IAS	X	X	X	X			X
Florideophyceae	*Dasysiphonia japonica*	North West Pacific	NEA, MED, NP	HF, WC, AQ, BW	IAS	X	X		X			X
Florideophyceae	*Grateloupia imbricata*	North West Pacific	-	-	NIS	X					X	
Florideophyceae	*Mesophyllum expansum*	Mediterranean Sea	-	-	NIS	X	X	X	X	X		
Polychaeta	*Dipolydora capensis*	South Africa	-	-	NIS	X		X	X	X		
Gymnolaemata	*Bugula neritina*	Tropical or Subtropical waters	NEA, SWA, NWA, SP, NP	HF, BW	IAS	X					X	
Florideophyceae	*Gelidium microdonticum*	Western Atlantic	-	-	NIS	X			X	X		
Florideophyceae	*Neogastroclonium subarticulatum*	Pacific coast of America	-	-	NIS	X		X	X	X		
Demospongiae	*Hymeniacidon gracilis*	Indonesia	-	-	NIS	X		X				
Florideophyceae	*Botryocladia wrightii*	North West Pacific	NEA, MED	AQ	IAS	X		X				

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Of the 14 non-indigenous species that were detected, 64.3% of them are native to the Northern Pacific. However, this region was the one with the lowest levels of traffic (Gijon Port Authority, 2017) regarding the gross tonnage of ships arriving at Gijon from this area (Figure 5a). On the other hand, the South west Atlantic was the zone with the highest traffic (mainly ships coming from Brazil), but only 21.4% of the species that were detected with metabarcoding were native to this biogeographic area. The analyses by station showed that station C received a much higher volume of traffic compared to its station counterparts in the analyzed period (Figure 5b).

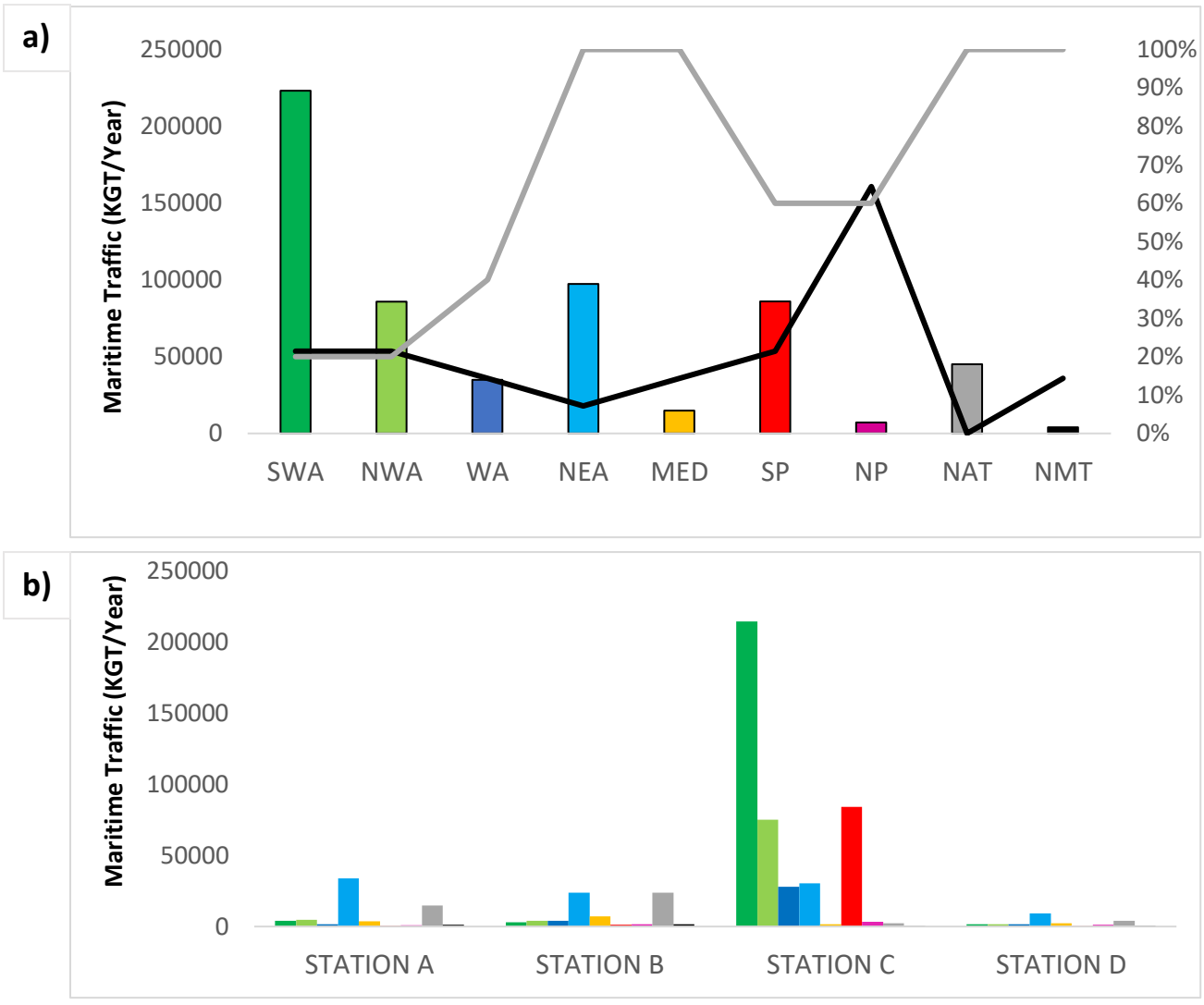

Figure 5. a) Different traffic origins and corresponding global ships tonnage arrived to the Port of Gijon (Period 2004-2017) from South West Atlantic (SWA), North West Atlantic (NWA), Western Africa (WA), North East Atlantic (NEA), Mediterranean (MED), Southern Pacific (SP), Northern Pacific (NP), national Atlantic traffic (NAT) and national Mediterranean traffic (NMT). Grey and black lines represent the percentages of non-indigenous species detected with metabarcoding and their native biogeographical area (primary dispersal: in black) or invaded areas (secondary dispersal: in grey) **b)** Marine traffic by stations in the Port of Gijon.

The ecological status of the port of Gijon following biotic indices and including Blue-gNIS.

Overall, the results from our eDNA-based sampling indicate that the port of Gijon has a high/good ecological status (Table 2). Although there are small discrepancies among stations or depending on the biotic index used to measure the environmental status, the results are uniform and indicate good environmental conditions in the port. It is remarkable that, regarding quantitative data (q), the corresponding index scores were mostly worse than those obtained with presence/absence data (p/a) (higher for gAMBI, Blue-gNIS and ALEX and lower for Bentix). However, none of the biotic indices did show statistically significant differences between one method and the other.

On the other hand, when comparing gAMBI and Blue-gNIS indices, both presence/absence data (p=0.012) and quantitative data (p=0.014) showed statistically significant differences. Blue-gNIS showed worse scores for all stations and for the global values when compared to gAMBI.

Table 2. Different biotic index scores obtained from presence/absence (p/a) and quantitative (q) data in the port of Gijon, Bay of Biscay. Water Framework Directive (WFD) ecological status is shown in parenthesis: High (H), Good (G) and Poor (P).

Area	Sample name	Blue-gNIS (p/a)	gAMBI (p/a)	Bentix (p/a)	ALEX (p/a)	Blue-gNIS (q)	gAMBI (q)	Bentix (q)	ALEX (q)
Port of Gijon	Station A	2.00(G)	1.13(H)	5.06(H)	0.55(H)	2.49(G)	1.84(G)	3.70(G)	0,58(H)
	Station B	1.82(G)	1.14(H)	4.94(H)	0.39(H)	2.15(G)	1.64(G)	4.40(G)	0,47(H)
	Station C	1.56(G)	1.15(H)	4.76(H)	0.33(H)	1.38(G)	0.73(H)	5.10(H)	0,42(H)
	Station D	1.95(G)	1.21(G)	4.66(H)	0.42(H)	2.55(G)	1.23(G)	2.20(P)	1,03(G)
	Station E	2.25(G)	1.28(G)	5.14(H)	0.50(H)	2.01(G)	1.48(G)	5.15(H)	0,25(H)
Global value		1.92(G)	1.18(H)	4.91(H)	0.44(H)	2.12(G)	1.38(G)	4.11(G)	0.55(H)

Correlations among index scores

The Blue-gNIS showed significant correlations with both ecology-based (AMBI) and invasiveness-based (ALEX) index scores. All significant correlations were positive, indicating that the Blue-gNIS responds in a similar way to the environmental factors (Table 3). From the 17 significant correlations that were found, 6 were significant when the Bonferroni correction was applied. The best correlation was found between the quantitative Blue-gNIS and gAMBI, which share the same formula; however, the Blue-gNIS also showed a strong correlation ($p<0.001$) with the ALEX. This way Blue-gNIS showed a strong correlation with an ecology-based biotic index (gAMBI) and an invasiveness-based biotic index (ALEX) that do not correlate each other (Table 3). Also, results indicate that Blue-gNIS responds in a similar way to Bentix (when considering quantitative data) due to their positive correlation, although it is not significant when applying Bonferroni correction (Table 3).

Table 3. Spearman rank correlations between Blue-gNIS, gAMBI, Bentix and ALEX scores obtained in the port of Gijon and the additional points from the Cantabrian Sea. Both, presence/absence (p/a) and quantitative data (q) were analyzed. For each pair, the correlation coefficients are presented in the first line and the p-value in the second line. Significant correlations are indicated in bold and the significant correlations after applying Bonferroni correction are indicated with an asterisk.

	Blue-gNIS (p/a)	gAMBI (p/a)	Bentix (p/a)	ALEX (p/a)	Blue-gNIS (q)	gAMBI (q)	Bentix (q)	ALEX (q)
Blue-gNIS (p/a)	-	**0.53** *0.012*	0.24 *0.679*	**0.65*** *0*	**0.53*** *0*	**0.34** *0.010*	0.28 *0.973*	**0.38** *0.02*
gAMBI (p/a)	-	-	**0.28** *0.016*	0.40 *0.112*	**0.59*** *0*	**0.53*** *0*	**0.40** *0.039*	-0.06 *0.432*
Bentix (p/a)	-	-	-	0.08 *0.946*	**0.29** *0.011*	**0.37** *0.035*	**0.53*** *0*	-0.29 *0.778*
ALEX (p/a)	-	-	-	-	**0.55** *0.006*	0.38 *0.074*	-0.03 *0.581*	**0.49** *0.009*
Blue-gNIS (q)	-	-	-	-	-	**0.74*** *0*	**0.52** *0.032*	0.19 *0.260*
gAMBI (q)	-	-	-	-	-	-	**0.44** *0.035*	-0.08 *0.708*
Bentix (q)	-	-	-	-	-	-	-	-0.08 *0.746*
ALEX (q)	-	-	-	-	-	-	-	-

DISCUSSION

DNA metabarcoding is a technique with a demonstrated effective cost-benefit ratio due to its high taxonomic resolution, species detectability and comparability across geographic regions (Pawlowski et al. 2018). The results from this work showed the high species detectability of metabarcoding since 141 marine OTUs belonging to a wide range of eukaryotic taxa were detected. This includes species-level assignments for Polychaeta, Nematoda and Demospongiae classes that require high levels of taxonomic expertise for identification based on visual traits. In this way, using environmental DNA, species could be detected with a high effectiveness and taxonomic resolution, supporting previous studies that propose metabarcoding as an innovative tool for the

evaluation of the ecological and environmental status of aquatic ecosystems (Chariton et al., 2015; Hering et al., 2018; Pawlowski et al., 2018).

The sampling strategy used in this work, which involved combining water and sediment sampling, allowed us to detect many benthic macroinvertebrate species that could not be detected in water samples. Thanks to this, the biotic indices could be calculated with a larger list of species. Therefore, our results are consistent with those of other authors (Holman et al., 2019) suggesting that both, water and sediment, should be considered in these types of environmental studies. Despite this, metabarcoding results must be carefully treated, especially when working with quantitative data. In our case, sediment and water data were pooled in each station because a very low percentage (4%) of species was detected in both of the substrates and the obtained biotic index scores were not statistically different when comparing sediment and water results. However, this is something that must be considered when performing these kinds of analyses, as species that are present in both type of samples can be overrepresented (the number of detected sequences is not proportional to the number of individuals) and bias the results.

Moreover, many problems associated with DNA barcode reference databases can also affect the metabarcoding results. Regarding the objective of this study, marine macroinvertebrates that are commonly used for biomonitoring are not completely covered in the reference databases, and these gaps could lead to erroneous environmental evaluations (Weigand et al., 2019). This is something that has been directly observed in this study when the list of macroinvertebrates used for gAMBI estimations was compared with our COI database; only a 17.44% of these macroinvertebrate species was represented. This is consistent with Aylagas et al., 2014 which found a 15% to 20% of macroinvertebrate representation in the databases. This data is useful to emphasize the urgent need to complete and update the databases in order to perform more accurate metabarcoding studies. Besides, invertebrate taxa may show lower detection outcomes than other species when employing eDNA due to factors such as differential sampling, primer affinities, incomplete databases or too stringent bioinformatic processing (Macher et al., 2018; Blackman et al., 2019), which also affects the number of species that can be detected. These can be some of the reasons why in this study two out of the nine macroinvertebrate species that were included in the positive control (and are included in the COI database) were not detected after the sequencing process, indicating the need of performing calibration experiments in order to achieve a standardized eDNA-based macroinvertebrate biomonitoring.

Blue-gNIS evaluation

The biotic indices calculated in this research and exclusively based on the detected macroinvertebrate species (Bentix, gAMBI, ALEX) classified the port of Gijon in a high/good ecological status. However, these indices are based on a single parameter, such as the species ecology (gAMBI, Bentix) or species invasion history (ALEX). New biotic indices that combine these two parameters are lacking, and the impacts that biological invasions cause on ecosystems are not considered when performing

environmental evaluations with current macroinvertebrate-based indices. This is even more necessary when evaluating the environmental status of ports that are main hot spots for the introduction of non-indigenous marine species.

Invasive species can impact native macroinvertebrate communities; for example, the introduction of primary producers (such as algae) affects native herbivorous macroinvertebrates. This is why the inclusion of invasive species in environmental assessments has the added value to give in-advance notice of the threat of invasive species for the ecosystems. This can be an excellent tool to take measures before non-indigenous and invasive species can affect macroinvertebrate communities (among others), as their presence will be reflected in the ecological quality, which can determine the need for starting management actions.

In this context, we propose the Blue-gNIS (significantly different from gAMBI), which combines species ecological information with their invasion history. Blue-gNIS classified the port of Gijon in a good ecological status with both presence/absence and quantitative data. It was calibrated by comparisons with the gAMBI, Bentix and ALEX indices and (although based on limited data) showed significant positive correlations, indicating that it responds in a similar way to the environmental factors. However, Blue-gNIS should also be tested in other regions where invasion rates are more intense than in the Cantabrian Sea, this way the correlation between Blue-gNIS and gAMBI in highly invaded areas could also be analyzed.

The basis for the generation of Blue-gNIS is the AZTI Marine Biotic Index (gAMBI), which has been previously calibrated and validated by many authors and which analyses exclusively macroinvertebrates to assess the ecological status (Muniz et al., 2005; Teixeira et al., 2012; Pelletier et al., 2018). Blue-gNIS's proposal is to add to this validated index (gAMBI) the ecological groups of the detected non-indigenous and exotic species without changing the original gAMBI, which is currently used as argument for conducting actions, and take measures in ports and that it is accepted and well known by institutions, states and at a supranational level.

One of the advantages of the Blue-gNIS is that not only macroinvertebrates are used for the environmental assessment, but any other taxa (with known distributional information and ecology) can also be included in the index calculations. For example, in this research, eight non-indigenous macroalgae species were included in the index calculation. At this point, special care must be taken to avoid potential future establishment and invasion events related to those NIS that were detected. These species may be new potential introductions in an early stage, so special attention should be taken in future samplings. If these organisms continue to be detected, this could indicate a possible establishment process that would need to start being managed. Moreover, many of these NIS were detected at different stations, which makes this finding more relevant. These results are useful to obtain an initial view of the potential biological invaders inhabiting the port of Gijon. In this case eight out of the fourteen NIS and IAS that were detected with metabarcoding in the port were macroalgae that commonly inhabit hard substrata. Considering these results, future morphology-based

monitoring should focus on hard substrata areas of the port in order to corroborate that these NIS are present and alive in the area.

NIS monitoring in the port of Gijon

Explaining the causes behind the current presence of these exotic species in an area is difficult. An analysis of the traffic volumes from different biogeographical areas was conducted to identify the potential origins of NIS. However, despite the very well demonstrated relationship between marine traffic and biological invasions events (Sardian et al., 2019; Lacarella et al. 2020), no clear correlation was observed regarding the native areas of these species, as the traffic levels arriving from these biogeographic zones were quite low. In fact, the South west Atlantic was the area with the highest traffic level coming to the Port of Gijon. However, only 21.43% of the detected NIS and IAS were native to that area. Thus, the spread from their native areas was unlikely to be the pathway by which these species arrived at the port of Gijon. The possibility that species could have arrived at the port through secondary dispersal from previously invaded areas was also considered. To date, five of the fourteen detected species have already successfully invaded areas outside of their native range (Board, 2015), and all of them are considered invasive (Table 1). These species are *Asparagopsis armata, Bonnemaisonia hamifera, Dasysiphonia japonica, Bugula neritina* and *Botryocladia wrightii*. Only one species, *Bugula neritina,* is invasive in the South western Atlantic, which is the area with the highest level of marine traffic coming into the port of Gijon. Remarkably, the Mediterranean area (with national and international traffic relevant to the port of Gijon) has previously been invaded by 100% of these invasive species. At the same time, 100% of these species have already invaded the North east Atlantic, which is the area with the second highest level of traffic to Gijon. This suggests that secondary dispersal from previously invaded areas that are geographically closer to Gijon could have been the origin of these species. Therefore, special attention must be paid to countries that are relatively closer to the recipient region (Gijon in this case) and that have established biological invaders in their ports.

Some of the NIS detected in this research (such as *Bonnemaisonia hamifera* and *Dasysiphonia japonica*) have been found in other morphological monitoring programs (Supplementary Table 7), showing that these species are already established in the area. The persistence of these NIS and IAS could be related to the high levels of human activities that can lead to a reduction in the resilience of ecosystems to invasion (Shea and Chesson, 2002; Miralles et al., 2016). Our results are consistent with this notion, as station D (the station most recently altered by the construction of new docks) showed the worst ecological status within the port (quantitative Blue-gNIS= 2,55). Comparing this value to the one obtained with gAMBI (1.23), Blue-gNIS score for station D was notoriously worse due to the presence of NIS. However, it is remarkable that this value, which is the worst environmental value obtained in this study is still in a good ecological status. This is why Blue-gNIS should be tested in other areas with higher exposures to NIS in order to evaluate its performance in a broader range and including those values that are closer to 6 in areas with poor or bad ecological status.

On the other hand, Blue-gNIS scores in the marina (station E) ranged between 2.01 and 2.25, whilst the scores for gAMBI were between 1.28 and 1.48. This increase is caused by the presence of non-indigenous and invasive species. Concretely, 1 NIS (*Oncaea waldemari*) and 4 IAS (*Asparagopsis armata, Bonnemaisonia hamifera and Dasysiphonia japonica*) were detected in the marina. These results suggest that more attention should be paid to marinas as recreational boating could effectively facilitate the spread of NIS and IAS (Ferrario et al., 2017; Martínez-Laiz et al., 2019). Our work reinforces the need to perform periodic environmental evaluations in ports to control non-indigenous species that may arrive and become invasive. By promoting the early detection of these species (which can effectively be conducted using metabarcoding techniques), easier and cheaper management plans can be designed to avoid the transmission of these species among ports (Mauremootoo et al., 2019; Rey et al., 2019).

In summary, innovative approaches for environmental evaluations that also consider biological invasions as a part of marine ecosystem quality assessments are urgent and necessary. The Blue-gNIS is a modification of gAMBI that proposes to include the NIS and IAS that affect the local biodiversity in environmental evaluations. In any case, there is still a long road ahead involving testing and implementing improvements to become an efficient and useful tool. Its use in other marine geographical areas facing much more pressure as a consequence of intense and periodic biological invasion events will undoubtedly give us more clues about its efficacy.

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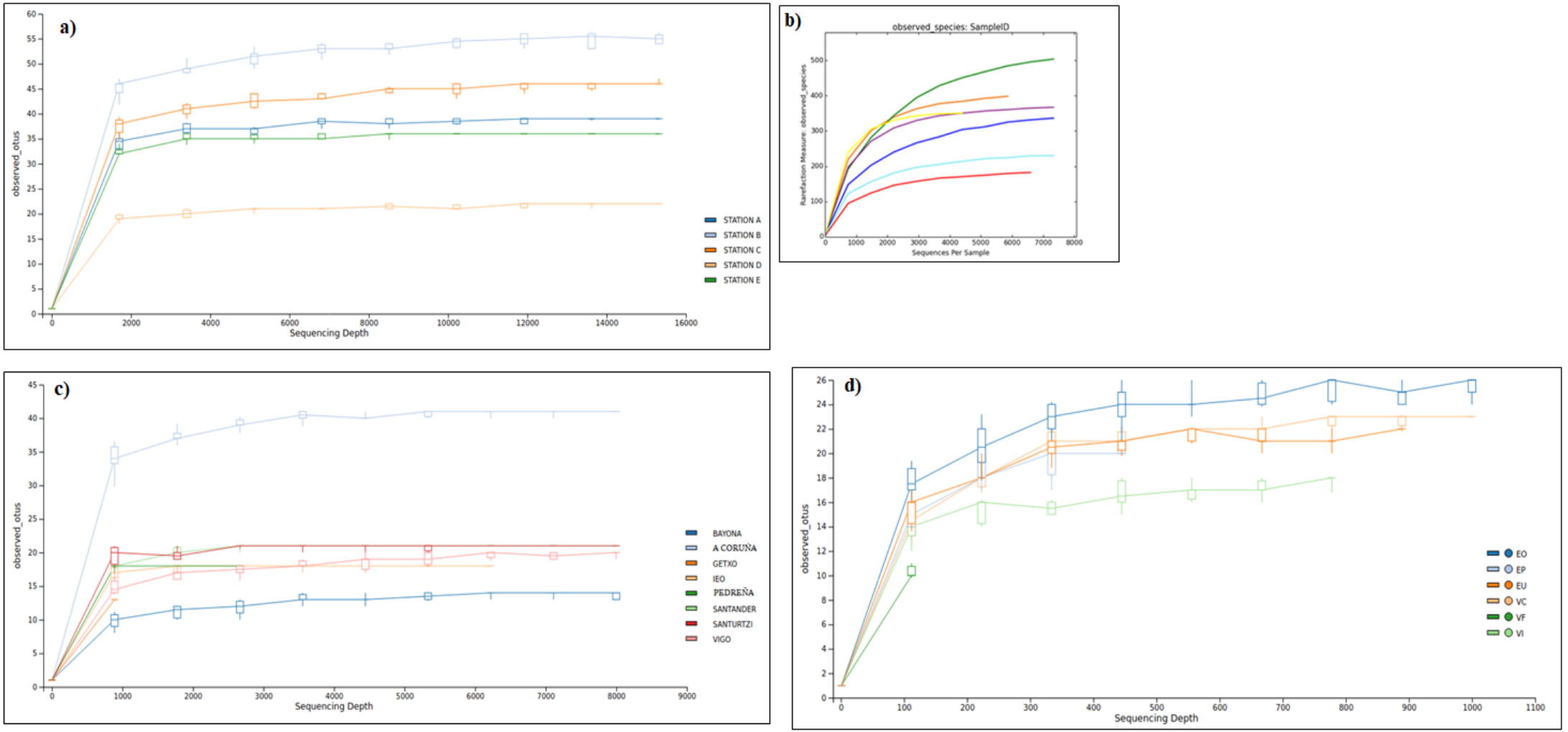
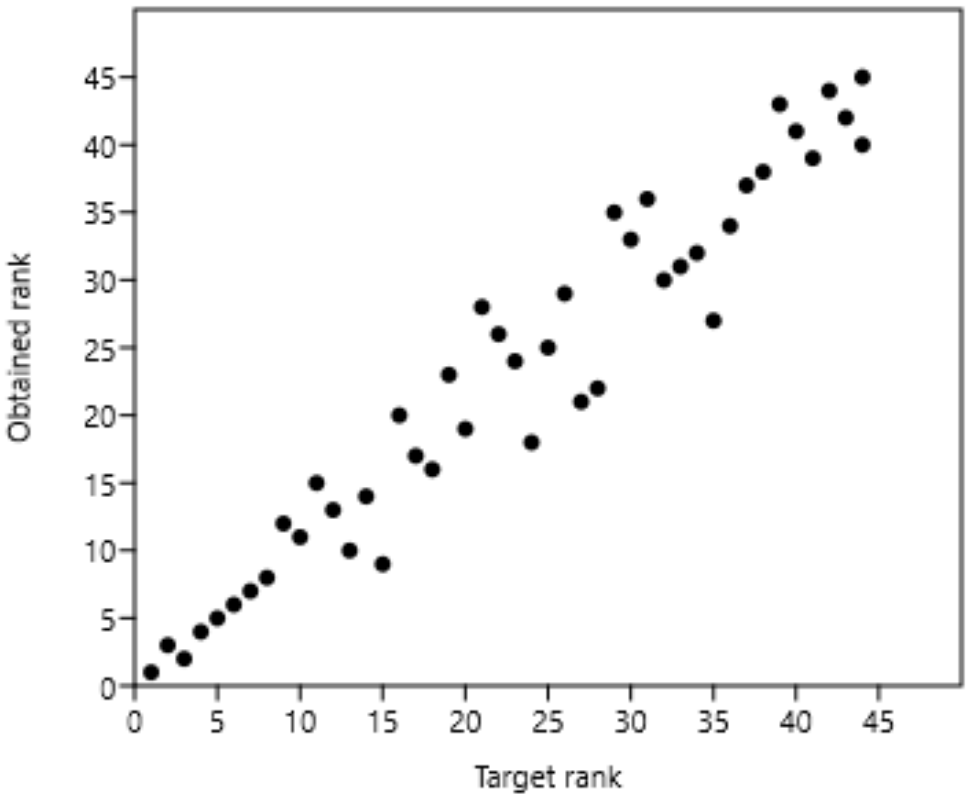
Supplementary Information



Supplementary Figure 1. Rarefaction plots of the metabarcoding results and the additional sampling points employed in the Blue gNIS index testing process. (a) The port of Gijon, (b) Asturian ports (Borrell et al., 2017), (c) the Cantabrian Sea (Miralles et al., 2019) and (d) Asturian estuaries (Borrell et al 2018).



Supplementary Figure 2. Sheppard plot showing the correlation of the similarity distances between original (target rank) and modified (obtained rank) data.

Supplementary Table 1. List of species employed in the positive control and the number of assigned sequences with 90% of minimum identity and E-value of 1e-50.

Species	**Number of assigned sequences**
Perforatus perforatus	583
Clibanarius erythropus	367
Paracentrotus lividus	1629
Magallana gigas	52
Patella depressa	506
Patella vulgata	7847
Gibbula umbilicalis	44
Mytilus galloprovincialis	0
Pollicipes pollicipes	0
Lepomis gibbosus	439
Micropterus salmoides	1653
Carassius auratus	4879
Leuciscus idus	3207
Pseudorasbora parva	1177
Gambusia holbrooki	6271
Platichthys flesus	12045
Oncorhynchus mykiss	5811
Ameiurus melas	2502
Silurus glanis	12173
Total assigned sequences	61185

Supplementary Table 2. Summary of Biotic Index (BI) values and their equivalences. Modified from Borja et al. (2003a).

gAMBI or Blue-gNIS value	Dominating Ecological Group	Benthic community health	Site Pollution Classification	Ecological status
0.0<BI≤0.2	1	Normal	Unpolluted	High status
0.2<BI≤1.2		Impoverished		
1.2<BI≤3.3	1	Unbalanced	Slightly Polluted	Good status
3.3<BI≤4.3	4-5	Transitional to pollution	Meanly Polluted	Moderate status
4.3<BI≤5.0		Polluted		Poor status
5.0<BI≤5.5	5	Transitional to heavy pollution	Heavily Polluted	
5.5<BI≤6.0		Heavily polluted		Bad status
Azoic (7.0)	Azoic	Azoic	Extremely Polluted	

Supplementary Table 3. Localization of the additional Metabarcoding samples employed for Blue-gNIS testing and the scores obtained with different biotic indices estimated with presence/absence data (p/a) and quantitative data (q). Water Framework Directive (WFD) ecological status is shown in parenthesis: High (H), Good (G), Moderate (M), Poor (P) and Bad (B).

Area	Reference	Sample name	Latitude	Longitude	Biotic index scores							
					gAMBI (p/a)	gAMBI (q)	Blue gNIS (p/a)	Blue gNIS (q)	Bentix (p/a)	Bentix (q)	ALEX (p/a)	ALEX (q)
Cantabrian Sea	Miralles et al. 2019	A Coruña	43º36'77''N	8º39'69''W	1.219 (G)	0.845 (H)	1.950 (G)	2.321 (G)	5.500 (H)	5,720 (H)	0.375 (H)	0,743 (H)
		Vigo	42º23'15''N	8º73'76''W	1.200 (G)	1.499 (G)	2.000 (G)	1.550 (G)	6.000 (H)	6,000 (H)	0.316 (H)	0,050 (H)
		IEO	43º46'82''N	3º75'77''W	0.750 (H)	0.199 (H)	1.091 (H)	0,288 (H)	5.556 (H)	5,543 (H)	0.167 (H)	0,056 (H)
		Santander	43º42'95''N	3º81'41''W	1.050 (H)	1.069 (H)	1.500 (G)	1.005 (H)	6.000 (H)	6,000 (H)	0.238 (H)	0,008 (H)
		Pedreña	43º44'86''N	3º77'06''W	2.250 (G)	2.883 (G)	3.900 (M)	3.173 (G)	4.000 (G)	2,312 (P)	0.611 (H)	0,030 (H)
		Santurtzi	43º32'89''N	3º02'86''W	1.200 (G)	0.336 (H)	1.350 (G)	0.527 (H)	4.800 (H)	5,882 (H)	0.285 (H)	0,236 (H)
		Bayona	43º52'59''N	1º50'41''W	7.000 (B)	2.994 (G)	5.250 (P)	4.409 (P)	6.000 (H)	0,000 (B)	0.667 (H)	0,036 (H)
Ports of Asturias	Borrell et al., 2017	Eo	43º53'98''N	7º03'63''W	1.800 (G)	2.672 (G)	1.800 (G)	2.672 (G)	5.000 (H)	4,000 (G)	0.000 (H)	0,000 (H)
		Luarca	43º54'66''N	6º53'23''W	1.500 (G)	1.500 (G)	1.500 (G)	1.500 (G)	0.000 (B)	0,000 (B)	0.000 (H)	0,000 (H)
		Cudillero	43º56'72''N	6º14'84''W	0.750 (H)	0.063 (H)	0.750 (H)	0.063 (H)	6.000 (H)	6,000 (H)	0.000 (H)	0,000 (H)
		Aviles	43º56'19''N	5º92'11''W	1.500 (G)	1.500 (G)	2.250 (G)	1.714 (G)	6.000 (H)	6,000 (H)	5.000 (B)	0,001 (H)
		Gijon	43º56'36''N	5º68'85''W	1.500 (G)	2.917 (G)	1.000 (H)	2.917 (G)	4.667 (H)	2,073 (P)	0.000 (H)	0,000 (H)
		Villaviciosa	43º52'56''N	5º38'93''W	0.000 (H)	1.200 (G)	0.000 (H)	1.200 (G)	6.000 (H)	6,000 (H)	0.000 (H)	0,000 (H)
		Ribadesella	43º46'35''N	5º06'28''W	1.200 (G)	1.500 (G)	0.750 (H)	1.500 (G)	5.000 (H)	5,984 (H)	0.000 (H)	0,000 (H)
		Llanes	43º42'03''N	4º75'32''W	2.667 (G)	3.520 (M)	3.000 (G)	3.704 (M)	3.500 (G)	2,694 (M)	1.000 (H)	0,013 (H)
Estuaries of Asturias	Borrell et al., 2018	VC	43º51'81''N	5º39'95''W	1.333 (G)	1.582 (G)	2.700 (G)	2.700 (G)	5.111 (H)	5.428 (H)	0.636 (H)	0.197 (H)
		VI	43º51'03''N	5º41'41''W	1.250 (G)	1.203 (G)	2.357 (G)	2.013 (G)	5.333 (H)	5.435 (H)	0.706 (H)	0.379 (H)
		EO	43º52'82''N	7º02'65''W	0.857 (H)	1.274 (G)	2.625 (G)	2.034 (G)	5.500 (H)	5.642 (H)	0.560 (H)	0.398 (H)
		EP	43º53'73''N	7º02'75''W	0.857 (H)	0.804 (H)	2.625 (G)	2.294 (G)	5.429 (H)	5.792 (H)	0.850 (H)	0.483 (H)
		EU	43º53'98''N	7º03'63''W	3.833 (M)	1.551 (G)	4.550 (P)	1.927 (G)	5.556 (H)	5.430 (H)	0.810 (H)	0.350 (H)

Supplementary Table 4. Environmental DNA concentrations obtained from water and sediment samplings inside the Port of Gijon, Bay of Biscay and used for posterior Metabarcoding analyses.

Station	Point	Replicate	Sampling Depth (m)	Original DNA concentration (ng µL-1)	Libraries DNA concentration (ng µL-1)
Station A	1	Water	Surface	14.73	74.23
		Water	Surface	13.68	70.93
		Sediment	6	15.00	3.15
		Sediment	6	12.50	40.03
	2	Water	Surface	9.25	72.29
		Water	Surface	11.61	67.87
		Sediment	16.2	12.62	15.73
		Sediment	16.2	9.47	7.37
Station B	1	Water	Surface	2.46	58.20
		Water	Surface	14.89	69.56
		Sediment	8	13.01	0.11
		Sediment	8	10.97	1.05
	2	Water	Surface	10.17	73.56
		Water	Surface	13.58	70.90
		Sediment	14	6.62	36.51
		Sediment	14	10.32	32.43
Station C	1	Water	Surface	14.30	73.71
		Water	Surface	8.92	71.86
		Sediment	19	6.49	13.55
		Sediment	19	5.68	16.55
	2	Water	Surface	15.07	73.91
		Water	Surface	10.37	71.38
		Sediment	20	4.17	15.45
		Sediment	20	2.09	9.71
Station D	1	Water	Surface	1.61	70.87
		Water	Surface	2.13	71.37
		Sediment	25	14.84	74.15
		Sediment	25	11.21	52.47
	2	Water	Surface	8.00	73.86
		Water	Surface	8.36	74.31
		Sediment	27.7	11.70	64.13
		Sediment	27.7	10.99	45.50
Station E	1	Water	Surface	9.71	76.82
		Water	Surface	12.07	75.82
		Sediment	3.3	9.98	0.21
		Sediment	3.3	10.86	0.01
	2	Water	Surface	7.73	79.79
		Water	Surface	5.14	80.55
		Sediment	5.3	7.98	62.44
		Sediment	5.3	12.33	59.57

Supplementary Table 5. Number of reads obtained for each taxon detected in the five stations of the port of Gijon. Species employed for Blue gNIS calculations are shown in bold. Species classified as NIS in the study region are shown with an asterisk and IAS are marked with double asterisk. The ecologic group assigned to each of the species employed for Blue-gNIS estimation is shown.

Class	species	Ecologic group	station A	station B	station C	station D	station E
Clitellata	***Tubificoides diazi***	5	0	0	0	0	1026
Polychaeta	***Capitella capitata***	5	0	0	332	139	0
Polychaeta	Euclymene sp.	-	107	0	0	0	0
Polychaeta	***Maldane glebifex***	1	0	0	464	135	0
Polychaeta	Polychaeta	-	223	0	0	0	0
Polychaeta	***Paradoneis ilvana***	3	7672	13853	3665	5811	0
Polychaeta	***Gyptis propinqua***	2	0	0	478	0	0
Polychaeta	***Syllidia armata***	2	860	500	622	0	164
Polychaeta	**Nephtys sp.**	2	32	0	0	723	0
Polychaeta	***Eumida ockelmanni***	2	0	14422	0	0	0
Polychaeta	***Eumida sanguinea***	2	0	0	512	0	0
Polychaeta	***Pterocirrus macroceros***	1	0	0	124	0	0
Polychaeta	***Spirobranchus triqueter***	2	0	14	0	0	0
Polychaeta	**Chaetopterus sp.**	1	248	2508	410	58	39
Polychaeta	***Spiochaetopterus costarum***	1	0	1129	0	0	0
Polychaeta	***Magelona minuta***	1	0	50	0	0	0
Polychaeta	***Dipolydora capensis****	4	89	181	590	0	0
Polychaeta	**Spionidae**	3	0	111	0	0	0
Polychaeta	***Sternaspis scutata***	3	2	0	8	0	32
Branchiopoda	***Evadne spinifera***	3	0	34	23	0	0
Branchiopoda	**Evadne sp.**	3	0	1394	0	41	0
Hexanauplia	*Acartia clausii*	-	1842	5638	2879	2081	124
Hexanauplia	*Acartia margalefi*	-	0	121	0	104	5952
Hexanauplia	*Paracartia grani*	-	0	0	0	0	313
Hexanauplia	*Calanus helgolandicus*	-	214	455	0	0	0
Hexanauplia	*Centropages typicus*	-	59	718	947	1669	0
Hexanauplia	*Clausocalanus jobei*	-	751	464	0	49	0
Hexanauplia	*Pseudocalanus elongatus*	-	0	666	0	0	0
Hexanauplia	*Paracalanus parvus*	-	431	2855	2529	1418	42
Hexanauplia	***Paracalanus quasimodo****	4	4967	10944	5745	12132	0
Hexanauplia	Paracalanus sp.	-	0	45	1479	0	0
Hexanauplia	*Euterpina acutifrons*	-	0	75	0	0	0
Hexanauplia	*Harpacticus flexus*	-	0	0	0	0	329
Hexanauplia	*Ditrichocorycaeus anglicus*	-	0	0	0	255	45
Hexanauplia	***Oncaea waldemari****	4	47	0	0	73	27
Hexanauplia	*Perforatus perforatus*	-	1572	130	0	817	607
Hexanauplia	***Chthamalus stellatus***	1	102	156	496	3528	0
Hexanauplia	***Verruca stroemia***	1	0	295	2493	0	0
Malacostraca	***Phtisica marina***	1	0	0	0	0	83

Malacostraca	***Monocorophium acherusicum***	3	60	0	0	0	0
Malacostraca	***Pilumnus hirtellus***	1	63	0	0	0	0
Malacostraca	***Pisidia longicornis***	1	0	0	8035	0	0
Malacostraca	***Porcellana platycheles***	1	23	0	0	0	0
Bacillariophyceae	Nitzschia sp.	-	8	0	0	0	8
Bacillariophyceae	*Psammodictyon panduriformis*	-	0	40	15	0	0
Bacillariophyceae	Pseudo-nitzschia sp.	-	0	3	5	0	0
Bacillariophyceae	Haslea sp.	-	0	162	0	0	0
Bacillariophyceae	*Pleurosigma strigosum*	-	0	0	17	0	0
Bacillariophyceae	Fallacia sp.	-	132	74	0	0	1012
Bacillariophyceae	Chaetoceros sp.	-	81	60	149	0	18
Bacillariophyceae	*Skeletonema marinoi*	-	31	5	21	0	17
Bacillariophyceae	Bacillariophyta	-	7598	669	1393	149	0
Gymnolaemata	***Bugula neritina*****	5	0	0	0	141	0
Gymnolaemata	***Electra pilosa***	2	0	222	0	0	0
Gymnolaemata	**Amathia sp.**	2	300	0	0	0	0
Actinopterygii	*Trachurus mediterraneus*	-	0	0	5	0	0
Actinopterygii	*Umbrina canariensis*	-	0	0	0	3	0
Actinopterygii	*Thunnus alalunga*	-	0	51	0	0	0
Actinopterygii	*Dentex dentex*	-	5	0	0	0	0
Anthozoa	***Anthopleura elegantissima***	2	0	0	47	0	0
Anthozoa	***Metridium senile***	1	116	334	3882	0	2146
Anthozoa	***Epizoanthus illoricatus***	1	0	0	1894	0	0
Anthozoa	***Nanozoanthus harenaceus***	1	52	246	0	0	0
Hydrozoa	***Bougainvillia muscus***	1	0	74	0	0	0
Hydrozoa	***Lizzia blondina***	1	0	0	0	268	0
Hydrozoa	***Clytia gregaria****	4	0	0	18	0	0
Hydrozoa	**Clytia sp.**	1	0	15	54	0	648
Hydrozoa	***Obelia geniculata***	2	0	0	673	843	0
Hydrozoa	**Obelia sp.**	2	4	2022	36	0	531
Hydrozoa	Campanulariidae	-	0	0	0	439	0
Hydrozoa	***Eutima gracilis***	1	0	0	0	8	0
Hydrozoa	**Leptothecata**	1	0	0	47	0	0
Hydrozoa	**Hydrozoa**	1	0	0	43	0	0
-	**Cnidaria**	1	250	4658	348	0	0
Asteroidea	***Marthasterias glacialis***	1	234	193	0	0	0
Echinoidea	***Brissopsis lyrifera***	1	0	0	702	0	0
Echinoidea	***Echinocardium cordatum***	1	474	0	277	0	277
Ophiuroidea	***Amphiura filiformis***	2	0	0	416	0	0
Ophiuroidea	***Ophiothrix fragilis***	1	1364	726	93	0	0
Bivalvia	***Corbula gibba***	4	0	20	0	0	0
Bivalvia	**Hiatella sp.**	1	0	358	197	122	0
Bivalvia	**Mytilus sp.**	3	617	1981	2	0	0
Bivalvia	***Nucula nitidosa***	1	0	18	11	9548	0

Bivalvia	***Ostrea edulis***	1	1138	0	132	0	4
Bivalvia	***Rocellaria dubia***	1	228	116	0	24	0
Bivalvia	***Spisula subtruncata***	1	0	55	0	0	48
Gastropoda	***Haminoea orteai***	2	0	403	0	0	0
Gastropoda	***Patella vulgata***	1	741	0	51	0	0
Gastropoda	***Mangelia attenuata***	2	0	0	0	0	7
Gastropoda	***Tritia incrassata***	2	0	63	0	0	0
Gastropoda	**Nassarius sp.**	2	261	105	0	0	0
Gastropoda	***Tritia reticulata***	2	38	0	0	0	666
Gastropoda	***Aeolidia papillosa***	1	0	0	1202	0	0
Gastropoda	**Doto sp.**	1	0	1483	0	0	0
Gastropoda	*Favorinus branchialis*	-	0	0	0	958	0
Polyplacophora	*Acanthochitona crinita*	-	0	5	33	0	0
Dinophyceae	*Azadinium caudatum*	-	0	134	0	0	0
Dinophyceae	*Protoceratium reticulatum*	-	550	650	190	108	130
Dinophyceae	Symbiodinium sp.	-	122	885	484	132	0
Florideophyceae	*Acrochaetium moniliforme*	-	0	48	0	0	82
Florideophyceae	*Acrochaetium secundatum*	-	0	0	0	0	9
Florideophyceae	Rhodochorton	-	0	0	13	0	0
Florideophyceae	***Asparagopsis armata*****	5	48	116	193	0	681
Florideophyceae	***Bonnemaisonia hamifera*****	5	1743	236	0	0	57
Florideophyceae	*Ceramium ciliatum*	-	0	2142	0	0	10
Florideophyceae	Pterothamnion	-	0	0	7	0	0
Florideophyceae	***Dasysiphonia japonica*****	5	0	74	0	0	12
Florideophyceae	Delesseriaceae	-	93	0	76	0	0
Florideophyceae	*Polysiphonia paniculata*	-	131	489	0	0	0
Florideophyceae	Rhodomelaceae	-	0	101	0	0	0
Florideophyceae	*Corallina caespitosa*	-	37	88	81	0	0
Florideophyceae	*Gelidium corneum*	-	0	0	6	0	0
Florideophyceae	***Gelidium microdonticum****	4	0	46	122	0	0
Florideophyceae	*Caulacanthus ustulatus*	-	264	9126	1998	0	0
Florideophyceae	*Chondracanthus acicularis*	-	0	55	246	0	0
Florideophyceae	*Peyssonnelia atropurpurea*	-	0	0	325	0	0
Florideophyceae	***Grateloupia imbricata****	4	0	0	0	8	0
Florideophyceae	***Mesophyllum expansum****	3	660	16	62	0	0
Florideophyceae	*Phymatolithon calcareum*	-	0	133	0	0	0
Florideophyceae	*Schizymenia dubyi*	-	189	0	0	0	0
Florideophyceae	*Plocamium lyngbyanum*	-	160	0	0	0	14
Florideophyceae	***Neogastroclonium subarticulatum****	4	45	142	171	0	0
Florideophyceae	***Botryocladia wrightii*****	5	107	0	0	0	0
Florideophyceae	*Rhodymenia pseudopalmata*	-	0	0	22	0	0
Phaeophyceae	*Feldmannia globifera*	-	0	45	0	0	0
Phaeophyceae	*Hincksia hincksiae*	-	0	0	0	0	2
Phaeophyceae	Chordariaceae	-	0	0	0	0	82

Phaeophyceae	*Planosiphon zosterifolius*	-	0	78	209	0	0
Phaeophyceae	*Scytosiphon lomentaria*	-	8361	0	0	0	0
Chromadorea	***Neochromadora poecilosomoides***	3	0	0	43	0	0
Chromadorea	***Terschellingia longicaudata***	3	13	0	0	0	82
Enoplea	**Trefusia sp.**	3	0	46	0	0	0
Hoplonemertea	***Tetrastemma candidum***	3	0	0	10	0	0
Piliophora	***Tenuilineus albocinctus***	3	0	0	691	153	0
Piliophora	***Hubrechtella dubia***	3	0	695	96	633	0
Demospongiae	**Cliona sp.**	1	117	165	0	0	0
Demospongiae	**Halisarca sp.**	1	511	0	0	0	0
Demospongiae	***Hymeniacidon gracilis****	3	33	0	0	0	0
Demospongiae	**Suberites sp.**	2	37	23	261	107	0
NA	Philactinoposthia sp.	-	8	213	23	0	0

Supplementary table 6. Results of the PERMANOVA analysis for the samples taken in different stations and substrates in the port of Gijon. Statistically significant values after applying Bonferroni correction are indicated in bold.

	Station A water	Station A sediment	Station B water	Station B sediment	Station C water	Station C sediment	Station D water	Station D sediment	Station E water	Station E sediment
STATION A water	-	**0.0007**	0.1722	**0.0001**	0.2337	**0.0001**	0.0798	**0.0001**	**0.0001**	**0.0081**
STATION A sediment	-	-	**0.0012**	0.8559	**0.0052**	0.4499	**0.0214**	0.5722	**0.0284**	**0.0277**
STATION B water	-	-	-	**0.0001**	0.4562	**0.0001**	0.5656	**0.0001**	**0.0001**	**0.0283**
STATION B sediment	-	-	-	-	**0.0001**	0.4026	**0.0002**	0.3493	**0.0018**	**0.0272**
STATION C water	-	-	-	-	-	**0.0001**	0.2255	**0.0001**	**0.0003**	**0.0001**
STATION C sediment	-	-	-	-	-	-	**0.0001**	0.3164	**0.0001**	**0.0289**
STATION D water	-	-	-	-	-	-	-	**0.0001**	**0.0277**	**0.0001**
STATION D sediment	-	-	-	-	-	-	-	-	**0.0002**	**0.0287**
STATION E water	-	-	-	-	-	-	-	-	-	**0.0002**
STATION E sediment	-	-	-	-	-	-	-	-	-	-

Supplementary Table 7. Non-indigenous (NIS) and invasive species (IAS) detected in the port of Gijon in this study and previous/posterior reports indicating the presence of these species in the area.

Species	**Status**	**Previous reports**	**Type of survey**	**Collected from**	**Localization**
Botryocladia wrightii	IAS	Bárbara et al., 2008.	Morphological	Rocky bottom	Cantabrian Sea
Asparagopsis armata	IAS	Montes et al., 2016	Morphological and Molecular	Jetties	Port of Gijon
Bonnemaisonia hamifera	IAS	Bárbara et al., 2019	Morphological	Seagrass meadow	Cantabrian Sea
Dasysiphonia japonica	IAS	Bárbara et al., 2019	Morphological	Seagrass meadow	Cantabrian Sea
Bugula neritina	IAS	Miralles et al., 2020	Morphological	Rocky bottom	Port of Gijon
Paracalanus quasimodo	NIS	-	-	-	-
Oncaea waldemari	NIS	-	-	-	-
Clytia gregaria	NIS	-	-	-	-
Neogastroclonium subarticulatum	NIS	-	-	-	-
Hymeniacidon gracilis	NIS	-	-	-	-
Grateloupia imbricata	NIS	Montes et al., 2016	Morphological and Molecular	Jetties	Port of Gijon
Mesophyllum expansum	NIS	Bárbara et al., 2019	Morphological	Rocky bottom	Cantabrian Sea
Dipolydora capensis	NIS	Miralles et al., 2020	Morphological	Rocky bottom	Port of Gijon
Gelidium microdonticum	NIS	Montes et al., 2020	Morphological and Molecular	Jetties	Port of Gijon