Universidad de Oviedo
*Universidá d'Uviéu*
*University of Oviedo*

Departamento de Biología Funcional

Programa Oficial de Doctorado en Ingeniería Química Ambiental y Bioalimentaria

# Estrategias científicas y educativas para una actividad portuaria sostenible ante invasiones biológicas

# Scientific and educational strategies for a sustainable port activity against biological invasions

Tesis Doctoral

Aitor Ibabe Arrieta

Oviedo, 2022

Universidad de Oviedo
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# RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

| **1.- Título de la Tesis** | |
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| Español/Otro Idioma: Estrategias científicas y educativas para una actividad portuaria sostenible ante invasiones biológicas. | Inglés: Scientific and educational strategies for a sustainable port activity against biological invasions. |

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**RESUMEN (en español)**

Una gran parte de la población mundial habita hoy en día en el entorno costero debido a la amplia variedad de recursos ecosistémicos que este ofrece. Los ecosistemas marinos pueden ser empleados como fuente de alimentos o energía, y suponen un recurso económico importante. El crecimiento de la población en zonas costeras implica también el aumento de las actividades antrópicas y de su impacto en el medio ambiente. Actualmente, las invasiones biológicas suponen uno de los principales factores asociados a la pérdida de la biodiversidad marina. Diversas acciones antropogénicas facilitan la introducción de especies exóticas a nuevas zonas donde, si se dan las condiciones necesarias, pueden establecerse y proliferar sin control, desencadenando graves daños ambientales y económicos. Ante esta situación, surge la necesidad de actuar y hacer frente a las invasiones biológicas, empleando para ello diferentes estrategias científicas y educativas que puedan servir para prevenir, limitar y reducir dichos impactos. Esta tesis doctoral centra su atención en el papel de los puertos como desencadenantes de eventos de invasiones biológicas, utilizando para ello como caso de estudio el Puerto de Gijón. El Objetivo general de esta tesis ha sido el desarrollo de estrategias científicas y educativas para la prevención de invasiones biológicas en los puertos. Los Objetivos específicos están relacionados con la prevención, detección temprana, evaluación de vectores de dispersión secundarios, mejora de los sistemas de evaluación ambiental y el desarrollo de estudios que fundamenten estrategias socioeducativas para generar concienciación en la población acerca del problema de las invasiones biológicas.

En esta tesis doctoral se ha desarrollado un análisis de predicción de especies no autóctonas para el puerto de Gijón, a partir del análisis de más de 380 especies, su historial de invasiones biológicas e impactos producidos, y de su idoneidad para adaptarse al hábitat. Para ello se ha creado un nuevo índice: NIS Invasion Threat Score (NIS-ITS), el cual podría ayudar a identificar las especies prioritarias que necesitan planes urgentes de detección temprana y erradicación.

Por otro lado, se llevaron a cabo evaluaciones ambientales en el Puerto de Gijón utilizando metabarcoding sobre ADN ambiental, y se estimó el gAMBI (Índice biótico marino desarrollado en el AZTI basado en datos genéticos). Los resultados indican un estado ecológico alto/bueno dentro del puerto. Sin embargo, se encontraron nueve especies no autóctonas y cinco

especies invasoras, y se propuso una modificación del gAMBI que incluye la invasividad de especies: Blue-gNIS. El índice se probó preliminarmente y clasificó el puerto en un buen estado ecológico y mostró su potencial utilidad para lograr evaluaciones más completas de la calidad del agua de los puertos.

La basura marina ha sido también foco de atención en esta tesis. Los organismos marinos pueden emplear la basura flotante como una superficie para adherirse y usarla como vector de propagación. En este trabajo se recogieron y clasificaron más de 1.000 elementos de basura de cinco playas cercanas al puerto de Gijón. Se identificaron un total de 717 organismos utilizando códigos de barras de ADN de los genes COI y 18S. En total se detectaron 23 NIS, 10 de ellas consideradas invasivas en la zona. La basura flotante mostró mayores densidades de especies nativas y exóticas que las superficies de las playas o los puertos. Esto puede indicar que la basura marina podría representar un nuevo hábitat para que las especies se dispersen en nuevas áreas.

Finalmente se realizaron encuestas a los ciudadanos de Gijón para determinar las principales fuentes de conocimiento empleadas para obtener información acerca del problema que suponen las especies invasoras. La educación y las redes sociales se clasificaron como principales fuentes de información para la población. Además, su conocimiento ambiental demostró estar basado fundamentalmente en una percepción visual, más que cognitiva, ya que la basura marina se considera un gran problema ambiental, mientras que las especies invasoras y el biofouling pasaban desapercibidos. Esto remarca la necesidad de elaborar estrategias socioeducativas efectivas que incrementen la concienciación ciudadana sobre el problema de las invasiones biológicas.

Los resultados recogidos en esta tesis doctoral demuestran cómo la incorporación de nuevos conocimientos y estrategias pueden ayudar a avanzar hacia una gestión eficaz de las invasiones biológicas en las zonas costeras donde se ubican puertos industriales y recreativos, permitiendo de esta forma disminuir sus impactos sobre bienes, servicios locales, tradiciones y patrimonios culturales.

**RESUMEN (en Inglés)**

A large part of the world's population lives today in the coastal environment due to the wide variety of ecosystem resources that it offers. Marine ecosystems can be used as a source of food or energy, and represent an important economic resource. The growth of the population in coastal areas also implies an increase in human activities and their impact on the environment. Currently, biological invasions are one of the main factors associated with the loss of marine biodiversity. Various anthropogenic actions facilitate the introduction of exotic species to new areas where, if the necessary conditions are met, they can establish themselves and proliferate without control, triggering serious environmental and economic damages. Given this situation, there is a need to act and deal with biological invasions, using different scientific and educational strategies that can serve to prevent, limit and reduce these impacts. This doctoral thesis focuses its attention on the role of ports as triggers of biological invasion events, using the Port of Gijon as a case study. The general objective of this thesis has been the development of scientific and educational strategies for the prevention of biological invasions in ports. The specific objectives are related to prevention, early detection, evaluation of secondary dispersal vectors, improvement of environmental evaluation systems and the development of studies that support socio-educational strategies to generate awareness in the population about the problem of biological invasions.

In this doctoral thesis, a prediction analysis of non-native species for the port of Gijón has been developed, based on the analysis of more than 380 species, their history of biological invasions

and impacts produced, and their suitability to adapt to the habitat. To this end, a new index has been created: NIS Invasion Threat Score (NIS-ITS), which could help identify priority species that need urgent early detection and eradication plans.

On the other hand, environmental assessments were carried out in the Port of Gijón using metabarcoding on environmental DNA, and the gAMBI (Marine Biotic Index developed at AZTI based on genetic data) was estimated. Results indicate a high/good ecological status within the port. However, nine non-native species and five invasive species were found, and a modification of gAMBI including species invasiveness was proposed: Blue-gNIS. The index was preliminarily tested and classified the port in a good ecological status and showed its potential utility to achieve more complete assessments of port water quality.

Marine litter has also been the focus of attention in this thesis. Marine organisms can use floating debris as a surface to attach to and use it as a propagation vector. In this work, more than 1,000 items of garbage from five beaches near the port of Gijón were collected and classified. A total of 717 organisms were identified using DNA barcoding of the COI and 18S genes. In total, 23 NIS were detected, 10 of them considered invasive in the area. Floating debris showed higher densities of native and exotic species than beach or harbor surfaces. This may indicate that marine litter could represent a new habitat for species to disperse to new areas.

Finally, surveys were carried out among the citizens of Gijón to determine the main sources of knowledge used to obtain information about the problem posed by invasive species. Education and social media were classified as the main sources of information for the population. In addition, their environmental knowledge proved to be fundamentally based on visual perception, rather than cognitive, since marine litter is considered a major environmental problem, while invasive species and biofouling went unnoticed. This highlights the need to develop effective socio-educational strategies that increase citizen awareness of the problem of biological invasions.

The results collected in this doctoral thesis demonstrate how the incorporation of new knowledge and strategies can help to move towards an effective management of biological invasions in coastal areas where industrial and recreational ports are located, thus allowing to reduce their impacts on goods, local services, traditions and cultural heritage.

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**Ibabe, A.**, Miralles, L., Carleos, C.E., Soto-López, V., Menéndez-Teleña, D., Bartolomé, M., Montes, H., González, M., Dopico, E., Garcia-Vazquez, E. & Borrell, Y. J. (2021). Building on gAMBI in ports for a challenging biological invasions scenario: Blue-gNIS as a proof of concept.

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**Ibabe, A.**, Rayón, F., Martínez, J.L.& Garcia-Vazquez, E. (2020). Environmental DNA from plastic and textile marine litter detects exotic and nuisance species nearby ports.

Publicado: *PloS one***.**

**Índice de impacto: 3.240**

Fernández, S., **Ibabe, A.**, Rayón, F., Ardura, A., Bartolomé, M., Borrell, Y.J., Dopico, E., González, M., Miralles, L., Montes, H., Pérez, T., Rodríguez, N.& Garcia-Vazquez, E. Flotsam, an overlooked vector for alien dispersal.

En revisión en *Estuarine, Coastal and Shelf Science*.

**Índice de impacto: 2.929**

**Ibabe, A.**, Borrell, Y. J., Knobelspiess, S.& Dopico, E. (2020). Perspectives on the marine environment and biodiversity in recreational ports: the marina of Gijon as a case study.

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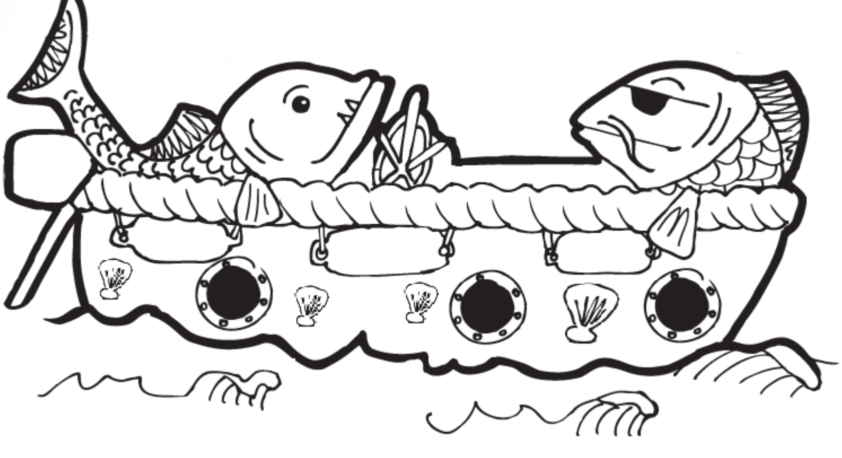
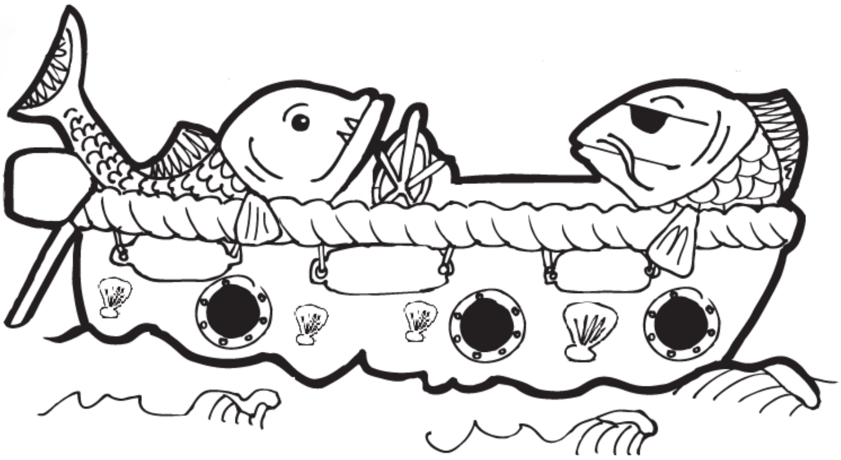
# Índice



# Resumen



**Resumen**

Una gran parte de la población mundial habita hoy en día en el entorno costero debido a la amplia variedad de recursos ecosistémicos que este ofrece al ser humano. El mar y los ecosistemas marinos pueden ser empleados como fuente de alimentos o energía, y suponen un recurso económico importante que genera empleos y sustenta modos de vida y tradiciones culturales en las regiones costeras. El crecimiento de la población en zonas costeras implica también el aumento de las actividades antrópicas y de su impacto en el medio ambiente. Actualmente, las invasiones biológicas suponen uno de los principales factores asociados al declive de las especies marinas y a la consecuente pérdida de la biodiversidad marina. Diversas acciones antropogénicas facilitan la introducción de especies exóticas a nuevas zonas donde, si se dan las condiciones necesarias, pueden establecerse y proliferar sin control, desencadenando graves daños ambientales y económicos. Ante esta situación, surge la necesidad de actuar y hacer frente a las invasiones biológicas, empleando para ello diferentes estrategias científicas, educativas y ciudadanas que puedan servir para prevenir, limitar y reducir el impacto de las introducciones y de la expansión de especies marinas no indígenas (NIS). Esta tesis doctoral centra su atención en el papel de los puertos y el tráfico marítimo asociado con ellos, como desencadenantes de eventos asociados a las invasiones biológicas. Para ello, utiliza como caso de estudio el Puerto de Gijón, localizado en Asturias, en la zona central del Golfo de Vizcaya, España. El *Objetivo general* de esta tesis ha sido el desarrollo de estrategias científicas y educativas para la prevención de invasiones biológicas en los puertos. Los *Objetivos específicos* de la tesis están relacionados con la prevención, detección temprana, evaluación de vectores de introducción y dispersión secundarios como la basura marina, mejora de los sistemas de evaluación ambiental en los puertos industriales, y finalmente, desarrollo de estudios que fundamenten futuras estrategias socio-educativas que desarrollen conciencia ciudadana y participación para enfrentar el acuciante problema de las invasiones biológicas.

En esta tesis doctoral se ha desarrollado un análisis de predicción de especies no autóctonas o NIS (non-indigenous species) para el puerto de Gijón, a partir de la identificación y análisis de más de 380 especies, su historial de invasiones biológicas e impactos producidos, y de su idoneidad para adaptarse al hábitat existente en este puerto. Para ello se ha creado un nuevo índice: NIS Invasion Threat Score (NIS-ITS). Este nuevo índice podría ayudar a identificar las especies prioritarias que necesitan planes urgentes de detección temprana y erradicación. Al mismo tiempo, y tras la realización de análisis morfológicos y genéticos de la biota encontrada en dos estudios de seguimientos anuales sucesivos llevados a cabo en las instalaciones y embarcaciones del puerto de Gijón, se identificaron 18 NIS, incluidos 6 de los NIS previstos a partir de NIS-ITS más altos. De hecho, el 80% (12 NIS) de las especies previamente identificadas como potencialmente más peligrosas (NIS-ITS > 90%) ya han sido detectadas en la zona del Golfo de Vizcaya. Por otro lado, el estado de los ecosistemas acuáticos ha sido monitoreado históricamente mediante el uso de índices bióticos. Sin embargo, pocas medidas bióticas consideran la presencia de especies no autóctonas

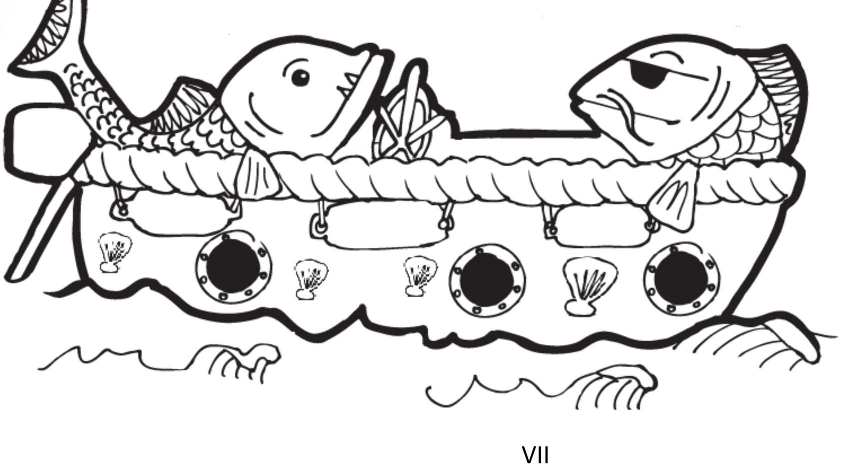
como un signo de contaminación antropogénica y perturbación del hábitat. En esta tesis, se llevaron a cabo evaluaciones ambientales en el Puerto de Gijón, utilizando metabarcoding sobre ADN ambiental, obtenido de muestras de agua y sedimentos, y se estimó el gAMBI (Índice biótico marino desarrollado en el AZTI basado en datos genéticos). Los resultados indican un estado ecológico alto/bueno dentro del puerto. Sin embargo, se encontraron nueve especies no autóctonas y cinco especies invasoras, y se propuso una modificación del gAMBI que incluye la invasividad de especies: Blue-gNIS. El índice se probó preliminarmente contra índices validados existentes como gAMBI, BENTIX (basado en la ecología de los macroinvertebrados) y ALEX (basado en la invasividad de la especie). Blue-gNIS clasificó el puerto en un buen estado ecológico y mostró su potencial utilidad para lograr evaluaciones más completas de la calidad del agua de los puertos.

Los vectores secundarios para las introducciones de especies exóticas marinas en los puertos, como pueden ser la basura marina existente en ellos, y en zonas aledañas, han sido también foco de atención en esta tesis doctoral. Los organismos marinos pueden emplear la basura flotante como una superficie para adherirse y usarla como vector de propagación. En este trabajo se recogieron y clasificaron más de 1.000 elementos de basura de cinco playas cercanas al puerto de Gijón. Se empleó la secuenciación de próxima generación para estudiar las comunidades de bioincrustaciones adheridas a elementos de diferentes materiales y se encontró una dominancia de ADN de Florideophyceae, Dinophyceae y Arthropoda, y se identificaron cuatro especies no autóctonas (NIS). Además, se estudió la composición y transferencia potencial de comunidades que habitan tres componentes ambientales diferentes: sustratos naturales y artificiales del Puerto de Gijón, seis localizaciones de tipo rocosas en playas próximas y basura flotante recogida en la costa adyacente. Se identificaron un total de 717 organismos utilizando códigos de barras de ADN de los genes COI y 18S. En total se detectaron 23 NIS, 10 de ellas consideradas invasivas en la zona. Los perfiles taxonómicos de los tres componentes ambientales fueron significativamente diferentes. Contrariamente a lo esperado, la basura flotante mostró mayores densidades de especies nativas y exóticas que las superficies de las playas o los puertos. Esto puede indicar que la basura marina podría representar un nuevo hábitat para que las especies se dispersen en nuevas áreas.

Finalmente, los puertos recreativos también son conocidos por ser fuentes de contaminación del medio marino costero debido al vertido de contaminantes o al traslado de especies invasoras a zonas aledañas. No obstante, la responsabilidad de proteger el medio marino no recae únicamente en las personas usuarias de los puertos, sino que también afecta al resto de la ciudadanía. Por ello, es necesaria una comunicación eficaz entre científicos/as y ciudadanos/as (incluyendo a las personas usuarias de los puertos) para impulsar la cooperación frente a estos problemas medioambientales. En este estudio (centrado en el puerto deportivo de Gijón, aledaño al Puerto industrial), se realizaron encuestas a la población y señalaban la educación y las redes sociales como sus principales fuentes de información, considerando raramente la divulgación y la literatura científica como fuentes de información óptimas. Además, su conocimiento ambiental demostró estar basado fundamentalmente en una percepción visual, más que cognitiva: consideraban, la basura marina como un gran problema ambiental, mientras que las especies invasoras y el biofouling les pasaban inadvertidas. Esto remarca la necesidad de elaborar estrategias socioeducativas y de comunicación efectivas a partir de las fuentes científicas, que incrementen la concienciación ciudadana sobre el problema de las invasiones biológicas.

Los resultados recogidos en esta tesis doctoral demuestran cómo la incorporación de nuevos conocimientos y estrategias pueden ayudar a avanzar hacia una gestión eficaz de las invasiones biológicas en las zonas costeras donde se ubican puertos industriales y recreativos, permitiendo de esta forma disminuir sus impactos sobre bienes, servicios locales, tradiciones y patrimonios culturales.

# Summary



**Summary**

A large part of the world's population lives today in the coastal environment due to the wide variety of ecosystem resources that it offers to human beings. The sea and marine ecosystems can be used as a source of food or energy, and represent an important economic resource that generates jobs and sustains ways of life and cultural traditions in coastal regions. The growth of the population in coastal areas also implies an increase in human activities and their impact on the environment. Currently, biological invasions are one of the main factors associated with the decline of marine species and the consequent loss of marine biodiversity. Various anthropogenic actions facilitate the introduction of exotic species to new areas where, if the necessary conditions are met, they can establish themselves and proliferate without control, triggering serious environmental and economic damage. Given this situation, there is a need to act and deal with biological invasions, using different scientific, educational and citizen strategies that can serve to prevent, limit and reduce the impact of introductions and the expansion of non-indigenous marine species (NIS). This doctoral thesis focuses its attention on the role of ports and the maritime traffic associated with them, as triggers of events associated with biological invasions. To do this, it uses the Port of Gijón, located in Asturias, in the central zone of the Bay of Biscay, Spain, as a case study. The *general objective* of this thesis has been the development of scientific and educational strategies for the prevention of biological invasions in ports. The *specific objectives* of the thesis are related to prevention, early detection, evaluation of secondary introduction and dispersion vectors such as marine litter, improvement of environmental evaluation systems in industrial ports, and finally, development of studies that support future strategies and socio-educational initiatives that develop citizen awareness and participation to face the pressing problem of biological invasions.

In this doctoral thesis, a prediction analysis of non-indigenous species (NIS) has been developed for the port of Gijón, based on the identification and analysis of more than 380 species, their history of biological invasions and impacts produced, and their suitability to adapt to the existing habitat in this port. For this, a new index has been created: NIS Invasion Threat Score (NIS-ITS). This new index could help to identify priority species that need urgent early detection and eradication plans. At the same time, and after carrying out morphological and genetic analyzes of the biota found in two successive annual monitoring studies carried out in the facilities and vessels of the port of Gijón, 18 NIS were identified, including 6 of the NIS predicted from higher NIS-ITS scores. In fact, 80% (12 NIS) of the species previously identified as potentially more dangerous (NIS-ITS > 90%) have already been detected in the Bay of Biscay area. On the other hand, environmental evaluations were carried out in the Port of Gijón, using metabarcoding on environmental DNA, obtained from water and sediment samples, and the gAMBI (Marine Biotic Index developed at AZTI based on genetic data) was estimated. The results indicate a high/good ecological status within the port. However, nine non-native species and five invasive species were found, and a modification of gAMBI including species invasiveness was proposed: Blue-gNIS. The new index was

preliminarily tested against existing validated indices such as gAMBI, BENTIX (based on macroinvertebrate ecology), and ALEX (based on species invasiveness). Blue-gNIS classified the port in good ecological status and showed its potential utility for more comprehensive assessments of port water quality.

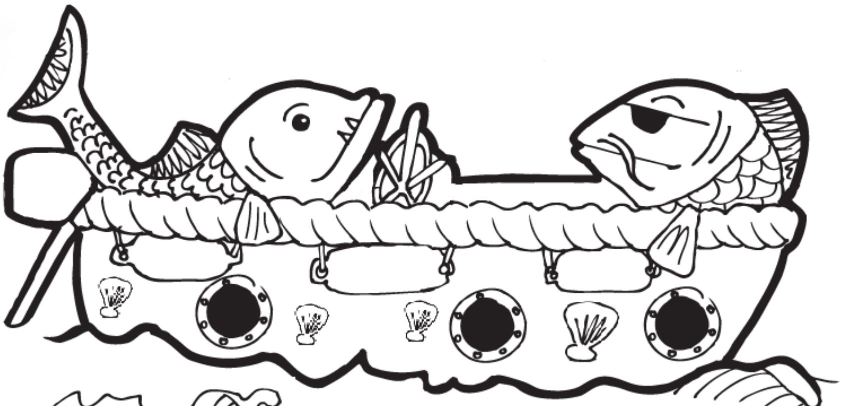
The secondary vectors for the introductions of exotic marine species in the ports, such as the marine litter present in ports and surrounding areas, have also been the focus of attention in this doctoral thesis. Marine organisms can use floating debris as a surface to attach to and use it as a propagation vector. In this work, more than 1,000 items of litter from five beaches near the port of Gijón were collected and classified. Next-generation sequencing was used to study biofouling communities attached to elements of different materials and a dominance of DNA from Florideophyceae, Dinophyceae and Arthropoda was found, and four non-indigenous species (NIS) were identified. In addition, the composition and potential transfer of communities that inhabit three different environmental components were studied: natural and artificial substrates of the Port of Gijón, six rocky locations on nearby beaches and floating litter collected on the adjacent coast. A total of 717 organisms were identified using DNA barcoding of the COI and 18S genes. In total, 23 NIS were detected, 10 of them considered invasive in the area. The taxonomic profiles of the three environmental components were significantly different. Contrary to expectations, floating litter showed higher densities of native and exotic species than beach or harbor surfaces. This may indicate that marine litter could represent a new habitat for species to disperse to new areas.

Finally, recreational ports are also known to be sources of contamination of the coastal marine environment due to the discharge of pollutants or the transfer of invasive species to surrounding areas. However, the responsibility to protect the marine environment does not fall only on port users, but also affects other citizens. Therefore, effective communication between scientists and citizens (including port users) is necessary to promote cooperation to face of these environmental problems. In this study (focused on the Gijón marina, adjacent to the industrial port), surveys were carried out among the citizens who rate education and social networks as the main sources of information for the population, rarely considering scientific literature as a valid source of information. In addition, their environmental knowledge proved to be fundamentally based on visual perception, rather than cognitive, since marine litter was considered a major environmental problem, while invasive species and biofouling went unnoticed. This highlights the need to develop effective socio-educational and communication strategies based on scientific sources, which increase citizen awareness of the problem of biological invasions.

The results collected in this doctoral thesis demonstrate how the incorporation of new knowledge and strategies can help to move towards an effective management of

biological invasions in coastal areas where industrial and recreational ports are located, thus allowing to reduce their impacts on goods, local services, traditions and cultural heritage.

# Introducción



## 1. El ser humano y los ecosistemas marinos

Los océanos proporcionan servicios fundamentales para la humanidad, como la regulación de la temperatura del planeta, la producción de casi la mitad del oxígeno de la atmósfera, protección contra la erosión y son además sumideros naturales de carbono (GLOBE, 2010). A lo largo de la historia, el ser humano ha estado siempre ligado a la costa y al mar. Los ecosistemas marinos y las especies que los habitan son necesarios para el desarrollo de muchas actividades humanas que se desempeñan en las zonas costeras y que incluyen actividades económicas como la pesca, el turismo, la acuicultura y el transporte marítimo. Las principales mega-ciudades que existen hoy están situadas en la zona costera. De hecho, más de un 40% de la población mundial habita cerca del mar, y existe una tendencia constante de migración costera, que está asociada con cambios demográficos globales (Hugo, 2011; Todd et al., 2019). Esto hace que el impacto humano sea cada vez mayor en estas zonas, lo cual deriva en la degradación de los ecosistemas marinos y en la pérdida de la biodiversidad (Hoffman y Broadhurst, 2016; Rai & Singh, 2020).

La intensificación de las actividades humanas ha sido la causa de la extinción global de diversas especies marinas a lo largo de la historia, incluyendo aves, mamíferos, peces, invertebrados y algas (Dulvy et al., 2003; Dulvy et al., 2014; Webb & Mindell, 2015; Ulman et al., 2020). Un ejemplo de ello es el caso de la vaca marina de Steller (*Hydrodamalis gigas*) extinta desde 1768 debido a intensas cacerías para obtener carne, grasa y piel de gran calidad. La sobreexplotación de las especies marítimas comerciales sigue siendo un problema hoy en día, ya que la demanda de alimentos crece junto con la población mundial. Se estima que en el Mediterráneo occidental más del 90% de las poblaciones de peces comerciales como el bacalao (*Gadus morhua*), la sardina (*Sardina pilchardus*), el besugo (*Pagellus bogaraveo*), la anchoa (*Engraulis encrasicolus*) o el lenguado (*Solea solea*), entre muchas otras, están sobreexplotadas mucho más allá de los límites biológicos seguros (STECF, 2019). La sobreexplotación de especies marinas implica la desaparición de dichas especies y su función en la cadena alimenticia, al igual que limitaría los servicios ecosistémicos para las generaciones futuras, que dispondrían de menos recursos naturales (Piroddi et al., 2017).

Otra consecuencia de las actividades antrópicas que afecta a la biodiversidad marina es la contaminación. El aumento de la población humana en las zonas costeras implica también el incremento de actividades que generan residuos, los cuales muchas veces, debido a una mala gestión, un sistema deficiente de depuración, o accidentes, se vierten al mar. Estos residuos pueden ser compuestos orgánicos (provenientes de la agricultura, de aguas residuales urbanas, actividades pecuarias, limpieza de embarcaciones, navegación de recreo o el turismo) o inorgánicos (principalmente provenientes de actividades industriales). La acumulación excesiva de estos residuos genera graves problemas en los ecosistemas marinos. Ciertas especies, ante la abundancia de residuos orgánicos, proliferan sin control, generando condiciones de hipoxia, por lo que muchos organismos mueren por falta de oxígeno (Wurtsbaugh et al., 2019). A esto se le suma el hecho de que los residuos inorgánicos pueden ser introducidos en la cadena alimentaria y afectar directamente al ser humano.

Una de las principales causas para la pérdida de biodiversidad junto con las previamente mencionadas y la cual es objeto de estudio en esta tesis doctoral, son las invasiones biológicas, las cuales representan, tras la pérdida del hábitat, la segunda causante principal de pérdida de biodiversidad a nivel global (Gurevitch y Padilla, 2004). Hoy es una evidencia indiscutida el que, como resultado de la actividad humana, los ecosistemas marinos y costeros de todo el mundo están siendo invadidos a un ritmo extraordinario, desencadenando graves consecuencias ambientales (Chan y Briski, 2017). Para lidiar con el problema de las invasiones biológicas y conservar la biodiversidad marina, es necesario entender cómo actúan las especies invasoras, cuáles son las consecuencias de su introducción en nuevos hábitats y qué estrategias existen para poder hacerles frente.

## 2. Invasiones biológicas en el medio marino y el factor humano como principal desencadenante.

En las invasiones biológicas generalmente una especie adquiere una ventaja competitiva, tras la desaparición de una barrera natural, que le permite expandirse rápidamente y conquistar nuevas áreas dentro de ecosistemas receptores en los que se convierte en población dominante (Simberloff, 2013). Este proceso puede darse de forma natural (Vermeij 1991), no obstante, debido a la acción humana, los eventos de invasión se han incrementado de manera exponencial (Sardain et al., 2019). En lo referente a hábitats marinos, esto se debe a que diferentes acciones antrópicas como el tráfico marítimo, el comercio de especies ornamentales para acuarios, la acuicultura, la construcción de canales o la modificación del hábitat eliminan muchas de las barreras naturales que impiden la expansión de las especies (Castellanos-Galindo et al., 2020; Merson et al., 2020; Brosse et al., 2021) (Tabla 1).

**Tabla 1**. Ejemplos de introducciones de especies exóticas en el medio marino facilitadas por el ser humano.

| **Vía/propósito de introducción** | **Tipo de introducción** | **Especie introducida** | **Fuente** |
|---|---|---|---|
| Producción de alimento en centros de acuicultura | Intencionada | *Magallana gigas*, *Ruditapes philippinarum* | Miossec et al., 2009; Suárez y Raven, 2020 |
| Ornamentación | Intencionada | *Lepomis gibbosus* *Elodea canadensis* | Leppäkoski y Olenin, 2000 |
| Aguas de lastre | No intencionada | *Carcinus maenas*, *Rhithropanopeus harrisii* | Briski et al., 2012 |
| Bioincrustaciones | No intencionada | *Kapraunia* schneideri | Wolf et al., 2018 |
| Construcción de canales | No intencionada | *Rhopilema nomadica* | Galil et al., 2015 |
| Escapes de acuarios | No intencionada | *Caulerpa taxifolia* | Jousson et al., 1998 |

Cada especie habita un ecosistema del cual es nativa y su rango natural está limitado por diferentes barreras, ya sean físicas o ambientales. El proceso de invasión biológica da comienzo cuando dichas barreras se atenúan o desaparecen (generalmente debido al factor humano). La principal causa de la desaparición de estas barreras es el transporte de dichas especies (voluntaria o involuntariamente) que pueden ser desplazadas en forma de organismos adultos, propágulos, larvas, esporas, etc. Una vez introducida la especie en los ecosistemas recipientes, dependiendo de su capacidad para adaptarse a los nuevos parámetros bióticos y abióticos y la continuidad del suministro de propágulos, la especie puede, o no, lograr sobrevivir al nuevo hábitat al que ha sido introducido (Cassey et al., 2018) (Figura 1). A estas especies que se encuentran fuera de su hábitat nativo se las denomina especies no indígenas o NIS (del inglés Non-Indigenous Species). En algunos casos, las especies serán capaces no solo de sobrevivir al nuevo entorno, sino de establecer una población reproductivamente viable. Dependiendo de las características de cada especie, esto podrá requerir un suministro continuo de propágulos o no. Una vez que la especie es capaz de reproducirse con éxito en el nuevo entorno, las poblaciones comienzan a establecerse y se vuelven independientes de sus poblaciones en el hábitat nativo.

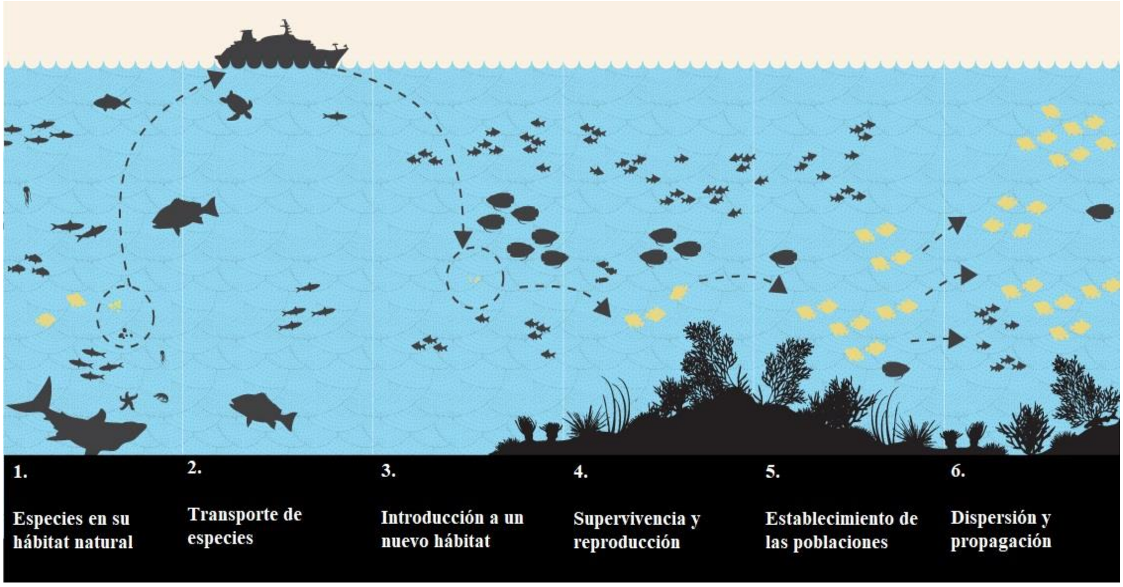



**Figura 1. El proceso de invasión biológica en sistemas marinos.** En la imagen se muestran las distintas etapas que debe superar una especie desde que se introduce en un nuevo hábitat hasta que se convierte en especie invasora causante de daños económicos y ambientales. Imagen adaptada de Namboothri et al. (2012).

Existen diversos factores que facilitan el proceso de establecimiento de las especies no indígenas, como las perturbaciones ambientales, la biodiversidad del área receptora, el alto éxito competitivo de la especie no indígena o la falta de depredadores naturales (Yannelli et al., 2017; Cuthbert et al., 2018; Kenworthy et al., 2018). Todos estos factores pueden estar estrechamente relacionados con la contaminación producida como consecuencia de distintas actividades humanas, las cuales hacen que disminuyan o desaparezcan ciertas especies sensibles en determinados ecosistemas (Fernández-Martínez et al., 2020; Owens et al., 2020). Esta pérdida de biodiversidad genera nichos ecológicos que pueden ser explotados por las especies no indígenas. De la misma forma, la contaminación puede eliminar posibles competidores (que empleen los mismos recursos que la especie no indígena) o depredadores que pudiera haber en el ecosistema recipiente. Es por ello por lo que los ecosistemas con mayor diversidad son más resistentes a las invasiones biológicas y por lo que la contaminación aumenta el éxito relativo de las especies oportunistas, incluyendo especies no indígenas (Stachowicz et al., 2002; Johnston y Roberts, 2009; Mayer-Pinto et al., 2010; Crooks et al., 2011; Mayer-Pinto et al., 2015). Junto con estos factores, también debe tenerse en cuenta la capacidad de las especies no indígenas de tolerar amplios rangos de condiciones ambientales, de explotar diversas fuentes de alimentación o la posesión de características como altas tasas de crecimiento y reproducción (Mannino et al., 2017). Estas características pueden desembocar en que finalmente, una especie no indígena se convierta en una especie invasora, logrando establecer poblaciones reproductivamente viables en el nuevo hábitat, dispersándose y proliferando de manera descontrolada, convirtiéndose en especies dominantes del ecosistema.

Las invasiones biológicas causan daños importantes en los ecosistemas marinos. Las especies invasoras pueden reducir la abundancia de algunas especies nativas mediante procesos de depredación, competición o hibridación. Por ejemplo, el pez león, *Pterois volitans*, es un depredador invasor que supone una grave amenaza para los ecosistemas marinos ya que se alimenta vorazmente de pequeños peces nativos (Morris y Akins, 2009; Peake et al., 2018). Similarmente, un ejemplo de competición es el caso del alga invasora *Caulerpa cylindracea* y el alga nativa *Posidonia oceanica,* las cuales compiten por el espacio y los recursos en el Mediterráneo (Boschi et al., 2020). Del mismo modo, la hibridación entre especies nativas y exóticas también puede afectar a los ecosistemas, como ocurre en el Pacifico norte con los híbridos de la especie invasora *Mytilus galloprovincialis* y la especie nativa *Mytilus trossulus* o como resulta en el caso de la hibridación entre las percas nativas e invasoras (*Micropterus coosae* y *Micropterus henshalli*) en Norteamérica (Brannock y Hilbish, 2010; Bangs et al., 2018). Además, las especies invasoras pueden introducir nuevos patógenos y enfermedades (a las cuales muchas veces ellas mismas son resistentes) que afectan a la biota local (McCallum et al., 2003; Randolph y Rogers, 2010; García-Vásquez et al., 2017).

No obstante, al hablar de invasiones biológicas no solo se deben tener en cuenta los impactos ecológicos, puesto que las especies invasoras también afectan gravemente al ser humano. Este impacto puede ser directo, como en el caso de introducciones de patógenos y nuevas enfermedades que pueden afectar a las personas, pero también puede ser indirecto; las invasiones biológicas pueden afectar al turismo o a los servicios ecosistémicos (por ejemplo, disminuyendo el stock disponible para la pesca), lo cual deriva en importantes pérdidas económicas. Un claro ejemplo del impacto de las especies invasoras en la economía es el caso del mejillón cebra (*Dreissena polymorpha*), una especie incluida en el Catálogo Español de Especies Exóticas Invasoras que tiene la capacidad de tapizar superficies artificiales como cascos de barcos (ralentizándolos y aumentando el consumo de combustible) o tuberías, obligando a invertir grandes cantidades de dinero para su mantenimiento y limpieza. Se estima que las pérdidas económicas asociadas a las invasiones biológicas en medios acuáticos han supuesto a la economía global un coste superior a los 345 mil millones de dólares (Cuthbert et al., 2021).

Las estrategias de detección temprana suponen la base para hacer frente a las invasiones biológicas. Mediante la puesta en marcha de planes de actuación que permitan llevar a cabo una detección de especies exóticas de manera temprana, es posible implementar las medidas necesarias para evitar el establecimiento y la propagación de las especies nuevamente detectadas. De hecho, la detección temprana es el método más eficaz para poder hacer frente a las consecuencias derivadas de las invasiones biológicas, ya que su objetivo es actuar de forma previa al establecimiento o naturalización de las especies exóticas (Devloo-Delva et al., 2016; Miralles et al., 2016). En los casos en los que falla la detección temprana, las especies exóticas son detectadas de forma tardía, habiendose establecido y propagado en el ecosistema recipiente de manera que su eliminación es más costosa y altamente improbable (Harris et al., 2018).

En resumen, las invasiones biológicas son una creciente amenaza para el ser humano y el medioambiente. Para poder hacer frente a este problema, es necesario desarrollar y poner a prueba nuevas técnicas que permitan hacer frente a las especies invasoras, ya sea mediante la prevención de nuevas introducciones o mediante el monitoreo y la detección temprana. No obstante, es necesario no sólo conocer las características biológicas, ecológicas o fisiológicas de las especies, sino que también es de vital importancia conocer cuáles son las principales vías de propagación o introducción de las especies exóticas e invasoras, para poder elaborar planes de actuación específicos que puedan evitar o minimizar posibles introducciones de especies no indígenas.

## 3. El transporte marítimo como vector principal de especies invasoras marinas

Las vías de introducción de especies exóticas en nuevos habitats son muy diversas. Entre ellas están la apertura de canales que sirven como corredores para el desplazamiento de las especies, o los escapes de centros de acuicultura y acuarios (Castellanos-Galindo et al., 2020; Forneck et al., 2021). No obstante, el principal vector de transmisión de especies en el medio marino es el tráfico marítimo.

El tráfico marítimo es la vía principal por la cual se mueven materiales y bienes en todo el mundo, y representa más del 80% del comercio mundial (UNCTAD, 2020). Existe una red global de transporte marítimo que conecta las distintas partes del mundo, y que es especialmente densa en el noreste Atlántico y en el mar Mediterráneo. Actualmente, exceptuando las zonas polares, apenas existen áreas costeras que no estén incluidas en esta red (Figura 2). Debido a esto, las especies pueden ser transportadas a casi cualquier lugar del mundo. En este contexto, se estima que el tráfico marítimo es el principal responsable de las invasiones biológicas marinas (Hewitt et al., 2009; Shuckmith y Shelmerdine, 2015; Letschert et al., 2021).

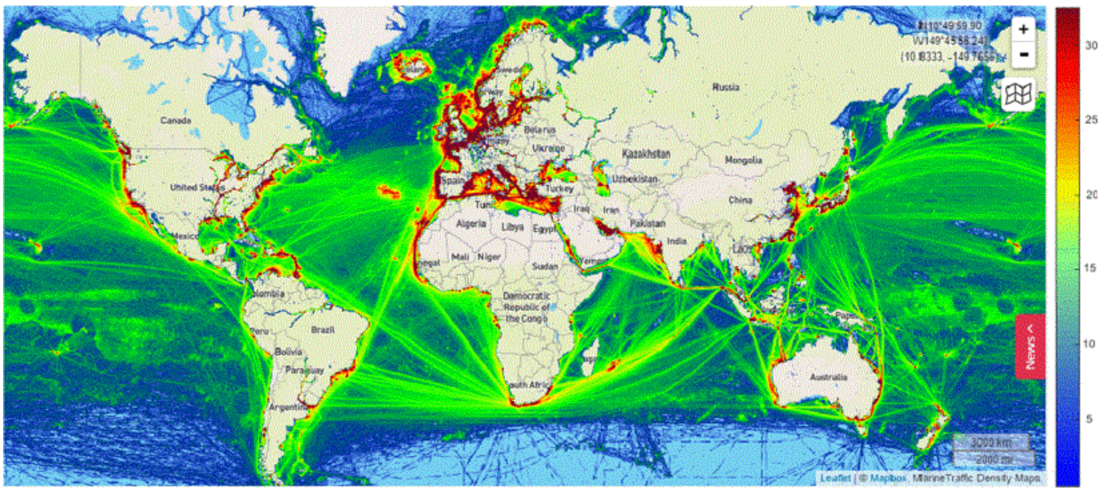



**Figura 2. Mapa de densidad de tráfico marítimo mundial.** En la imagen se muestran las principales rutas comerciales del mundo. La densidad se evalúa como el número de barcos por celda de cuadrícula de 1 ° × 1 ° por día. El fondo cian indica regiones sin tráfico marítimo. Imagen adaptada de Di Simone et al. (2017).

El transporte de especies marinas asociado al tráfico marítimo ocurre fundamentalmente por dos vías: aguas de lastre e incrustaciones en los cascos de las embarcaciones (Figura 3). El objetivo principal del agua de lastre es proporcionar estabilidad al barco durante la navegación. Para ello, se recoge agua del entorno y se introduce en unos depósitos estancos situados en el interior del casco de los barcos donde se almacena y se expulsa, según las necesidades del momento. Durante el proceso de llenado de los depósitos es muy frecuente que la biota que habita en el agua sea arrastrada al interior. Las especies introducidas en los depósitos de la embarcación permanecerán allí hasta que el agua de lastre sea nuevamente expulsada. En el momento en el que se vacían los depósitos, los organismos acumulados por absorción son liberados al exterior junto con el agua. Este proceso suele llevarse a cabo al finalizar la navegación, por lo que en muchos casos se transportan especies a un nuevo entorno que puede estar a miles de kilómetros de su origen. A pesar de que las condiciones en estos depósitos de agua de lastre son extremas (falta de luz, agotamiento del oxígeno, cambios de temperatura y salinidad), se ha confirmado que muchas especies pueden sobrevivir dentro de ellos largos períodos de tiempo en forma de larvas, huevos, propágulos o esporas (Deagle et al., 2003; Flagella et al., 2007; Ardura et al., 2015; Wu et al., 2019) demostrando su potencial de propagarse a nuevas zonas fuera de su rango nativo, donde podrían establecerse y comenzar un proceso de invasión biológica (Cabrini et al., 2019).

La segunda vía por la que las especies marinas pueden ser transportadas es mediante las incrustaciones en los cascos de las embarcaciones. Muchas especies tienen la capacidad de adherirse a estructuras artificiales como boyas, anclas o cuerdas, donde pueden asentarse y formar colonias. Esto también incluye los cascos de las embarcaciones que proporcionan un substrato para el asentamiento de especies asociadas a comunidades incrustantes (Meloni et al., 2021). Estas superficies pueden ser ocupadas por una amplia variedad de organismos que pueden recorrer grandes distancias adheridos a las embarcaciones. Por ejemplo, especies como *Ceramium gardneri* (Rhodophyta), *Diadumene leucolena* (Anthozoa), *Crepidula onyx* (Gastropoda), *Balanus amphitrite* (Cirripedia), *Bugula neritina* (Bryozoa) y *Microcosmus squamiger* (Ascidiacea) fueron observadas en Hawaii formando incrustaciones en los cascos de embarcaciones provenientes de California, y habiendo sobrevivido a un viaje de más de 3.000 kilómetros (Godwin, 2003).

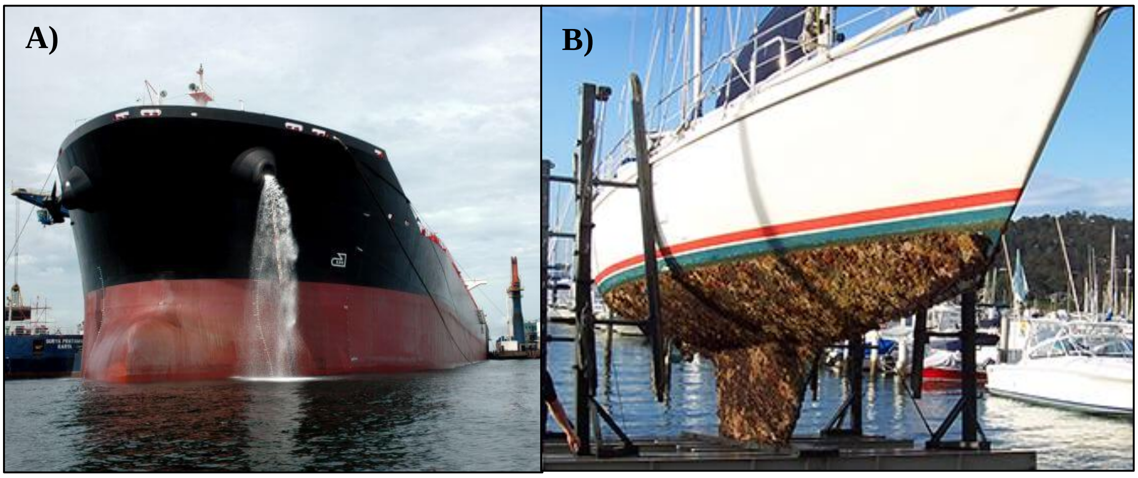



**Figura 3. Métodos de dispersión de especies marinas asociadas al tráfico marítimo.** A) Agua de lastre (Fuente de la imagen: https://www.marineinsight.com/). B) Incrustación en el casco de la embarcación (Fuente de la imagen: https://www.agriculture.gov.au/).

No obstante, el tráfico marítimo de tipo industrial y de largas distancias no es el único que puede transportar especies invasoras. En los últimos años se ha visto que las embarcaciones de recreo juegan un papel muy importante en la propagación de estas especies. Se consideran embarcaciones de recreo aquellas que tengan eslora de casco comprendida entre 2.5 y 24 metros y que estén destinadas para fines recreativos y deportivos (Real Decreto 1434/1999). Las velocidades relativamente bajas de estas embarcaciones (en comparación con los barcos comerciales), las convierten en vectores ideales para las especies incrustantes. De esta manera, muchas especies pueden ser transportadas de una marina a otra mediante la navegación recreativa, favoreciendo la dispersión secundaria y la colonización de nuevas áreas (Drake et al., 2017; Cole et al., 2019). Por ejemplo, las embarcaciones de recreo han estado implicadas en la introducción de varias especies de algas tales como *Undaria pinnatifida* y *Codium fragile* spp. *Tomentosoides* y diferentes especies de bivalvos como *Perna viridis* y *Dreissena polymorpha* en la Columbia Británica (Clarke Murray et al., 2011).

Ante la situación de alarmante aumento en el número de invasiones biológicas a nivel global, se han elaborado diferentes acuerdos y protocolos para evitar la dispersión de especies invasoras mediante el tráfico marítimo y de esta manera proteger los ecosistemas marinos y su biodiversidad. Uno de ellos es el Convenio internacional para el control y la gestión del agua de lastre y los sedimentos de los buques (Convenio BWM, del inglés Ballast Water Management Convention), que tiene como objetivo prevenir la propagación de organismos acuáticos nocivos de una región a otra (IMO, 2004). En este convenio se establecen una serie de normas como la expulsión obligatoria del agua de lastre a una distancia mínima de 200 millas náuticas de la costa, o un límite de 10 organismos viables mayores o iguales a 50 micrómetros por cada metro cúbico de agua (IMO, 2004). Mediante las enmiendas aplicadas a este convenio, a partir de octubre de 2019 se ha formalizado un cronograma de implementación de estas normas, estableciendo el año 2024 como fecha límite (IMO, 2019). Junto al Convenio

BWM, también está en vigor el reglamento IAS (del inglés Invasive Alien Species), aplicado en Europa desde 2015 y que proporciona un conjunto de medidas para la prevención, detección temprana y manejo de especies invasoras en la Unión Europea, incluyendo planes de acción que involucran la ciencia ciudadana como una herramienta complementaria (Reglamento de ejecución UE 1143/2014). Para poder cumplir con los objetivos que se recogen en estos reglamentos, es necesario elaborar protocolos de actuación y estrategias efectivas para hacer frente al problema de las invasiones biológicas. Dichos protocolos no solo deben centrarse en evitar la introducción de estas especies, sino que también deben establecer herramientas para evitar su proliferación y propagación descontrolada una vez detectadas fuera de su rango nativo.

## 4. Estrategias científicas para hacer frente a las invasiones biológicas

El primer paso para hacer frente a las invasiones biológicas es la prevención. Considerando que la amplia mayoría de las invasiones biológicas marinas están causadas por el tráfico marítimo, existen diferentes técnicas para evitar que las especies puedan emplear las embarcaciones como medio de propagación. Algunos de los tratamientos de las aguas de lastre incluyen: esterilización por ozono, luz ultravioleta, corrientes eléctricas, tratamientos térmicos y biocidas. Estos procedimientos evitan o disminuyen la supervivencia de los organismos presentes en los depósitos de agua de lastre (Tsolaki y Diamadopoulos, 2010). En cuanto a las incrustaciones en los cascos, existen pinturas con función biocida (por ejemplo, pinturas basadas en cobre) que eliminan gran parte de los organismos adheridos a los barcos (Hopkins y Forrest, 2008). No obstante, gran parte de estas técnicas emplean substancias con altos niveles de toxicidad, por lo que muchas han sido prohibidas (Amara et al., 2018). Para hacer frente a este problema, se han propuesto nuevas alternativas que se basan en compuestos de cobre como el óxido cuproso ($Cu2O$) y el tiocianato de cobre ($CuSCN$), con suplementos de biocidas potenciadores para controlar los organismos incrustantes resistentes al cobre. Estos biocidas están destinados a ser menos dañinos para el medio ambiente, sin embargo, el problema de la toxicidad sigue persistiendo en varias especies marinas (Mochida et al., 2010).

Los puertos marítimos necesitan también, por si mismos, estrategias efectivas para hacer frente a aquellas especies que puedan sobrepasar las barreras naturales existentes e introducirse en nuevas regiones. La introducción de especies exóticas en nuevos hábitats es un proceso altamente estocástico, por lo que su predicción es muy difícil (Melbourne y Hastings, 2009). Es por ello por lo que la clave del éxito a la hora de enfrentarse a las invasiones biológicas radica en la combinación de técnicas de prevención con detección temprana. Esto se debe a que durante el proceso de introducción y antes de establecerse, el número de individuos suele ser bajo (Drake y Lodge, 2006; Tobin et al., 2011). Es en este momento, antes de que se produzca la proliferación y dispersión descontrolada, cuando las estrategias de erradicación son más efectivas. Desarrollar maniobras prácticas de prevención en los puertos industriales, implica, en cualquier caso, métodos de control mucho más exhaustivos y una mayor inversión económica (Hulme, 2021).

Tradicionalmente se han empleado métodos basados en el muestreo físico, la captura de individuos y su posterior identificación taxonómica basada en las características morfológicas (Pont et al., 2021). No obstante, esta metodología tiene ciertos inconvenientes. Habitualmente, las especies se propagan en una etapa ontogenética temprana (por ejemplo, huevos, larvas o propágulos de algas) y no son visualmente identificables, por lo que los individuos no indígenas pueden permanecer sin ser detectados hasta que ya son adultos y comienzan a reproducirse y dispersarse (Jerde et al., 2011). Además, la identificación basada en la morfología del organismo requiere taxonomistas expertos especializados en los taxones a analizar y, a menudo (especialmente en las primeras etapas de desarrollo) la identificación en base a las características morfológicas supone un trabajo muy laborioso (Garcia-Vazquez et al., 2021).

En los últimos años se han desarrollado herramientas genéticas basadas en el ADN ambiental, que pueden ser complementarias a las metodologías convencionales, para detectar de forma temprana la biota que pueda estar siendo introducida en un área geográfica determinada (Ruppert et al., 2019). Estas técnicas están basadas en la obtención de muestras del entorno (por ejemplo, muestras de agua o sedimento), donde las especies que lo habitan liberan moléculas de ADN que pueden ser procesadas para identificar la especie de la que provienen (Gold et al., 2021). Para ello es necesario un proceso de purificación de la muestra recogida (donde se separan las moléculas de ADN de otros contaminantes), amplificación (donde se copia y aumenta la cantidad de moléculas de ADN empleando marcadores genéticos), secuenciación (donde se identifica la secuencia de nucleótidos de cada grupo de moléculas de ADN) y posterior análisis bioinformático (en el que se aplican filtros de calidad sobre las secuencias, se comparan con las secuencias depositadas en bancos de datos genéticos y se asignan las secuencias de ADN a la especie a la que pertenecen). A esta técnica se la denomina Metabarcoding, y es hoy en día una de las principales herramientas que se emplea para la detección temprana de especies exóticas e invasoras a nivel global (Borrell et al., 2017; Trebitz et al., 2017; LeBlanc et al., 2020; Thomas et al., 2020). Un mayor conocimiento acerca de las especies exóticas e invasoras, así como de las principales vías de expansión que estas emplean, puede suponer una gran diferencia a la hora de hacer frente a los eventos de invasión.

## 5. El papel de la basura marina como vector de dispersión de especies exóticas.

En las últimas décadas se ha empezado a tener en cuenta otro factor de origen humano que, como el tráfico marítimo, también puede facilitar la expansión de especies exóticas: la basura marina. Se considera basura marina todo aquel material manufacturado o procesado sólido y persistente, eliminado o abandonado en la costa o en el mar (UNEP, 2009). Debido a la intensa actividad humana, se estima que cada año entre 4 y 8 millones de toneladas de basura acaban en el mar (Jambeck et al., 2015), lo cual ha causado que hoy en día la basura marina esté distribuida por todos los océanos del mundo (Galgani et al., 2015). De esta forma, en el escenario global actual se pueden observar grandes concentraciones de plásticos en los principales giros subtropicales de los hemisferios norte y sur, a los cuales se les denomina “islas de plástico” debido a su gran tamaño (Eriksen et al., 2014).

La basura marina supone un grave problema para los ecosistemas, ya que puede causar la muerte de diversos organismos, ya sea por asfixia al ingerirla, o al quedarse atrapados en objetos como redes de pesca (Rolland et al., 2019; Petry et al., 2021). Además, se ha visto que también puede afectar a la salud humana por medio de los microplásticos (pequeñas partículas de un tamaño inferior a 5mm que se generan debido a la degradación de los residuos plásticos de mayor tamaño), que, al ser transferidos por la cadena trófica, pueden llegar al ser humano y generar diversas patologías (Vethaak y Legler, 2021).

Otro de los impactos relevantes causados por la basura marina es su influencia en la dispersión de especies, puesto que puede facilitar una superficie a la que los organismos puedan adherirse y desplazarse empleando las corrientes marinas (Figura 4). Este fenómeno puede darse con especies autóctonas, pero también con especies exóticas e invasoras (Carlton et al., 2017; Miralles et al., 2018; Mantelatto et al., 2020). A pesar de que, a priori, se pueda pensar que la basura marina no puede recorrer distancias tan largas como las embarcaciones, esta afirmación parece ser completamente errónea. Más de 100 especies (de las cuales una gran parte han sido reportadas por haber causado eventos de invasiones conocidas más allá de su hábitat nativo) fueron encontradas en las costas de Hawaii y Estados Unidos, habiendo cruzado el Pacífico norte y sobrevivido a un viaje de más de 7000 kilómetros tras el tsunami ocurrido en Japón en el año 2011 (Chapman et al., 2018; Miller et al., 2018).

**Figura 4**. Cirripedos adheridos a una botella de plástico (Fuente de la imagen https://www.galapagosreport.org/).

Recientemente, se han acuñado nuevos conceptos que apuntan a una situación comprometida con respecto a la cantidad de basura marina en los océanos. Hoy en día se habla de "comunidades neopelágicas", refiriéndose a los conjuntos de especies que habitan de forma persistente en basura que flota en el océano abierto, y la cual representa un nuevo hábitat para estos organismos, que de forma natural no podrían habitar estas áreas (Haram et al., 2021). Fenómenos como este podrían desencadenar graves consecuencias ecológicas debido a la llegada de nuevas especies a otros ecosistemas. Es decir, la basura marina puede servir no sólo como vector, sino que también puede suponer, por si misma, un nuevo hábitat que puede favorecer las invasiones biológicas. En este contexto, se hace evidente la necesidad de elaborar nuevas metodologías para mejorar el tratamiento de los deshechos y disminuir la cantidad de residuos generados, especialmente los residuos plásticos, los cuales pueden llegar a representar hasta un 80% de la basura flotante (Suaria y Aliani, 2014). Además, los estudios realizados a nivel global sobre la basura marina evidencian la necesidad de entender su papel, no sólo como contaminante ambiental, sino como un factor que propicia la dispersión de especies que pueden dañar los ecosistemas.

## 6. Información, educación e implicación ciudadana para hacer frente a las invasiones biológicas.

Las actuaciones para hacer frente a las invasiones biológicas pueden llegar a ser procesos largos y laboriosos, por lo que, en muchos casos, es necesario emplear estrategias complementarias a los monitoreos. La ciencia ciudadana puede ser una herramienta poderosa en la que apoyarse para la detección temprana de especies invasoras, debido a su potencial para recolectar grandes volúmenes de datos sobre grandes áreas (Crall et al., 2010). Gracias a la recopilación de datos mediante la ayuda de la acción ciudadana, es posible realizar un seguimiento y monitoreo exitoso de las expansiones de rango de muchas especies no indígenas, como en el caso de la introducción del pez león (*Pterois volitans)* en México, cuya primera introducción en un Parque Nacional fue detectada gracias a la colaboración de los ciudadanos (López-Gómez et al., 2014).

Uno de los principales desafíos de esta estrategia para la prevención de invasiones biológicas reside en la fiabilidad y la calidad de los datos producidos por observadores/as no profesionales (Bonney et al., 2014; Steger et al., 2017). Los proyectos que utilizan ciencia ciudadana señalan que los datos pueden tener cobertura desigual dependiendo del área geográfica, además de que es necesario tener conocimientos taxonómicos a la hora de identificar una especie de forma correcta. Esto puede conducir a falsos positivos o a sesgos en el muestreo (Dickinson et al., 2010). A pesar de estas inconveniencias, diversos estudios han demostrado que, con una cantidad limitada de formación, observadores/as no profesionales pueden ser casi tan eficaces como los/as profesionales (Gallo y Waitt, 2011; Larson et al., 2020; Pernat et al., 2021). De hecho, el proceso de recopilación de datos puede ser estandarizado y validado por expertos/as, lo cual contribuye a una considerable mejora de la calidad de los datos obtenidos y permite aumentar drásticamente la cantidad de observaciones de especies disponibles para la investigación de la biodiversidad (Silvertown, 2009; Johnson et al., 2020).

No obstante, la ciencia ciudadana necesita de la participación activa de la sociedad. Para ello, es necesario que la población tenga un nivel alto de concienciación sobre las invasiones biológicas y los peligros que estas conllevan. De hecho, la falta de compromiso público puede limitar u obstaculizar la eficacia de las acciones tomadas por las autoridades (Peng et al., 2017). Es aquí donde entra el papel de la educación y la transmisión de conocimientos a la sociedad. La identificación de las principales vías de transmisión más efectivas supone un gran avance para hacer llegar el conocimiento científico al resto de la sociedad. Mediante la adquisición de dichos conocimientos se pueden mejorar las actitudes de la población (Queiruga-Dios et al., 2020) y de esta manera, impulsar una mayor concienciación e implicación, no solo con el problema de las invasiones biológicas, sino con el medioambiente en general.

## 7. El puerto internacional de Gijón

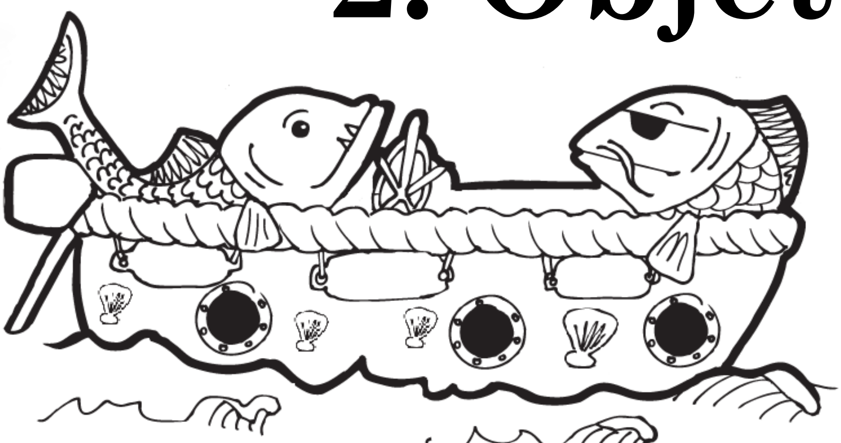
Situado en la costa cantábrica, el puerto de Gijón, conocido como *El Musel*, es uno de los principales puertos marítimos del Arco Atlántico y el primer puerto de España para el movimiento de graneles sólidos. Ocupa 415 hectáreas y dispone de más de 7.000 metros lineales de muelles, además de contar con dos pequeñas marinas con embarcaciones de recreo ubicadas fuera de los muelles principales (Puerto Deportivo y Marina Yates). El puerto de Gijón recibe cada año más de 1.200 embarcaciones de procedencia nacional e internacional que superan el tonelaje total de 18.000.000 GT anuales (Autoridad Portuaria de Gijón, 2019). Debido a la gran actividad que alberga, el puerto de Gijón, se sitúa entre los principales puertos de España, así como del Golfo de Vizcaya, ya que es receptor de más de 16.000 embarcaciones anuales procedentes de diversas partes del mundo, principalmente de America del Sur y Australia (Autoridad Portuaria de Gijón, 2020). La zona portuaria también cuenta con instalaciones de acuicultura con una plataforma de pre-engorde de moluscos y un acuario situado cerca del puerto que conforma un complejo con más de 400 especies acuáticas.

Debido a sus características, el puerto de Gijón supone un escenario de gran potencial para sufrir invasiones biológicas. El intenso tráfico marítimo puede introducir especies no indígenas en la zona mediante aguas de lastre o incrustaciones en los cascos. Las instalaciones de acuicultura trabajan con especies no indígenas de bivalvos como la almeja japonesa (*Ruditapes philippinarum*) procedente del Pacífico occidental y presente ya en zonas aledañas (Arias Rodríguez, 2012; Suárez y Raven, 2020). Los escapes en la acuicultura son una vía también relevante en la introducción de especies exóticas (Casimiro et al., 2018; Garcia et al., 2018). De la misma manera, los escapes de individuos de acuarios también pueden ser una posible fuente para la introducción de nuevas especies (West et al., 2019).

Para hacer frente a los problemas ambientales, el puerto de Gijón dispone de una estrategia medioambiental, la cual comprende varias líneas de acción que incluyen el control de emisiones atmosféricas y de vertidos, el tratamiento de residuos domésticos y las medidas de acción para evitar la contaminación acuática. Asimismo, también se señalan las distintas sanciones existentes para aquellos usuarios del puerto que no cumplan con la normativa vigente (Autoridad Portuaria de Gijón, 2014). Adicionalmente, a nivel estatal existen diversas guias de actuación y buenas prácticas para los diferentes tipos de usuarios de los puertos del Estado (Puertos del Estado, 2011). Sin embargo, las menciones acerca de las medidas para hacer frente a las invasiones biológicas son mucho más escasas y no se recoge ninguna estrategia estatal para la prevención y detección temprana de especies exóticas. Además, a pesar de que los usuarios de los puertos puedan influir en la dispersión de las especies exóticas (ya sea mediante embarcaciones recreativas o el tratamiento indebido de residuos domésticos), las guías de buenas prácticas actuales no recogen información sobre los diferentes comportamientos que podrían ayudar a evitar este problema ambiental.

En este contexto, en el año 2017, el Ministerio de Economía y Competitividad financió el proyecto Blueports. Este proyecto, en el cual se enmarca esta tesis doctoral, tiene el objetivo principal de elaborar y evaluar diferentes estrategias científicas y educativas para hacer frente a las invasiones biológicas.

# 2. Objetivos



**Objetivos**

El *Objetivo General* de esta tesis ha sido el desarrollo de estrategias científicas y educativas para la prevención de invasiones biológicas en los puertos. La *Hipótesis* de partida se definió sobre la base de que el uso de herramientas socioeducativas y de nuevas técnicas de detección de especies usando ADN ambiental, en combinación con muestreos tradicionales, podrían permitir el desarrollo de nuevas metodologías para la prevención de invasiones biológicas en los puertos. Diferentes *Objetivos Específicos* (y tareas) se han desarrollado en esta tesis doctoral:

**1-** **Prevención**: Desarrollar una estrategia que, teniendo en cuenta el tráfico marítimo, características ambientales de las zonas portuarias y requerimientos ecológicos de especies con un historial previo significativo en cuanto a invasividad e impactos medioambientales, permita crear nuevas herramientas científicas eficaces en la identificación de especies no nativas con altas probabilidades de ser introducidas, y de establecerse en las zonas portuarias. Esta estrategia permitiría preparar acciones preventivas y de erradicación especie-específicas.

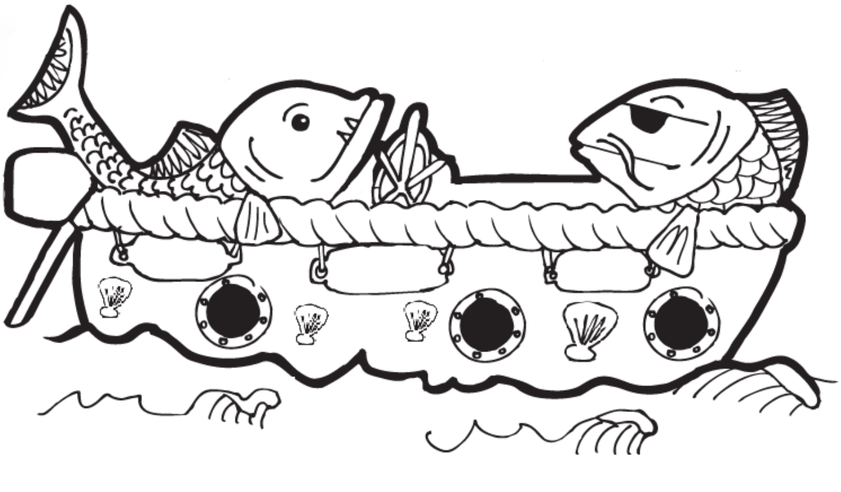
2- **Detección temprana**: Comprobar la eficacia del uso del ADN ambiental y el metabarcoding como técnicas novedosas para la detección temprana de especies exóticas e invasoras en puertos industriales.

3- **Evaluaciones ambientales**: Desarrollar nuevos índices bióticos que tengan en cuenta las especies exóticas e invasoras y sus consecuencias en el medioambiente a la hora de evaluar el estado ambiental de un ecosistema como los puertos industriales y recreativos.

4- **Vectores de dispersión secundaria**: Estudiar y evaluar la influencia de vectores secundarios para la dispersión de especies exóticas e invasoras como pueden ser la basura marina presente en puertos industriales, recreativos y localidades adyacentes.

5- **Concienciación ciudadana**: Evaluar el nivel de conocimientos existente en la sociedad acerca del problema de las invasiones biológicas marinas e identificar las principales fuentes de información que se emplean en la adquisición de conocimientos en este ámbito para promover nuevas estrategias eficaces en la concienciación, y participación ciudadana, en el enfrentamiento colectivo a este desafio medioambiental.

# Objectives



## Objectives

The *General Objective* of this thesis has been the development of scientific and educational strategies for the prevention of biological invasions in ports. The starting *hypothesis* has been defined on the basis that the use of socio-educational tools and new species detection techniques using environmental DNA, in combination with traditional sampling, could allow the development of new methodologies for the prevention of biological invasions in ports. Different Specific Objectives (and tasks) have been developed in this doctoral thesis:

1- **Prevention:** To develop a strategy that, taking into account maritime traffic, environmental characteristics of port areas and ecological requirements of species with a significant previous history in terms of invasiveness and environmental impacts, allows the creation of new effective scientific tools for the identification of non-native species with high probability of being introduced, and of establishing themselves in port areas. This strategy would make it possible to prepare species-specific preventive and eradication actions.

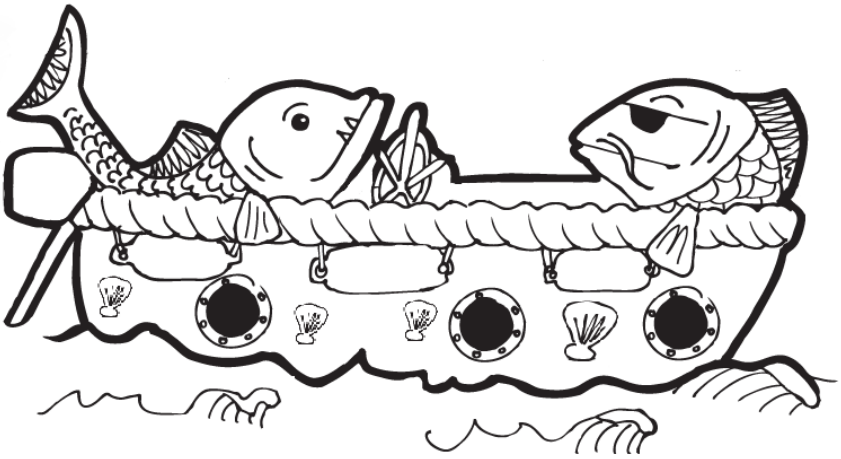
2- **Early detection:** To test the effectiveness of the use of environmental DNA and metabarcoding as novel techniques for the early detection of exotic and invasive species in industrial ports.

3- **Environmental assessments:** To develop new biotic indices that take into account exotic and invasive species and their consequences on the environment when evaluating the environmental status of an ecosystem such as industrial and recreational ports.

4- **Secondary dispersal vectors:** To study and evaluate the influence of secondary vectors for the dispersion of exotic and invasive species such as marine litter present in industrial and recreational ports and adjacent locations.

5- **Citizen awareness:** To assess the level of knowledge existing in society about the problem of marine biological invasions and identify the main sources of information used in the acquisition of knowledge in this area to promote new effective strategies in raising awareness, and citizen participation, in the collective confrontation to this environmental challenge.

# Resultados



# Resultados

**Capítulo 1:** Miralles, L., Ibabe, A., González, M., García-Vázquez, E., & Borrell, Y. J. (2021). "If You Know the Enemy and Know Yourself": Addressing the Problem of Biological Invasions in Ports Through a New NIS Invasion Threat Score, Routine Monitoring, and Preventive Action Plans. *Frontiers in Marine Science,* 8, 242.

**Capítulo 2:** Ibabe, A., Miralles, L., Carleos, C.E., Soto-López, V., Menéndez-Teleña, D., Bartolomé, M., Montes, H., González, M., Dopico, E., Garcia-Vazquez, E. & Borrell, Y. J. (2021). Building on gAMBI in ports for a challenging biological invasions scenario: Blue-gNIS as a proof of concept. *Marine Environmental Research*, 169, 105340.

**Capítulo 3:** Ibabe, A., Rayon, F., Martinez, J. L., & Garcia-Vazquez, E. (2020). Environmental DNA from plastic and textile marine litter detects exotic and nuisance species nearby ports. *PloS one*, 15(6), e0228811.

**Capítulo 4:** Fernández, S., Ibabe, A., Rayón, F., Ardura, A., Bartolomé, M., Borrell, Y. J., Dopico, E., González, M., Miralles, L., Montes, H., Pérez, T., Rodríguez, N. & Garcia-Vazquez, E. Flotsam, an overlooked vector for alien dispersal. *Estuarine, Coastal and Shelf Science*, en revision.

**Capítulo 5:** Ibabe, A., Borrell, Y. J., Knobelspiess, S. & Dopico, E. (2020). Perspectives on the marine environment and biodiversity in recreational ports: the marina of Gijon as a case study. Publicado: *Marine Pollution Bulletin*, 160, 111645.

# Capítulo 1

**"If you know the enemy and know yourself": Addressing the problem of biological invasions in ports through a new NIS invasion risk score, routine monitoring and preventive action plans.**

Miralles, L., Ibabe, A., González, M., García-Vázquez, E., & Borrell, Y. J.

Frontiers in Marine Science

8 (2021), 242.

# “If you know the enemy and know yourself”: Addressing the problem of biological invasions in ports through a new NIS invasion risk score, routine monitoring and preventive action plans.

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## ABSTRACT

Invasive alien species (IAS) are currently considered one of the greatest threats to global marine ecosystems. Thus, ships and maritime activity have been identified as the main factors responsible for the vast majority of accidental species translocations around the world, implying that prevention should be the core of environmental port policies. Preventive port strategies should include analyzing risks based on traffic origins and volumes, revising port policies for inspections, estimating probabilities of non-indigenous species (NIS) appearance, monitoring routine species within ports, and finally implementing management plans and focused actions. Here, we conducted a comprehensive NIS prediction analysis for the port of Gijon (northern Spain), one of the largest ports in the south Bay of Biscay, as a case study that can be extrapolated to other international seaports. An extensive bibliographic search (1953–2020) was conducted and we identified 380 species that have been transported through hull fouling and ballast water around the world. We evaluated their likelihood of arriving (from 14 years of traffic data) and becoming established (from habitat suitability and demonstrated impacts and invasion ability) within the Gijon port, creating a new NIS Invasion Threat Score (NIS-ITS). This new index could help to identify target species that are likely invaders for early detection and prevention policies within the port. The results showed that 15 NIS had >90% likelihood of becoming a biological invasion problem in Gijon Port. At the same time, we reported morphological and genetic analysis of biota found in two successive annual monitoring surveys of Gijon port and ships ($n$ = 612 individuals) revealing 18 NIS, including 6 of the NIS predicted from high NIS-ITS. Actually, 80% (12 NIS) of those potentially most dangerous species (NIS-ITS > 90%) have already been detected in the Bay of Biscay area. We propose the use of this new tool for a risk-reduction strategy in ports, based on accurate predictions that help in promoting specific early detection tests and specific monitoring for NIS that have a high chance of establishment. All international seaports can adopt this strategy to address the problem of biological invasions and become "blueports" in line with EU policy.

**KEYWORDS:** Invasive species, genetic barcoding, ports strategies, blueports, NIS assessment, invasion likelihood.

## INTRODUCTION

The distribution of marine taxa is limited by natural barriers to their dispersal (Elton, 1958). However, over the past centuries, human activities have modified those barriers (Vemeij, 1978) and, consequently, the distribution of many species (Kotta et al., 2016). At present, based on data from the European Alien Species Information Network (EASIN), there are approximately 14,000 non-indigenous species (NIS) recorded in Europe (EASIN Catalog)1 and invasive alien species (IAS) represent a major threat to the ecological, economic and social values of marine environments (Vanderploeg et al., 2002; Molnar et al., 2008). They are considered one of the greatest threats to global marine ecosystems, affecting their structure and function (Costello et al., 2010), with negative socioeconomic consequences (e.g., Gherardi et al., 2011). Ballast water and hull fouling, associated with maritime transport, are responsible for the vast majority of accidental marine translocations around the world (Carlton, 2001; Hewitt et al., 2009; Seebens et al., 2013), however, there are other human-mediated pathways that can be used by species to spread to new habitats, such as the opening of new corridors that facilitate the spread of species (Steger et al., 2019; Castellanos-Galindo et al., 2020) or escapes from aquaculture facilities (Thorvaldsen et al., 2015; Ju et al., 2020) among others.

At a global scale, ships are widely recognized as the primary vector of aquatic biological invasions (Carlton, 1985; Ruiz et al., 2000; Hewitt et al., 2004; Gollasch, 2006). Additionally, ships contain sub-vectors, including ballast water (Carlton, 1985), ballast sediments (Bailey et al., 2005), hull biofouling (Allen, 1953; Ashton et al., 2016), internal tank biofouling (Drake et al., 2005), sea chests (Coutts, 1999), and internal seawater piping systems (Carlton, 1985; Lewis and Dimas, 2007). As a result, all ships are different in terms of vector risk, and there are differences in species entrainment, transfer and release among different ship types (Davidson et al., 2018).

Risk analyses and management actions to reduce ship-mediated species dispersal around the world are being developed and have increased as marine biosecurity concerns grow (Davidson et al., 2018). There are several international legislative frameworks focused on managing the introduction and spread of potential NIS. Within the European Union, the main policy and legislative drivers to target marine NIS are the Marine Strategy Framework Directive (MSFD), the Biodiversity Strategy, and EU Regulation no. 1143/2014 on Aquatic Invasive Species (EC, 2014). Within this legislative framework, the EU blue growth strategy advocates for a blue economy that exploits natural resources in a sustainable way and protects the environment (European Commission, 2012). This includes the *Blueports objective*, a strategy that aims to create an adequate management of resources along with new strategies to avoid biological invasions and the environmental or economic impacts that these can produce (Dopico Rodríguez and Borrell Pichs, 2020). Recently, the first EU-scale Horizon Scanning (HS) focusing on marine NIS was performed, aiming to deliver a ranked list of species that should be of high priority for risk assessment (Tsiamis et al., 2020). These lists of species with invasive potential can be used to update the EU regulation 1143/2014, which shows a very limited coverage of marine invasive species and, this way, preventive actions against these potentially harmful species can be promoted (Tsiamis et al., 2020). These global initiatives are extremely relevant, although they can lose effectiveness if they are not properly interpolated and adjusted to the characteristics of the different marine areas in a much more concrete or specific way within the European Union (areas, countries, even ports). On the other hand, it is necessary to develop accessible and friendly methodologies for stakeholders (usually not experts in biological invasions) with the responsibility of making decisions that can prevent these events (e.g., port authorities).

Outside of Europe, a set of port monitoring guidelines have been developed to maintain up-to-date records of the distribution of NIS, such as those for Australian ports (Hayes et al., 2019). Similarly, in New Zealand, a series of targeted (species-specific) surveys are now implemented in 11 harbors every 6 months (Arthur et al., 2015). Moreover, in order to avoid species introductions via marine traffic, some Australian ports require arriving ships to be inspected and treated for marine pests before they can enter a marina. These inspections are compulsory for vessels arriving from international ports (DPC, 2013).

Recently, the Ballast Water Management Convention (BWM) entered into force. All international sea-going ships under this convention must comply with standards that require discharging ballast water from the last port and replacing it with new sea water at a minimum of 200 nautical miles from shore before arriving at a new port (IMO, 2004, 2011). Despite this, several studies have indicated that in comparison to ballast water, biofouling likely accounts for more NIS introductions (e.g., Fofonoff et al., 2003; Davidson et al., 2009; Hewitt and Campbell, 2010). In this context, other projects such as the GloFouling partnership project (a collaboration among the International Maritime Organization, the United Nations Development Programme and the Global Environment Facility) have been developed with the objective of building capacity in developing countries for implementing the IMO Biofouling and other relevant guidelines for biofouling management and to catalyze overall reductions in the transboundary introduction of biofouling-mediated IAS. Thus, biofouling should be included in a marine NIS risk assessment regardless of the currently low capacity to predict inoculation rates and difficulties associated with sampling (Ware et al., 2014).

Although a management and legislative framework and a marine vector-based risk assessment methodology are well-established in the literature (Campbell and Hewitt, 2011; Keller et al., 2011; Chan et al., 2012; Floerl et al., 2013; Ware et al., 2014), both have many limitations. First, NIS monitoring surveys are not yet performed in all international ports consequently, there is not a global and updated information source about the current state of biodiversity in ports (Blueports Interreg Project Advisory Committee, pers. comm.) and there are not complete baselines for temporal and spatial intra-/inter-port comparisons worldwide. Secondly, vessel arrival details are a poor proxy since the substantial variety in ships and shipping routes make the process stochastic (Verling et al., 2005; Lawrence and Cordell, 2010; Ruiz et al., 2013; Ware et al., 2014).

In this study, an initial biological invasion preventive strategy has been developed for the international port of Gijon, located in the Bay of Biscay, which is the leading port for solid bulk movement in Spain. The approach consisted on a global NIS occurrence likelihood assessment based on an exhaustive review of worldwide previously reported species introductions through shipping in the last 70 years (biofouling and ballast water) and combined with, the habitat suitability for each species in the given port conditions, species invasiveness and traffic data analyses. Together, these factors were considered to produce a specific NIS invasion threat score (NIS-ITS) for the port of Gijon. Two consecutive years of physical monitoring surveys of Gijon Port’s biota were performed, and different hull ships were sampled to analyze the biofouling community with the aim of comparing predicted and actual NIS scenarios.

## MATERIALS AND METHODS

### “El Musel" the International Industrial Port of Gijon

Located on the Cantabrian coast (5°41.9496′W, 43°33.5120′N), the port of Gijon is one of the main seaports in the Atlantic arc and the leading port for solid bulk movement in Spain2. The port of Gijon covers 415 hectares and has more than 7,000 linear meters of docks. The spaces provided by the port of Gijon are structured in areas divided by each type of traffic (solid bulk, liquid 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 (Figure 1).

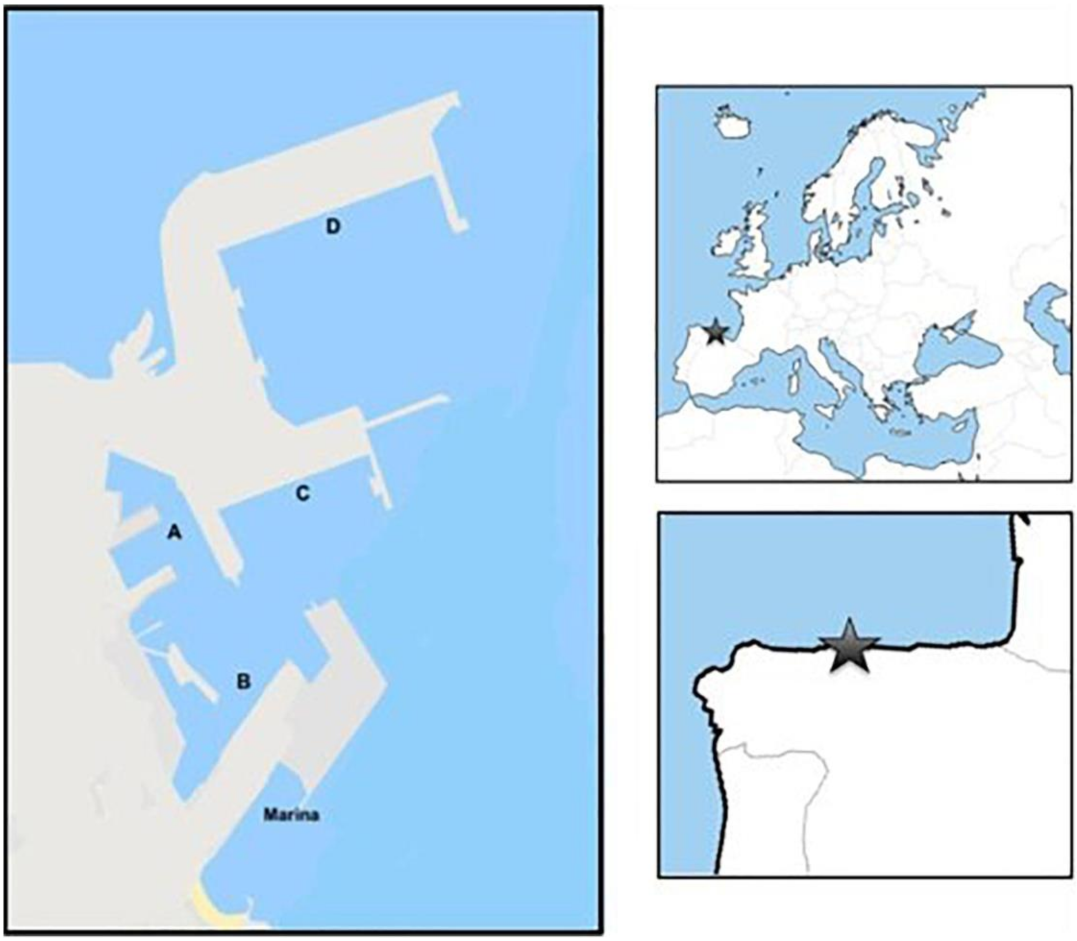


**Figure 1.** Map of "El Muse1" international industrial port of Gijon, Bay of Biscay, Spain. (A–D) correspond to the different sampling areas inside the port divided by the type of traffic received.

To identify and control environmental quality, the Port Authority of Gijon has established and implemented an Environmental Surveillance Plan. This plan was developed taking into account the applicable regulations and the Program of Recommendations for Maritime Works (ROM) and based on the standards of the International Standard ISO 14001. For this reason, the Port Authority of Gijon is focused on identifying its most significant environmental impacts and acting on them by continuously improving its management strategies3.

**Expected NIS scenario**

To evaluate the threats of invasions in the port of Gijon, we created a NIS-Invasion Threat Score (NIS-ITS). A review of the scientific literature from 1953 to 2020 was conducted considering reports of species dispersal via maritime traffic. This search was conducted considering all living organisms, from microscopic species such as dinophytes that have been studied traveling in ballast tanks to macroscopic organisms such as bryozoans or ascidians that have been observed traveling via hull fouling. All translocation reports were collected, and a list of species with evidence of using marine traffic as a vector for expansion was generated. This dataset was analyzed for the case study of the port of Gijon and species were categorized as native, NIS, IAS or cryptogenic in the area. These classifications were carried out by using the Invasive Species Specialist Group (ISSG) global database of the International Union for Conservation of Nature4, the DAISIE (Roy et al., 2020) and the AquaNIS (AquaNIS, 2015) databases. Once this categorization was done, the NIS-ITS was calculated for the NIS in the area under study. On the other hand, those species with evidences of using maritime traffic as a vector, but classified as native in the area under study were listed apart in order to facilitate a checklist to other regions where these species could arrive and might become a NIS.

The NIS-ITS was calculated using three criteria. The first criterion was the suitability of the environmental conditions of the port of Gijon for each of the potentially arriving species. This information was obtained from the SeaLifeBase webpage (Palomares and Pauly, 2020). Values for habitat suitability (Hs) range from 0 (totally unsuitable habitat) to 1 (perfect environmental conditions for the species). These values are calculated for each species and geographic location by using estimates of environmental preferences with respect to depth, water temperature, salinity, primary productivity, sea ice concentration and distance to land (Ready et al., 2010). Each species-specific environmental preferences are matched against local environmental conditions to determine the suitability of a given area in the ocean for a particular species.

By combining the information for these parameters, an environmental envelope is generated for each species, which is essentially a response curve that describes the habitat usage of such species or its preferences with respect to certain environmental parameters. If the mean environmental conditions fall within the preferred parameter range of the species, the habitat suitability will be the highest (Hs = 1.00). However, when values fall outside this range, it will decrease linearly toward the species’ absolute minimum or maximum parameter thresholds and it is set to zero (Hs = 0.00) beyond the absolute threshold values (Ready et al., 2010). The suitability is first calculated for each of the individual environmental parameters and the product is then used to determine the overall habitat suitability in a given location, that is:

$$Hs = S_{depth} \times S_{temperature} \times S_{salinity} \times S_{prymaryproduction} \times S_{iceconcentration} \times S_{landdistance}$$

This multiplicative approach allows each environmental predictor to act as a “knock-out” criterion. For instance, if the salinity in a given location exceeds the salinity preference of a particular species, the probability of occurrence with respect to salinity will be zero ($S_{salinity} = 0.00$). The overall habitat suitability for the species in that given location will then be zero (Hs = 0.00), even if all other environmental attributes are within the preferred range of that species. Habitat suitability data can be found for specific geographic regions and for particular species and it is accessible at https://www.aquamaps.org/search.php. Georeferenced values for the mentioned parameters are provided at 0.5° resolution (Kaschner et al., 2019). For this case study, data from the point 43.75°, −5.75° were employed since the port of Gijon is located inside this area.

The second criterion was the known invasion history of the species. A numeric score is given to each species depending on the following situations: (a) the species is only present in its native habitat and there are no reports of its introduction in any other area, (b) the species has been introduced in areas outside its native range but there is no information on any relevant impact, (c) the species has invaded new areas and causes various impacts of different magnitudes. The score for each of these conditions is set based on the six EICAT (Environmental Impact Classification for Alien Taxa) impact categories from Hawkins et al. (2015) by assigning an ascending numerical value according to the level of impact of the species (Supplementary Table 1). A score of zero was given to those species that are present only in their native habitat without any known introduction event. A score of 0.2 was given to those species that have been introduced in areas out of their native range but do not cause any relevant impact. This score corresponds to the “minimal concern” EICAT impact category which includes species with “small inconsequential changes,” as suggested by Roy et al. (2019),. An invasion history score of 1 was given to those species that have been introduced and produce environmental or economic impacts outside their native areas. This score corresponds to the rest of categories with higher EICAT impact magnitudes (minor, moderate, major and massive) which are all merged due to the lack of specific information to differentiate between these categories with high accuracy.

Finally, the third criterion employed for the calculation of the NIS Invasion Threat Score (ITS) was the maritime traffic. In order to calculate the maritime traffic score for a given species, its native range is determined based on bibliography. Results are reported as presence or absence of the species in each of the seven biogeographic areas defined by Menéndez-Teleña (2019) (see Supplementary Table 2). Once the native range for a species is assessed, it is also checked if it has stablished populations in other biogeographic areas. If so, these areas are also considered as potential donors of species and included in the traffic score calculation. The specific dispersal method that may be employed by the species (e.g., ballast water or hull fouling) is not taken into account. If the species is present in a biogeographic zone, the maritime traffic originating from this area is analyzed and summed to other potential areas where the species is present and therefore may arrive to the port of Gijon using the ships as vectors. The traffic score is calculated by using available traffic data for the port under study. Depending on the case, this data availability may vary. However, this score can be calculated in any case without a minimum data limitation. This means that the score can be calculated, for example, annually in those cases with scarce traffic data, but it can also employ data from a longer period, such as in this case study, in which 14 years of traffic in the port of Gijon were used. Once the global distribution of the species is determined, the relative proportions of traffic from native and invaded areas (the ratio between the volumes of traffic from the area divided by the total traffic arriving at Gijon) are estimated and then summed to obtain a unique total traffic score. Areas with heavy maritime traffic will show a high traffic ratio. Then, species that are present in these areas will show high maritime traffic scores. Values for maritime traffic also range from 0 (total absence of a species in all of the biogeographic areas with marine traffic to Gijon) to 1 (a species that was present in all the areas with maritime traffic to the port of Gijon).

Following the methods used to calculate invasion risk scores by different authors (Bomford, 2008; Gallardo et al., 2016; Tsiamis et al., 2020), the NIS-ITS can be calculated by the sum of the specific criteria, which in this case are the habitat suitability, invasion history and maritime traffic. All of them are considered equally important as in Gallardo et al. (2016) and references therein. This method (unlike multiplicative ones) allows obtaining non-zero scores when one of the parameters is null. In summary, the index can be calculated by the following formula:

$$\text{NIS-ITS} = \text{HabitatSuitability} + \text{InvasionHistory} + \text{MaritimeTraffic}$$

The maximum NIS-ITS that a species could obtain after combining all the criteria is 3 (examples of ITS calculations can be seen in Supplementary File 1A). A NIS-ITS = 3 (100% of the maximum ITS) indicates that the port of Gijon presents perfectly suitable environmental conditions for the specific species (habitat suitability = 1), that the species has shown previous capacity to impact ecosystems out of its native range and biologically invade elsewhere (invasion history = 1), and the species is present in all the biogeographic areas from which the port of Gijon receives marine traffic (total traffic risk = 1). On the contrary, a species could also obtain an NIS-ITS = 0 when: environmental conditions are outside the species' absolute minimum or maximum parameter thresholds on at least one of the environmental parameters (habitat suitability = 0), the species is only present on its native range, without any known introduction or invasion event (invasion history = 0) and when the species is not present in any of the biogeographic areas with maritime traffic with the port under study (maritime traffic= 0).

This method does not take into account if vessels employ techniques for the prevention of biological invasions, such as techniques to release ballast water away from ports or to avoid fouling by using paints that cover the hulls. In these cases, where these preventive measures are taken, the NIS-ITS should be lower than in cases where they are not. Due to the lack of data, this method cannot be implemented in this case study, but we

provide an example for its calculation that could be considered for future cases (see Supplementary File 1B).
The port of Gijon can also be a donor of species, so it is also important to understand which species that are present within the port can be transferred via maritime traffic. Thus, from the initial list of species containing species with the potential of using maritime traffic as a vector for expansion, those that are native to the area studied in this work (the port of Gijon) were analyzed. These species were categorized as IAS (when the species has invaded new habitats and causes impacts and environmental damages), NIS (when the species has been reported out of its native range, but there is not information about any impact in these habitats) or NA (no alien populations). It is expected that these data can help destination ports complete their own and specific NIS-ITS information with data on species habitat suitability and maritime traffic for their own conditions.

**Port Biota and Biofouling Samplings**

The port biota study was focused on macroinvertebrates attached to port facilities (rocky ground and port structures). Targeting macroinvertebrates is the typical sampling procedure routinely followed in most ports to estimate biotic indices for environmental quality assessments of marine ecosystems (Borja et al., 2000, 2009) and is recommended in both the EU Water Framework Directive (WFD, Directive 2000/60/EC) and the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC) (Borja et al., 2015). Sampling was performed in two consecutive years (2016 and 2017) during the highest spring tide. Two years of sampling were selected to optimize the surveillance design, in terms of effort vs. resolution, by following the recommendations for sampling aquatic invasive species in Hoffman et al. (2016).

The sampling was divided into the structured areas of the port: A, B, C, D, and marina (Figure 1). In each area, two locations were studied, except for in the marina, where only one place was sampled due to its small area. At each location, sampling occurred at three different tidal levels (high, low and medium). A rapid assessment survey approach (RAS; Minchin, 2007) and the sampling protocol from Miralles et al. (2016) were adapted and conducted on the assemblages living on artificial port structures. A team of five researchers (two postdoc and three predoc) expert in marine science did the 2 years sampling. Before starting the sampling and to avoid biases due to patchy spatial distributions, a visual inspection to determine the different types of organisms present at the sampling site was conducted for approximately 10 min. To standardize the sampling effort, the surface sampled at each site within the area of the port was approximately 200 $m^2$, and the duration of the sampling was 30 min. Roughly 1% of the animals visually detected attached on that surface were collected at random. The number of individuals picked of each morphotype (group of organisms phenotypically different) was approximately proportional to the abundance of such morphotype in the area. A maximum of 50 individuals per location were selected and were placed in buckets with seawater for further morphological, taxonomical and genetic analyses.

All sampled individuals were immediately transported to the laboratory of Genetics of Natural Resources at the University of Oviedo. In the laboratory, individuals were identified based on their morphology to the species or to the lowest possible taxonomic level. Then, a piece of tissue from each individual was preserved in absolute ethanol for further genetic analysis. The taxonomic nomenclature of all identified species was verified on the World Register of Marine Species (WoRMS, 2020).

Apart from the biota on the port structures, species attached on the hulls of ships arriving at the port of Gijon were analyzed. To carry out this effort, during July 2017, 22 ships from 12 different countries were contacted directly through the Port Authority of Gijon to ask them to participate in this study. Sampling consisted of scratching all biofouling organisms from a quadrat of approximately 30 × 30 cm in three different places along the draft of the vessel: stern, mid-ship and bow. Sampling was performed under strict safety procedures with professional divers. All divers were fastened by wiring and had audio and visual connections with the supporting ship where the scientists were placed located. The general procedure of diving was based on the Collective Agreement of Professional Diving and Hyperbaric Means; the Spanish Ministerial Order of October 14, 1997; the Modification to the Collective Agreement of Professional Diving and Hyperbaric Means of October 18, 2016; and the UNE-EN 15333-1. Samples were placed in bags with seawater and transported to the University of Oviedo. In the laboratory, individuals were identified based on morphology, and a piece of tissue from each individual was preserved in absolute ethanol for further genetic analysis. The taxonomic nomenclature of all identified species was verified on the World Register of Marine Species (WoRMS, 2020).

The list of NIS found in these samplings was enhanced by data from previous studies of macroinvertebrates and algae (such as Arias et al., 2014; Miralles et al., 2016) and from metabarcoding data (Borrell et al., 2017; Ibabe et al., 2020a,b) to generate an accurate and complete NIS scenario for the port of Gijon.

### Genetic Species Identification

Genetic species identification was carried out to confirm the morphological taxonomic identification following Miralles et al. (2016). DNA was extracted from ethanol-preserved individuals using a Chelex® resin (Bio-Rad Laboratories Inc., United States) protocol (Estoup et al., 1996) or the EZNA Mollusc DNA Kit (Omega Bio-Tek Inc., United States). The genetic barcode employed was the mitochondrial cytochrome oxidase subunit I coding region (COI gene), with additional genes (RNAr18s or RNAr16s) to improve resolution or to determine species when the database references were insufficient (Miralles et al., 2016; Pejovic et al., 2016). The obtained DNA sequences were visually inspected and edited with BioEdit v7.2.5 (Hall, 1999). All sequences were compared with online public databases using nBLAST in NCBI5 and Bold Systems6. The sequence with a maximum score and at least a 97% nucleotide identity and an E-value <e-100 was considered the reference for genetic species identification.

All sampled port biota and ship biofouling individuals were classified as native species, NIS, IAS or cryptogenic species. The Invasive Species Specialist Group (ISSG) global database of the International Union for Conservation of Nature (IUCN), the DAISIE (Delivering Alien Invasive Species Inventories from Europe), and the AquaNIS (information system on aquatic non-indigenous and cryptogenic species database) were the references used to confirm the introduced and invasion status of each NIS when sufficient information was available. Species that are unable to be identified as native or NIS were classified as cryptogenic (Carlton, 1996). Once species identity was confirmed, species abundances for statistical analyses were calculated based on the number of individuals with confirmed identification and the total number of species identified in each sampling (2 years separately).

### Statistical Analyses

Since samplings were conducted in different port areas and years, a multivariate non-metric multidimensional scaling (nmMDS) analysis was conducted to determine whether the distribution of the species (including both, native and NIS) was strongly clustered by area or year or whether they were more or less scattered. The Bray-Curtis dissimilarity index was applied, and a stress <0.2 was considered acceptable. The Shepard plot was inspected to determine the linearity of the index in the dataset. A SIMPER analysis was conducted (only for the non-indigenous biota) to assess the species that most contributed to the differences from 1 year to the other and detect any potential expansion or population growth. Comparisons between years and areas as well as diversity indexes (Shannon and Simpson), tests and plots were performed with PAST version 3.8 (Hammer et al., 2001).

## RESULTS

### Expected NIS Scenario

Eighty research articles recording worldwide evidences of species introductions and translocations through shipping from approximately 70 years of marine research were compiled in this work. These reports were used to create a list of species that were able to travel by ship and have the potential to be future invaders in any port. A large species taxonomical representation was obtained from that list of 380 species. From this dataset, 252 species could be considered as NIS (if found in the port of Gijon), whereas 128 were classified as native in accordance with their natural geographic distributions. NIS habitat suitability data for the port of Gijon were available only for 71 out of the 252 NIS. This information was combined with the invasiveness of each species and the corresponding port of Gijon’s maritime traffic score to estimate the NIS-ITS for each individual species (Supplementary Table 3). Although NIS-ITS cannot be estimated for those species lacking habitat suitability information, scores for maritime traffic and invasion history were also estimated for these species (Supplementary Table 4).

A maximum invasion threat score (NIS-ITS = 3.00) was obtained by 3 out of the 71 species [Bugula neritina (Linnaeus, 1758), *Ficopomatus enigmaticus* (Fauvel, 1923), and *Botryllus schlosseri* (Pallas, 1766)]. On the contrary, some species such as *Corynactis californica* Carlgren, 1936 (NIS-ITS = 0.153) showed low scores, indicating a lower likelihood of being a successful invader in the area (Supplementary Table 3). Most of the analyzed species were crustaceans (Arthropoda), ascidians (Chordata), bivalves and gastropods (Mollusca). All species belonging to Rhodophyta and Ctenophora phyla showed invasion threat scores above 70%. Similarly, four polychaetes showed also more than a 70% score and two out of four bryozoans scored above 90% (Figure 2). A total of fifteen NIS showed more than 90% of invasion likelihood (NIS-ITS > 2.70) (Table 1). Most of them were meroplanktonic animals belonging to classes such as Malacostraca, Hexanauplia, Polychaeta, or Ascidiacea. The only exception was *Hypnea musciformis* Lamouroux, 1813, which is a seaweed belonging to the class Florideophyceae. All these species with a NIS-ITS higher than 90% showed an invasiveness score of 1, meaning that all of them had previously invaded areas out of their native ranges and produced environmental impacts. Special care and attention should be given to these species to avoid their establishment in the port of Gijon. In fact, most of the species with an invasion history score of 1 showed a NIS-ITS above 70% whilst species that are only known to be present in their native habitat (invasion history score = 0) showed low NIS-ITS values (Figure 3).

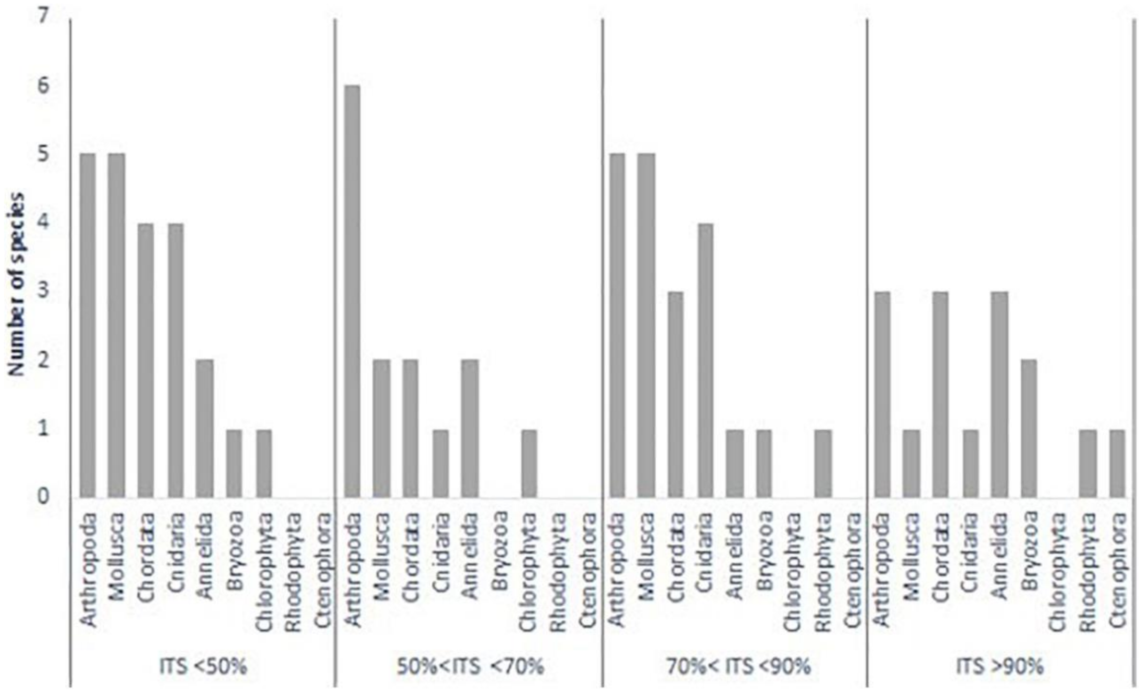


**Figure 2.** Count of species belonging to different phyla grouped based on their invasion threat scores (NIS-ITS).

**Table 1.** Non-indigenous species (NIS) with more than a 90% invasion threat score (NIS-ITS) in the port of Gijon.

| n | Class | Species | NIS-ITS (%) | References for the species report |
|---|---|---|---|---|
| 1 | Ascidiacea | ***Botryllus schlosseri*** (Pallas, 1766)* | 100 | Nydam et al., 2017 |
| 2 | Gymnolaemata | ***Bugula neritina*** (Linnaeus, 1758)* | 100 | This study |
| 3 | Polychaeta | ***Ficopomatus enigmaticus*** (Fauvel, 1923) | 100 | Miralles et al., 2016 |
| 4 | Ascidiacea | ***Diplosoma listerianum*** (Milne Edwards, 1841)* | 99.97 | This study |
| 5 | Anthozoa | ***Diadumene lineata*** (Verrill, 1869) | 97.87 | Altuna, 2015 |
| 6 | Polychaeta | *Polydora cornuta* Bosc, 1802 | 97.87 | Not found |
| 7 | Hexanauplia | ***Acartia tonsa*** Dana, 1849 | 94.30 | Aravena et al., 2009 |
| 8 | Gymnolaemata | *Schizoporella errata* (Waters, 1878) | 94.03 | Not found |
| 9 | Ascidiacea | ***Styela plicata*** (Lesueur, 1823)* | 92.67 | Miralles et al., 2016 |
| 10 | Malacostraca | ***Callinectes sapidus*** Rathbun, 1896 | 92.63 | Arias et al., 2014 |
| 11 | Malacostraca | ***Rhithropanopeus harrisii*** (Gould, 1841) | 92.63 | BOE, 2013 |
| 12 | Bivalvia | ***Mytilopsis leucophaeata*** (Conrad, 1831) | 92.20 | BOE, 2013 |
| 13 | Tentaculata | ***Mnemiopsis leidyi*** Agassiz, 1865 | 92.20 | BOE, 2013 |
| 14 | Florideophyceae | ***Hypnea musciformis*** Lamouroux, 1813 | 91.63 | Arias et al., 2014 |
| 15 | Polychaeta | *Marenzelleria viridis* (Verrill, 1873) | 91.30 | Not found |

*Those species detected in previous reports in the Bay of Biscay (the geographic area of the port) are shown in bold. Species already detected in the port of Gijon in this and previous studies are shown with an asterisk.*

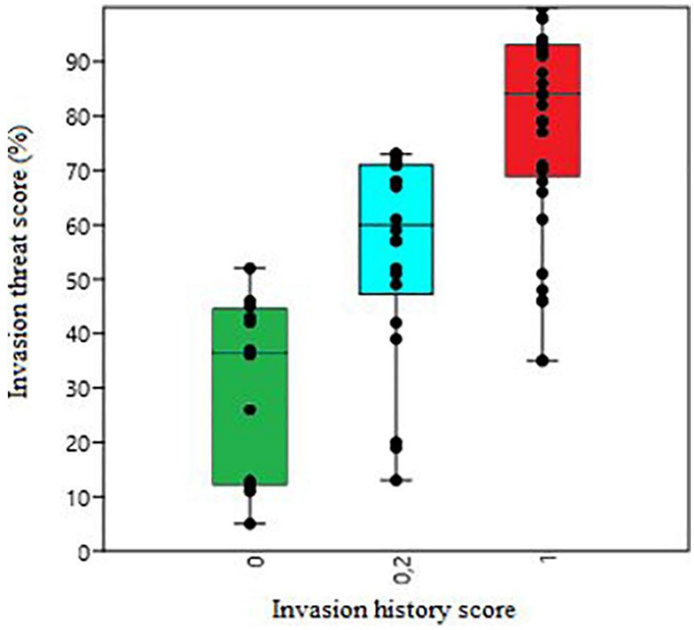


**Figure 3**. The NIS invasion threat score percentages obtained for each of the groups based on species invasion history score. Group 0 (for those species only present in their native habitat and without known introduction events), group 0.2 (for species that have been introduced outside their native range but do not cause any known impact) and group 1 (for those species that have invaded new areas and cause various impacts of different magnitudes).

On the other hand, 128 species capable of using maritime traffic as a vector were found to be native to Gijon (Supplementary Table 5). Sixteen of these species have already invaded other areas and produced environmental impacts outside their native range. Similarly, 32 species that are native to the area under study, despite having no records of producing environmental impacts, have already been detected outside their native habitats. These species are mainly dinophytes transferred by ballast water or bryozoans transferred by hull fouling (Supplementary Table 5).

**Port Biota Biofouling**

A total of 522 individuals were genetically identified to 82 taxa in the 2 years samplings of the port of Gijon. In general, genetic analyses confirmed the previous morphological identification. The observed communities were mainly composed by polychaetes, bivalves and gastropods (GenBank accession numbers MN185333-MN185374 for COI gene, MN164033-MN164046 for 18s RNA gene, and MN164346-MN164348 for 16s RNA gene; Supplementary Table 6).

The global biodiversity in the port of Gijon was not significantly different between 2016 and 2017 (diversity t-test for Shannon index $t = -1.108$ and $p = 0.268$; Simpson index $t = 0.901$ and $p = 0.368$; Figure 4A). Regarding the observed NIS, IAS and cryptogenic species, significant differences between years were found (diversity t-test for Shannon index $t = -2.475$ and $p = 0.015$; Simpson index $t = 2.61$ and $p = 0.010$).

The rarefaction plot revealed higher NIS richness in 2017 than in 2016 (Figure 4B). SIMPER analysis showed that the NIS that mainly accounted for the differences between 2016 and 2017 communities were *Magallana gigas* (Thunberg, 1793), *Mytilaster minimus* (Poli, 1795), *Watersipora subtorquata* (d'Orbigny, 1852), and *Mytilus trossulus* (Gould, 1850), which accounted for 70% of the dissimilarities. The first two species decreased, while *W. subtorquata* (7.74% of the contribution to the dissimilarities between 2016 and 2017), and *M. trossulus* (5.31% of the contribution to dissimilarities) increased in observed specimens from 2016 to 2017. In fact, *W. subtorquata* was found at all five stations in 2017 (found only at stations A and B and the marina in 2016), and *M. trossulus* was found in stations A and C and the marina in 2017 while it was present only at station C in 2016 (Supplementary Table 6).

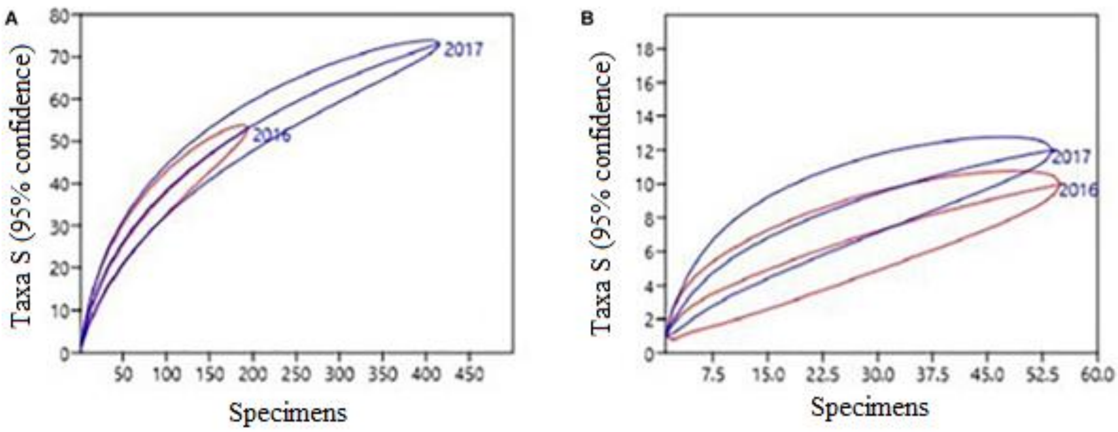


**Figure 4**. Diversity rarefaction plots of (A) all observed species and (B) non-indigenous species from the port of Gijon, Bay of Biscay, Spain.

Biodiversity comparisons among the main industrial docks (A, B, C, and D) showed that they clustered together regardless of the sampling year (Figure 5, stress = 0.080). However, the marina always appeared slightly distant from the remainder of the industrial docks both years, and notable differences were found when comparing communities inhabiting the marina in 2016 and 2017 (Figure 5).

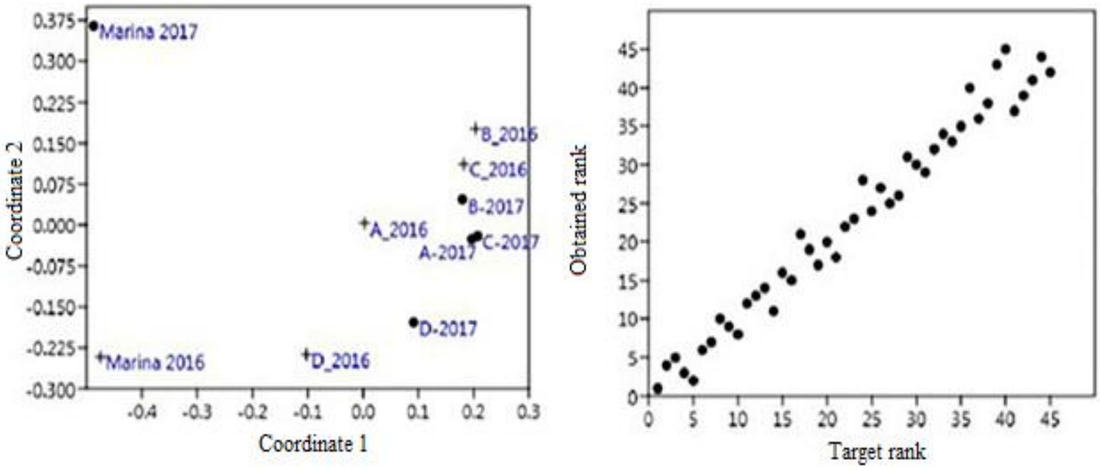


**Figure 5.** Two-dimension non-metric multidimensional scaling (nmMDS) plot of the areas under study in the Port of Gijon, Bay of Biscay, Spain in 2016 and 2017 and its Shepard plot. Crosses represent samples collected in 2016 and dots represent samples from 2017. The name of the area is given (A–D and Marina). Stress value is 0.080.

Apart from analyzing the biota inhabiting the different areas of the port of Gijon, the species attached to the hulls of three ships docked in the port were also studied. Notably, out of the 22 ships contacted, these three ships were the only ones that agreed to participate in the study. To maintain anonymity, the sampled vessels were labeled ships 1, 2, and 3. Ship 1 was an international vessel that arrived to Gijon from Russia and its destiny was United Kingdom. Ship 2 was a dredge vessel that used to work along the Cantabrian Sea (South Bay of Biscay) but it was stopped due to maintenance in Gijon for 13 days before sampling. Finally, Ship 3 was an inactive vessel docked in Gijon for 3 months (details about the study vessels in Supplementary Table 7). A total of 73 specimens and 15 different species, including native but also 5 NIS, were found fouling the hulls of the ships (Table 2). Most of the species were natives already observed in the samplings from other port areas. However, some species were only found on the vessels. For instance, Ship 3 had been docked in Gijon for the longest period, and almost all the fouling species were already described in the sampled areas of the port. Nevertheless, a NIS [*Diplosoma listerianum* (Milne Edwards, 1841)] that did not appear in the port samplings was collected from this ship. Two new potentially dangerous species (which were not reported in the 2 years previous monitoring surveys) appeared on Ship 2: *Monocorophium acherusicum* (Costa, 1853) and *Spirobranchus taeniatus* (Lamarck, 1818), in addition to other NIS, *Spirobranchuslatiscapus* (Marenzeller, 1884) and *Dipolydora capensis* (Day, 1955) that had already been observed in the port samplings (Table 2). Thus, the risk of being a vector of NIS through biofouling was not the same for the three vessels. Ship 1 could be classified as “no invasion risk” (because there was no attached biota), while ships 2 and 3 could represent a “potential vector of invasions” since new species were present in their hull (and not in the port).

**Table 2.** The number of non-indigenous (NIS) and invasive (IAS) specimens observed in macroinvertebrates samplings conducted in two consecutive years (2016, 2017) in the port of Gijon, Bay of Biscay, Spain.

| n | Class | Species | Status in Gijon | Sampling | | |
|---|---|---|---|---|---|---|
| | | | | 2016 | 2017 | Vessels |
| 1 | Anthozoa | *Anthopleura anjunae* Den Hartog and Vennam, 1993 | NIS | 1 | 1 | |
| 2 | Anthozoa | *Anthopleura elegantissima* (Brandt, 1835) | NIS | 2 | 2 | |
| 3 | Gymnolaemata | *Bugula neritina* (Linnaeus, 1758)$^{+}$ | IAS | 1 | 1 | |
| 4 | Gymnolaemata | *Crassimarginatella papulifera* (MacGillivray, 1882)$^{+}$ | NIS | 1 | | |
| 5 | Gymnolaemata | *Watersipora subtorquata* (d'Orbigny, 1852)$^{+}$ | IAS | 3 | 9 | |
| 6 | Ascidiacea | *Diplosoma listerianum* (Milne Edwards, 1841)$^{+}$ | IAS | | | 3 |
| 7 | Ascidiacea | *Microcosmus squamiger* Michaelsen, 1927 | NIS | | 1 | |
| 8 | Polychaeta | *Dipolydora capensis* (Day, 1955) | NIS | | 1 | 1 |
| 9 | Polychaeta | *Phyllodoce groenlandica* Örsted, 1842 | NIS | 1 | 2 | |
| 10 | Polychaeta | *Spirobranchus latiscapus* (Marenzeller, 1884) | NIS | | 2 | 1 |
| 11 | Polychaeta | *Spirobranchus taeniatus* (Lamarck, 1818) | NIS | | | 1 |
| 13 | Bivalvia | *Magallana gigas* (Thunberg, 1793) | IAS | 18 | 14 | |
| 15 | Bivalvia | *Mytilus trossulus* Gould, 1850 | NIS | 1 | 5 | |
| 16 | Bivalvia | *Ostrea stentina* Payraudeau, 1826 | NIS | 2 | | |
| 17 | Bivalvia | *Talochlamys multistriata* (Poli, 1795) | NIS | | 2 | |
| 18 | Hexanauplia | *Balanus trigonus* Darwin, 1854 | NIS | | 2 | 6 |
| | **Total** | | | **30** | **42** | **12** |

*Those species marked with plus symbol (+) are colonial species. In these cases, the number of specimens is the number of representative units of each colony (size 2 $mm^3$, number of individuals can vary).*

**Expected vs. Observed NIS Scenario**

Complete data to estimate the proposed NIS-ITS were available only for approximately a quarter (28.2%) of the potential invaders. Four out of the fifteen species (26.7%) with NIS-ITS values above 90% have been already detected in the port of Gijon (Table 1). Two of them [*Bugula neritina* (Linnaeus, 1758) and *Diplosoma listerianum* (Milne Edwards, 1841)] were found in the samplings from this study, and the other two were found in samplings from a previous report for the same port [*Botryllus schlosseri* (Pallas, 1766) (Nydam et al., 2017) and *Styela plicata* (Lesueur, 1823) (Miralles et al., 2016)]. Moreover, eight other NIS (Ficopomatus enigmaticus (Fauvel, 1923); *Diadumene lineata* (Verril, 1869); Acartia tonsa Dana, 1849; Callinectes sapidus Rathbun, 1896; *Rhithropanopeus harrisii* Gould, 1841; *Mytilopsis leucophaeata* (Conrad, 1831); *Mnemiopsis leidyi* Agassiz, 1865; and *Hypnea musciformis* Lamouroux, 1813) have also been reported in the Bay of Biscay in previous studies (Table 1). Therefore, 80% (12 NIS) of the potentially most dangerous species have already been detected in the Bay of Biscay area. In addition, two other NIS [*Watersipora subtorquata* (d'Orbigny, 1852) ITS = 2.593 (86%) and *Balanus trigonus* Darwin, 1854 ITS = 2.199 (73%)] that were also included in the expected NIS scenario have also been found in the samplings from this study (Table 2). Globally, from the total list of 252 NIS (including species with and without habitat suitability information), 12 species have already been reported in Gijon (6 were species detected in the samplings from this work) and 52 species (25.6%) in the Bay of Biscay (Supplementary Tables 3, 4).

## DISCUSSION

In this work, a representative case study in one of the main seaports in the Atlantic arc (Port of Gijon) was conducted as a proof of concept to be implemented in other international seaports with the aim of reaching real "blueports" (ports with an adequate management of resources and wastes that put in practice new strategies to avoid biological invasions) according to EU policy (European Commission, 2012). Prevention and early detection of NIS must be at the core of current port policies since they are the most cost-effective strategies for NIS management (CBD, 1992). However, some species may slip through prevention and detection efforts and might establish and become abundant pests (Lodge et al., 2006). Prioritization of efforts for particular "target species," defined as species previously identified as high invasion risks within a port, is indispensable for the correct management of biological invasions (McGeoch et al., 2016). On the other hand, biological port surveys set a baseline of native and non-indigenous port biodiversity. This knowledge can help in both, early NIS detection avoiding the proliferation and spread of NIS, and in the establishing of effective IAS management strategies (e.g., Ojaveer et al., 2014, 2018; Miralles et al., 2016, 2018; Marraffini et al., 2017).

The present study represents the first step to establish a NIS threat assessment procedure in the Port of Gijon and in all ports connected with it via maritime traffic. This biodiversity baseline can be consulted in future NIS assessments for the port. As a result, a list of potential NIS ranked by their Invasion Threat Scores (NIS-ITS) is now available. The biota list is now ready to be used by stakeholders and researchers, and should be updated from further samplings, and include species detected in the area from other studies. Fixing a specific score to consider a species a threat will always be a raw approximation or a little bit subjective decision. For example, Gallardo et al. (2016) set the threshold in 12 points out of the maximum 16 that a species can obtain.
Similarly, Tsiamis et al. (2020) set the limit in 38 points out of 48, so that species above this score will be considered as high-risk or high-likely invaders. Both studies use expert judgment to establish this threshold. In the case of NIS-ITS, authorities should pay special attention to those species over the 90% NIS-ITS (high likely invaders). Even more, most of the species with known invasive potential (invasion history score = 1) showed invasion threat scores over 70% (Figure 3). In fact, a posteriori analyses revealed that those species that according to the Spanish catalog of invasive species (BOE, 2013) are classified as IAS are all above the 70% threshold, except *Caulerpa taxifolia* Agardh, 1817, which obtained a 60% score due to very low habitat suitability in the Bay of Biscay area (Supplementary Table 3). These results indicate the importance of carefully studying the potential threat of those species with more than 70% NIS-ITS, which show specific traits that can make them especially dangerous.

Given this information, it is expected that specific port prevention strategies would be enriched and environmental monitoring would be developed in advance and thus improved. Currently, in addition to physical surveys, environmental DNA (eDNA) assays could also be a useful alternative for determining the presence of species based on organism vestigial particles that remain in the environment, negating the need to catch or observe the organism (Darling and Mahon, 2011; Borrell et al., 2017). It is also possible to design species-specific primers for the rapid, early detection of certain species of interest within a seawater community using eDNA (e.g., Devloo-Delva et al., 2016; Miralles et al., 2019).

The new NIS-ITS can be implemented and used by any other international port as a first line of defense against biological invasions without the need of experts. The index contains three components, and two of them (habitat suitability and invasiveness/impact) are based on joint efforts by researchers and are currently public and available for all stakeholders concerned about this challenge. The AquaMaps7 and the Global Invasive Species Database8 websites are constantly updated and improved, and they have incorporated relevant tools such as the Hawkins et al.
(2015) and Palomares and Pauly (2020) approaches, respectively. The third NIS-ITS component is a relatively easy-to-create port database that compiles all the destinations and cargo origins (traffic) within a port (e.g., 15 years for our case study at the port of Gijon). However, this score can be calculated in any case without a minimum data limitation. This last component could also be improved if the complete, and accurate,

itinerary of the cargo were available in all port registries and if treatments of the vessels hulls and ballast water management procedures were known. The full history of ships beyond immediate cargo origin can be important since hull foulers can be entrained in all locations visited. In the case of ballast water, knowing the origin and history or the water being discharged into Gijon is important and its origin is likely not where ships sourced cargo. Unfortunately, complete history of the vessels travels and treatments/procedures are not always available. Despite this, we propose how to incorporate BW and Hull Fouling treatments (if available) in our maritime traffic element when calculating the NIS-ITS in future studies.

This work is consistent with previous studies (Cardeccia et al., 2018) that indicate the need to collect data about the biological and ecological traits of NIS. These data can be used to generate habitat suitability information, which is necessary to calculate indices such as NIS-ITS that may facilitate the prevention of biological invasions. In fact, due to the lack of this information, we obtained only approximately 30% of the complete habitat suitability data for all the potential NIS travelers identified from the literature screening. At the same time, for some species with wide and abundant distributions in the Bay of Biscay, such as *Magallana gigas* (Thunberg, 1793), very low values of habitat suitability were found (0.110) (Supplementary Table 3). Most likely, aquaculture practices, which could imply diverse sources and sizes of the inoculums, periodic introduction events and differential extents of bottleneck processes in the new environments (Roman and Darling, 2007), could be a modulating factor currently not considered (but relevant) when predicting habitat suitability for those species. In the same way, although the IUCN Environmental Impact Classification for Alien Taxa (EICAT) was developed a few years ago and it is in use, data related to the impacts that are produced in the invaded areas are still lacking for many relevant IAS (Ojaveer and Kotta, 2015). One of the main recommendations that arises from this work is that more efforts and initiatives must be developed (and funded) to collect biological data for the main IAS and NIS around the world and that this information must be integrated into databases that are easily accessible for any type of stakeholder.

Another possible biological invasion risk for the port of Gijon, which was not considered in the newly proposed NIS-ITS, could originate from other surrounding marinas. There have been multiple cases where recreational vessels, yachts and boats (not industrial traffic) have been cited as either the primary introduction vector or secondary spread vector for an invasive species (Floerl and Inglis, 2005; Davidson et al., 2010; Clarke-Murray et al., 2014; Ulman et al., 2019; Iacarella et al., 2020). For large industrial ports, traffic data are often accessible and public but recreational vessel traffic data are hard to collect and compile (Le Tixerant et al., 2018). A relevant tool to integrate this information into biological invasion predictions could be tracking ship movements with an automatic identification system (AIS) (EU, 2002; Merchant et al., 2012; Ulman et al., 2017). Coastal and offshore area data from satellite-based AIS (Loretta, 2016) could provide information about the spatial and temporal distribution of shipping (Shelmerdine, 2015). However, AIS transmitters are only required on larger ships, passenger vessels and large fishing vessels. Thus, millions of recreational vessels worldwide are not required to have an AIS, which makes it difficult to develop AIS-based models (Hermannsen et al., 2019). Despite this issue, it is clear that in the future, NIS Invasion Threat Scores should include recreational vessel traffic data from surrounding marinas when estimating a specific port's ITS and predicted scenarios.

Despite the previously mentioned limitations, in the predicted port of Gijon NIS scenario based on the new ITS, we found 15 marine species with values above the 90% ITS. After sampling macroinvertebrates on the port structures and in the hulls of vessels (as conducted during routine water quality inspections within ports; see Borja et al., 2015), 26.7% of these high-threat species were observed in the port. The outcome revealed that eight other NIS have also been reported in or near the port of Gijon (south Bay of Biscay) (Table 1). These results increase the percentage of NIS findings with regard to the previously described expected NIS scenario with an 80% coincidence level. This scenario could indicate that expected species arrivals based on the NIS-ITS could be, even with the current limitations, close to reality and should be considered at the time of developing prevention/management port strategies. In the case of Gijon, specific management plans must be developed for the species already present in the port. This is also necessary for those NIS that are not present in the port yet but were classified as high-threat species by the NIS-ITS, indicating their potential to become future invaders that are spread by port industrial activity and traffic (Colautti et al., 2006; Floerl et al., 2009; Bishop and Hutchings, 2011; Ojaveer et al., 2018).

One important discovery here was that three species were found on boat hulls and not in the port where they were sampled in. Considering that only 3 out of 22 ships authorized hull samplings, it seems that Spanish port policies should be updated in correspondence with the great threat that biological invasions are for biological diversity. When vessels move from one port to another, organisms can fall off the hulls and may become introduced into a new host environment (Dobroski et al., 2017). Thus, mandatory routine monitoring, along with periodic inspections on arriving boats should be implemented to detect new arrivals of NIS (Bishop and Hutchings, 2011), and marine stakeholder participation (authorities, ship owners, scientists and citizens) will be fundamental.

The port of Gijon is not only a destination for exotic species, it can also be the origin of new introductions to other regions. For example, native species that commonly inhabit the port structures can be transported to other areas outside their native range where they can display invasive features. This study also provides a list of native species at risk of being inoculum (port of Gijon as a donor) to spread/start new invasion processes overseas, since the results of the bibliographic search showed that they have the ability to use maritime traffic as a vector for expansion. As an example, *Carcinus maenas* (Linnaeus, 1758) is native to the European coasts (where the port of Gijon is located) but has been listed as one of the "100 worst invasive species" as it has become invasive in areas like the northwest Pacific or the southwest Atlantic because it is a voracious predator that affects epibenthic and infaunal species through predation, competition and burrowing activities (Pollard and Hutchings, 1990; Bravo et al., 2007; Briski et al., 2011; Edgell and Hollander, 2011; Lim et al., 2017). This is why this list can be very useful in other regions and these species can be considered in future risk assessments.

Globalization has increased international marine traffic, particularly the shipping industry (Tournadre, 2014; Halpern et al., 2015), by moving not only people and goods but also NIS around the world (Hulme, 2009). It is urgent to develop effective prevention strategies and management plans of biological invasions. Each international port should develop its expected NIS scenario and prepare themselves for early/rapid detection of high-risk species. Thus, the use of the new NIS-ITS index proposed here (where there is not the need of experts) and the implementation of species-specific detection tests using eDNA or species-specific physical sampling strategies within ports will be of help for that. Finally, annual biota monitoring within ports is fundamental and must be mandatory. These data will help update global data about port biodiversity (and its risks) and to obtain substantially more insights into NIS colonization dynamics and a clearer picture of the real influence of maritime traffic on marine biological invasion processes worldwide.

Together with these ecological studies, it is necessary to put into practice other strategies related to the management of shipping activities that facilitate the spread of NIS, such as the implementation of mandatory periodic hull cleanings, discharges of ballast water at least 200 nautical miles from the nearest land or the implementation of specific policies related to the boat treatments in recreational boating that can avoid the dispersal of species (Anwar, 2016; IMO, 2018). The combination of these methods can lead to a better understanding and management of biological invasions and a significant reduction of environmental risks, leading to a real blue economy.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MN185333-MN185374; MN164033-MN164348.

## AUTHOR CONTRIBUTIONS

LM: methodology, formal analysis, investigation, writing—original draft, and writing—review and editing. AI: methodology, formal analysis, investigation, visualization, and writing—review and editing. MG: project administration and funding acquisition. EG-V: conceptualization, supervision, project administration, and funding acquisition. YB: methodology, investigation, conceptualization, supervision, project administration, and funding acquisition, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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## CONFLICT OF INTEREST

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


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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.633118/full#supplementary-material.

## FOOTNOTES

1. https://easin.jrc.ec.europa.eu/
2. https://www.puertogijon.es/en/
3. https://www.puertogijon.es/en/port/sustainable-port/
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# Capítulo 2

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

Ibabe, A., Miralles, L., Carleos, C.E., Soto-López, V., Menéndez-Teleña, D., Bartolomé, M., Montes, H., González, M., Dopico, E., Garcia-Vazquez, E. & Borrell, Y.J.

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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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## HIGHLIGHTS

•eDNA Metabarcoding showed to be an effective tool for conducting environmental evaluations in ports and coastal waters.

•The Blue-gNIS index is proposed, which considers exotic species to perform environmental assessments of marine ecosystems.

•A preliminary calibration showed significant correlations among Blue-gNIS and other existing indices such as gAMBI or ALEX.

•Fourteen exotic species were detected and the port of Gijon was classified in a good ecological status by the Blue-gNIS.

## 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.



**KEYWORDS:** Metabarcoding; Invasive species; AMBI; Biotic index; Blue-gNIS; Blueports


## 1.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, 2015; McCauley 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 and 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, andunstandardized 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.

## 2. MATERIALS AND METHODS

### 2.1. 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 ha and has more than 7000 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 (Fig. 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 90 cm2 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.

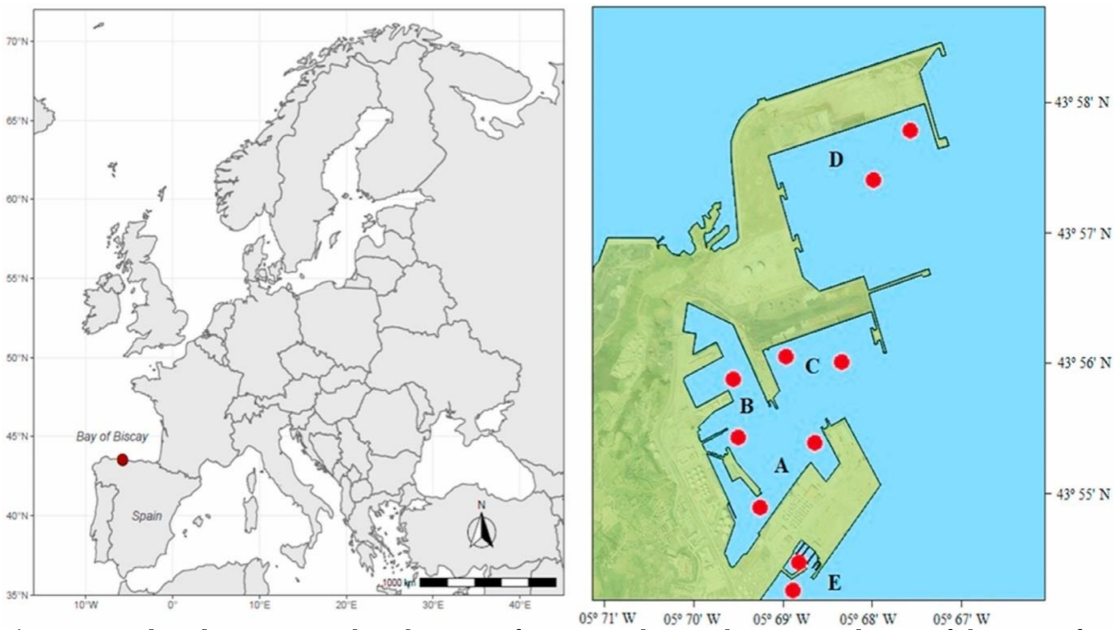

**Fig. 1**. Sampling locations within the Port of Gijon in the southern central area of the Bay of Biscay (5°41′W, 43°34′N).

### 2.2. 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 1 L 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 5 °C) 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 (50 ng μL-1) belonging to 9 different macroinvertebrate species and another 10 additional species (Supplementary Table 1).

### 2.3. 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, 1×: 95 °C for 3 min; 25×: 95 °C for 30 s, 44.7 °C for 30 s and 72 °C for 30 s; and finally, 1×: 72 °C for 5 min followed by a 4 °C hold. Conditions for the index PCR were: 1×: 95 °C for 3 min; 8×: 95 °C for 30 s, 44.7 °C for 30 s and 72 °C for 30 s; and finally, 1×: 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 and 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., 2019). 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.

## 2.4. 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 (Fig. 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. Editorial Board, 2015), DAISIE (Roy et al., 2019) ISSG (http://www.issg.org/database), GRIIS (http://www.griis.org), CABI (CABI, 2019), Algaebase (Guiry and 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 (Fig. 2).

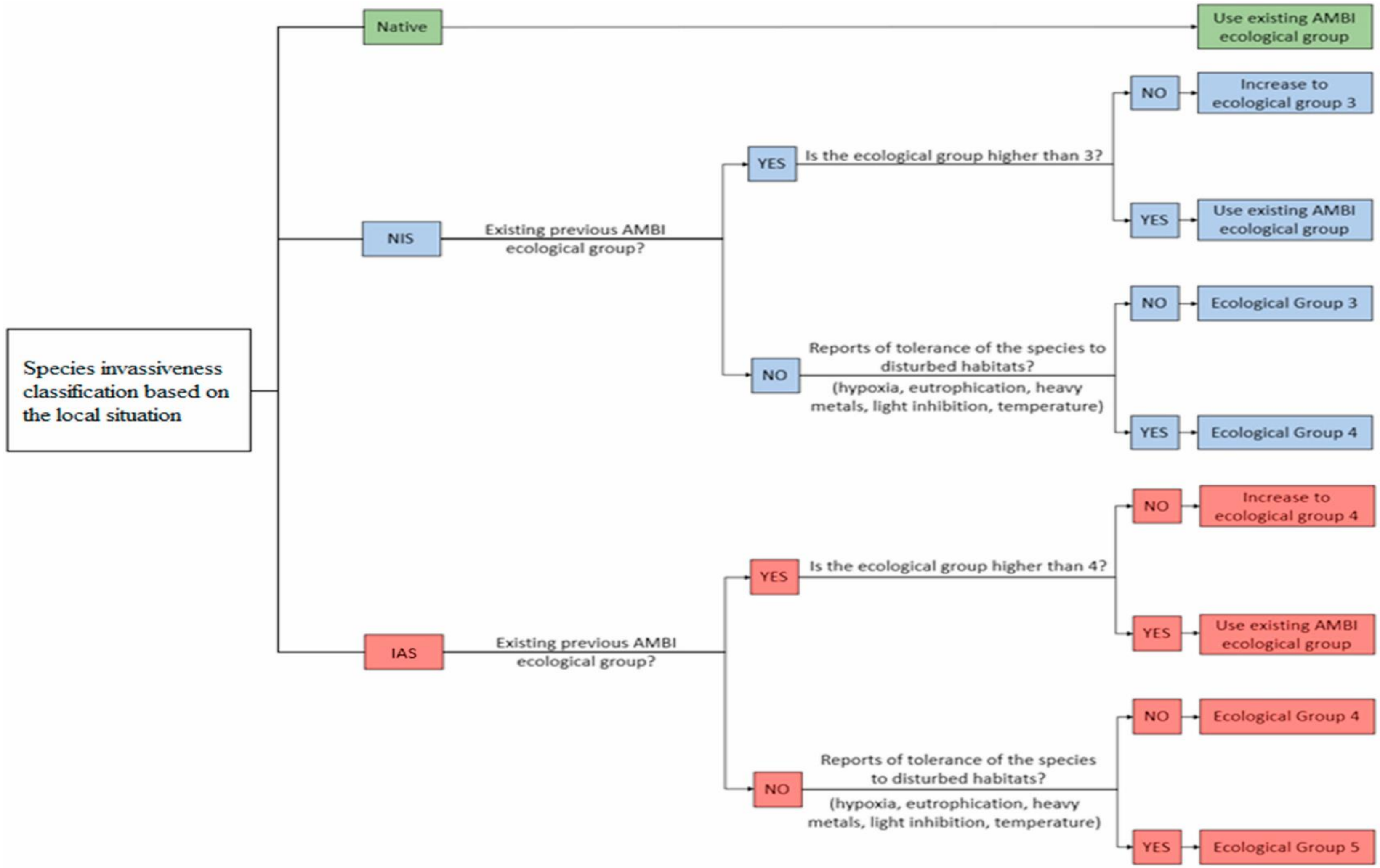


**Fig. 2**. Criteria for the classification of species into ecological groups for Blue-gNIS estimations.

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 (Fig. 2).

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).

### 2.5. 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).

### 2.6. 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.

## 3. RESULTS

### 3.1. 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 r2 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 (Fig. 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).

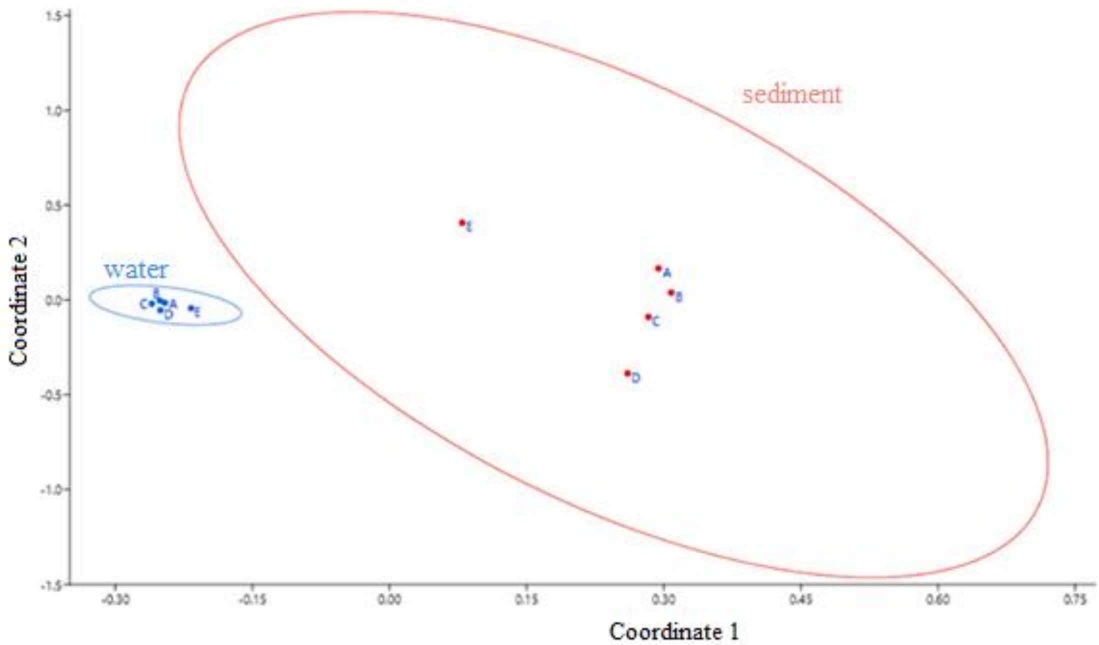


**Fig. 3**. Non-metric Multidimensional Scaling of the metabarcodes found in each station for water (blue dots) and sediment (red dots) samplings in the Port of Gijon, Bay of Biscay. Circles indicate 95% confidence ellipses. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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 (Fig. 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.

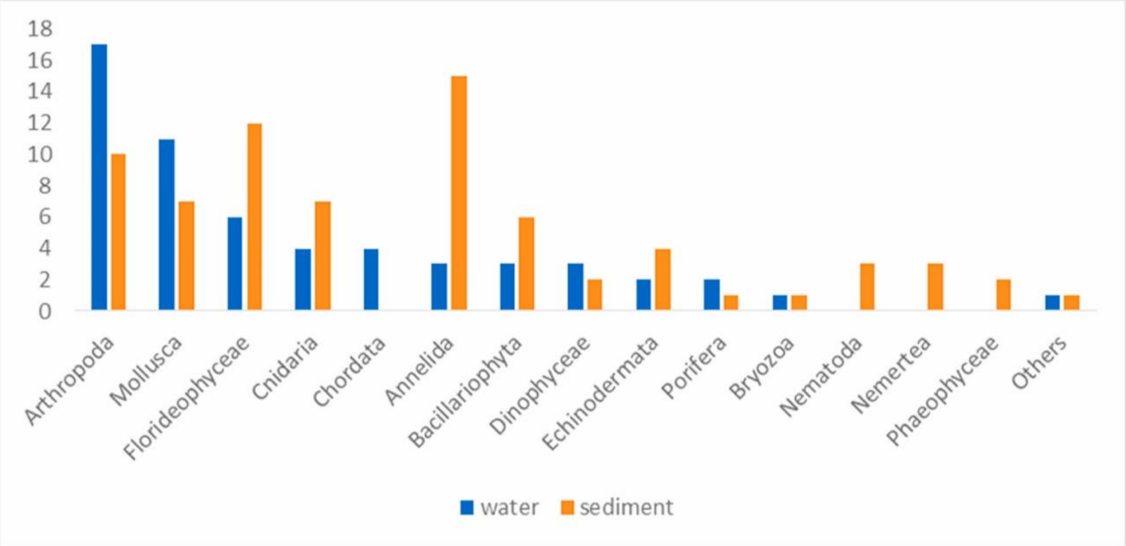


**Fig. 4**. Number of OTUs detected using metabarcoding for major taxa in sediment and water samples from the Port of Gijon, Bay of Biscay.

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 Afrika | - | - | 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 | | | | |

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 (Fig. 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 (Fig. 5b).

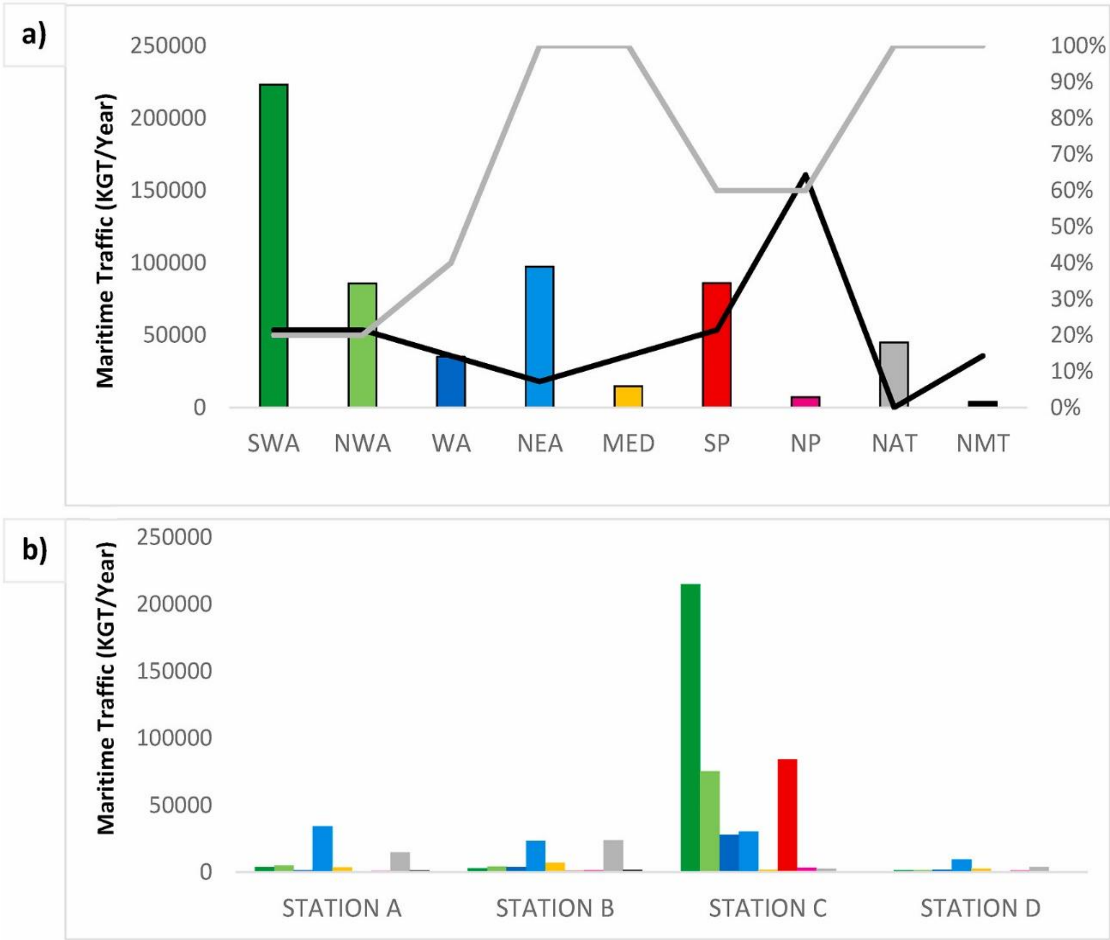


**Fig. 5**. a) Different traffic origins and corresponding global ships tonnage arrived to the Port of Gijon (Period, 2004–2017) from South West Atlantic (SWA), North West Atlantic (NWA), Western Africa (WA), North East Atlantic (NEA), Mediterranean (MED), Southern Pacific (SP), Northern Pacific (NP), 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.

### 3.2. 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) |

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

## 4. 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%–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.

### 4.1. 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.

### 4.2. 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 (AquaNIS. Editorial 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.

## CRediT AUTHORSHIP CONTRIBUTION STATEMENT

**A. Ibabe**: Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **L. Miralles**: Methodology, Investigation, Formal analysis, Writing – original draft. **C.E. Carleos**: Methodology, Writing – review & editing. **V. Soto-López**: Methodology, Investigation. **D. Menéndez-Teleña**: Methodology, Investigation. **M. Bartolomé**: Methodology, Investigation. **H.J. Montes**: Methodology, Investigation. **M. González**: Methodology, Investigation. **E.**

**Dopico**: Conceptualization, Project administration. **E. Garcia-Vazquez**: Conceptualization, Supervision, Project administration, Funding acquisition. **Y.J. Borrell**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

## 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://www.sciencedirect.com/science/article/pii/S0141113621000969?casa_token=wwxSq_G65loAAAAA:ekDNlpm31CT9JL8x_TrKaO2m8Svds4Y6JLv4f6g6M-VAUitPVXCNZY5Fc6Vwf1tWN-6fdCFXJ54#appsec1

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# Capítulo 3

**Environmental DNA from plastic and textile marine litter detects exotic and nuisance species nearby ports.**

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# Environmental DNA from plastic and textile marine litter detects exotic and nuisance species nearby ports.

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## ABSTRACT

Marine debris is currently a significant source of environmental and economic problems. Floating litter can be employed by marine organisms as a surface to attach to and use as spreading vector. Human activities are promoting the expansion of potentially harmful species into novel ecosystems, endangering autochthonous communities. In this project, more than 1,000 litter items were collected and classified from five beaches eastwards the port of Gijon, in Asturias, Spain. Next generation sequencing was employed to study biofouling communities attached to items of different materials. A dominance of DNA from Florideophyceae, Dinophyceae and Arthropoda was found, and four non-indigenous species (NIS) were identified. Results showed a clear preference of Florideophyceae and Bryozoa to attach on textile surfaces versus plastic ones. Considering that these taxa contain several highly invasive species described to date, these data emphasize the potential of textile marine debris as a vector for dispersal of NIS. Moreover, the closest beaches to the port contained a more similar biota profile than the farther ones, confirming that both plastic and textile marine litter can be vectors for species dispersal from ports.

**KEYWORDS**: Marine debris; Biofouling; Next generation sequencing; non-indigenous species

## INTRODUCTION

Human activities have been triggering environmental changes all over the world since the beginning of intensive production methods. Human activities such as agriculture, fisheries, or industry, overexploit natural resources, and as a result, rates of species extinction are now 100 to 1000 times higher than prior to human influence [1]. A huge amount of the waste produced from this excessive human activity is ending up in the ocean, altering marine ecosystems. These materials are known as marine debris or marine litter. This problem has led to a difficult situation, not only for the conservation of marine ecosystems, but also for human health and economic activities. Plastic litter that is floating on the oceans is an important cause of mortality for many animals such as marine mammals, seabirds or turtles, either because they ingest it [2–3] or because they get entangled [4–6]. In addition, marine litter causes important economic losses in industries, such as fisheries, because of the time spent cleaning the debris from nets and net losses. As an example, marine plastics cost between $15 million and $17 million per year to the Scottish fishing industry [7]. Tourism can also suffer negative impacts due to the presence of marine litter on the coasts, which can affect the public perception of the quality of the surrounding environment and lead to a loss of income for this sector [8]. Besides, the degradation of plastic debris produces microplastics that can be transferred into the food chain and affect humans that consume them indirectly via contaminated marine food; this exposure to microplastics can result in chromosome alteration which can lead to infertility, obesity and cancer [9–10].

The role of marine debris as a dispersal vector of invasive organisms is of special concern [11]. Marine litter promotes the establishment and dispersal of NIS. It can provide a surface for colonizing species, facilitating their spread to new habitats [12]. Newly entered colonizers can get stablished and become alien invasive species (AIS) that alter the local ecosystem affecting the native organisms in several ways (competition, predation, habitat alteration, transmission of exotic diseases to local species) [13–15]. In addition to the impacts on local biodiversity, AIS have also severe impacts on the economy. In the United States, more than $138 billion are used every year to control new colonizers or to avoid infections of non-indigenous diseases [16]. Aquaculture industries are also affected by AIS that can alter the productivity, as in the case of Undaria pinnatifida which forms dense mats and obstructs light inhibiting shellfish growth [17], or Carcinus maenas which consumes native commercially important clams in Tasmania [18].

Identifying the biota that arrives in the local ecosystem is the only way to detect alien species and to control invasions. However, quite often invaders are spread in an early ontogenetic stage (e.g. eggs, larvae or algae propagules) and they are not visually identifiable, thus non-indigenous individuals may remain undetected until they are already adults and start reproducing and expanding [19–20]. Exhaustive monitoring is needed, but there is low probability of finding NIS because of their low density [21–22]. Identification based on organism morphology requires expert taxonomists specialized on the taxa to be analyzed, and often (especially in early development stages) identification cannot be done to a species level, limiting it to higher groups such as genus or family, which would not be useful for non-indigenous species identifications [23].

More recently, new techniques have been developed and species identification can be done based on sequencing and analyzing nucleic acids extracted from environmental samples [24], also called environmental DNA or RNA (eDNA, eRNA). Metabarcoding is a well-established method for the detection of NIS and for biosecurity applications [25–28]. In fact, techniques based on eDNA are advantageous when detecting species with low densities (such as exotic species at their arrival and before establishing), as very low DNA concentrations may be enough to find a species when the individuals are still very scarce and/or small [29–30].

Predicting invasions requires understanding the process of the invasion [31–32]; it is therefore crucial to understand how marine debris is spread, and to study the organisms with the capacity of attaching to these surfaces. Among some of the extensive work done on NIS transport via marine debris [33–40], some studies have shown the ability of biota to perform extreme transoceanic travels and survive over years attached to floating litter. For example, in 2011 a massive tsunami launched debris from the Japanese coast to Hawaii and North American shores. More than 280 living organisms native to Japan were documented attached to debris [41].

However, to our knowledge no research has examined the role of textile litter as a vector. We considered as textile litter, the disposed waste created during fiber and clothing production and the waste created by consumers use and disposal of textile products (including certain parts of sanitary pads that were classified as textile litter in this study). Ports are potential donors of both, marine litter and invasive species, therefore, studies on possible vectors employed by biota inhabiting ports are needed, in order to predict potential future invasions and dispersions from these areas.

In this study, biota attached to litter items of different artificial materials were characterized by using next generation sequencing of DNA extracted from the biofilm, to analyze the composition of the communities inhabiting the marine debris. In order to assess a potential origin for biota found on litter, it was compared to the one growing on structures in the Port of Gijon (central south Bay of Biscay, Spain), where several NIS and AIS have been reported [42].

## MATERIALS AND METHODS

### Sampling

Beaches east of Gijon port were selected for our study for two reasons: (1) Gijon is a potential donor of marine invasive species; (2) in the winter, at the time of the study, dominant currents flow eastward along the coast [43], likely depositing debris from Gijon on beaches to the east. Therefore, five beaches located east of Gijon port were selected for litter sampling: Arbeyal, El Rinconin, Peñarrubia, Cagonera and La Ñora (Fig 1). No special access permits were needed as all samples were collected in public beaches.

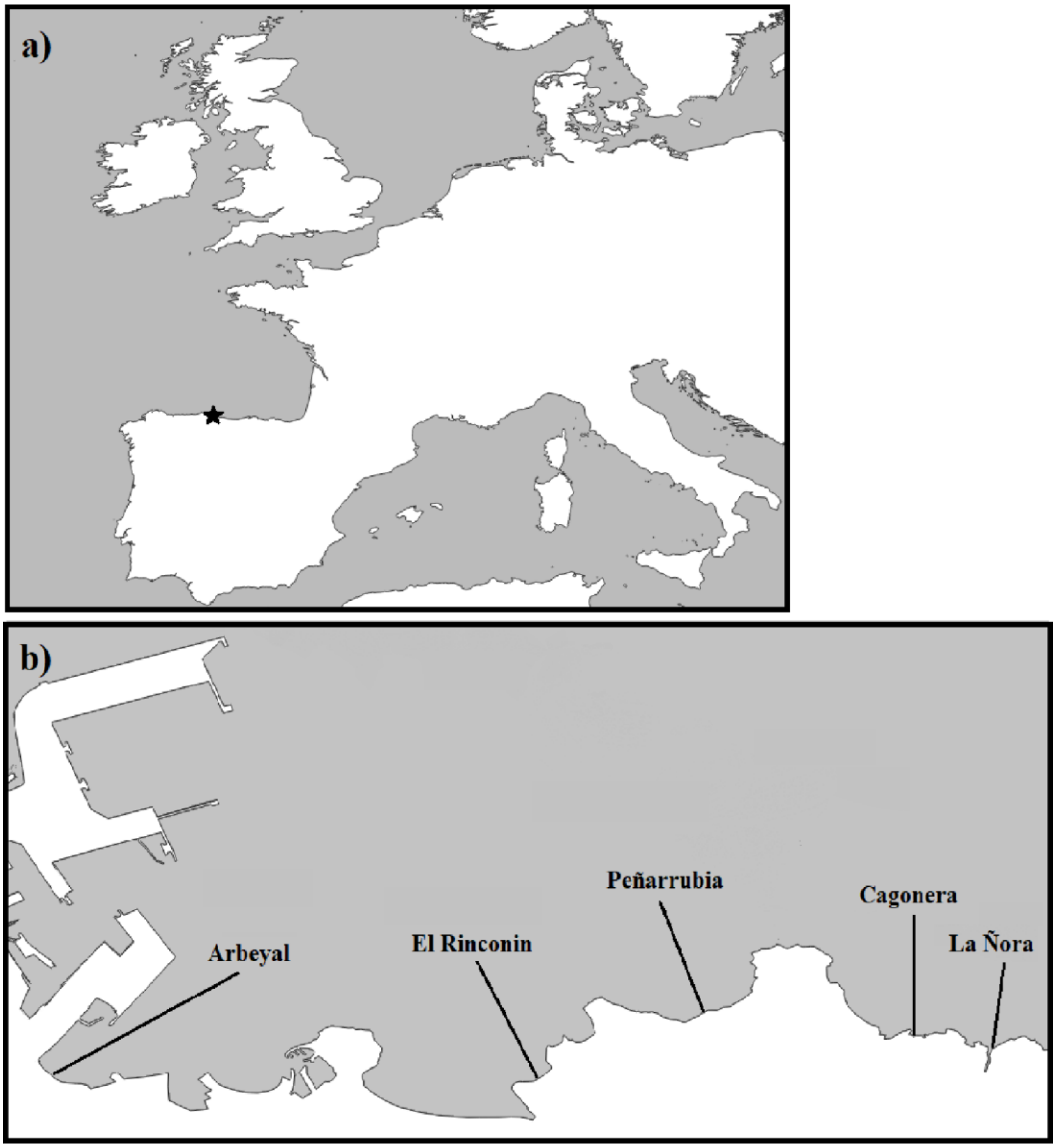


**Fig 1**.a) Location of the sampled area in the Northern coast of Spain. b) Location of the five sampled beaches eastwards the port of Gijon.

From 13th to 17th of January 2017, litter items were collected from the five beaches. Sampling was carried out during the lowest diurnal tide (starting 2 hours before and ending 2 hours after) in order to increase the beach surface available to sample. The whole beach surfaces were sampled during these high-coefficient low tides and all litter pieces bigger than 5cm were taken. No transects nor quadrats were employed, as all the surface was analyzed and every litter piece was collected.

For a posterior characterization of the beaches, the litter was classified in situ in different types: sanitary pads, textiles, plastic bags, plastic bottles, expanded polystyrene (EPS) fragments, fishing gear, and others. After classification, only litter items containing visible biofilm were stored for posterior analyses. Samplings were taken in winter, at temperatures below 10°C and most of the collected litter items were discarded and recycled due to the lack of biofilm. A total of 16 items or item fragments from the beaches which were representative of the litter profile on each beach (approximately 0.25% of the total litter surface), were collected in sterile tubes and stored in ethanol for further biofilm sampling and extraction of eDNA.

## Taxonomy

For the names of the species, we followed the taxonomic nomenclature from the World Register of Marine Species [44]. Regarding the status of the species detected visually and employing DNA, NIS and AIS were identified from the European Network of Invasive Alien Species Database NOBANIS [45].

## Environmental DNA extraction and Metabarcoding

From all the litter items that were stored in ethanol, only 16 items belonging to different beaches and different types of material were selected for the eDNA extraction as the rest of the collected litter did not show any biofilm attached. Sterile swabs and gauzes were used to collect the attached biofilm from the litter (Fig 2) by scratching the surface. Sterile DNA/RNA free distilled water was used to rinse and clean the surface.

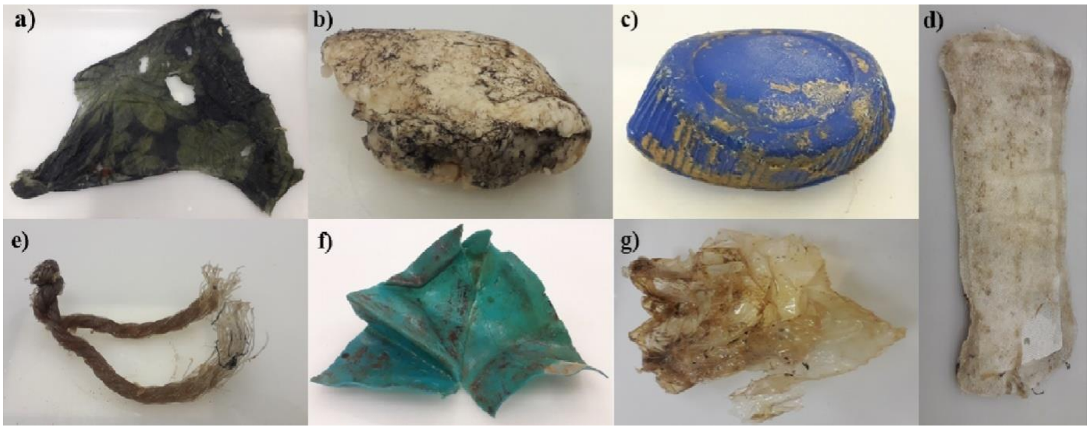


**Fig 2**. Different sampled litter types showing biofilm that was scratched for eDNA extraction. a) Fabric piece b) Expanded polystyrene c) Plastic bottle d) sanitary pad e) Fishing gear f) Plastic fragment g) Plastic bag.

After the biofilm was recovered from the litter, the cotton extremes of the swabs were cut and collected with the gauzes in 15ml Falcon tubes with the water that was also employed to remove the biofilm. Then they were macerated for 2 minutes using a Stomacher 80 biomaster (Seward, UK) which was cleaned after each use with different samples to maintain sterility. A negative control was prepared for this whole procedure, by using sterile swab and gauze extremes and by suspending them into Sterile, DNA/RNA free distilled water. Once the Stomacher finished, excess liquid was squeezed from the swabs and gauzes and the suspension was pelleted by centrifugation (3000 x g 15 min) following the procedure reported by Pochon et al. (2015) [46]. The supernatant was discarded and then DNA was extracted from the pellet using an E.Z.N.A® Soil DNA Kit (Omega Bio-tek, USA) following the manufacturer's instructions.

The primers mICOIintF and jgHCO2198 [47] were employed to amplify a fragment of ≈300bp within the COI gene (miniCOI). Both primers were modified to include the specific sequences needed for Ion PGM libraries. A single common forward primer was used. Reverse primers were modified to include barcodes for each of the samples, so 16 different barcoded reverse primers were used. Each barcode has a known sequence to identify the samples after the whole process. Before sequencing, the quantity and quality of the DNA from PCR products was measured using Bioanalyzer (Agilent technologies). The PCR reactions were performed using negative controls to monitor possible contamination. Thermocycling conditions were: 1x: 95°C for 5 min; 35x: 95°C for 1 min, 48°C for 1 min and 72°C for 1 min; 1x: 72°C for 5 min and 4°C on hold. The amplicons were analyzed directly in the platform Ion Torrent PGM (ThermoFisher Scientific, USA), in the Unit of DNA Analysis of the Scientific & Technical Services of the University of Oviedo.

**Bioinformatics pipeline for analysis of NGS data**

Bioinformatics analyses were performed using QIIME1.9, an open-source bioinformatics pipeline [48]. Firstly, an initial screening was carried out in order to select reliable sequences, with a quality value > 20 and a length >200 bp. For taxonomic assignment, instead of using the whole GenBank as a reference, a specific database containing only eukaryotic COI sequences was generated with the script entrez.qiime (Chris Baker. ccmbaker@fas.harvard.edu. Pierce Lab, Department of Organismic and Evolutionary Biology, Harvard University). An initial assignment was made considering a minimum identity of 97% and an E-value of 1e-10 as these conditions were considered enough to obtain reliable species identification from COI barcodes [49]. In addition, assignments were also done employing minimum identity of 95% and E-value of 1e-50, to compare results. From the operational taxonomic unit (OTU) table obtained after the assignment, only marine and brackish taxa were retained for further statistical analysis. A subset of 50 sequences assigned to a species level from each parameter set were randomly taken from the OTU table. To double-check the reliability of the taxonomic identification of these sequences, they were assigned manually against GenBank using NCBI´s BLAST web browser (NCBI webpage, accessed July 2019).

**Statistical analyses**

The statistical analysis was carried out with parametric or non-parametric tests done in PAST program [50] after checking normality in the dataset. For beach litter composition, the proportion of each type of debris was compared among beaches using non-parametric contingency Chi-square, confirmed from Monte Carlo procedure (n = 9999 permutations). The litter composition was compared between pairs of beaches using Euclidean distance, and the results visualized in a plot constructed from non-metric multidimensional scaling (nmMDS) analysis after checking stress and r2 in a Shepard plot.

The DNA dataset was analyzed with the following variables: the number of species of each taxon, the total number of species, the proportion of exotic species over the total number of species in each sample. Sequences assigned to terrestrial species and assignment artifacts (singletons and wrong species assignments due to the scarcity of reference sequences for certain taxa on NCBI database) were excluded from the analysis. Comparisons of the average number of species on plastics (as plastic bags, plastic bottles, buoys and expanded polystyrene) and textile objects (including sanitary pads and fabric pieces) were done using non-parametric Mann-Whitney tests. The community inferred from metabarcoding was compared between pairs of items using Gower's general similarity coefficient for presence-absence of each species, and nmMDS analysis was conducted as above. The same PAST software by Hammer et al. (2001) was employed.

## RESULTS

### Beach litter

Beach surface area ranged from 2500 m2 in El Rinconín to 17500 m2 in La Ñora. A total of 1023 litter objects were found on the beaches; the corresponding densities were between 1.26 and 4.57 items/m2 in Arbeyal and Peñarrubia respectively (Table 1). Considering the litter surface area, it was between 2.46 cm2 of litter/m2 of beach in the cleanest Arbeyal to 18.6 cm2 of litter/m2 in the most littered Peñarrubia (Table 1). For litter surface La Ñora joined the group of more polluted beaches together with Peñarrubia and Rinconín, while for the number of items La Ñora beach was closer to the least polluted Arbeyal and Cagonera showing that few but big litter pieces were found on this beach.

**Table 1**. Characteristics of the beaches sampled from the central south Bay of Biscay.Beach surface in m2. The litter density is given in surface as cm2 of litter per $m^2$, and as litter items per $m^2$.

| | Arbeyal | Rinconín | Peñarrubia | Cagonera | La Ñora |
|---|---|---|---|---|---|
| **Type of beach** | Urban | Urban | Rural | Rural | Rural |
| **Substrate** | Sand | Sand | Pebble | Pebble | Sand |
| **River** | No | No | No | No | Yes |
| **Beach surface area** | 14000 | 2500 | 8250 | 10625 | 17500 |
| **Latitude** | 43.5445N | 43.5483N | 43.5518N | 43.5501N | 43.5471N |
| **Longitude** | 5.6934W | 5.6390W | 5.6237W | 5.6100W | 5.5897W |
| **Litter density ($cm^2$ of liter /$m^2$ of beach)** | 2.46 | 9.01 | 18.61 | 2.93 | 10.46 |
| **Litter density (number of items/$m^2$ of beach)** | 1.26 | 4.20 | 4.57 | 1.29 | 1.30 |

The majority of litter (61.9%) was plastic, 33.9% was textile and only 43 objects (4.2%) were other materials. Textile items were mostly clothes but also sanitary pads (compresses) were included in this group although they are mainly composed by plastic. This is explained because eDNA for extraction was only taken from their textile part, which was the one with macroscopically observable biofilm. The five beaches were significantly different from each other for the type of litter ($\chi2$ = 837.94; d. f. 40; p = 6.31x10-150; Monte Carlo p = 0.0001). For example, in Cagonera there were more textile items, while in La Ñora the predominant litter was small plastic pieces (Fig 3). Abandoned, lost or otherwise discarded fishing gears (plastic ALDFG) were found in all the beaches except in Arbeyal (the urban beach closer to Gijón port). None of this fishing gear showed any metallic parts (they were completely composed by plastic), in fact, metallic objects, like cans, were scarce in all beaches. They were found only on Rinconin beach (Fig 3).

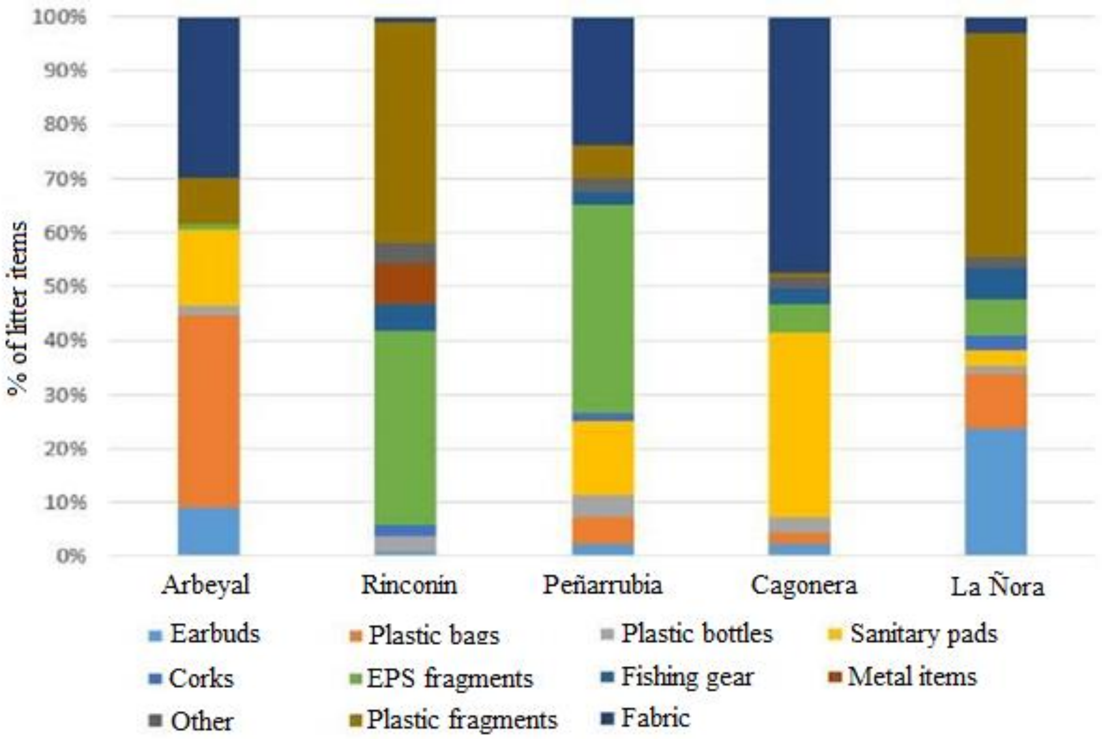


**Fig 3**. Litter composition in the five beaches analyzed in this study, presented as proportion of each type of item.

The nmMDS based on Euclidean distances had stress of 0, r2 of 0.865 and 0.002 for the axis 1 and 2 respectively. Beaches were similarly connected in the minimum spanning tree regarding both, similarities for litter composition (Fig 4A), and similarities for biota composition (Fig 4B). Beaches that were richer in plastics (Rinconín and La Ñora) were quite proximate to each other but separate from those rich in textile (Arbeyal and

Cagonera). Peñarrubia was isolated. The same connection was obtained for biota comparisons, with Peñarrubia beach again being the most different one.

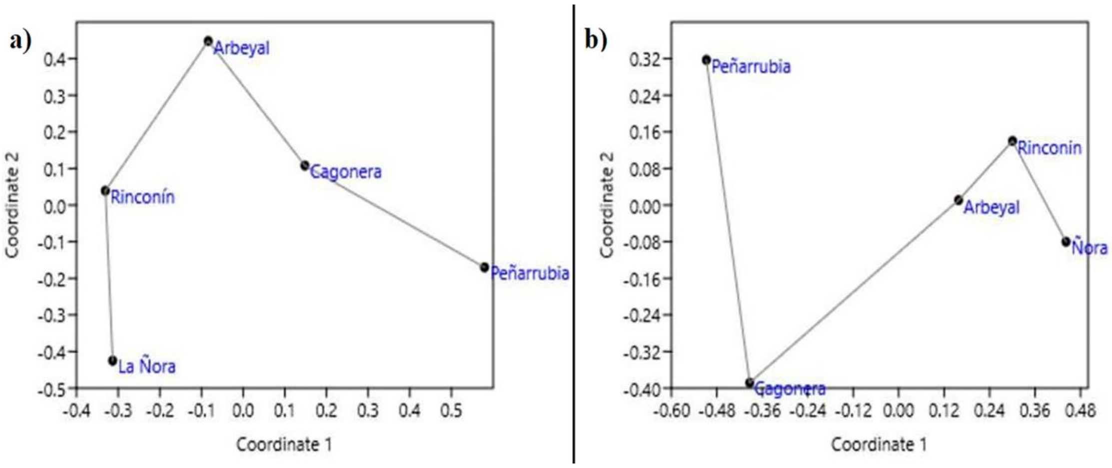


**Fig 4**. Non-metric multidimensional scaling analysis of the litter composition (a) and the litter biofouling biota identified from DNA (b), in the five analyzed beaches. Scatter plots constructed from pairwise Gower distances. The minimum spanning tree is presented.

### Biota on litter items identified with next generation sequencing

The surfaces sampled for biofilm and their composition are presented in Table 1. from all the litter items that were found (more than 1000), only the ones containing visible biofilm were stored for eDNA sequencing. In total they corresponded to 16 litter items from the different beaches, accounting for approximately 0.25% of the total litter surface. Only biofilms from 12 samples (from the initial 16 samples) provided DNA of quality to be successfully PCR-amplified and sequenced (Table 2). DNA sequences were not obtained from four expanded polystyrene pieces. For the 12 remaining biofilm samples, nine were from plastic objects and three were from textiles.

The initial screening left 278 124 sequences (Table 2) that were useful for species assignments since they passed the quality filter (sequences >200bp and with a quality value >20). Although the same DNA amount of each sample library was employed for next generation sequencing, results were dissimilar, as for some samples much more sequences were obtained than from others (Table 2). The polystyrene piece from Peñarrubia (P-P3) was the sample from which more sequences were obtained (> 90000), while the plastic fragments from Cagonera (C-P4) provided the smallest number of sequences. After OTU assignment 66% of the sequences in P-P3 were lost (still remaining > 30000 sequences), and for the sample C-P4 none of the sequences assigned to a species with the employed BLAST criteria. So finally, biofilm communities were inferred from only 11 samples.

**Table 2**. Raw and filtered NGS results. Litter surfaces used for biofilm analyses with the codification for each liter item (initial letter of the beach; P for plastic, T for textiles) concentration of eDNA as ng/μL, number of reads obtained before and after quality filters, and number of sequences assigned taxonomically after the final BLAST.

| Beach | Litter type | Material | Code | eDNA concentration (ng/μL) | Before quality filter (number of sequences) | After quality filter (number of sequences) | After BLAST (%97, $<E^{-50}$) (number of sequences) |
|---|---|---|---|---|---|---|---|
| La Ñora | Plastic Bottle | Plastic | Ñ-P1 | 2.66 | 57596 | 8476 | 969 |
| | Plastic Bag | Plastic | Ñ-P2 | 1.45 | 110931 | 33004 | 3417 |
| | Sanitary pad | Textile | Ñ-T1 | 2.09 | 8024 | 5915 | 97 |
| | Expanded polystyrene | Plastic | Ñ-P3 | - | - | - | - |
| Cagonera | Plastic fragment | Plastic | C-P4 | 1.88 | 1597 | 540 | 0 |
| | Fishing gear | Plastic | C-P5 | 2.22 | 99716 | 38098 | 1379 |
| | Sanitary pad | Textile | C-T1 | 1.98 | 93725 | 38877 | 3137 |
| | Expanded polystyrene | Plastic | C-P3 | - | - | - | - |
| Peñarrubia | Fabric piece | Textile | P-T2 | 1.79 | 14414 | 8501 | 1193 |
| | Expanded polystyrene | Plastic | P-P3 | 5.63 | 370143 | 91354 | 30241 |
| | Plastic Bottle | Plastic | P-P1 | 3.54 | 103369 | 24871 | 366 |
| Rinconín | Buoy | Plastic | R-P5 | 2.09 | 88324 | 19754 | 53 |
| | Expanded polystyrene | Plastic | R-P3 | - | - | - | - |
| Arbeyal | Plastic Bag | Plastic | A-P2 | 2.17 | 5332 | 707 | 81 |
| | Plastic fragment | Plastic | A-P4 | 3.45 | 21964 | 8027 | 131 |
| | Expanded polystyrene | Plastic | A-P3 | - | - | - | - |

Species assignments made with a minimum identity of 90% and an e-value of 1e-10 retrieved many hits (S1 Table), but the reliability was too low because 82% of the manual individual BLAST did not assign the OTU to the same species. For >97% identity with the same e-value of 1e-10, despite much fewer significant hits retrieved, 45% of the sequences checked manually were assigned to a different species using manual BLAST. With a more stringent e-value of 1e-50 and 90% identity, the number of discrepancies between QIIME pipeline and the manual BLAST assignations was 22%. Finally, with an e-value of 1e-50 and 95% identity, all the putative species identified from QIIME coincided with those retrieved from manual BLAST. However, in order to increase the number of assignments to species level and not only to genus, a minimum identity of 97% was chosen, so that the final employed conditions were a minimum identity of 97% and e-value of 1e-50. Although 85% of the initial sequences were lost due to these highly stringent parameters the identifications obtained were very robust, as deduced from total coincidence with the manual BLAST.

In total, 122 species were identified from the eDNA present in the sampled litter. Homo sapiens and other non-marine species were detected, such as insects, mammals and freshwater organisms, but they were not considered for posterior analyses (S2 Table). Since we were working with debris like sanitary pads or plastic bottles, which are in contact with humans, we expected to obtain a lot of human sequences. Potential contamination with human DNA throughout the processing of samples can be discarded since no DNA amplification was detected in the negative controls. Insect species (specially Diptera) and big mammals like Bos taurus (cattle) and Sus scrofa (wild boar) were found in rural beaches like Cagonera where it is likely that runoffs had carried the eDNA from inland. In the case of insects, there is also the possibility that DNA belongs to eggs laid by adults on the debris such as it has been seen in the case of the marine insect Halobates sericeus that is known to lay eggs on marine debris and has been shown to benefit from the increase in marine debris in recent years [51].

Considering only marine and brackish taxa, 86 species classified into 17 major groups were identified from the analyzed samples. The putative taxa were not equally distributed in all the samples and beaches (Fig 5). In fact, some items showed a higher number of taxa than others. Sanitary pads from Cagonera (C-T1) provided more species (44 species) than the rest. On the other hand, biofilm from a plastic bag from Arbeyal (A-P2) only appeared to have a phaeophycean alga (Petalonia fascia).

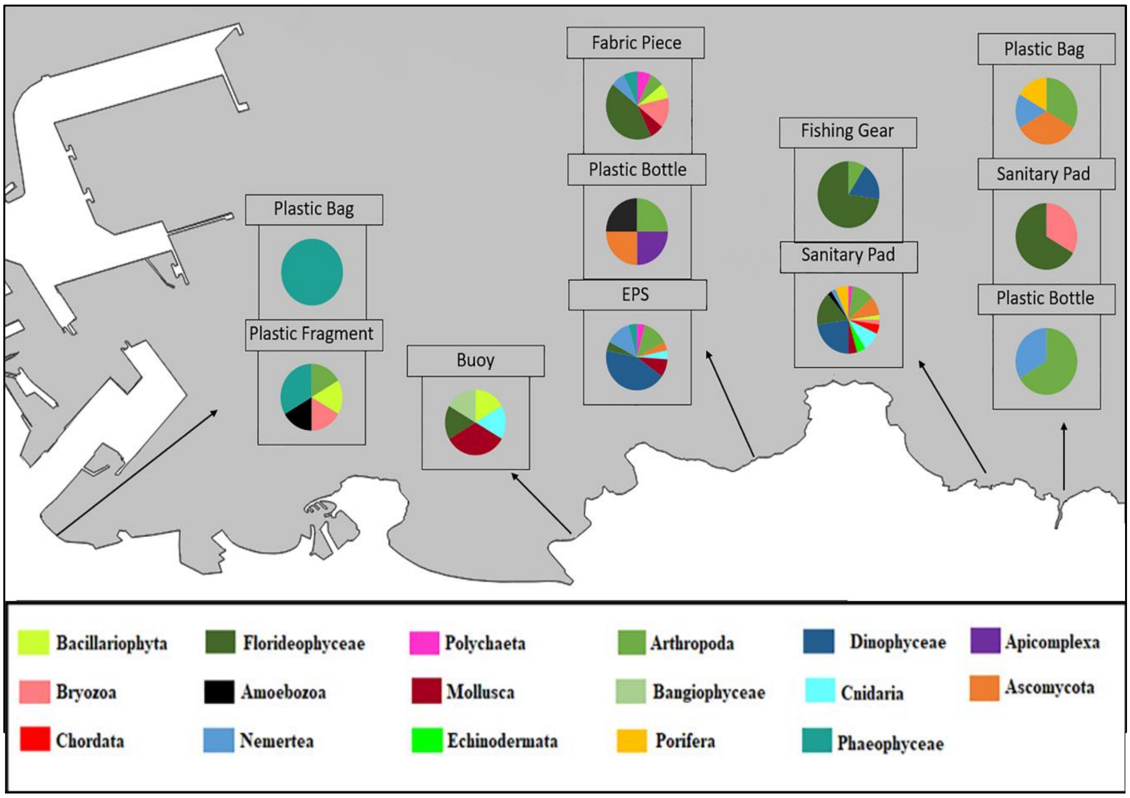


**Fig 5**. Composition of the main taxa found on each litter sample.

The non-metric scaling analysis arranged the beaches from their fouling biota in an order similar to that found on the litter items (Fig 4A and 4B), with La Ñora, Rinconin and Arbeyal connected closer than Cagonera and finally Peñarrubia. This was connected with different types of biota found in biofilm from textile and from plastic litter. For example, more Florideophyceae (red algae) species were found on textile samples than on plastic ones (13 species were found on textile samples and only 9 on plastic; Fig 6A and 6B). For Dinophycea, more species were found on plastic litter (Fig 6A) than in textiles (Fig 6B). Only one species of Bangiophycea appeared, which was found on plastic from Rinconin beach. On the other hand, the two species of Echinodermata and the DNA of two species of Chordata (two Perciformes) that were found, only appeared on textile litter.

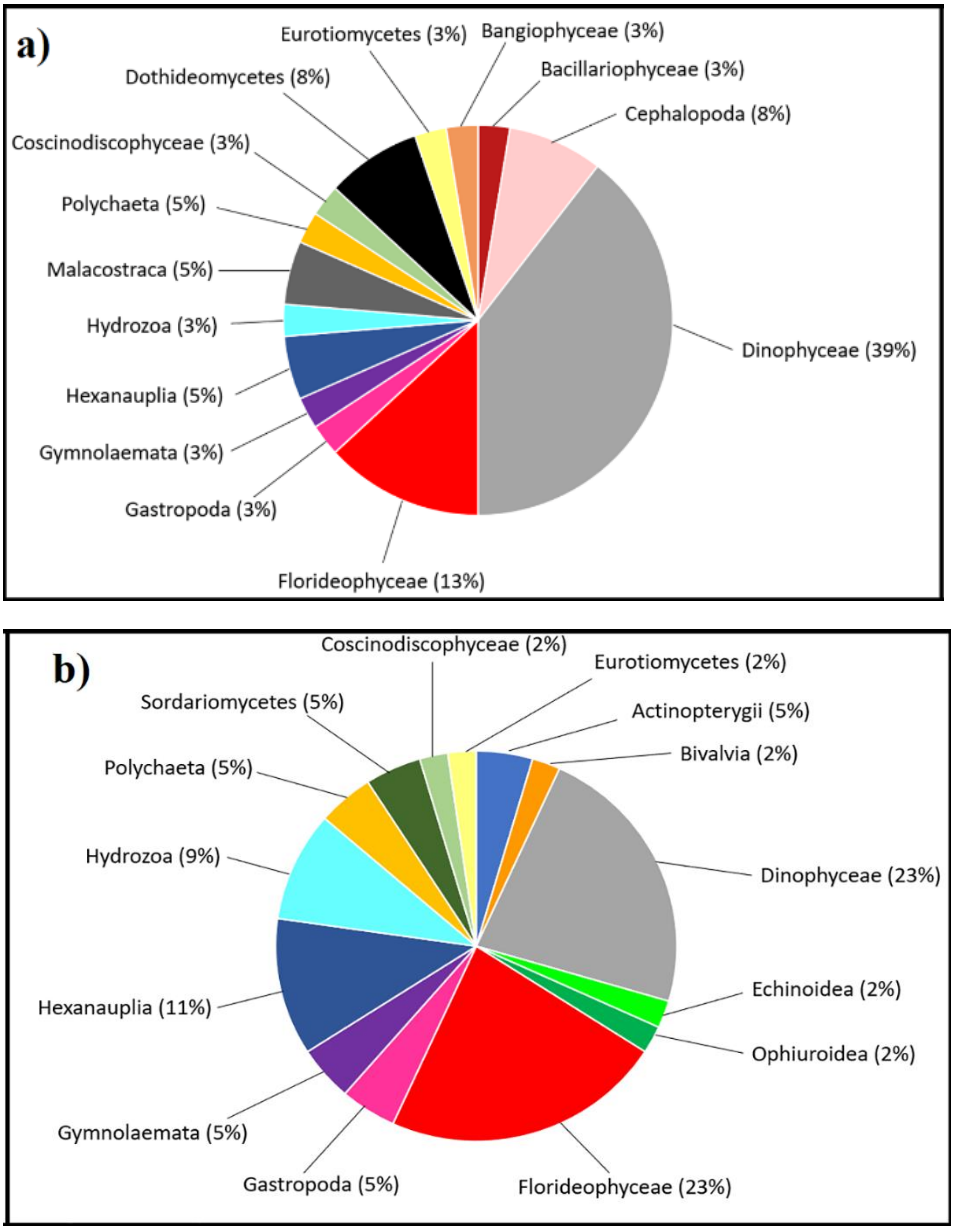


**Fig 6**. a) Composition of biota occurring on plastic litter surfaces. b) Biota occurring on textile litter surfaces.

Textiles and plastics were compared for the number of species of each taxonomic group. Statistically significant differences between the two groups of litter items were found only for Bryozoa and Florideophyceae DNA, as a significantly higher number of species of these taxa occurred on textile items than on plastic ones (Mann-Whitney $U = 0.5$ with $z = 2.764$, $P = 0.006$; and $U = 3$ with $z = 2.062$, $P = 0.03$, for Bryozoans and red algae respectively). In fact, for Bryozoans most plastic litter samples showed no species and only a single species was detected in one plastic piece (standard deviation = 0.333) while for most textile samples two Bryozoan species where found (standard deviation 0.577). On the other hand, most plastic samples showed no Florideophyceae species attached, although one sample recorded 8 different species (standard deviation = 2.645). Nevertheless, all textile samples had more than 4 different Florideophyceae species (standard deviation = 1) However, focusing only on the macrofauna profiles analyzed in previous studies in the region (i.e. number of species of the phyla Annelida, Arthropoda, Bryozoa, Chordata, Cnidaria, Echinodermata, Mollusca, Porifera published in Miralles et al. 2016), there was no significant difference between textile and plastic ($\chi 2$ = 7.885, 6 d.f., P = 0.247, and Fisher's exact test with P = 0.249 > 0.05, not significant).

DNA belonging to three exotic species was found in the dataset, including two species that are currently considered IAS (they are already stablished species that alter local ecosystems) in the study region: the brown alga Sargassum muticum (found on an EPS from Peñarrubia beach) and the signal crayfish Pacifastacus leniusculus. DNA assigned to Pacifastacus leniusculus, which is from brackish or fresh waters, was found on biofilm from a plastic bottle in La Ñora beach near the river (Table 3). The third species (*Illex argentinus*, NIS in the study area) was also detected in the EPS from Peñarrubia, a fragment of a box typically employed to transport fishing products. Thus, since the origin of DNA was likely from seafood catch remains and not true South American squid larvae, it was not taken into consideration.

**Table 3**. Non-indigenous and nuisance species which DNA was found attached to beached litter objects; Shaded in grey, species native from the study region that have been described as NIS or AIS elsewhere.

| Taxon | Species | Reason for concern | Sample | Reference |
|---|---|---|---|---|
| Malacostraca | *Pacifastacus leniusculus* | AIS | Ñ-Plastic Bottle | [52] |
| Cephalopoda | *Illex argentinus* | NIS | P- Polystyrene | [53] |
| Phaeophyceae | *Sargassum muticum* | AIS | P- Polystyrene | [52] |
| Apicomplexa | *Isospora* sp. | Human parasite | P- Plastic Bottle | [54] |
| Ascomycota | *Cladosporium herbarum* | Asthmatic outbreaks and allergies | Ñ-Plastic Bag | [55] |
| | | | P- Polystyrene | [55] |
| | | | P- Polystyrene | [55] |
| Ascomycota | *Penicillium digitatum* | Rare pneumonia cases | C- Sanitary pad | [56] |
| Ascomycota | *Fusarium solani* | Infection of human cornea | C- Sanitary pad | [57] |
| Cnidaria | *Muggiaea atlantica* | AIS in Germany | C- Sanitary pad | [58] |
| Bivalvia | *Mytilus edulis* | NIS in the Black Sea | C- Sanitary pad | [59] |
| Dynophyceae | *Alexandrium catenella* | Paralytic shellfish poisoning | P- Polystyrene | [60] |
| Dynophyceae | *Karenia brevis* | Respiratory irritation | C- Sanitary pad | [60] |
| Dynophyceae | *Peridinium* sp. | Toxic blooms | P- Polystyrene | [60] |
| Dynophyceae | *Alexandrium ostenfeldii* | Paralytic shellfish poisoning | C- Sanitary pad | [60] |
| Dynophyceae | *Karlodinium* sp. | Toxic blooms | C- Sanitary pad | [60] |
| Dynophyceae | *Alexandrium minutum* | Toxic PSP blooms | C- Sanitary pad | [60] |
| | | | C-Fishing Gear | [60] |
| Dynophyceae | *Alexandrium sp.* | May produce toxic blooms | P- Polystyrene | [60] |
| Dynophyceae | *Azadinium poporum* | Azaspiracid shellfish poisoning | C- Sanitary pad | [60] |
| Dynophyceae | *Prorocentrum micans* | Shellfish killing blooms | P- Polystyrene | [61] |
| Dynophyceae | *Scrippsiella sp.* | May produce high density blooms | P- Polystyrene | [62] |
| Dynophyceae | *Alexandrium affine* | NIS in China, Ukraine, California | C-Fishing Gear | [15] |
| Florideophyceae | *Plocamium cartilagineum* | NIS in the Mediterranean Sea | C- Sanitary pad | [15] |
| Florideophyceae | *Ellisolandia elongata* | NIS in the Belgian coast | R-Buoy | [63] |
| Florideophyceae | *Jania rubens* | NIS in the Mediterranean Sea | P-Fabric piece | [64] |
| Florideophyceae | *Chondrus crispus* | NIS in the United Kingdom | C-Fishing Gear | [65] |
| Florideophyceae | *Gymnogongrus crenulatus* | NIS in the Australian coast | C-Fishing Gear | [66] |
| Phaeophyceae | *Leathesia marina* | NIS in the Mediterranean Sea | A-Plastic fragment | [67] |

Apart from these NIS and AIS, several native species were also found attached to the litter. Many of these species are considered potentially harmful because some strains can form toxic blooms (case of some dinoflagellate species), or produce diseases or allergies (Table 3). Some species do not cause any known toxicity or nuisance effects, but they are considered potentially dangerous in different places around the world where they are non-indigenous (NIS) or even invasive species (AIS). We detected Florideophycea species such as *Plocamium cartilagineum*, *Jania rubens* (aliens in the Mediterranean Sea), *Chondrus crispus* (alien in the United Kingdom) and *Gymnogongrus crenulatus* (alien in the Australian coast); Mollusca (*Mytilus edulis*; alien in the Black Sea); and Cnidaria (*Muggiaea atlantica*; invasion reports in Germany).

**Litter as a vector for species dispersal from Gijon port**

For exploring the possibility of marine litter being a vector of dispersal from ports, the taxonomic profiles found in this study from beach litter were compared with published data from the port of Gijon. The comparison was done using the subset of marine macroscopic animal species only, because only macroscopic sessile animals were sampled in Miralles et al. (2016).

These samples were taken from three different sites in the port of Gijon; one near the port mouth, one in the inner section and another one half way between these two. Approximately 200m$^2$ of artificial pot structures were sampled in each site, where visual inspections were done prior to sampling in order to detect phenotypically different organisms and to identify as many different species as possible, targeting all macroscopic biota inhabiting the port of Gijon. A total of 24 species were published in the port [42] (S3 Table) which were identified by visual and also genetic methods (barcoding of COI gene).

The number of shared species across taxonomic groups found in litter biofilm from beaches and in the port was four out of a total of 44 macroscopic animal species, corresponding to Platynereis dumerilii and Syllis gracilis (Polychaeta) the mussel Mytilus edulis, and the limpet Patella vulgata. For further analysis, the macrofaunal species fouling on litter (whatever litter type, since no significant differences were found between textile and plastic for macrofauna species profiles) were organized by proximity to the port, considering together the beaches located in the same bay, Arbeyal, Rinconin and Peñarrubia on one group, Cagonera and La Ñora on the other (see Fig 1).

The profile of the fouled macroscopic fauna of the port and the litter found on closer beaches was more similar to each other than the biota of the litter found on farther beaches (Fig 7). The macrofauna profile of Gijon port published by Miralles et al. (2016) was not significantly different of that found on litter from the three closer beaches ($\chi2 = 7.797$; 5 d.f.; $p = 0.168$, and Fisher's exact test with $p = 0.193$). In contrast, the taxonomic profile of the litter macrofauna found from the farther La Ñora and Cagonera beaches was highly significantly different from the port fauna reported by Miralles et al. (2016) ($\chi2$ = 27.051;7 d.f.; p = 0.0003, and Fisher's exact test with p = 1.91x105).

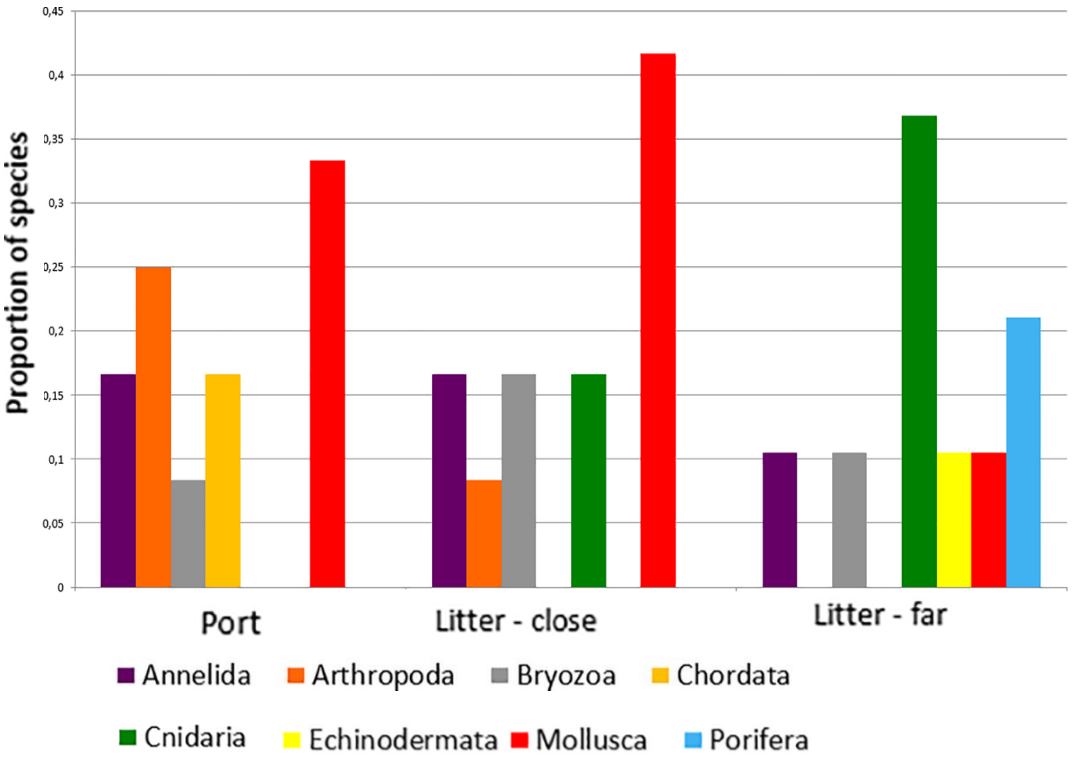


**Fig 7.** Proportion of species of different animal groups fouling Gijon old port piers and litter from beaches near (Litter-close) or apart (Litter-far) the port.

## DISCUSSION

Although based on a modest number of items, this study provided a number of results of importance in the field of environmental biosecurity. In the biofouling communities, as expected, some of the species detected from DNA are microscopic, such as dinophytes which are the most likely to survive attached to plastics or debris. For the macroscopic species, DNA was probably provided from free eDNA of macroscopic organisms, or from their microscopic life stages (early larvae, eggs, fragments). It is not possible to define if the detected species were alive organisms fouling marine litter, because DNA can persist for extended periods in the environment making discrimination of living versus dead organisms difficult [68]. Thus, we cannot confirm whether these results reflect an ability of the detected species to colonize new materials or not.

Environmental RNA (eRNA) is an increasingly employed molecular tool for metabarcoding based environmental characterization, and is being considered for biosecurity applications because it can be employed to distinguish living biodiversity [69–70]. However, some disadvantages related to eRNA are: overrepresentation of organisms with complex genomes and numerous copies of transcriptionally active marker genes [71], or different artifacts that can occur during RNA processing and PCR amplification [72]. Thus, further research is needed to achieve appropriate methodologies for metabarcoding based biodiversity characterizations.

Nevertheless, the presence of a wide variety of species was detected in this study, including non-animal taxa such as Florideophyceae, Phaeophyceae and Dinophyceae. These results are consistent with previous studies that confirm Cytochrome Oxidase I (COI) gene as an effective tool for the barcoding and identification of algae and Dinophyte species [73–76]. In fact, many of these algae sequences were randomly reviewed with individual BLAST and gave a robust assignment with >97% identity and high scores, so our study aligns with other authors who found COI to be a good tool to sequence red algae such as Florideophyceae [77].

A result to be highlighted was DNA of a significantly higher number of red algae and Bryozoa species found in textile debris than in plastic litter. Taking into account that both red algae [78–79], and Bryozoa [80–81] contain a high proportion of AIS, it seems that textile debris would have the potential to be a reservoir of potentially invasive species. Moreover, some of the species found in textiles are dangerous for public health because they may cause red tides (e.g. Alexandrium minutum) or produce infections (e.g. Fusarium solani, Cladosporium herbarum), thus the role of textile litter as a reservoir of species should be carefully taken into account. Previous studies have assessed marine plastic litter as a vector for nuisance species including human pathogens such as vibrio genus, or the dinoflagellates Ostreopsis sp., Coplia sp. And Alexandrium taylori, known to form harmful algal blooms under favorable conditions [82–83]. Considering that 45% of the potential nuisance species that we detected were fouling textile litter, our data suggest that this type of litter could also be employed as a vector by these species, facilitating their spread into new habitats.

Fabric floatability is in principle lower than that of plastics, thus having lower dispersal capacity. However, on beaches with high litter accumulation the species accumulated in textile may pass on plastic items and eventually navigate offshore. This is why future studies should consider also other types of litter–in addition to plastics- in order to fully understand the role of marine litter as reservoir and dispersal vector of nuisance species.

Regarding the litter profile that was found in each beach, Cagonera showed a very high proportion of textile litter with many sanitary pads. This can be explained from a malfunctioning of the domestic wastewater treatment in the neighborhood. The neighbors were consulted about this and explained that the local wastewater treatment plant was temporarily closed and the toilets flushed directly to the beach. Thus, the large proportion of textile litter in that beach is likely not representative of the common beach state. Campaigns for not disposing this type of objects in toilets should be conducted in this area. On the other hand, Peñarrubia beach showed to be different to the rest of the beaches regarding litter composition and biota. This can be explained as

Peñarrubia, unlike the other beaches, is not a sheltered beach, and it is also the only pebble beach located outside the city of Gijon (thus, it is not cleaned during winter). This could explain the high differentiation (regarding both, litter and biota) that this beach shows from the other ones.

Another interesting result was that the biota profile found on litter closer to the port was more similar to that of the port's macrofauna than litter collected further away. This can be considered a signal of species dispersal from the port using marine litter as a vector. In some cases, ports can be sinks as well as donors of species. However, the received species can be newly transferred to neighboring areas after arriving into a port [84]. In the case of the port of Gijon, marine currents flow eastwards in winter, so the port could be sink of litter coming from the west. However, this litter and species attached to it could be afterwards spread to the east from the port, which is why we consider it as the origin of the sampled litter items, as all the beaches are located eastwards the port. The macrofauna species found on litter were all native or cosmopolitan, suggesting that litter could not only transport alien species from Bay of Biscay ports [38] but also serve as a vector for the dispersal of native species, as it was found in Swedish waters [85].

Cnidaria were detected in great proportions on litter from far beaches and were not found on the port sampling; the detected species were millimetric polyps [86] that could adhere to floating debris (on adult or larvae stages). In fact, in the case of this type of hydroid fauna that was found, the sessile hydroid is more likely responsible for long range dispersal than the planktonic medusa stage [87].

The increase in the proportion of Cnidaria on distant beaches could be due to the fact that most of the species detected on the litter (such as Clytia gracilis or Clytia paulensis) are very sensitive to disturbance [88] and these areas have a lower human impact than those closer to the city of Gijon. Similarly, it is also remarkable that species belonging to Porifera and Echinodermata phyla (classified as very sensitive to pollution) were only detected in distant beaches.

On the other hand, detected Annelida and Bryozoa species were all indifferent to pollution, and were found inside the port and in near and far beaches. These species have microscopic larval stages [89–90] that can adhere to litter items when floating as plankton and employ its surface in the adult phase (due to their size commonly smaller than 1mm) when they become benthic, leaving DNA traces that would be detected posteriorly. Some of the detected Mollusca were benthic species that also show a planktonic larval stage, that settle when the shell size is still smaller than 1mm [91] thus, early life stages could have employed marine litter to attach and spread.

Moreover, some of the native species that were found attached to marine litter (mainly Florideophyceae) are considered NIS or AIS in many other zones around the Globe, therefore, our results show that marine litter could be used as a spreading vector, facilitating exotic-species to reach and colonize new habitats.

Regarding local NIS and AIS, in the NGS results we detected DNA of several species, including an alga (*Sargassum muticum*), a cephalopod (*Illex argentinus*), and a freshwater crayfish (*Pacifastacus leniusculus*). NIS tend to be very difficult to identify in the initial phases of colonization, because their population size is normally small. This is an important issue because their eradication is easier in the first introduction stages when the population is not too big [92]. Sequences with low frequency occurrence, like those found in this study, should be taken into account, as they might be the key to anticipate or avoid possible future invasions. Following this approach, a deeper analysis is needed to correctly interpret the presence of DNA of exotic species on the particular litter objects analyzed in this study. The polystyrene piece sampled in Peñarrubia carried 15 DNA sequences identified as Illex argentinus. Individual BLASTs were made with some of the sequences belonging to Illex argentinus and confirmed that they were all correctly assigned. However, the Argentinean squid has no sessile life stages, and this species has never been detected in the Bay of Biscay. The origin of the polystyrene could explain this result; this material is employed in fishing vessels—polystyrene boxes are used to store the catch. Probably a polystyrene box used to store that squid ended on the sea and arrived in Peñarrubia beach, still containing remains of squid DNA in the biofilm. This data show that although eDNA is an important tool for an effective detection and identification of species, it does not guarantee detection of live organisms.

In contrast with Illex argentinus, the other two exotic species are considered invasive in Spanish waters. *Sargassum muticum* is a brown seaweed that has been already detected in Asturias [93] and alters local biodiversity triggering the decline of some native species such as *Gelidium spinosum* [94]. Our results suggest small propagules of this species could be transported attached to marine litter, using it as a spreading vector to colonize new environments. On the other hand, the presence of DNA of the freshwater signal crayfish (*Pacifastacus leniusculus*) in a plastic bottle (household origin) from La Ñora beach can be easily explained. This beach is in the estuary of River La Ñora, and eggs, larvae or naked DNA from freshwater organisms can arrive from the river, as rivers are conveyor belts of DNA diversity [95]. The species *Pacifastacus leniusculus* has been reported from River La Ñora [96] and our results are consistent with it, having a representation of the species living upstream.

On the technical side, next-generation sequencing was carried out with miniCOI amplicons in this study as COI is a largely studied gene and large amount of sequences are available [97]. Reference databases for the 18S gene are currently growing and the gene has been incorporated for example in BOLD (Barcoding of Life Diversity); however, the number of reference sequences is still smaller for 18S gene [98]. For this reason, we based our study only on COI, that is one of the most represented DNA barcodes in public databases [99].

A problem for the use of Metabarcoding in biodiversity inventories is the unbalanced coverage of different taxonomic groups in current reference databases, especially in aquatic species [73]. For example, three sequences from a sanitary pad were assigned to Squamamoeba japonica (S1 Table) which is a deep-sea Pacific amoeba [100]. This could be an assignment artifact due to the scarcity of references because in July 2019 the only sea amoebas represented in GenBank with COI gene were of this species. It is possible that some DNA sequences of other marine amoebas were erroneously assigned to it. These assignment artifacts may happen not only with amoebas but with any other species that are not well defined on databases. So, in order to avoid this type of errors on future studies, the need of constructing global well referenced databases is remarkable.

### Conclusions and management recommendations

In this study, potentially dangerous species for ecosystem and for human health have been found employing DNA analysis of biofilm fouling litter objects. Textile objects, although likely less mobile than plastic ones, carried a significantly higher proportion of nuisance species. On the other hand, the macrofauna profile of litter objects found on beaches seemed to be associated with distance from the port, the closer the beach the more similar the macrofauna profile of litter. From the results obtained in this study, we consider that together with the general public concern about plastics and microplastics, more attention should be paid to textile litter. Preventing litter dispersal from ports is another important recommendation for avoiding exotic species spread.

### Supporting information

The Supplementary Material for this article can be found online at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0228811#sec014

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# Capítulo 4

**Flotsam, an overlooked vector for alien dispersal.**

Fernández, S., Ibabe, A., Rayón, F., Ardura, A., Bartolomé, M., Borrell, Y. J., Dopico, E., González, M., Miralles, L., Montes, H., Pérez, T., Rodríguez, N. & Garcia-Vazquez, E.

Estuarine, Coastal and Shelf Science

(en revision)

# Flotsam, an overlooked vector for alien dispersal.

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## HIGHLIGHTS

• Ports and marinas are sources of non-indigenous species

• Marine litter carries non-indigenous and invasive species

• Species are shared between port, flotsam and nearby beaches in Bay of Biscay

• Flotsam litter is a vector of species between ports and natural spaces nearby

## ABSTRACT

Anthropogenic litter is considered a potential vector for the dispersal of nonindigenous species (NIS) in marine ecosystems. Using the bay of Gijon (Southwestern Bay of Biscay) as a case study, we studied the composition and potential transfer of the communities inhabiting three different environment components: 1) natural and artificial substrates from the international port of Gijon, 2) six proximate rocky beaches and 3) floating litter collected in the adjacent coast. A total of 717 organisms were morphologically identified and DNA barcoded using COI and 18S genes. In total 23 NIS were detected, 10 of them considered invasive in the area. The taxonomic profiles of the three environment components were significantly different, flotsam containing higher proportions of Hexanauplia and less mollusks, echinoderms and polychaetes than ports and beaches. Contrary to expectations, floating litter showed higher densities of

native and exotic species than beaches or port surfaces. This and shared haplotypes between port, flotsam and beaches in some invasive species may indicate that marine litter could represent a new habitat for species to disperse into new areas.

**KEYWORDS**: Marine litter; Non-indigenous species; Species dispersal; Community diversity.

## 1. INTRODUCTION

Marine ecosystems are a precious asset for humans since they are an important source of goods and services (FAO, 2020; Datta et al., 2015; Spalding et al., 2014; Cusack et al., 2021). In a scenario of increasing anthropic pressures such as overfishing, hábitat destruction and pollution, marine ecosystems are facing a global degradation (Halpern et al., 2015). This problem has been well assessed in Europe, where human pressures affect practically the entire marine environment (Korpinen et al., 2019). Another important factor that has been gaining importance over the years and that is nowadays considered a major threat for marine ecosystems is marine anthropogenic litter, which is defined as "any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment" (UNEP 2009). This anthropogenic litter is another crucial factor that affects marine ecosystems and is one of the major obstacles to achieve the Sustainable Development Goal (SDG) #14 "Life under water", that aims to conserve and sustainably use the oceans, seas and marine resources (Goal 14, United Nations Sustainable Development Goals). Synthetic polymers (commonly known as plastics) have become a recognized global environmental problem. It is estimated that approximately 8 million metric tons of plastic enter the marine environment annually (Jambeck et al., 2015) and the production is continuously increasing (Geyer et al., 2017).

This situation is the cause of the severe environmental damages in marine ecosystems that harm aquatic organisms and endangers marine biodiversity (Deudero & Alomar, 2015; Fossi et al., 2018), consequently affecting the sustainability of fishing resources worldwide, from the Baltic (Depellegrin et al., 2020) to Oceania (Smith, 2012) and Africa (Masiá et al., 2022). After entering the marine environment, floating litter can easily be ingested or entangled by the inhabiting species, increasing their mortality (Steer et al., 2017; Rizzi et al., 2019). Also, when breaking in smaller fragments like microplastics (those plastic fragments smaller than 5mm long), plastic litter can release chemical substances or even accumulate persistent organic pollutants and other substances of concern that can be transferred to the food web (Hahladakis et al., 2018; Chen et al., 2019).

In addition to its adverse effects as a macro-pollutant, marine litter is a vector of biopollution as well. Biopollution (i.e., harmful and invasive species) poses a threat to native diversity in all oceans (Galgani et al., 2015; Ryan, 2015; Williams & Rangel-

Buitrago, 2019; Occhipinti-Ambrogi, 2021) as species which are non-native to an ecosystem (known as exotic species, alien species or non-indigenous species) can establish new populations and cause severe impacts on these new habitats. At this point, following the terminology from Iannone et al. (2021), the classification of these species is modified from exotic to invasive species. Flotsam litter could be seen as a temporary niche of biopollutant species that move from one to another location attached to those light artificial substrates that can be passively transported far away (Kiessling, 2015; Whichmann et al., 2019; Maclean et al., 2021), even to remote islands (Rech et al., 2018a). In fact, the role of flotsam to increase connectivity between islands is well known; some NIS travel long distances, even crossing oceans, attached to floating litter (Miller et al., 2018; Therriault et al., 2018; Garcia-Gomez et al., 2021). In this scenario, marine litter has been recommended to be classified as a "transport-stowaway" pathway in the Convention on the Biological Diversity Pathway Classification framework as it can help fouling species to spread rapidly using marine currents (Pergl et al., 2020).

Urban areas and maritime ports are of special interest because they are main exporters of marine litter to open waters (Barnes et al., 2009; Chen, 2015; Rayon-Viña et al., 2022). Simultaneously, maritime ports are hubs of exotic species that are mainly introduced by maritime traffic (Molnar et al., 2008) and due to the high litter density, can be used as a key location for biopollutants to disperse to other areas. Rech et al. (2016) warned about the likelihood of port-exported flotsam to be a key contributor of invasive species dispersal worldwide. Garcia-Vazquez et al. (2018) found that litter concentrated nearby port areas at the entry of the Baltic Sea contains a higher proportion of non-indigenous species than litter deposited on farther beaches. In the Cantabrian Sea, invasive species attached to beached plastic bottles and ropes are more abundant near ports (Miralles et al., 2018a) and eDNA biota profiles found in beached marine litter show clear similarities with the biota inhabiting ports (Ibabe et al., 2020).

Despite these evidences, the role of marine litter as a vector of NIS between ports and surrounding natural areas is still unclear. It is possible that invasive species restricted to ports, especially if they have limited mobility, or if surrounding areas exhibit sufficient native biodiversity for aliens might not to be able to find empty niches to colonize (Shea & Chesson, 2002; Knight & Reich, 2005; Crooks et al., 2011). In recent years, different plans such as the EU Marine Strategy Framework Directive (MSFD, 2008/56/EC) have been produced to deal with the problem posed by marine litter. However, there is still a long road ahead in order to better understand the consequences of litter in the marine environment (either as a macropollutant or a vector for biopollutants).

In this work we studied the contribution of floating litter to the dispersal of invasive species, using the Southwestern Bay of Biscay as a case study. Macroscopic animals attached to natural and artificial substrates were sampled from three different environment components: 1) the main international port of the region, 2) floating objects collected outside the port in front of the adjacent coast, and 3) the six main rocky beaches in that part of the coast. DNA barcoding was used to identify biopollutants in the three environments, and the relative density and species sharing between environments to determine the role of flotsam in biopollution dispersal between the port and natural coastal areas. Expectations were: i) flotsam will act as a vector between ports and beaches; ii) only a fraction of biopollutants –those preferring plastic substrate- will be transferred from port to beaches; iii) consequently the species density will decrease in the transfer vector (flotsam) in comparison to the donor (port) and recipient habitats (natural rocks).

## 2. MATERIAL AND METHODS

### 2.1. Ethics statement

This study was approved from the Research Ethics Committee of the Principality of Asturias with the reference 166/19. The general procedure of diving works was based on the Collective Agreement of Professional Diving and Hyperbaric Means (Ministerial Order of October 14, 1997, Spain), modified by the Collective Agreement of Professional Diving and Hyperbaric Means of October 18, 2016 accomplishing the directive UNE-EN 15333-1.

### 2.2. Study area

The study region selected was the Bay of Gijón (coordinates 43.573 – 43.551, -5.728 – -5.588; southwestern Bay of Biscay) and its surroundings. The international Port of Gijón has the local name of El Musel (Figure 1), and has an adjacent marina between the port and Arbeyal beach. Beaches were selected for having a large surface of rocky substrate: Xivares, Arbeyal, El Rinconin, Peñarrubia, Cagonera and La Ñora (Fig. 1). Between Arbeyal and the city of Gijón there is another marina. Touristic sandy beaches were not considered since targets were species fouling on hard substrates. Structures of the port of Gijón including the marina, floating marine litter and rocks from the six beaches mentioned were sampled for attached biota. All samplings were done between January and July of 2017.

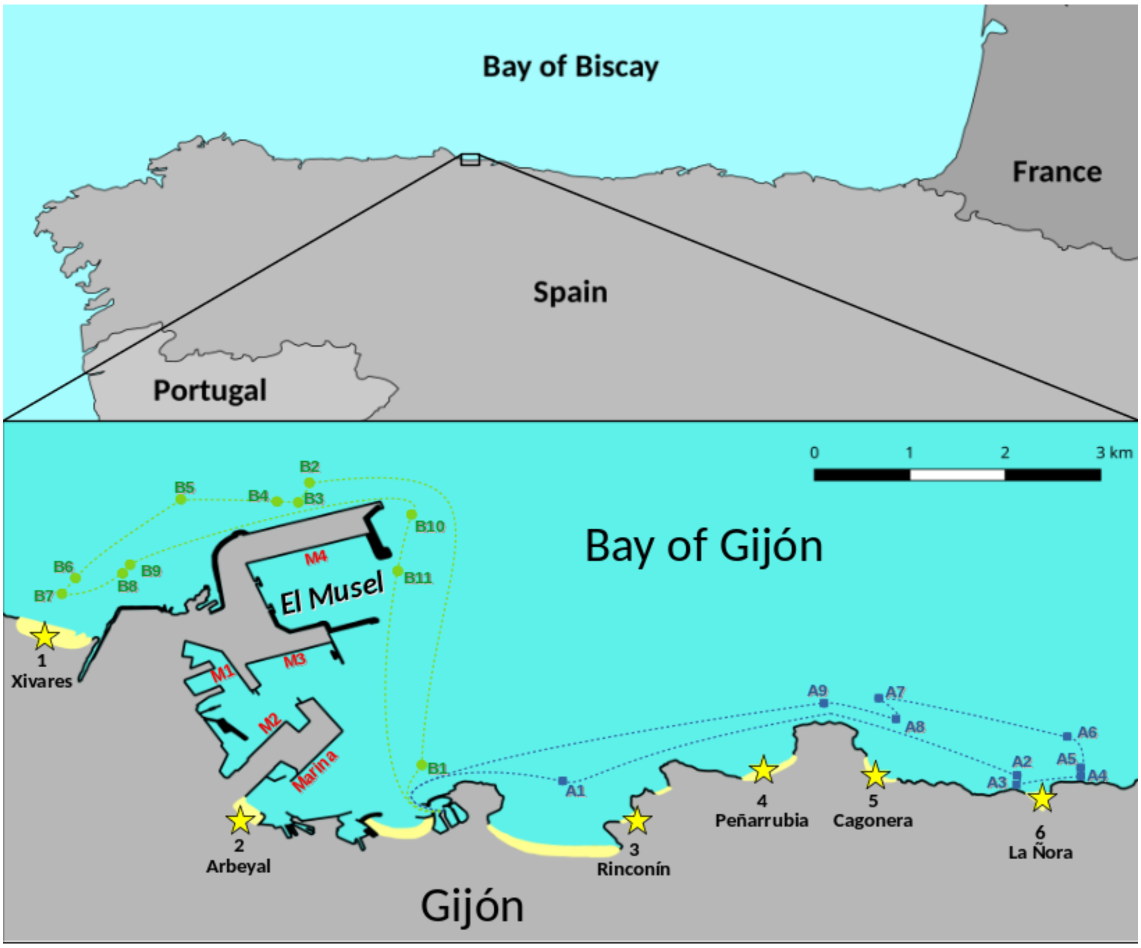



**Figure 1.** Map showing the locations sampled in this study within southwest Bay of Biscay. The sites where floating objects were collected are marked on two routes departing from Gijón marina (eastward A1-A9 and westward B1-B11). The sampled beaches, marked with yellow stars, are from 1 to 6: Xivares, Arbeyal, Rinconin, Peñarrubia, Cagonera and La Ñora, respectively.

### 2.3. Sampling procedures

#### 2.3.1. Beach rocks and beached marine litter

In beaches, sampling was performed in transects on natural hard substrate (rocks, pebbles). Starting 30min before low tide, three perpendicular transects of 1m width from the water line to the green line (where vegetation starts) were deployed in each beach. These transects were separated evenly at least 100m one from each other to avoid confounding and to cover the whole surface of the beaches, whose surfaces differed greatly from each other. Therefore, the area sampled varied depending on the beach size being approximately between 300-400 m2 per beach. The total Surface sampled summing all beaches was approx. 2200 m2. All visible animals found inside transects were sorted morphologically by phenotype for taxonomic identification. Up to 10 individuals per phenotype (putative species) were selected for the genetic analysis and preserved in 100% ethanol for further DNA identification.

The beaches were sampled for beached marine litter too. During low tide, the ítems bigger than 2.5 cm found in the transects described above were collected, categorized by material and type of object (plastic, metal, glass, textile; ear buds, bags, bottles, cans, sanitary pads etc.) and photographed. Every item was checked for any visible macrobiota, fouling individuals were isolated, preserved in 100% ethanol and transported to the laboratory for further genetic identification. Litter objects were then thrown into recycle bins. The total surface of these litter items was approx. 42.5m2. The surface estimation includes front and back surfaces of the materials. These samplings were performed between the 13th of January and the 27th of March, to avoid cleanings made by the administrations that could affect the results.

### 2.3.2. Port sampling

The international Port of Gijón is the largest in the Southwestern Bay of Biscay. Sampling was carried out in four sections within the commercial port (two locations within each section) and in the adjacent marina (one location) in July 2017. Sampling procedure is described in detail in Miralles et al. (2021). Briefly, first a visual inspection was conducted during low tide to locate biota patches and determine, by using quadrats, the different types of organisms attached to hard substrates, including rocky ground and concrete structures. To standardize the sampling effort, the surface sampled from each quadrat was approximately 200m2. Macroscopic animals were sampled proportionally to their relative abundance for 30 min per location. Individuals were scraped using spatulas when necessary and then introduced in ambient water (contained in 10L buckets) after being separated by morphotype for posterior visual identifications. The total surface sampled from port surfaces was approximately 1800 m2. In addition, three quadrats of approx. 30x30cm were scratched from each of the three vessels (one of them arrived from Russia and the other two from Spanish ports) long-docked in the port to have a representation of all the artificial surfaces carrying organisms. These quadrats were placed in three different zones along the draft of the vessel: stern, mid-ship and bow. Theses samplings were done by professional divers and all the collected individuals were stored and then classified by morphotype for posterior visual identifications. The individuals collected were transported to the laboratory in coolers. Once in the laboratory, individuals were sorted *de visu*, and preserved in 100% ethanol for further genetic identification.

### 2.3.3. Flotsam sampling

Two trips were performed in the waters of the bay of Gijón, one eastward and other westward (see Figure 1), following the coastline and traveling at an average of 10 knots in July 2017. In each trip visual sightings of floating objects were made by four people on the deck of the vessel. Every flotsam item traced down was collected along with any associated solid material, its location registered with a GPS, isolated in individual empty bags and transported in coolers to the laboratory. In the laboratory, litter ítems were listed, measured, photographed and examined for macroscopic individuals fouling on or carried inside the object, that were picked and preserved in 100% ethanol in the laboratory of Genetics of Natural Resources at the University of Oviedo. A total of 45 objects were found (principally of plastic), summing in total approx. 2.8 m2 of surface. As for beached litter, the surface estimation included front and back surfaces of the materials.

### 2.4. Taxonomic analysis

#### 2.4.1. Species identification from morphology

Visual identification based on morphological traits was performed following the Guide to Seashores and Shallow Seas (Campbell, 2005) to the lowest possible taxonomic level (species whenever possible; genera, family or order in some cases). The specimens were double-checked with the help of experts in the laboratory, using magnifying glasses when needed. Only those classified down to a species or genera level were retained for further analysis. The taxonomic nomenclature followed the World Register of Marine Species (WoRMS Editorial Board, 2020). The invasiveness capacity of the species was assessed according to the Invasive Species Compendium CABI (2021), the Global Invasive Species Database (Pagad et al., 2015), and the native distributions as in WoRMS. Species were classified as native (those that occur naturally in a geographic area), NIS (those that do not occur naturally in a specific geographic area but do not cause any known impact; considered synonym of exotic, alien and nonnative) or invasive (those that are nonnative to a specific geographic area and cause environmental or economic harm or harm to humans) following Iannone et al. (2021).

#### 2.4.2. Genetic analysis

To confirm the visual identification and perform haplotype analysis, DNA was extracted from small pieces of muscular tissues (approx. 2 $mm^3$), following (Estoup et al., 1996) protocol. In the case of very small individuals, the full organism was used. For the individuals that were not successfully extracted following Estoup et al (1996) we used E.Z.N.A® Mollusc DNA kit (Omega Bio-tek) following manufacturer's instructions.

DNA barcoding was done by PCR-amplifying a fragment of the cytochrome oxidase subunit I gene (COI) and 18S rDNA (18S). COI was amplified by using jgLCO1490 and jgHCO2198 primers and the PCR program and conditions detailed in Geller et al. (2013). For the identification of the species attached to the beached litter COI and 18S rRNA genes were employed. 18S was amplified using 18S-EukF and 18S-EukR primers and conditions described in Medlin et al. (1988). For the polychaete species from these samples COI was preferred. PCR products were analyzed on 2% agarose gels stained with SimplySafe to confirm amplification before sequencing. Amplicons were quantified by using a Qubit dsDNA HS kit (ThermoFisher scientific, USA) and remaining primers and nucleotides were removed with Clean Sweep kit (ThermoFisher scientific, USA). Sequencing of the PCR products was performed principally in Macrogen company (Macrogen, 2017). Part of the sequences were obtained in the DNA

Analysis Facility of Scientific Technical Services, Oviedo University using BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher scientific, USA) and an ABI Prism 3100xl Genetic Analizer (ThermoFisher scientific, USA). Individuals from beached litter were forward and reverse sequenced and the rest only forward sequenced.

Final sequences were edited with SnapGene Viewer software and contrasted with BLAST software (Dumontier and Hogue, 2002) in NBCI (National Center of Biotechnology Information) within GenBank. The threshold for species assignation was a maximum E- value = 1e-100 and at least 97% nucleotide identity, which is considered the level at which species differ in the case of COI (Hebert et al., 2003) and 18S (Brown et al., 2016).

DnaSP v6 (Rozas et al., 2017) was used to obtain haplotypes from the sequences of each species. MEGA software version X (Kumar, Stecher, Li, Knyaz, and Tamura 2018) was employed to reconstruct a Maximum Parsimony Tree using COI haplotypes from *Magallana gigas* and sequences from different regions downloaded from GenBank. A haplotype network was constructed from *Magallana gigas* sequences using the software Network 10.2.0.0 (available at http://www.fluxus-technology.com) applying default settings in Median Joining.

*2.5. Statistics*

Parametric or non-parametric tests were done after checking normality in the dataset using Shapiro-Wilk test. The number of species per phylum, the total number of species, and the proportion of NIS (NIS individuals over the total number of individuals, or NIS over the total number of species) were measured in each sample. To check differences between components (specifically, if biota is significantly different in flotsam, ports and beaches), first we looked at the distribution of species per phylum (i.e., the taxonomic diversity), then at the proportion of NIS. Comparison between environments for the distribution of NIS versus native species, and for the distribution of shared versus exclusive species, was done using contingency Chi-square analysis.

Next, we looked at the density of exclusive (species that were only found in one of the three environment components) and shared species (the ones that were found in different environment components). Species density was calculated as the number of species divided by the square meters sampled. Two-way ANOVA without replication was employed to test the effect of compartment (port, beaches, flotsam) and species type (exclusive versus shared) on the species density.

Communities were finally compared using Bray-Curtis distance and 9999 permutations for the presence (1) / absence (0) of each species (because the surface sampled from each environment was not the same), and a plot was constructed from nonmetric multidimensional scale analysis (NMDS), checking stress and r2 of axis 1 and 2.

PAST software by Hammer et al. (2001) was employed for statistical tests and construction of NMDS plots.

## 3. RESULTS

### *3.1 Overview of animal biota in the three environment components*

Animals sampled were taxonomically identified down to the species level whenever possible. Their DNA barcodes can be found in GenBank with the accession numbers MT258923- MT258975 for samples from beaches and flotsam, and MN185333-MN185374, MN164033-MN164046 for port samples.

In Gijon port 427 individuals from 73 species were barcoded (Table 1) 20 from the marina, 56 from the commercial port and 15 attached to ship hulls (Supplementary Table 1). Port dataset details can be found in Miralles et al. (2021).

In beach transects a total of 218 organisms were taxonomically identified from morphology and confirmed from DNA, belonging to 54 different species. The number of species per beach ranged from 11 in Arbeyal to 29 in Rinconin (Table 1, Supplementary Table 1). Only a few macroscopic organisms (five individuals of 4 species) were found attached to beached litter, the great majority of ashore objects being apparently recently abandoned with no observable biota attached. Identification to species level from DNA barcoding confirmed the presence of acorn barnacle *Perforatus perforatus* (on a plastic in Cagonera), the tunicate *Didemnum vexillum* (also on Cagonera), the oyster *Neopycnodonte cochlear* (on hard plastic in Peñarrubia) and the polychaete *Sabellaria spinulosa* (on hard plastic in Rinconín).

**Table 1.** Summary of biota diversity in the three environment components considered (in bold), included the beached litter. Number of DNA-barcoded individuals in the sampling locations and litter (N); diversity as number of species (Species richness) and Shannon diversity index H (Shannon); number and proportion of non-indigenous individuals (%NIS individuals) and species (%NIS).

| | N | Species richness | Shannon | NIS individuals(%) | NIS(%) |
|---|---|---|---|---|---|
| Xivares (st.1) | 42 | 19 | 2.73 | 5 (11.9) | 1 (5.3) |
| Arbeyal (st. 2) | 22 | 11 | 2.31 | 6 (27.3) | 2 (18.2) |
| Rinconin (st. 3) | 48 | 29 | 3.27 | 2 (4.2) | 2 (6.9) |
| Peñarrubia (st. 4) | 29 | 20 | 2.95 | 1 (3.4) | 1 (5) |
| Cagonera (st. 5) | 33 | 17 | 2.74 | 0 (0) | 0 (0) |
| La Ñora (st. 6) | 34 | 16 | 2.64 | 2 (5.9) | 1 (6.2) |
| **All beaches** | **218** | **54** | **3.62** | **16 (7.3)** | **4 (7.4)** |
| **Flotsam** | **67** | **26** | **3.00** | **7 (10.4)** | **6 (23.1)** |
| **Gijon port** | **427** | **73** | **3.65** | **100 (23.4)** | **21 (28.8)** |
| **Beached litter** | **5** | **4** | **1.33** | **1 (20)** | **1 (25)** |

Regarding floating marine litter (Figure 2), 80% of the objects sampled (36 out of 45) carried visible fouling biota (Supplementary Table 2). We counted 67 macroscopic animals that could be visually observed on 19 of those objects (42.2%), representing a total of 26 species (Table 1, Supplementary Table 1); the rest of objects carried algae.


**Figure 2.** Examples of floating objects sampled from point #21 (site B11 on westward route).

The species living on the three environment components did not exhibit the same taxonomic profile (Figure 3), and the difference between them was highly significant ($\chi2$ = 47.7, 22 d.f., $p$ = 0.001). The community travelling on flotsam contained more Hexanauplia (barnacles) while less molluscs, echinoderms and polychaetes than ports and beaches. Considering the differences in surface sampled, Shannon diversity (Table 1) was not much different in the three different environment considered. The exception was the beached litter, not included as a habitat in this study, with a logical very low diversity.

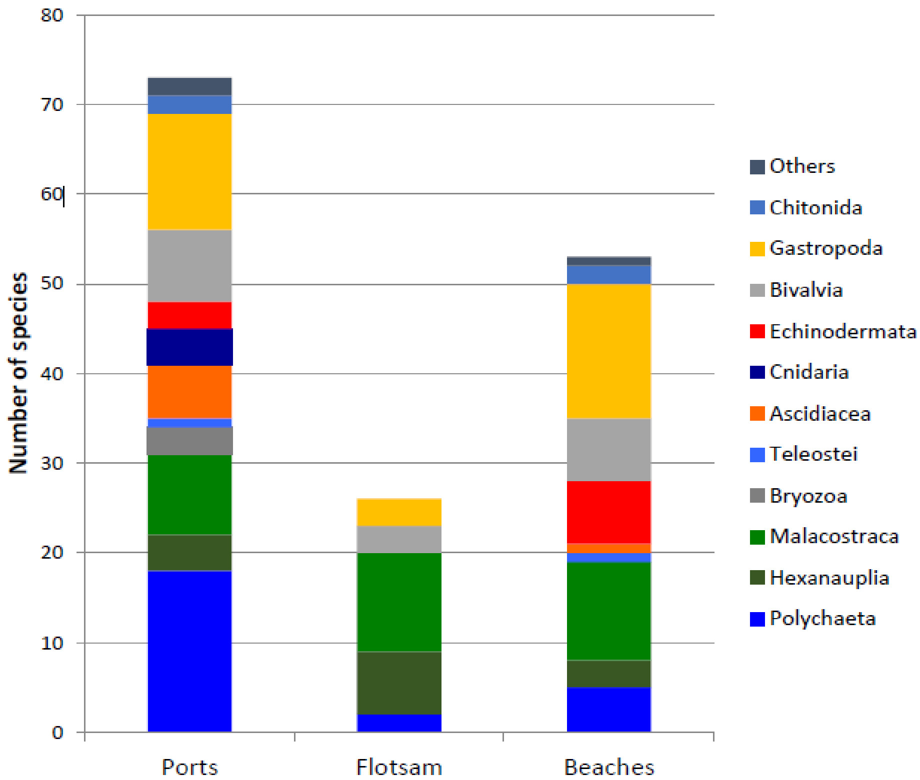



**Figure 3.** Taxonomic profile of the species sampled from beaches, ports and floating litter.

Many species (31.5% of the total) were shared between the three components (Figure 4) eight among the three environments, 20 between the port and beaches, five between the port and floating litter, and two between flotsam and beaches. In the port 54.8% of the species were exclusive (found only therein), while this percentage was 44.4% in beach rocks and flotsam.

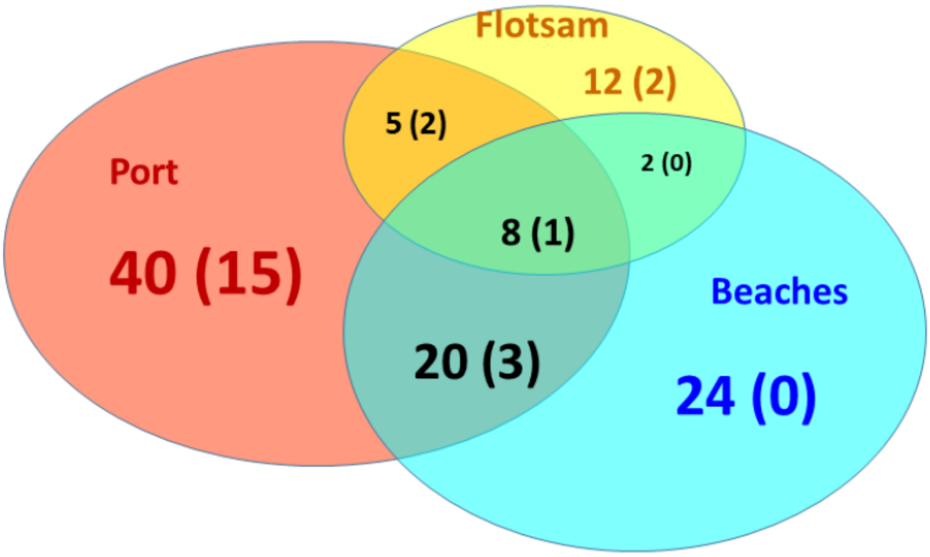



**Figure 4.** Share of macroscopic animal species attached to hard substrate in the three environments analyzed. Number of species is given for each environment (from which NIS in parenthesis).

The majority of species shared between environments belonged to five taxonomic groups that accounted for 28 of the 35 shared species (Supplementary table 1) gastropods (9), bivalves (6), malacostraceans (5), polychaetes (4) and Hexanauplia –barnacles- (4). Then followed echinoderms (3), chitons (2), ascidians (1) and decapods (1). Considering the proportion of species of each big taxonomic group that occupy different environments (environment sharing), mollusks (53.1% of species), followed by echinoderms (42.9%) were by far the most shared (Table 2), followed by arthropods, annelids and chordates species that were shared by at least two environments in these samplings. The distribution of species sharing environments (versus exclusive of an environment) in the considered taxonomic groups was significantly different ($\chi 2$ = 15.09, 7 d.f., $p = 0.03$), indicating that, in this study, the species of some taxonomic groups moved between environments (namely, port, flotsam and beaches) more frequently than the species of other groups.

**Table 2.** Species occupying different environments (environment sharing) by taxonomic groups. The total number of species in each taxonomic group (NSp) and the number of species shared between different environments are shown. Regarding each taxonomic group, the proportion of species occupying more than one environment (%multi-habitat) is indicated.

| | Environment sharing | | | | | |
|---|---|---|---|---|---|---|
| | NSp | Port & Beach | Port & Litter | Litter & Beach | All environments | % multi-environment |
| Annelida | 22 | 2 | 2 | 0 | 0 | 0.182 |
| Arthropoda | 32 | 1 | 3 | 2 | 4 | 0.313 |
| Bryozoa | 3 | 0 | 0 | 0 | 0 | 0 |
| Chordata | 8 | 1 | 0 | 0 | 0 | 0.125 |
| Cnidaria | 4 | 0 | 0 | 0 | 0 | 0 |
| Echinodermata | 7 | 3 | 0 | 0 | 0 | 0.429 |
| Mollusca | 32 | 13 | 0 | 0 | 4 | 0.531 |
| Other | 3 | 0 | 0 | 0 | 0 | 0 |

Two-way ANOVA without replication (Table 3) revealed significant differences between environment components for species density ($F_{2,5}$ = 140, *p* = 0.007). Litter flotsam -despite much smaller surface- contained similar species richness to that of individual beaches (Table 1), and its density of exclusive and shared species was 0.26 and 0.31 species/m2 respectively. These densities were respectively 0.023 species/m2 and 0.017 species/m2 in the port, and 0.011 species/m2 and 0.014 species/m2 in beach rocks. This means that species density was ten-fold higher in flotsam than in hard substrates of beaches and in the port. On the other hand, the factor “species type by sharing” (shared versus exclusive of an environment) was not significant (Table 2), meaning that the density of shared and exclusive species was not significantly different in these environments.

**Table 3.** Two-way ANOVA without replication showing the effect of the factors environment type (litter, port, beaches) and species sharing (shared versus exclusive species) on the density of species in this study, measured as number of species per square meter.

| Factor | Sum of squares | df | Mean square | F | P (same) |
|---|---|---|---|---|---|
| Environment type: | 0.098 | 2 | 0.049 | 140 | 0.007 |
| Sharing: | 0.0003 | 1 | 0.0003 | 0.837 | 0.457 |
| Error: | 0.0007 | 2 | 0.0003 | | |
| Total: | 0.099 | 5 | | | |

*3.2 Non-indigenous species in the ecosystem components analyzed*

In total 124 individuals of 23 NIS were found and barcoded in this study (Table 4, Supplementary table 1). They belonged to eight classes: Polychaeta, Ascidiacea (tunicates) and Bivalvia, with four species of each class; Hexanauplia (barnacles), Gymnolaemata (bryozoans) and Anthozoans (two sea anemones and one coral), with three species in each class; one Malacostraca (an isopod); one Actinopterygii (*Acanthogobius*, a fish). Ten of the 23 NIS are invasive species (Table 4): two barnacles, the three bryozoans, three of the four tunicates, the fish and the Japanese oyster *Magallana gigas*.

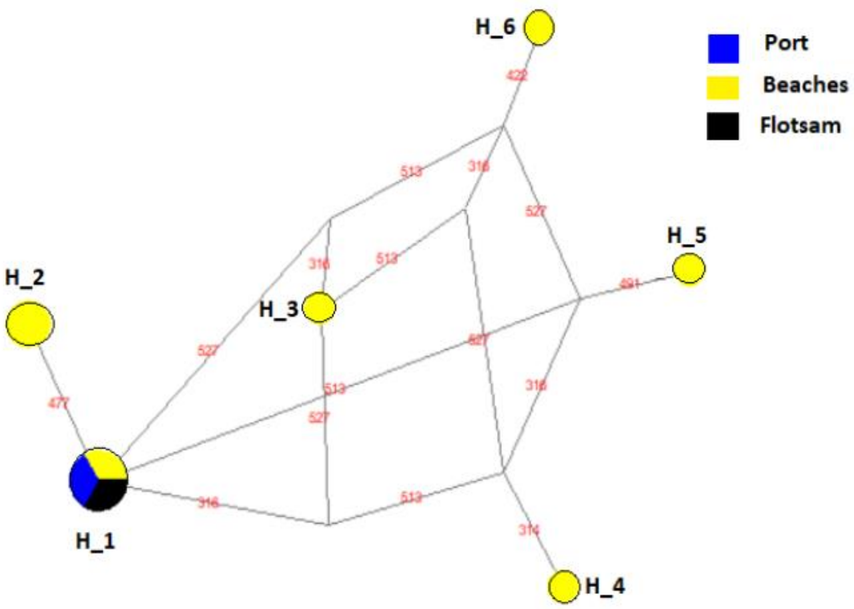
**Table 4.** Non-indigenous species found in this study in the three environments analyzed, and their invasive status as recognized in the Invasive Species Compendium (CABI, 2021) and National Estuarine and Marine Exotic Species Information System (NEMESIS, 2021). N=number of individuals barcoded. The density of NIS in each environment is presented.

| Species | Class | Native distribution | Status | N | Port | Flotsam | Beaches |
|---|---|---|---|---|---|---|---|
| *Phyllodoce groenlandica* | Polychaeta | Other North Atlantic waters | Exotic | 2 | 1 | 0 | 0 |
| *Spirobranchus latiscapus* | Polychaeta | Red Sea | Exotic | 4 | 0.75 | 0.25 | 0 |
| *Spirobranchus taeniatus* | Polychaeta | Australia | Exotic | 2 | 0.5 | 0.5 | 0 |
| *Dipolydora capensis* | Polychaeta | South Africa | Exotic | 2 | 1 | 0 | 0 |
| *Austrominius modestus* | Hexanauplia | Australasian | Invasive | 2 | 0 | 1 | 0 |
| *Balanus trigonus* | Hexanauplia | Pacific | Invasive | 9 | 0.89 | 0.11 | 0 |
| *Chamaesipho columna* | Hexanauplia | New Zealand | Exotic | 1 | 0 | 1 | 0 |
| *Livoneca redmanii* | Malacostraca | Caribbean | Exotic | 5 | 1 | 0 | 0 |
| *Bugula neritina* | Gymnolaemata | Unknown | Invasive | 1 | 1 | 0 | 0 |
| *Amathia verticillata* | Gymnolaemata | Unknown | Invasive | 7 | 1 | 0 | 0 |
| *Watersipora subtorquata* | Gymnolaemata | Unknown | Invasive | 12 | 1 | 0 | 0 |
| *Acentrogobius cf. pflaumii* | Actinopterygii | North Pacific | Invasive | 1 | 1 | 0 | 0 |
| *Diplosoma listerianum* | Ascidiacea | Unknown | Cryptogenic | 3 | 1 | 0 | 0 |
| *Microcosmus squamiger* | Ascidiacea | Australia | Invasive | 4 | 0.5 | 0 | 0.5 |
| *Botryllus schlosseri* | Ascidiacea | Unknown-Pacific? | Cryptogenic | 1 | 1 | 0 | 0 |
| *Styela plicata* | Ascidiacea | Atlantic - Western central | Invasive | 3 | 1 | 0 | 0 |
| *Anthopleura anjunae* | Anthozoa | Indo-Pacific | Exotic | 1 | 1 | 0 | 0 |
| *Anthopleura elegantissima* | Anthozoa | Pacific | Exotic | 2 | 1 | 0 | 0 |
| *Caryophyllia grayi* | Anthozoa | South Pacific | Exotic | 1 | 1 | 0 | 0 |
| *Mytilaster minimus* | Bivalvia | East Mediterranean | Exotic | 27 | 0.89 | 0 | 0.11 |
| *Mytilus trossulus* | Bivalvia | Northwest Atlantic - Baltic | Exotic | 7 | 0.71 | 0 | 0.29 |
| *Magallana gigas* | Bivalvia | Northwest Pacific | Invasive | 25 | 0.56 | 0.08 | 0.36 |
| *Talochlamys multistriata* | Bivalvia | South Africa/ Mediterranean | Exotic | 2 | 1 | 0 | 0 |
| | | | NIS number | | 21 | 6 | 4 |
| | | | NIS density | | 0.012 | 0.132 | 0.002 |

The proportion of NIS (Tables 1 & 4, Figure 4) in the three environments was significantly different ($\chi 2 = 8.9$, 2 d.f., $p = 0.01$ for the distribution of NIS versus native species). The port contained the highest proportion of NIS (28.8% of species), followed by flotsam (22.2% of species) and finally beaches (7.4% of species). Of the three environments, floating litter contained the highest density of NIS (Table 4), ten times higher than that of the port and 60 times higher than NIS density in beaches.

All the four NIS found from beaches were shared with ports (principally) and flotsam (Table 4). The majority of NIS of this study were found only within the port (15 species, the 65% of the total number of NIS). Two barnacles (*Austrominius modestus* and *Chamaesipho columna*) were found only on flotsam, and none was exclusive of beaches. A total of six NIS (the 26%) were shared between environments (Figure 4, Table 4). Only one species, the Japanese oyster *Magallana gigas* (Supplementary Table 1), was present in the three environments considered (port, litter and natural substrate in beaches; Table 4). Different haplotypes were found for this species (Figure 5). The most frequent Haplotype 1 was shared between the three environments (Figure 5A). According to sequence databases, this haplotype and is also present in other regions (Figure 5B) that are connected with the port of Gijon by maritime traffic: North America, North Sea and Mediterranean Sea (Miralles et al., 2018b). This would suggest transference of this species between environments, probably in the direction port – flotsam - beaches. Five more haplotypes were found only in beaches, which is expected given repeated introductions of *Magallana gigas* in the region via aquaculture (Semeraro et al., 2016).

A)



B)

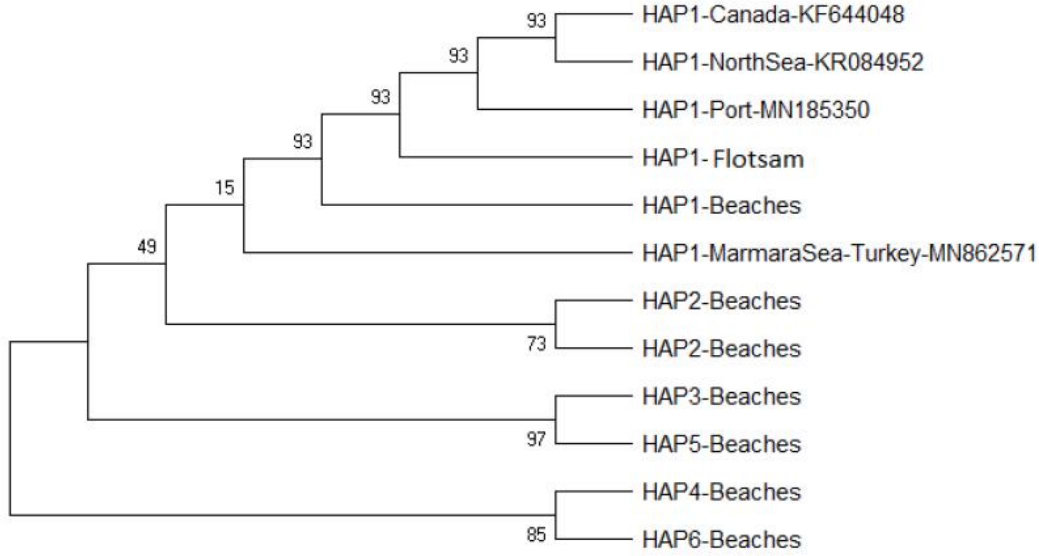



**Figure 5. A:** Median-joining network showing the relationships among the *Magallana gigas* haplotypes defined by COI sequence variation. H_1-H_6 are the haplotype names. Areas in circles are proportional to the frequency of each haplotype. Color codes as blue, ports; yellow, beaches; black, flotsam. **B:** Maximum Parsimony analysis of haplotypes from *Magallana gigas.* Tree #1 out of 10 most parsimonious trees (length = 122) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Sequences from Canada, the North Sea and Marmara Sea were included from NCBI databases (Accession numbers are provided).

Similar haplotype analysis could not be done in other species due to insufficient information, because the majority of the shared species had too few haplotypes. For example, *Balanus trigonus* sequences had the same haplotype in port and flotsam individuals, and *Mytilus trossulus* had only two haplotypes, one in the port and other in two individuals found from beaches, which is not much informative.

Focusing specifically on the 10 invasive NIS, all of them were in the port except the acorn barnacle *Austrominius modestus* that was found only on flotsam in these samplings (Table 4). The majority were restricted to the port with a few exceptions: *Balanus trigonus* (present in litter too), *Microcosmus squamiger* (also in beaches) and *Magallana gigas* (in the three environments as explained above). The proportion of species shared between environments, three of 10 (30%) was similar to that of total NIS (7 of 23, 30.4%) and that of native species (29 of 88, 33%). From these data, NIS/invasive NIS do not move more frequently among environments than native species.

NMDS analysis (low stress of 0.011, r2 of axis 1 = 0.68 and r2 of axis 2 = 0.11) supported a possible role of flotsam as an intermediary between the port and surrounding beaches for species dispersal. The scatter plot showed flotsam biota located between the port and the beaches (Figure 6). Arbeyal and Xivares, the closest beaches to flotsam in the NMDS plot, are the closest beaches to the port (Figure 1).

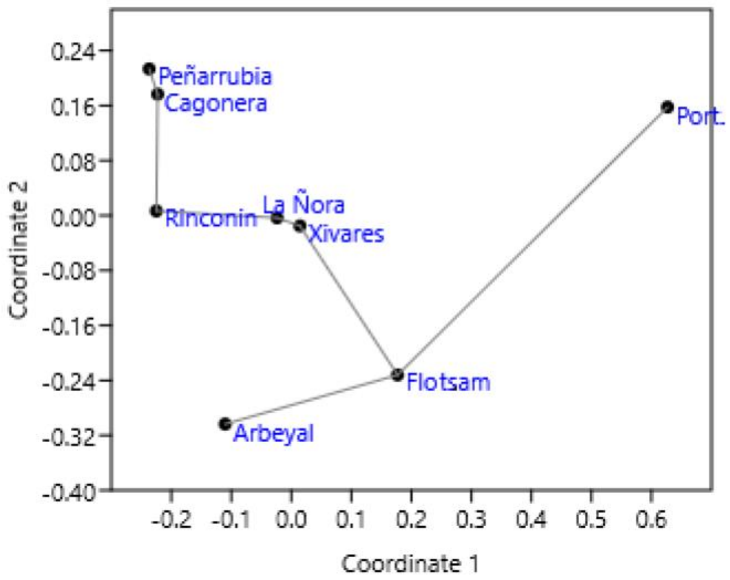



**Figure 6.** Scatter plot of NMDS with minimum spanning tree, constructed from presence-absence of the species found in this study using Bray-Curtis distance and 9999 permutations.

## 4. DISCUSSION

The first novelty of this study is the evidence of a higher density of species in flotsam than in port structures and natural substrates. At first, the opposite could be expected, because only a fraction of biopollutants (those preferring plastic substrate) would be transferred from port to beaches using flotsam as a vector. A high density from taxa that prefer plastic substrates could be expected on flotsam, as in the case of goose and acorn barnacles (Whitehead et al., 2011) or the bryozoan *Bugula neritina* (Li et al., 2016), but more species per surface unit was a really unexpected result, contradicting Hypothesis iii) that would expect a decrease in species density in the transfer vector (flotsam) in comparison to the donor (port) and recipient habitats (natural rocks). The reason could lie in a diversity of shapes, colors and materials in floating objects (illustrated in Figure 2), that have sufficient space to shelter many small individuals of the same or different species. The sum of such heterogeneous objects under the denomination of "floating litter habitat" makes it a quite diverse environment.

The results from this study showed the expected differences in the taxonomic composition of the three environments analyzed, thus confirming Hypothesis ii) (only a fraction of biopollutants that prefer plastic substrate will be transferred from port to beaches). In fact, the plastic flotsam here analyzed looks like a partially separated world, enriched in species belonging to the classes Malacostraca and Hexanauplia that make up a community that strongly differs from those observed in the port or on the beaches. These results are consistent with previous studies that indicate that marine litter will be fouled by a fraction of the total organisms inhabiting an environment, which will depend on a series of abiotic and biotic factors (Gibson et al., 2006). These factors include the ecological characteristics of the encrusting biota, such as having a long larval cycle, or being suspension feeders, which will be helpful to survive in abiotic substrates like marine litter where organisms depend on food resources from the surrounding environment (Kiessling et al., 2015). In the same way, the characteristics of marine litter (material, size, roughness) can also limit biofouling (Bravo et al., 2011), with large plastic fragments generally allowing the adherence of a great diversity of species (Goldstein et al., 2014; Shabani et al., 2019).

This study also revealed similar density of shared and exclusive species in all the environment components, indicating that species shared are not denser in any environment than exclusive species. In other words, there are still exclusive species but many are shared regardless of the type of environment. Likewise, NIS and invasive species were not more frequently shared between environments than native species. This can be interpreted as artificial substrates in ports and flotsam are habitats (and vectors in the case of flotsam) of both NIS and native species in this region, as shown in the Baltic Sea (Garcia-Vazquez et al., 2018). The invasive condition would not determine the likelihood of occupation of artificial litter, which would act as a generic way of transport of any species (native or NIS) able to attach to it.

The high proportion of NIS found in the port (28.8% of the species) was consistent with previous results in the same area (Miralles et al., 2016), and also with results in very distant and ecologically different regions like Polynesia (Ardura et al., 2021). As expected, the port of Gijón was the environment component where more NIS were detected. This is due to the fact that ports are hotspots for the introduction of exotic species because of the intense maritime traffic, which is considered one of the most important pathways for the dispersal of marine species (López-Legentil et al., 2015; Ulman et al., 2017; Orlando-Bonaca et al., 2021). On the other hand, although having a lower proportion of NIS (22.2%), flotsam showed the highest densities of biopollutants, even surpassing the densities in the port surfaces. While all the NIS that were found on the beaches were also present in the port or in flotsam, the species attached to floating litter were shared in a great proportion with both natural and port areas and few of them were exclusive to flotsam. These results show the potential of the port of Gijon as a donor of NIS to the adjacent areas and remark the role of marine litter as a facilitator of their dispersion (Miralles et al., 2018a). Marine litter can be used by invasive species as a vector for the colonization of new areas. Clear examples in our study are the invasive species *Balanus trigonus* and *Magallana gigas* which were found alive fouling different litter items floating adrift on the coast near the port of Gijón. Moreover, the same COI haplotype was found in the port, flotsam and some nearby beaches for *Magallana gigas*, and in the port and flotsam for *Balanus trigonus*, reinforcing the idea of flotsam as a vector of invasive species between the port and other areas (Miralles et al., 2018a).

In our results, the beaches and beached objects seem to harbor different communities from those present in the flotsam. Interestingly, only a few beached objects had visible attached biota, while a large proportion of flotsam objects carried living macroscopic animals. Specimens might have been removed from the beached litter due to scavengers, desiccation, etc. The fauna associated to flotsam is still alive while the one on the beached objects might have been there but then washed away. It is worth mentioning that Arbeyal beach was not connected to the rest of the beaches in the NMDS (Figure 5). This may be due to the influence of marine litter and NIS that may come from the marinas located near this beach. Previous studies have shown that the communities that inhabit marinas are slightly different from those found in ports (Miralles et al., 2021) and that non-industrial traffic from these marinas can also be a vector for the expansion of NIS to new areas. Marinas should also be considered when carrying out studies on the pathways of propagation that biopollutant species may employ for their dispersal.

The introduction and uncontrolled proliferation of exotic species can have serious environmental consequences in the ecosystems which include competition for resources with native species, predation or the transmission of novel pathogens among others (Vilcinskas, 2015; Miaud et al., 2016; David et al., 2017; Yu et al., 2018). In this context, biological invasions can encompass threats to the sustainability of fishing resources and therefore, compromise the Sustainable Development Goal #14. Both marine litter and all the species found in this study should be taken into account for control and management to meet Sustainable Development Goal #14 in the Bay of Biscay. In order to avoid further deterioration of marine ecosystems and deal with biological invasions, there is an urgent need to reduce those pathways used by invasive species to disperse and enter new habitats, including marine litter. Among different actions that have been developed to reduce the presence of marine litter in aquatic ecosystems, the EU Marine Strategy Framework Directive (MSFD, 2008/56/EC) establishes a framework for the protection and sustainable use of marine ecosystems.

This regulation also contemplates marine litter under the descriptor #10 that contains different considerations and proposals discussing the effectiveness of measures leading to reductions in marine litter (Galgani et al., 2013). At a more local scale, campaigns such as "*Mójate por un mar sin residuos*" (Get involved in a sea without waste) or "*Basura a mares*" (Litter everywhere) have been carried out in Asturias, with the objectives of removing litter from the sea by fishermen or raising awareness among citizens about the problem posed by the litter that reaches the marine environment (Autoridad Portuaria de Gijón, 2018). In fact, to face the problem of marine litter, it is also necessary to generate citizen awareness to give rise to a more cooperative and involved society (Locritani et al., 2019). The reduction of marine litter will not only solve the problems that it causes to biota (intoxications, entanglements), but also prevent it from being used as a vector for the expansion of biopollutant species.

## ACKNOWLEDGMENTS

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## SUPPLEMENTARY INFORMATION

Supplementary table 1. Individuals found in the sampling points considered and litter objects.

| | | Gijon port | | | Beaches | | | | | | Litter | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Taxonomic classification | Species | Marina | Port | Ships | Xivares | Arbeyal | Rinconin | Peñarrubia | Cagonera | La Ñora | Flotsam | Beached litter |
| Annelida, Polychaeta, Dorvilleidae | *Ophryotrocha puerilis* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Eunicidae | *Lysidice ninetta* | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Eunicidae | *Leodice harassii* | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Lumbrineridae | *Scoletoma funchalensis* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Nereididae | *Nereis splendida* | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Nereididae | *Nereis pelagica* | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Nereididae | *Platynereis dumerilii* | 13 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Phyllodocidae | *Eulalia clavigera* | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Phyllodocidae | *Eulalia viridis* | 0 | 3 | 0 | 2 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| Annelida, Polychaeta, Phyllodocidae | *Eumida sanguinea* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| Annelida, Polychaeta, Phyllodocidae | *Nereiphylla lutea* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Phyllodocidae | *Phyllodoce groenlandica* | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Sabellariidae | *Sabellaria alveolata* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Annelida, Polychaeta, Sabellariidae | *Sabellaria spinulosa* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 |
| Annelida, Polychaeta, Serpulidae | *Serpula concharum* | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Serpulidae | *Spirobranchius sp.* | 0 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Serpulidae | *Spirobranchus latiscapus* | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Annelida, Polychaeta, Serpulidae | *Spirobranchus taeniatus* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Annelida, Polychaeta, Serpulidae | *Spirobranchus triqueter* | 0 | 4 | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Spionidae | *Dipolydora capensis* | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Syllidae | *Syllis gracilis* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Annelida, Polychaeta, Terebellidae | *Terebella lapidaria* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Arthropoda, Decapoda, Grapsidae | *Pachygrapsus marmoratus* | 0 | 5 | 0 | 0 | 1 | 1 | 0 | 2 | 0 | 0 | 0 |
| Arthropoda, Hexanauplia, Balanidae | *Perforatus perforatus* | 2 | 23 | 26 | 1 | 0 | 0 | 0 | 0 | 0 | 4 | 1 |
| Arthropoda, Hexanauplia, Austrobalanidae | *Austrominius modestus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| Arthropoda, Hexanauplia, Balanidae | *Balanus trigonus* | 0 | 2 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Arthropoda, Hexanauplia, Balanidae | *Perforatus perforatus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Arthropoda, Hexanauplia, Chthamalidae | *Chamaesipho columna* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Arthropoda, Hexanauplia, Chthamalidae | *Chthamalus montagui* | 0 | 18 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 3 | 0 |
| Arthropoda, Hexanauplia, Chthamalidae | *Chthamalus stellatus* | 0 | 28 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 3 | 0 |
| Arthropoda, Hexanauplia, Lepadidae | *Lepas anatifera* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 |
| Arthropoda, Malacostraca, Ampithoidae | *Ampithoe rubricata* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Arthropoda, Malacostraca, Carcinidae | *Carcinus maenas* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Arthropoda, Malacostraca, Corophiidae | *Monocorophium acherusicum* | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Arthropoda, Malacostraca, Cymothoidae | *Livoneca redmanii* | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Arthropoda, Malacostraca, Dexaminidae | *Dexamine spiniventris* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Arthropoda, Malacostraca, Diogenidae | *Clibanarius erythropus* | 0 | 0 | 0 | 0 | 1 | 3 | 3 | 4 | 0 | 1 | 0 |
| Arthropoda, Malacostraca, Gammaridae | *Gammarus crinicornis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Arthropoda, Malacostraca, Gecarcinidae | *Gecarcoidea natalis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Arthropoda, Malacostraca, Grapsidae | *Planes minutus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 0 |
| Arthropoda, Malacostraca, Hippolytidae | *Hippolyte varians* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 |
| Arthropoda, Malacostraca, Idoteidae | *Idotea balthica* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 6 | 0 |
| Arthropoda, Malacostraca, Melitidae | *Abludomelita obtusata* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| Arthropoda, Malacostraca, Niphargidae | *Niphargus laticaudatus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Arthropoda, Malacostraca, Palaemonidae | *Palaemon elegans* | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 0 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Arthropoda, Malacostraca, Palaemonidae | *Palaemon serratus* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Arthropoda, Malacostraca, Pilumnidae | *Pilumnus hirtellus* | 1 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Arthropoda, Malacostraca, Pinnotheridae | *Pinnotheres pisum* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Arthropoda, Malacostraca, Polybiidae | *Liocarcinus corrugatus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 0 |
| Arthropoda, Malacostraca, Porcellanidae | *Porcellana platycheles* | 0 | 5 | 0 | 0 | 0 | 2 | 1 | 1 | 0 | 1 | 0 |
| Arthropoda, Malacostraca, Porcellanidae | *Pisidia longicornis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Arthropoda, Malacostraca, Talitridae | *Orchestia mediterranea* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Arthropoda, Malacostraca, Talitridae | *Talorchestia martensii* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Arthropoda, Malacostraca, Xanthidae | *Lophozozymus incisus* | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 |
| Bryozoa, Gymnolaemata, Bugulidae | *Bugula neritina* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Bryozoa, Gymnolaemata, Vesiculariidae | *Amathia verticillata* | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Bryozoa, Gymnolaemata, Watersiporidae | *Watersipora subtorquata* | 3 | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chordata, Actinopterygii, Gobiesocidae | *Lepadogaster lepadogaster* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 |
| Chordata, Actinopterygii, Gobiidae | *Acentrogobius sp.* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chordata, Ascidiacea, Didemnidae | *Diplosoma listerianum* | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chordata, Ascidiacea, Polyclinidae | *Morchellium argus* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chordata, Ascidiacea, Clavelinidae | *Clavelina lepadiformis* | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chordata, Ascidiacea, Pyuridae | *Microcosmus squamiger* | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| Chordata, Ascidiaceae, Styelidae | *Botryllus schlosseri* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chordata, Ascidiaceae, Styelidae | *Styela plicata* | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cnidaria, Anthozoa, Actiniidae | *Anemonia viridis* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cnidaria, Anthozoa, Actiniidae | *Anthopleura anjunae* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cnidaria, Anthozoa, Actiniidae | *Anthopleura elegantissima* | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cnidaria, Anthozoa, Caryophylliidae | *Caryophyllia grayi* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Echinodermata, Asteroidea, Asteriidae | *Coscinasterias tenuispina* | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 |
| Echinodermata, Asteroidea, Asteriidae | *Marthasterias glacialis* | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Echinodermata, Asteroidea, Asterinidae | *Asterina gibbosa* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 |
| Echinodermata, Echinoidea, Parechinidae | *Paracentrotus lividus* | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| Echinodermata, Holothuroidea, Synaptidae | *Oestergrenia digitata* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Echinodermata, Ophiuroidea, Ophiotrichidae | *Ophiothrix fragilis* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| Echinodermata, Ophiuroidea, Ophiotrichidae | *Ophiothrix sp* | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 |
| Mollusca, Bivalvia, Gryphaeidae | *Neopycnodonte cochlear* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Mollusca, Bivalvia, Limidae | *Limaria hians* | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 |
| Mollusca, Bivalvia, Mytilidae | *Mytilaster minimus* | 11 | 13 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Bivalvia, Mytilidae | *Mytilus galloprovincialis* | 0 | 8 | 3 | 1 | 0 | 3 | 0 | 0 | 1 | 2 | 0 |
| Mollusca, Bivalvia, Mytilidae | *Mytilus sp* | 32 | 6 | 4 | 4 | 0 | 3 | 2 | 0 | 5 | 0 | 0 |
| Mollusca, Bivalvia, Mytilidae | *Mytilus trossulus* | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| Mollusca, Bivalvia, Noetiidae | *Striarca lactea* | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Bivalvia, Ostreidae | *Magallana gigas* | 0 | 14 | 0 | 5 | 4 | 0 | 0 | 0 | 0 | 2 | 0 |
| Mollusca, Bivalvia, Ostreidae | *Ostrea edulis* | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Bivalvia, Pectinidae | *Talochlamys multistriata* | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Chromodorididae | *Felimare villafranca* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Fionidae | *Fiona pinnata* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Mollusca, Gastropoda, Haliotidae | *Haliotis tuberculata* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Littorinidae | *Melarhaphe neritoides* | 0 | 7 | 0 | 3 | 0 | 2 | 0 | 0 | 3 | 0 | 0 |
| Mollusca, Gastropoda, Muricidae | *Nucella lapillus* | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Muricidae | *Ocenebra erinaceus* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Muricidae | *Ocinebrina sp.* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mollusca, Gastropoda, Muricidae | *Stramonita haemastoma* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Nassariidae | *Tritia reticulata* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Nassariidae | *Nassarius incrassatus* | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Patellidae | *Patella aspera* | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Patellidae | *Patella depressa* | 0 | 11 | 0 | 3 | 1 | 3 | 3 | 3 | 3 | 2 | 0 |
| Mollusca, Gastropoda, Patellidae | *Patella sp* | 9 | 0 | 0 | 0 | 0 | 1 | 2 | 3 | 3 | 0 | 0 |
| Mollusca, Gastropoda, Patellidae | *Patella ulyssiponensis* | 0 | 4 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| Mollusca, Gastropoda, Patellidae | *Patella vulgata* | 0 | 10 | 0 | 1 | 4 | 3 | 1 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Pleuribranchidae | *Berthellina edwardsii* | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Trochidae | *Phorcus lineatus* | 0 | 5 | 0 | 5 | 0 | 4 | 0 | 1 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Trochidae | *Phorcus sauciatus* | 0 | 2 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Trochidae | *Steromphala cineraria* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mollusca, Gastropoda, Trochidae | *Steromphala umbilicalis* | 0 | 10 | 0 | 2 | 3 | 1 | 2 | 6 | 7 | 4 | 0 |
| Mollusca, Polyplacophora, Acanthochitonidae | *Acanthochitona crinita* | 0 | 8 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Mollusca, Polyplacophora, Lepidochitonidae | *Lepidochitona cinerea* | 0 | 1 | 0 | 1 | 3 | 2 | 0 | 0 | 0 | 0 | 0 |
| Nemertea, Pilidiophora, Lineidae | *Riseriellus occultus* | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| Porifera, Demospongia, Halichondriidae | *Hymeniacidon sp.* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sipuncula, Phascolosomatidea, Phascolosomatidae | *Phascolosoma granulatum* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Number of individuals | 99 | 250 | 73 | 42 | 22 | 48 | 29 | 33 | 34 | 66 | 5 |
| | Number of species | 20 | 56 | 15 | 19 | 11 | 29 | 20 | 17 | 16 | 26 | 4 |

Supplementary table 2. Floating litter objects. GPS coordinates, sampling point, type of object, main types of attached biota, and sample codes for further barcoding, are presented. NI, not identifiable.

| Coordinates | Sampling point | | Material | Biota type | Sample code |
|---|---|---|---|---|---|
| 43.5495667 -5.6519667 | P1 | | Ice cream wrap | No biota | -- |
| 43.5568167° - 005.6201667° | P2 | | Cuttlebone | No biota | -- |
| 43.5481500° - 005.5930333° | P3 | | White plastic | Green algae | P3A |
| | | | | Red algae | P3B |
| | | | | Brown algae | P3C |
| | | | | Brown algae | P3D |
| | | | | Red fouling | P3D |
| 43.5481500° - 005.5930333° | P4 | | Sanitary pad | Red algae | P4A |
| | | | | Green algae | P4B |
| | | | | Brown algae | P4C |
| | | | | Transparent algae | P4D |
| | | | | Small crustacean | P4E |
| | | | | Brown/black algae | P4F |
| 43.5502000° - 005.5848000° | P5 | | White plastic bag | Red algae | P5A |
| | | | | Brown algae | P5B |
| | | | | Red algae | P5C |
| 43.5503333° - 005.5848000° | P6 | | White plastic bottle | Small crustaceans | P6A |
| | | | | Brown algae | P6B |
| | | | | Red algae | P6C |
| 43.5554833° - 005.5865500° | P7 | | Plastic film | No biota | -- |
| 43.5605667° - 005.6109833° | P8 | P8.1 | Blue plastic rod | No biota | -- |
| | | P8.2 | Transparest plastic paper | No biota | -- |
| | | P8.3 | White plastic piece | Very small crustaceans | P8.3A |
| | | P8.4 | Sunflower seed shell | Very small crustaceans | P8.4A |
| | | P8.5 | Plastic bottle cap | Fouling algae | P8.5A |
| | | P8.6 | Big black plastic foil | Acorn barnacle | P8.6A |
| | | | | Small crustaceans | P8.6B |
| | | | | Prawns | P8.6C |
| | | | | Isopod | P8.6D |
| | | | | Fouling NI | P8.6E |
| | | | | Tube worm | P8.6F |
| 43.5578167° - | P9 | P9.1 | Red hard plastic | No biota | -- |
| | | P9.2 | Earbud stick | No biota | -- |

| | | | | | |
|---|---|---|---|---|---|
| 005.6087500 ° | | P9.3 | White hard plastic | Small crustaceans | P9.3A |
| | | | | Red fouling (Bryozoan) | P9.3B |
| 43.5578833° - 005.6180833 ° | P10 | | Plastic starfish toy | Bryozoan | P10A |
| | | | | Prawns | P10B |
| | | | | Small crustaceans | P10C |
| | | | | Fouling NI | P10D |
| 43.5517500° - 005.6702500 ° | P11 | | Snacks plastic bag | No biota | -- |
| 43.5892500° - 005.6848333 ° | P12 | | Plastic sand shovel toy | Shrimp | P12A |
| | | | | Fouling NI | P12B |
| 43.5866167° - 005.6862667 ° | P13 | | Candle | Brown algae | P13A |
| | | | | Red algae | P13B |
| | | | | Fouling NI | P13C |
| 43.5867167° - 005.6890833 ° | P14 | P14.1 | Green plastic scrap | Fouling NI | P14.1 |
| | | P14.2 | Red plastic scrap | Shrimp | P14.2A |
| | | | | Brown algae | P14.2B |
| | | | | Small crustaceans | P14.2C |
| | | | | NI fouling | P14.2D |
| 43.5870000° - 005.7015333 ° | P15 | P15.1 | White plastic prope | Brown algae | P15.1A |
| | | | | Small crustaceans | P15.1B |
| | | | | Shrimp | P15.1C |
| | | | | Fouling NI | P15.1D |
| | | P15.2 | Actimel bottle | Very small crustaceans | P15.2A |
| | | | | Small crustaceans | P15.2B |
| | | | | Transparent NI fouling | P15.2C |
| | | | | Brown algae | P15.2D |
| | | P15.3 | White plastic straw | Amphipoda NI | P15.3A |
| | | | | Very small crustaceans | P15.3B |
| | | | | Brown fouling | P15.3C |
| | | P15.4 | Sanitary pad | Very small crustaceans | P15.4A |
| | | | | Small crustaceans | P15.4B |
| | | | | Small crustaceans | P15.4C |
| | | | | Algae | P15.4D |
| | | P15.5 | Tetra brick lid | Algae | P15.5A |
| 43.5783333° - 005.7081000 ° | P16 | | Plastic bottle cap | Brown algae | P16A |
| | | | | Red fouling NI | P16B |
| | P17 | | | Acorn barnacle | P17A |

| | | | | | |
|---|---|---|---|---|---|
| 43.5771667° - 005.7090333 ° | | | Big green tupperware lid | Brown fouling NI | P17B |
| 43.5744833° - 005.7169333 ° | P18 | P18.1 | Soap/wax piece | No biota | -- |
| | | P18.2 | Plastic paper | Small crustaceans | P18.2A |
| | | | | Fouling NI | P18.2B |
| | | P18.3 | White plastic scrap | Fouling NI | P18.3A |
| | | P18.4 | White plastic scrap | Small crustaceans | P18.4A |
| 43.5765833° - 005.7152000 ° | P19 | | Coke label | No biota | -- |
| 43.5774833° - 005.6734000 ° | P20 | | Big brown plastic cap | White fouling NI | P20A |
| 43.5850167° - 005.6716000 ° | P21 | P21.1 | White plastic bottle cap | Shrimp | P21.1A |
| | | P21.2 | Red plastic cap | Brown fouling NI | P21.2A |
| | | P21.3 | Plastic bag | Small crustaceans | P21.3A |
| | | | | Shrimp | P21.3B |
| | | | | Very small crustaceans | P21.3C |
| | | | | Small crustaceans | P21.3D |
| | | | | NI fouling | P21.3E |
| | | P21.4 | Plastic scrap | No biota | -- |
| | | P21.5 | Earbud stick | No biota | -- |
| | | P21.6 | Fishing gear | Very small crustaceans | P21.6A |
| | | | | Orange fouling | P21.6B |
| | | P21.7 | Plastic scrap | Brown fouling | P21.7A |
| | | P21.8 | Plastic paper | No biota | -- |
| | | P21.9 | Plastic cap | No biota | -- |
| | | P21.10 | Fishing gear | Algae | P21.10A |
| | | P21.11 | Small bottle | NI fouling | P21.11A |

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# Capítulo 5

**Perspectives on the marine environment and biodiversity in recreational ports: the marina of Gijon as a case study.**

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# Perspectives on the marine environment and biodiversity in recreational ports: the marina of Gijon as a case study.

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## HIGHLIGHTS

•Citizens from Gijon show low knowledge about Marine Biodiversity and Biosecurity.

•Education and social media are considered the main information sources by citizens.

•Science outreach lacks an effective communication with Gijon citizens.

•Citizens perceive environmental problems visually rather than on a cognitive way.

## ABSTRACT

Recreational ports are known to be sources of pollution to the coastal marine environment due to the pouring of pollutants or the transfer of invasive species to neighboring areas. Nonetheless, the responsibility of protecting the marine environment does not lie solely on the users of the ports, but also affects the rest of citizens. Thus, an effective communication is necessary between scientists and citizens to avoid the lack of knowledge and boost cooperation against these environmental problems. In this study, (focused on the marina of Gijon, Northwestern Spain) citizens set education and social media as the main sources of information, rarely considering science outreach. Also, their environmental knowledge showed to be based on a visual perception, rather than on a cognitive one, as marine litter was considered a great environmental problem, while invasive species and biofouling went unnoticed, remarking the lack of an effective communication from scientific sources.

**KEYWORDS**: Marine biodiversity; Science literacy; Blue economy; Invasive species

## 1. INTRODUCTION

Marine ecosystems occupy two thirds of our planet. They represent a dynamic ecosystem of constant interaction of living organisms. The biodiversity they harbor is one of their main characteristics, but marine ecosystems are also an important source of services, as they support different economic activities that are necessary for human well-being. Marine species are used as providers of food, shelter, medicines and livelihoods and are also sources for economic activities like tourism and fishing. Moreover, sea outside, in the land zones, marine coastal environments and their biodiversity are a fundamental base for many ecosystem services, as they support the 90% of marine exploitation resources (Barnabe and Barnabe-Quet, 2000).

However, due to the increase of human pressures, global biodiversity indicators of marine ecosystems are showing an accelerated decline all over the world (Halpern et al., 2008; Butchart et al., 2010; McCauley et al., 2015). Overexploitation is one of the most important threats to the marine environment; fisheries and their continued resource consumption has led to a situation where the 33.1% of world fish stocks are subject to overfishing (Food and Agriculture Organization, 2018), triggering drastic reduction in species population sizes like it happened in the case of the Bluefin tuna (*Thunnus thynnus*) in the Mediterranean Sea (Block, 2019). Similarly, anthropogenic litter has become another serious problem for marine ecosystems, as debris ends up in the sea where marine life is harmed: some species can get strangled by nets, macroplastics can cause death due to indigestions and microplastics (plastics degraded into particles smaller than 5 mm) can enter the food chain and become sources of toxic chemicals that are released to the environment (Vélez-Rubio et al., 2018; Liu et al., 2019). All along with this, our oceans are facing many other threats that alter the ecosystems, such as climate change that is causing global declines in tropical and subtropical coral species (Hughes et al., 2017) or shipping-associated pollution that causes mortality among many marine species (Walker et al., 2019).

Not only open seas, but also coastal marine environments are critically affected by human activities. Due to the rapid human population growth, new land is being reclaimed from the sea, causing severe habitat destruction and biodiversity loses (Lai et al., 2015; Tay et al., 2018). Within coastal areas, human populations are typically constructed around ports where activities related to the marine environment and its services are carried out. Commercial ports are receptors of ships that travel around the world, and alter marine ecosystems by generating air pollution, greenhouse gases, oil and chemical spills, garbage or underwater noise pollution (Christensen et al., 2018; Wan et al., 2018; Papaefthimiou et al., 2016; Tidau and Briffa, 2019; Walker et al., 2019). In addition, shipping also facilitates the transfer and spread of invasive species (via ballast water or biofouling), which cause biodiversity losses all over the world (Bellard et al., 2016; Doherty et al., 2016). These species colonize new habitats and affect the local ecosystem by competing or predating and can also affect humans by bringing new infectious diseases and economic loses (Molnar et al., 2008; Walsh et al., 2016; Bayliss et al., 2017). Maritime shipping is known to be the first pathway for marine invasions both when moving people and goods, and when ballast water is loaded or unloaded (Zaiko et al., 2015), so that ports are very vulnerable to be colonized by invasive species (Drake and Lodge, 2004). Prevention is the most effective way to fight these invasions: once the alien species is stablished in an ecosystem, its eradication is a very complicated and expensive process (Simpson et al., 2009), this means that an early detection of arriving species must be done, when the population is still manageable.

In coastal urbanizations we can find, in addition to commercial ports, marinas where recreational boaters (local or foreign people) sail along the coast with leisure purposes. Vessel activities occurring inside these recreational docks also serve as inputs of boating-associated pollutants that can alter the local coastal marine ecosystem. The main pollution sources from recreational boating are fuel, oil and other chemicals discharged from powered boats (Burgin and Hardiman, 2011). These pollutants can be discharged due to engine activities, affecting species present in the ecosystem (Whitfield and Becker, 2014) but also by dilution from antifouling paints employed on ship hulls to prevent fouling by marine organisms (Schiff et al., 2004). Actually, as these treatments contain toxic chemicals for some organisms, they also have an effect against biological invasions via recreational boating, which has been classified as an important vector for secondary dispersal of non-indigenous and invasive species (Clarke Murray et al., 2011; Drake et al., 2017). Apart from this, recreational boating has also shown to be a source of plastic litter that ends in the marine environment, contributing to environmental degradation in the area (Milliken and Lee, 1990; Mehlhart and Blepp, 2012). Moreover, as these ports are typically located inside cities (to be close to local citizens that are its main users), they have a strong interaction with urban areas and land-based activities that can also be sources of pollution, such as rubbish that can reach water from urban runoff or landfills and affect the marine ecosystem (Walker et al., 2006; Munari et al., 2016).

At this point, an urgent solution is needed to fight this complex of environmental problems. To this day, several policies and protocols have been developed, in order to protect marine ecosystems and their biodiversity; MARPOL (The International Convention for the Prevention of Pollution from Ships) is one of them. Its main objectives are to prevent pollution by oils, harmful substances, dumping and air pollution produced by all kinds of vessels. The IAS (Invasive Alien Species) regulation enforced in Europe in 2015 (European Union, 2014) is another regulation that provides a set of measures for the prevention, early detection and management of invasive species in the European Union including plans that involve citizen science. Similarly, the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWMC) aims to prevent the spread of harmful aquatic organisms from one region to another, by establishing standards and procedures for the management and control of ships' ballast water and sediments (International Maritime Organization, 2004). Finally, the European Marine Strategy Framework Directive (EU MSFD) that was adopted in 2008 is focused on the protection of biodiversity and the achievement of good ecological status of the European marine waters by 2020 (European Commission, 2008).

In order to accomplish these regulations and meet the established objectives, the collaboration of all the groups of society is required. Stakeholders and policy makers need to be aware of the existing problems and potential ways to manage them, in order to put in practice necessary actions. In the same way, public support can be critical for future projects (McKinley et al., 2017) and it is necessary to understand the present attitudes within the society, in order to develop educational activities where scientific information can be transferred to citizens.

Access to this information that can raise awareness about environmental sustainability is produced through various channels: education, scientific dissemination, personal contact with the environment, media… and all these information flows can provide useful knowledge and awareness when adequate receptivity is obtained. However, there is still a gap of communication and collaboration among scientists, citizens, policy makers and stakeholders that hinders the preservation of coastal ecosystems and their resources (Young et al., 2016).

There are many studies about science literacy, and how the knowledge level of a population affects the public opinion about scientific projects or findings. Facts like religious and political identities determine attitudes of individuals, such as in the case of global warming issue (McCright and Dunlap, 2011; Maibach, 2015), however, it has been seen that a higher education level and scientific knowledge may suppose a higher support for scientific research motivated by personal nonscientific concerns (Drummond and Fischhoff, 2017). This is why it is necessary to identify and support the best sources of information that can improve the level of knowledge among different groups that compose society. This way, a better management of environmental issues could be achieved, aiming the development of a blue economy, based on the sustainable consumption of marine resources and the protection of biodiversity (Silver et al., 2015).

Here we present the case study of the Gijon's marina, a leisure port with 780 moorings distributed in four docks that is located in the city of Gijon, Asturias. This port has a regulation directed for all users of the marina: In order to avoid water pollution, all vessel users must comply with MARPOL directive, by using specific containers for the deposit of garbage, oils, bilges, fecal wastes or any other kind of wastes (IMO, 2011). Outside the leisure port, citizens are also responsible for maintaining the coastal environment on a good status; it is important to determine their level of awareness and the potential sources of information that contribute to a better comprehension of the potential environmental problems that the port may face. This study has the aim of defining the current perception that citizens show about the environmental conditions in the port and the potential impacts that can threaten the local marine biodiversity, also assessing the existing level of knowledge and the main sources of information that contribute it.

## 2. MATERIAL AND METHODS

A questionnaire was designed and personal and individually handed to random walkers at different parts of the city of Gijon, located in Asturias, Northwestern Spain (43° 20′ N, 6° 0′ W) (Fig. 1). The questionnaire was composed by 13 items, including questions about the port of Gijon and its environmental status, about the knowledge level of respondents and their perception about environmental stressors (Supplementary Table 1). All items were designed to be answered employing the Likert scale, with values from 1 to 5 excepting items 7, 8 and 9. The survey was carried out within October 2018 and April 2019 with a total of 200 respondents that were classified by sex, age, study level and by the frequency of their visits to the port area. The questionnaire was validated in a pilot trial with 15 volunteers; Cronbach's alpha was calculated for all the 13 items and for 10 items (excluding items 7, 8 and 9, that are not in Likert scale) obtaining values of 0,6564 and 0,6791 respectively.

**Fig. 1**. Geographical location of the Port of Gijon in Europe.

In order to determine citizen's general attitude towards the port, items 9, 11 and 12 were employed as they are related with the perception about the environmental status of the port and the effectiveness of its current environmental management strategies. As question 9 (is marine pollution a problem in this port?) is not in Likert scale, numerical values were given to responses, giving the value 1 to the answer “yes”, 5 to the answer “no” and 3 to answers “probably” and “I don't know”. This way a mean value for each respondent was built from these three items, resulting in a value from 1 to 5 that reflects the perception about the port, it's activities and environmental status.

Statistical analyses were carried out with non-parametric tests done in PAST program (Hammer et al., 2001) after checking normality in the dataset. Responses given by each population groups were compared using the non-parametric Mann-Whitney test and p values were estimated using Bonferroni correction.

## 3. RESULTS

Out of the 200 surveyed people, 117 (58,5%) were women and 83 (41,5%) were men. The 48% were younger than 30, and only 30 people were over 60 years of age. The education level was higher in most of the respondents (66,5%) and regarding the port visit frequency, the 62,5% of the answerers (125 people) affirmed that they seldom visited such areas, being the option “sometimes” the next most chosen one (39 people) (Fig. 2).

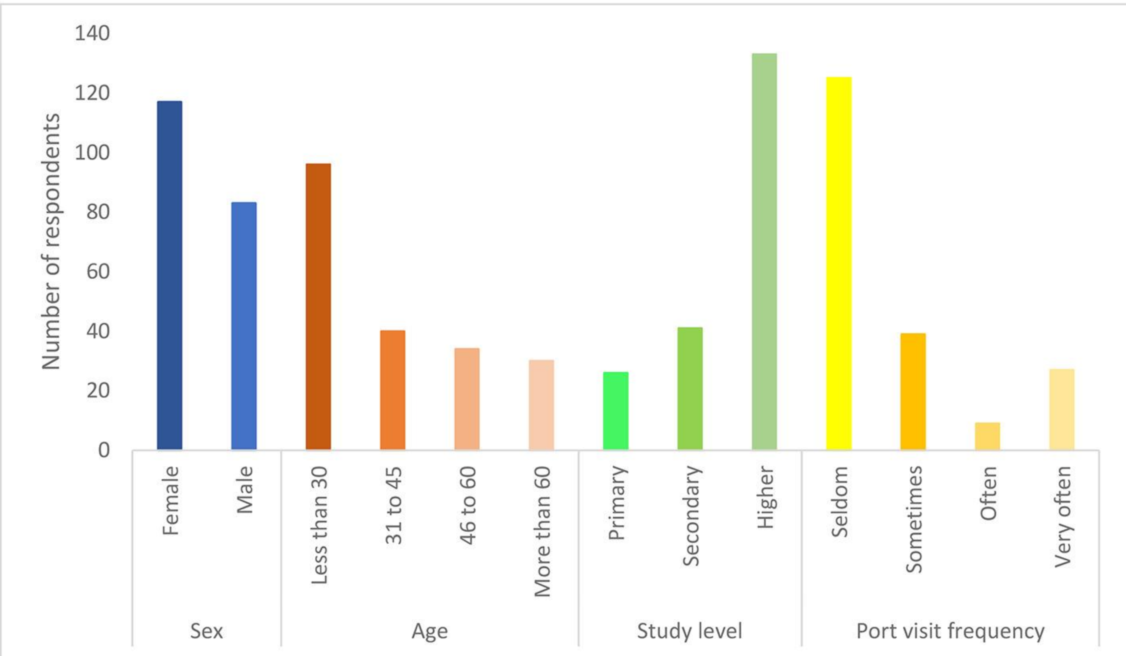


**Fig. 2**. Grouping of the surveyed population by age, sex, study level and port visit frequency and the number of respondents belonging to each option.

Considering the whole dataset, the opinion that citizens have about the port resulted to be considerably negative, with a mean value of 1,92 out of 5. Citizens think that marine pollution is a problem in the port area (mean value 1,3 out of 5); although some people

(9%) think that the port is in excellent or very good conditions, overall, citizens think that the marine ecosystem and its biodiversity are not in a healthy state in the port of Gijon (2,36 out of 5) and that there should be more effective measures to protect the environment (2,11 out of 5).

Regarding the population groups, statistically significant differences were found: those with higher studies showed a more negative point of view about the port, its regulation and environmental status than people with basic studies ($p = 0,0042$). On the other hand, women had a more negative perception of the port than men ($p = 0,0076$). Age also resulted to be a factor affecting the perception about the port, as older people (more than 60 years) showed to have the more positive opinion about the port when comparing to people between 46 and 60 years ($p = 0,0058$), 31–45 years ($p = 0,0026$) and people younger than 30 ($p = 0,0032$) who showed a negative perception about the environmental status and the effectiveness of management strategies in the area.

In order to assess the level of knowledge/awareness of the population, respondents were asked about terms biodiversity and marine biosecurity. The level of knowledge about them turned out to be quite low in the population (2.76 on the Likert scale). The term marine biosecurity obtained a lower score than the term biodiversity ($p = 0.0001$), showing a greater level of ignorance towards it.

When analyzing the different population groups, statistically significant differences were found in many of them. As expected, people with a higher educational level showed a greater knowledge than those with basic studies ($p = 0,0008$). Regarding age, it was seen that people over 60 years old showed the lowest level of knowledge (mean values of 2,2 out of 5), with significant differences with the population under 30 years (mean values of 2,82 out of 5) ($p = 0.0035$) and with people between 46 and 60 years (mean values of 2,69 out of 5) ($p = 0.0003$). In addition, men (mean knowledge level of 2,96) showed a higher level of knowledge than women (mean knowledge level of 2,62) ($p = 0.0265$). Regarding the frequency of visits to the port area, no significant difference was detected between groups.

Once the level of knowledge was established for each population group, the next question aimed to identify the main source of information that citizens consider best to raise awareness about biodiversity and marine biosecurity. Education was selected as the most important one, with 32.90% of the votes, followed by social media (21.22%). It is remarkable that literature and science outreach were rarely voted as sources of information, comparing with the 200 votes that education obtained, science outreach obtained 118 and literature only obtained 46 votes.

Regarding the different groups present in the population, almost all agreed that education is the main source of knowledge about the subject, however, people between 31 and 45 years believe that social media are the most important source nowadays, and people over 60 believe that other media such as press or television have more importance (Table 1).

**Table 1**. Number of votes that received each source of information from each population group. Shaded in grey the most voted sources of information for each group.

| | | Education | Literature | Science outreach | Social media | Others |
|---|---|---|---|---|---|---|
| **Sex** | Male | 70 | 23 | 52 | 58 | 49 |
| | Female | 130 | 23 | 66 | 71 | 66 |
| **Study level** | Basic | 39 | 7 | 16 | 9 | 14 |
| | Secondary | 30 | 7 | 21 | 29 | 29 |
| | Higher | 131 | 32 | 81 | 91 | 72 |
| **Port visit frequency** | Seldom | 137 | 25 | 75 | 66 | 73 |
| | Sometimes | 41 | 12 | 32 | 33 | 17 |
| | Often | 9 | 2 | 6 | 5 | 4 |
| | Very often | 13 | 7 | 5 | 25 | 21 |
| **Age** | Less than 30 | 114 | 16 | 48 | 58 | 48 |
| | 31 to 45 | 23 | 11 | 26 | 36 | 26 |
| | 46 to 60 | 35 | 9 | 33 | 27 | 11 |
| | More than 60 | 28 | 10 | 11 | 8 | 30 |

Next, citizens were asked about the main factors that are causing pollution in the port area. Several options were handed for them (oil pollution, marine litter, invasive species, biofouling and others) to define the dangerousness for each one in Likert scale. Marine litter was the factor considered more dangerous by respondents, with a mean value of 4,28 out of 5 followed by oil pollution ($\bar{x}$ =3,76) and other factors such as carbon spillage or industry ($\bar{x}$ = 3,55). Marine litter was considered much more dangerous than invasive species (p = 1,72 E-33) or biofouling (p = 3,01 E-31). In fact, these factors were the ones considered to be less important by respondents, with considerably lower mean scores in Likert scale: invasive species ($\bar{x}$ = 2,69) and biofouling ($\bar{x}$ =2,73) (Fig. 3).

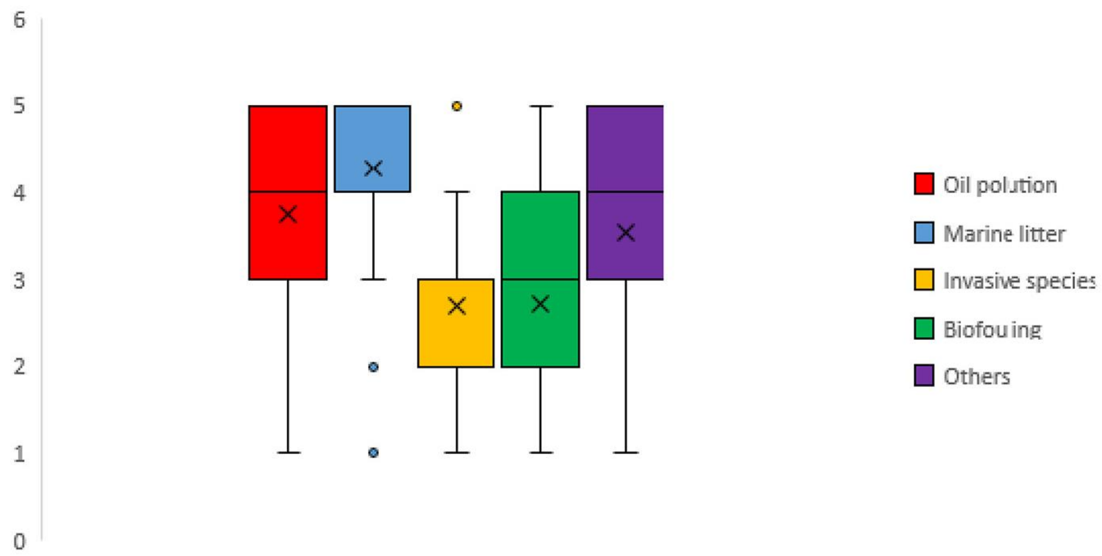


**Fig. 3**. General opinion about the dangerousness of the environmental stressors causing problems in the port area (Likert scale).

Considering the population groups, the study level of respondents showed to affect the perception of the different environmental stressors (Fig. 4); Invasive species are considered much more dangerous by people with basic studies than people with secondary ($p = 0{,}0015$) or higher studies ($p = 0{,}0077$). The same happens with other factors (including carbon spillage and industry), which are considered more dangerous by respondents with basic studies than those with secondary ($p = 0{,}021$) and higher studies ($p = 0{,}016$). In short, people with basic studies gave more importance to each and every environmental stressor than those with a more complete education.

Regarding age, people older than 60 years perceive oil pollution to be more dangerous than people younger than 30 ($p = 0{,}033$). The same happens with marine litter which is considered very dangerous ($\bar{x} = 4{,}60$) by people older than 60, but not that much by people between 46 and 60 years ($p = 0{,}0065$). In the same way, people above 60 give a higher level of dangerousness to invasive species than those below 30 years old, that consider invasive species much less dangerous ($p = 0{,}0069$). The level of dangerousness for biofouling and other factors did not show statistically significant differences between ages (Fig. 5).

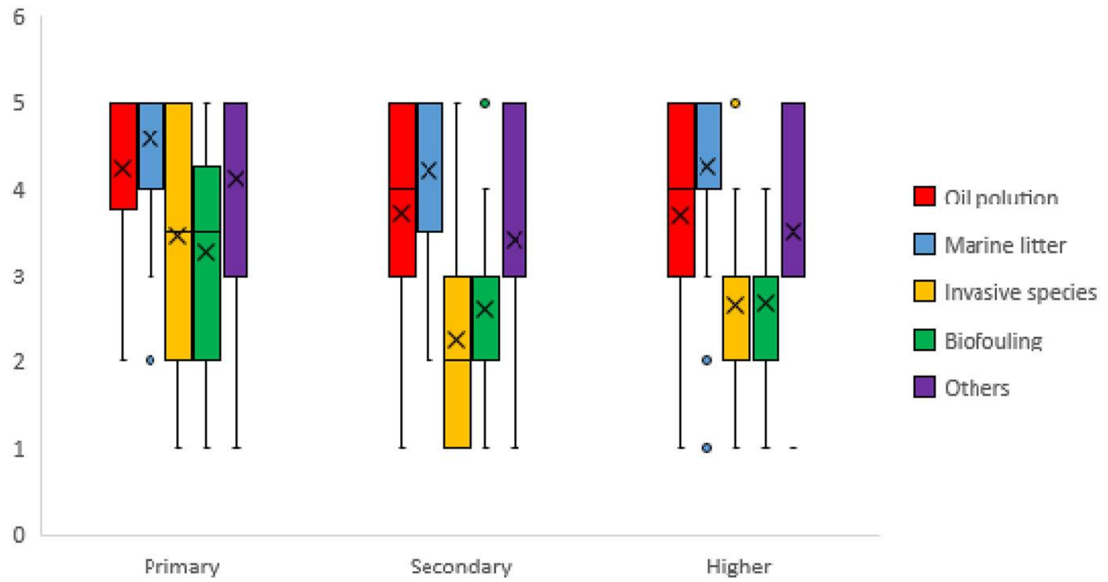


**Fig. 4**. Level of dangerousness given to the environmental stressors (Likert scale) by respondents grouped by their study level.

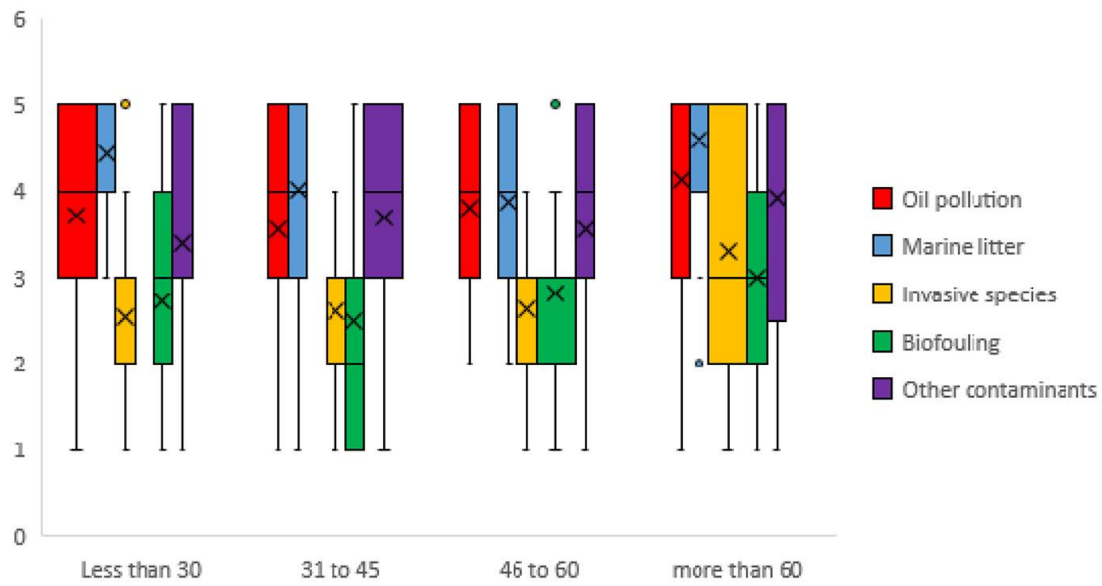


**Fig. 5**. Level of dangerousness given to the environmental stressors (Likert scale) by respondents grouped by age.

Regarding sex, women gave a higher level of dangerousness to oil pollution (p = 0,0047), invasive species (p = 0,014), biofouling (p = 0,026) and other contaminants (p = 0,014) than men. For marine litter, both sexes showed a similar opinion, valuing it as the most problematic factor in the area (Fig. 6).

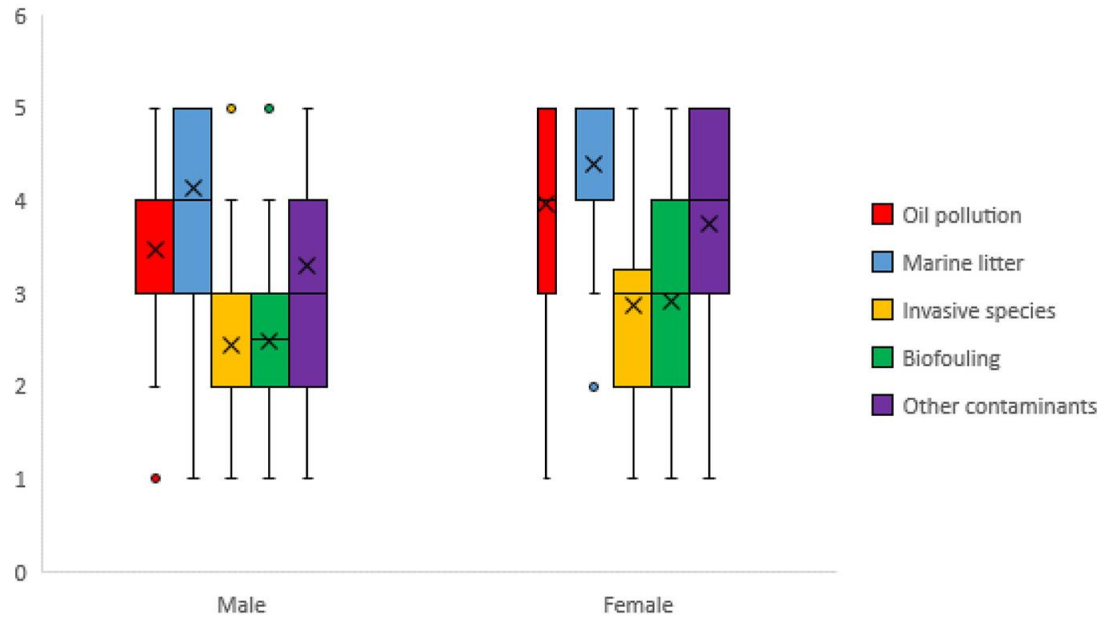


**Fig. 6**. Level of dangerousness given to the environmental stressors (Likert scale) by respondents grouped by sex.

Statistically significant differences were also found when considering the visiting frequency to the port area. In fact, people going seldom to the port showed a higher concern about the potential dangers of oil pollution than those that visit the port very often (p = 0,0078) (Fig. 7). In the same way, people that visit the port area very often show a lower level of concern about the dangerousness of biofouling than those that visit the area seldom (p = 0,0068).


**Fig. 7**. Level of dangerousness given to the environmental stressors (Likert scale) by respondents grouped by port visit frequency.

## 4. DISCUSSION

The results provide an overview of the opinion of the population about the leisure port of Gijon and their level of knowledge and awareness about the coastal marine environment. The surveyed people showed a general negative attitude regarding the port, qualifying it in a poor environmental status and considering as ineffective the management measures that are carried out for its protection. This attitude was more negative in those people with higher studies than in those with only basic studies, showing a higher level of awareness about the potential problems that the port may face, probably triggered by the higher level of knowledge obtained in the education process. These results show citizen concern about the environmental status of the port, which can be helpful at the time of designing management plans, as citizens with a high level of awareness and knowledge can give support for new regulation and policies (Bremner and Park, 2007; Owen and Parker, 2018).

However, despite confirming the critical reflection on the port by citizens, results show a low knowledge level about basic biological terms which could be a reflection of the lack of communication between scientists and citizens. These results are consistent with previous reports that highlight the need for increasing science literacy among the general public (Carley et al., 2013). In fact, people older than 60 years showed the lowest knowledge level related with marine biosecurity and biodiversity, which could be explained due to the high percentage (56,70%) of respondents with only a basic education level within this age range (see Supplementary Fig. 1). On the other hand, regarding sex, surveyed men showed a higher level of knowledge than women when asked about biodiversity and marine biosecurity, which can be also explained with the education level for each sex, since the 4,87% of men had only basic studies, while for women, the percentage of individuals that only reached a primary education level was much higher (18,64%), that is, the more studies the more concern.

Our results show that literature and science outreach are the information sources considered to be less important or effective by respondents. This is consistent with previous studies that remarked the low level of effectivity for communication from these information sources (Gelcich et al., 2014) suggesting that there is a need to develop new methods to achieve an effective scientific communication. The low effectiveness of these information sources may be related with the low level of knowledge that showed the population. The specific communicative channels employed by these information sources (scientific articles, books, congress, seminars…) may be the reason of their low effectiveness, as a very technical and difficult to understand language is used, mostly directed to experts.

To address this problem, changes must be done in the communication methods that are being employed by literature and science outreach, such as using a more understandable lexicon directed to a public with basic knowledge about the covered topics or also, the implementation of STEM education (integrated learning of Science, Technology, Engineering and Mathematics) in school centers, which is a method that can serve as a basis to promote an effective learning process that could be reflected in a population with a higher level of knowledge and, therefore, more aware and collaborative citizenship. Besides, there are reports showing that government employees and stakeholders employ official websites as main sources of scientific information (Young et al., 2016), which, along with scientific profiles tags on social media, is something to be considered as an alternative to traditional communication forms of scientific publications, since it hands an effective way to get the information to this part of the population that is implied in the elaboration and funding of management plans. To the date, studies have shown that social media are a great tool for science communication, for example, for bringing audience to oceanic exploration (Mitchell et al., 2019) or to the field of space science (Hwong et al., 2017).

It is important to remark that social media were considered as the most important information source by people between 31 and 45 years (which was the second group with the lowest level of knowledge after people older than 60). Nowadays, in the internet age, an alarming rise of fake news related to social media (Twitter, Facebook…) has been reported as they can be spread with ease by liking, sharing or also employing social bots (automated accounts impersonating humans) (Lazer et al., 2018). This leads to a situation where misinformation originated by fake news is getting more present within the users of social media. As we have seen in this study people attach great importance to this source of information, thus, it is important to take measures against these fake news in a way that prevents their spread. To do this it is necessary to elaborate methods for empowering individuals in order to be able of detecting these kinds of news, or preventing exposure of individuals to them.

Respondents that were older than 60 years old classified television and press as the main sources of scientific information followed by education. These media, specially science television have been reported as one of the most trusted sources of scientific information (Brewer and Ley, 2013) showing their potential of becoming a powerful channel for communication about the environment to the public, in this case, mainly to people above 60 years old.

The results obtained show that public perception about threats to the marine environment differs from the perception of scientific experts. This could be explained with the differences in the information sources employed by the public and scientists as it has been seen in this study: while literature and science outreach are sources of information rarely employed by citizens, scientific knowledge is mainly based on these kind of studies and data (Rubin et al., 2020).

This way, respondents classified marine litter and oil pollution as the main factors affecting the environment in the area; these factors appear to be the top marine environmental threats considered by the public, as seen in previous studies (Lotze et al., 2018). This importance given to marine litter and oil pollution indicates that citizens' knowledge is based on a visual perception of the environment that surrounds them, rather than on a cognitive perception (based on scientific information sources). Marine litter is something easily seen or detected by the passers-by who come to the port, as well as the oil stains that the boats release, however, other factors such as invasive species and biofouling, not being so visible, go unnoticed by the public. Indeed, our results show that people who seldom visit the port, give more importance to biofouling (they consider the accumulation of living organisms in the hulls of ships or in the docks as more dangerous) than those who visit it very often, which give much more importance to factors such as oil pollution (that can be visually perceptible in the waters of the port). This effect has been seen in other areas, such as In Scotland, for example, where the public saw oil spills as a greater threat than marine professionals, likely because oil spills are highly visible events and receive major press coverage (Howard and Parsons, 2006).

It is important to remark the perception that citizens show about the environmental threat of invasive species. Our results are consistent with (Colton and Alpert, 1998; Kleitou et al., 2019) that also concluded that there is a lack of awareness about this problem among citizens. Until now, it has been seen that leisure ports and recreational boating can be sources and vectors for the secondary dispersal of invasive species (Hirsch et al., 2016), but, as we have seen in this study, these invasion events go unnoticed by citizens that show much more awareness for other problems such as marine litter or oil pollution. It is necessary to transmit information about the danger of biological invasions to the population in order to raise awareness about this problem. To that end, it is necessary to continue investigating about biological invasions, since media attention seems to be associated with the production of scientific research (Geraldi et al., 2019) and more scientific dissemination strategies need to be developed in order to raise awareness about this problem in the local population.

## 5. CONCLUSIONS

Recreational ports are areas of leisure and economic activity that favor the cities. Even so, they are a focus of biological dangers (transport of invasive species in boats, dumping of waste to the sea, marine garbage …) and it is necessary to develop measures to protect the marine ecosystem with the collaboration of all the parties.

In this study it has been seen that there is a critical attitude towards the Gijon marina and its management, but it has also been found that the level of knowledge about marine biodiversity and biosecurity is very low. Although the importance of education as the main source of information is stressed, it is mentionable that social media are also considered one of the main fount of knowledge, often with manipulated information that is very difficult to control and that can send erroneous information to users.

Finally, citizens also showed a visual perception about the problems that may affect the marine ecosystem, since factors such as marine litter, or oil contamination (conditions that can be perceived visually) are the ones considered to be most dangerous, comparing with other factors, also very problematic, as invasive species and biofouling, that being less visible factors, go unnoticed in the population that shows an evident lack of knowledge about these problems present in leisure ports.

## CRediT AUTHORSHIP CONTRIBUTION STATEMENT

A. Ibabe: Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. Y.J. Borrell: Conceptualization, Supervision, Project administration, Funding acquisition. S. Knobelspiess: Methodology, Investigation. E. Dopico: Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

## 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://www.sciencedirect.com/science/article/pii/S0025326X20307633?casa_token=ltxnREoFEXQAAAAA:PK9CKw3bB8T5uQqmOD0BOSM-qBKB3r848I4Nm_I90uj1jTYO3z6VS9fOvFMEP2x1LWZ8T1iUwIo#s0040

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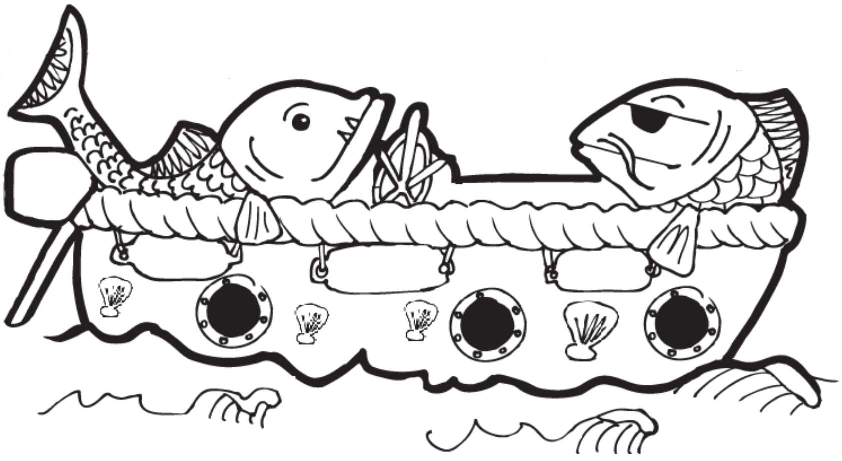
# Discusión general



## 1. El contexto legislativo y el puerto de Gijón en un escenario de invasiones biológicas

Los ecosistemas marinos suponen un bien preciado para el ser humano ya que proveen alimento y promueven la actividad económica (Selig et al., 2019). Sin embargo, las invasiones biológicas suponen un problema global que altera estos ecosistemas y causa graves impactos, no solo ambientales, sino económicos e incluso en la salud humana (Hoffmann & Broadhurst, 2016; Occhipinti-Ambrogi, 2021; Rai & Singh, 2020). En relación a la protección de los ecosistemas marinos, el objetivo principal de la directiva UE 845/2017 de la Comisión de 17 de mayo de 2017 (la cual modifica la directiva UE 2008/56/CE) es establecer un marco de acción para la consecución del Buen Estado Ambiental de los mares. En España, esta normativa se recoge en la Ley 41/2010, de 29 de diciembre, de Protección del Medio Marino y en el Real Decreto 957/2018, de 27 de julio. En el anexo 1 de dicha directiva se muestran 11 descriptores del buen estado ambiental, dentro de los cuales se incluye el descriptor 2: "especies alóctonas invasoras". De esta forma, la legislación española recoge la importancia de hacer frente a las invasiones biológicas para poder garantizar un buen estado ambiental de los mares.

En este marco legislativo, se divide el medio marino español en cinco demarcaciones marinas (Noratlántica, Sudatlántica, Estrecho y Alborán, Levantino-balear y Canaria) y se establecen una serie de estrategias marinas para cada demarcación, las cuales deben ser actualizadas en un período de 6 años. Actualmente, se dispone de los resultados obtenidos para cada una de las demarcaciones durante el primer ciclo de estrategias marinas (Ministerio de Agricultura, Alimentación y Medio ambiente, 2012). Según estos informes (con un retraso temporal significativo), el número de citas de nuevas especies exóticas descritas por primera vez en las costas españolas (estimado a partir de publicaciones que aportan datos concretos sobre taxones específicos detectados en una localidad determinada) aumenta año tras año (Figura 5). Cabe destacar el gran aumento en el número de reportes en la demarcación noratlántica, específicamente entre los años 2003 y 2004 (aunque lo reportes llegan hasta el año 2011) (Figura 5). Esto se debe a los estudios exhaustivos que se realizaron en este periodo en el País Vasco, los cuales detectaron una gran variedad de especies exóticas que no habían sido previamente descritas en el área estudiada (Martínez y Adarraga, 2005; Martínez y Adarraga, 2006). Esta información resalta la necesidad de llevar a cabo estudios periódicos actualizados para poder tener una mejor representación de la situación en los ecosistemas marinos españoles. Como se ha mencionado anteriormente, la detección temprana en los procesos de invasiones biológicas es fundamental.

Es importante resaltar que los datos provenientes de las cinco demarcaciones marinas ya señaladas se basan, sobre todo, en estudios puntuales no estandarizados, lo cual puede producir sesgos significativos. La puesta a punto de planes de monitoreos periódicos y con metodologías estandarizadas en todas las demarcaciones contribuiría a disminuir este sesgo. No obstante, los datos muestran una tendencia creciente, y preocupante, para la introducción y dispersión de especies exóticas en todas las demarcaciones (Figura 5).

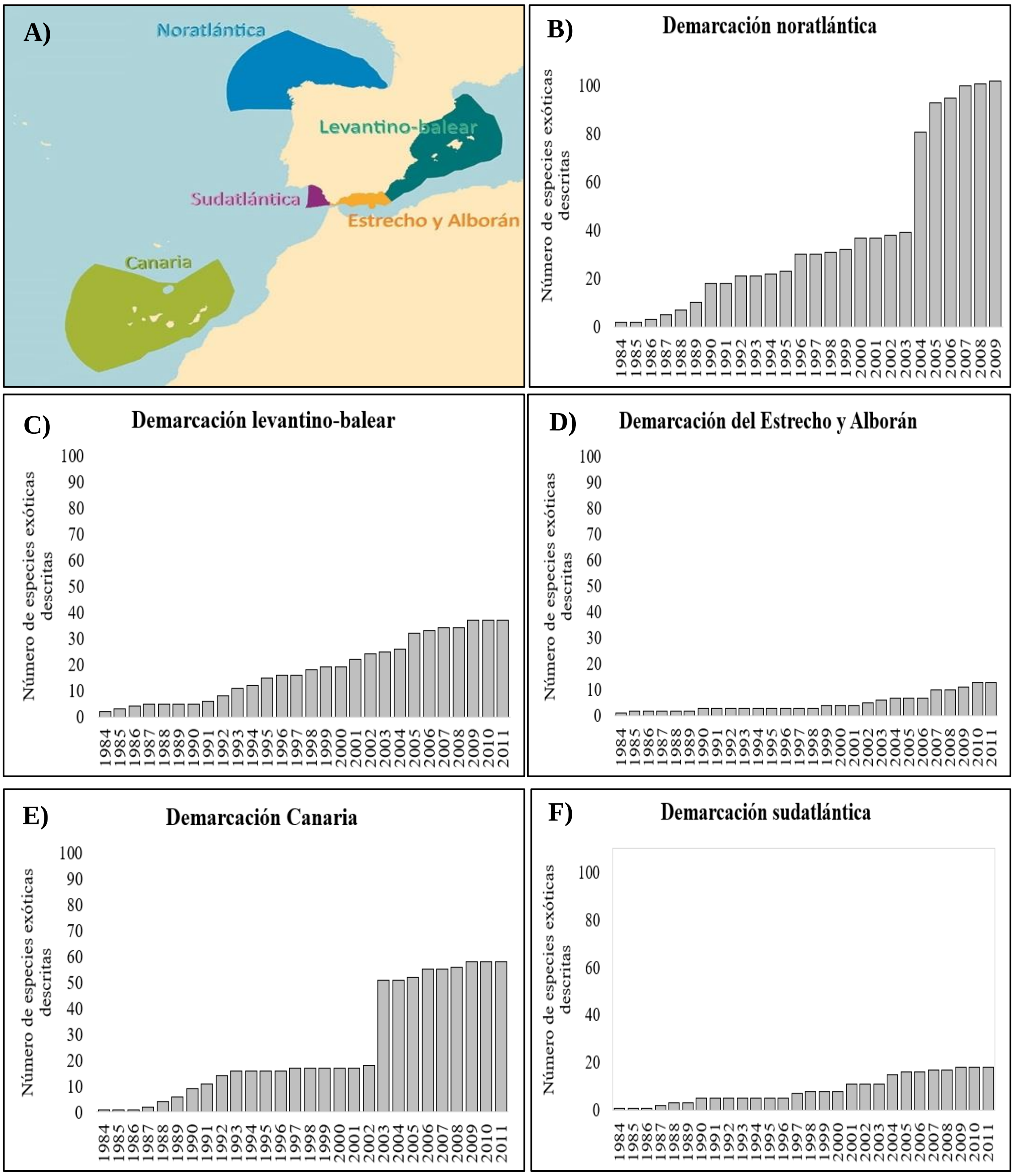



**Figura 5. Evolución temporal del número de especies exóticas descritas en las distintas demarcaciones del territorio español y sobre las que se dispone de información concreta sobre la fecha en que se produjeron las primeras citas**. No representa el total de especies exóticas detectadas. A) localización geográfica de las 5 demarcaciones. B) Demarcación noratlántica. C) Demarcación Levantino-Balear. D) Demarcación del Estrecho y Alborán. E) Demarcación Canaria. F) Demarcación sudatlántica. (Imágenes adaptadas de: https://www.miteco.gob.es/es/costas/temas/proteccion-medio-marino/estrategias-marinas/).

El rol específico de los puertos no se menciona en estos reportes, aunque es sabido que son considerados como los principales focos para la introducción y dispersión de especies exóticas e invasoras debido al tráfico marítimo (Chapman et al., 2013; Chan et al., 2015; Chainho et al., 2015). De hecho, el transporte mediante aguas de lastre (así como los escapes de acuarios y zonas de acuicultura/maricultura) están considerados entre las 12 vías principales de entrada y dispersión no intencionada de especies, según la normativa europea (Reglamento de ejecución UE 1454/2017). Como se decía antes, el puerto de Gijón es un foco potencial para la introducción de nuevas especies exóticas, dado que mantiene un intenso tráfico marítimo nacional e internacional, así como centros de acuicultura donde se cultivan diferentes especies no-indígenas.

A lo largo del desarrollo de la presente tesis, se han llevado a cabo diferentes tipos de muestreos en el puerto de Gijón que aportan nuevos datos sobre la situación actual en cuanto a especies exóticas e invasoras. Considerando todos los muestreos realizados, bien en las propias instalaciones del puerto o en zonas aledañas, se han podido detectar un total de 41 especies que no son nativas de la zona (Tabla 2). Además, 17 de estas especies no habían sido reportadas con anterioridad en la demarcación noratlántica definida en la Ley 41/2010, por lo que suponen citas nuevas para estas especies en el área. De hecho, todas ellas, a excepción de tres (*Chamaesipho columna*, que se encontró adherida a basura marina flotante, *Illex argentinus*, que se detecto en un fragmento de poliestireno y *Didemnum vexillum*, hallada en un fragmento de basura de las playas), fueron detectadas en el propio puerto de Gijón. Estos resultados son consistentes con los estudios que apuntan a los puertos como focos relevantes en los procesos de invasiones biológicas.

Durante el desarrollo de la presente tesis se han evaluado y puesto a prueba diferentes estrategias que pueden ser empleadas para lograr un manejo exitoso de las invasiones biológicas. Estas estrategias están basadas en tres ejes principales: prevención, detección temprana y participación ciudadana, que combinadas, pueden suponer un avance relevante en cuanto a la protección de los ecosistemas y de la biodiversidad marina.

**Tabla 2**. Listado general de las especies marinas no indígenas detectadas en los diferentes muestreos llevados a cabo a lo largo de la presente tesis en el puerto de Gijón y las zonas aledañas.

| Phylum | Especie | Lugar de detección | | | Método de detección | | Reportes previos en la demarcación noratlántica |
|---|---|---|---|---|---|---|---|
| | | Puerto | Basura marina | Playas | ADN ambiental | Muestreo físico y barcoding | |
| Annelida | *Dipolydora capensis* | X | | | X | X | N/A |
| Annelida | *Nereiphylla lutea* | X | | | | X | N/A |
| Annelida | *Phyllodoce groenlandica* | X | | | | X | [18] |
| Annelida | *Spirobranchus latiscapus* | X | X | | | X | [21] |
| Annelida | *Spirobranchus taeniatus* | X | X | | | X | [17] |
| Arthropoda | *Austrominius modestus* | | X | | | X | [1], [5],[6],[7] |
| Arthropoda | *Balanus trigonus* | X | X | | | X | [8] |
| Arthropoda | *Chamaesipho columna* | | X | | | X | N/A |
| Arthropoda | *Livoneca redmanii* | X | | | | X | [1] |
| Arthropoda | *Oncaea waldemari* | X | | | X | | N/A |
| Arthropoda | *Paracalanus quasimodo* | X | | | X | | N/A |
| Bryozoa | *Amathia verticillata* | X | | | | X | [1] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Bryozoa | *Bugula neritina* | X | | X | X | X | [1] |
| Bryozoa | *Crassimarginatella papulifera* | X | | | | X | N/A |
| Bryozoa | *Watersipora subtorquata* | X | | | | X | [1],[22] |
| Chordata | *Acentrogobius pflaumii* | X | | | | X | N/A |
| Chordata | *Botryllus schlosseri* | X | | | | X | [12] |
| Chordata | *Didemnum vexillum* | | X | | | X | N/A |
| Chordata | *Diplosoma listerianum* | X | | | | X | [1] |
| Chordata | *Microcosmus squamiger* | X | | X | | X | [1] |
| Chordata | *Styela plicata* | X | | | | X | [1] |
| Cnidaria | *Anthopleura anjunae* | X | | | | X | N/A |
| Cnidaria | *Anthopleura elegantissima* | X | | | | X | N/A |
| Cnidaria | *Caryophyllia grayi* | X | | | | X | N/A |
| Cnidaria | *Clytia gregaria* | X | | | X | | N/A |
| Mollusca | *Magallana gigas* | X | X | X | | X | [1],[6],[7],[17] |
| Mollusca | *Mytilaster minimus* | X | | X | | X | [23] |
| Mollusca | *Mytilus trossulus* | X | | X | | X | [1],[6],[7] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Mollusca | *Ostrea stentina* | X | | | | X | [1],[17],[23] |
| Mollusca | *Talochlamys multistriata* | X | | | | X | N/A |
| Mollusca | *Illex argentinus* | | X | | X | | N/A |
| Ochrophyta | *Sargassum muticum* | | X | | X | | [2],[4],[19],[20] |
| Porifera | *Hymeniacidon gracilis* | X | | | X | | N/A |
| Rhodophyta | *Asparagopsis armata* | | | X | X | | [2],[3],[4] |
| Rhodophyta | *Bonnemaisonia hamifera* | X | | | X | | [3],[4],[9],[10],[11] |
| Rhodophyta | *Botryocladia wrightii* | | | X | X | | [13] |
| Rhodophyta | *Dasysiphonia japonica* | X | | | X | | [4],[14],[15] |
| Rhodophyta | *Gelidium microdonticum* | X | | | X | | N/A |
| Rhodophyta | *Grateloupia imbricata* | | | X | X | | [16] |
| Rhodophyta | *Mesophyllum expansum* | X | | | X | | [11],[24] |
| Rhodophyta | *Neogastroclonium subarticulatum* | X | | | X | | N/A |

[1] Miralles et al., 2016
[2] Arias et al., 2014
[3] Gorostiaga et al., 2014
[4] Peteiro, 2014
[5] Barnes & Barnes, 1968
[6] Gómez Agenjo, 2016
[7] Miralles et al., 2018
[8] Zorita et al., 2013
[9] Salvador Soler et al., 2006
[10] Barbara et al., 2012
[11] Diez et al., 2012
[12] Castège et al., 2014
[13] Barbara et al., 2008
[14] Pena Martin et al., 2011
[15] Gallardo et al., 2016
[16] Montes et al., 2016
[17] Rech et al., 2018
[18] Serrano et al., 2006
[19] Salinas et al., 2011
[20] Lamela et al., 2012
[21] Muñoz-Colmenero et al., 2018
[22] Reverter-Gil & Souto, 2019
[23] Pejovic et al., 2016
[24] Muguerza et al., 2017

## 2. La prevención como estrategia inicial para hacer frente a las invasiones biológicas

Las estrategias de control y erradicación de especies invasoras se centran en la eliminación de una población alóctona ya residente, mientras que las estrategias de prevención eficaces pueden evitar la introducción de nuevas especies exóticas. Al aplicar estrategias que permitan la detección temprana, las probabilidades de erradicación exitosa de las especies exóticas aumentan (Rout et al., 2011). La prevención de invasiones biológicas supone una estrategia proactiva que permite diseñar planes de actuación antes de que estas ocurran. Por el contrario, una vez la especie exótica es introducida en el nuevo hábitat, el costo de su manejo es más elevado según avanza el proceso de invasión (Harris et al., 2018). En aquellos casos en los que la actuación es demasiado tardía (debido a la falta de prevención o estrategias de detección temprana), la erradicación de la especie invasora resulta altamente improbable debido al aumento del número de individuos y al área invadida, derivando en una gestión a largo plazo que implica un coste económico mucho más elevado (Figura 6). No obstante, a lo largo de los años se ha centrado el esfuerzo en la erradicación y el control post-invasión de especies; por el contrario, las medidas de prevención de invasiones biológicas han sido generalmente empleadas de forma más limitada (Horan et al., 2002).

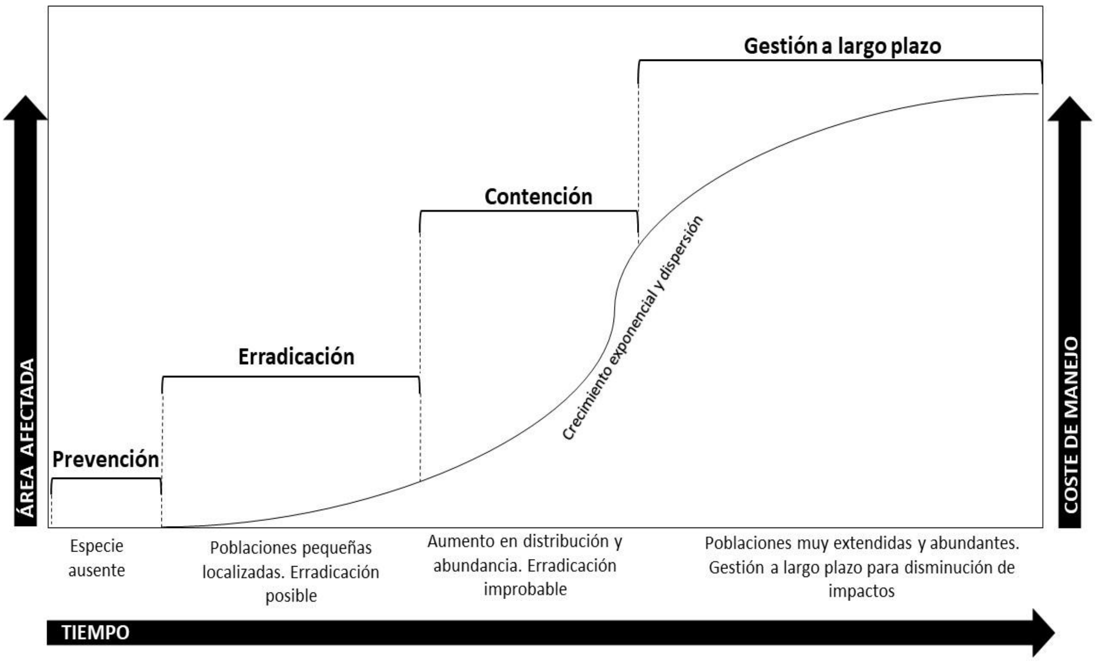



**Figura 6**. Curva de invasión generalizada y las diferentes medidas de gestión adecuadas a las distintas etapas de invasión.

La principal limitación a la hora de prever la introducción de especies exóticas en un nuevo entorno es la estocasticidad del proceso de invasión (Van Poorten et al., 2019). Es decir, de forma general, podría establecerse que las especies con amplios rangos nativos y mayor presión de propágulos tendrían una probabilidad más elevada de invadir nuevas áreas (Shah et al., 2011; Cassey et al., 2018). No obstante, debido a la estocasticidad del proceso, unas condiciones ambientales incompatibles podrían impedir la invasión de una especie con un amplio rango nativo, o de la misma forma, un único propágulo podría desencadenar una invasión (Duncan et al., 2014). Ante esta situación, surge la necesidad de identificar patrones generales que puedan ayudar a predecir y manejar las invasiones biológicas (Novoa et al., 2020). Con este objetivo, los estudios recientes se han centrado en la identificación de vectores de introducción de especies (Saul et al., 2017), taxones con alta probabilidad de convertirse en invasores (Bacher et al., 2018) y hábitats que pueden ser particularmente susceptibles a invasiones biológicas (Guo et al., 2015).

En los ecosistemas marinos, el tráfico marítimo ha sido identificado como el vector principal en la dispersión de especies (Castro et al., 2020). Por lo tanto, las medidas de prevención se centran en aquellas especies con características que las hagan capaces de sobrevivir a los viajes hasta el ecosistema receptor (presumiblemente un puerto) (Tempesti et al., 2020). Es por esto por lo que se han adoptado medidas de prevención a nivel mundial como el Convenio Internacional para el Control y la Gestión del Agua de Lastre y los Sedimentos de los Buques (Convenio BWM), el cual pretende establecer una serie de directrices para evitar el transporte involuntario de especies mediante el tráfico marítimo (IMO, 2004). Dicho convenio entró en vigor en 2017, no obstante, aún no se ha implementado por completo y el número de invasiones biológicas marinas sigue incrementando año tras año (Sardain et al., 2019).

En el desarrollo de esta tesis se ha elaborado un instrumento que puede servir como complemento a las estrategias de prevención ya existentes: NIS-ITS (Índice para medir la amenaza de invasión de especies no indígenas). Esta herramienta puede usarse para definir, dentro de un rango, el potencial de una especie exótica para invadir un área específica. El NIS-ITS se puso a prueba en el caso de estudio del puerto de Gijón, y se puede afrmar que una de las principales ventajas que presenta esta herramienta es que puede ser empleada en cualquier otro puerto del mundo. Esto se debe a que los parámetros que se tienen en cuenta a la hora de puntuar el potencial invasor de una especie son 1) la idoneidad del hábitat receptor 2) la invasividad de la especie y 3) el tráfico marítimo procedente de las áreas en las que está presente la especie. Por lo tanto, el potencial de invasión de una especie puede ser estimado en cualquier puerto del mundo siempre y cuando existan datos disponibles.

El NIS-ITS puede ser utilizado como un sistema de alerta para la prevención de invasiones biológicas. Esto se ha podido comprobar en el estudio de caso llevado a cabo en el puerto de Gijón en la presente tesis. Los resultados obtenidos tras analizar 380 especies con capacidad de emplear el tráfico marítimo como vector de expansión mostraron que, de las 15 especies que fueron clasificadas como potencialmente peligrosas (con valores del NIS-ITS superiores a 0.90), 12 han sido ya reportadas en la demarcación noratlántica española. Es más, 4 de estas especies ya han sido detectadas en el propio puerto de Gijón (ver capitulo 1, tabla 1). Estos resultados indican que el NIS-ITS puede ser de gran ayuda para identificar con antelación aquellas especies que en un futuro puedan generar eventos de invasión. Para ello es necesario poner en marcha esta estrategia preventiva de forma temprana. En nuestro caso, el que coincidieran la previsón y la confirmación de la presencia de estas especies en la zona, fue utilizado como un mecanismo de comprobación de la utilidad predictora de la herramienta.

Es importante recalcar que para algunas especies invasoras muy importantes (por ejemplo, *Sargassum muticum, Codium fragile, Ruditapes philippinarum*) no existen datos sobre la tolerancia, características del hábitat donde se desarrollan o daños ocasionados por los procesos de invasión, lo que puede constituir un factor limitante en la herramienta NIS-ITS. Por otro lado, en algunos casos los datos sobre tráfico marítimo no son públicos o no están del todo disponibles. Sin embargo, parece ser que la información acerca de la idoneidad del hábitat receptor es la información más limitante o escasa para un número elevado de especies invasoras. En nuestro trabajo, no todas las especies estudiadas disponían de una información completa y documentada.

Es determinante en el desarrollo de estrategias de prevención, el impulsar el estudio de las características fisiológicas de las distintas especies que puedan ser introducidas en un hábitat determinado. De esta forma, se podría evaluar su peligrosidad a la hora de prevenir posibles invasiones biológicas futuras. Mediante una búsqueda proactiva de especies con potencial invasor y empleando el NIS-ITS como herramienta, es posible generar un listado de especies potencialmente peligrosas para un entorno ecológico concreto. Esto puede impulsar el desarrollo de los estudios centrados en dichas especies como, por ejemplo, la elaboración de marcadores genéticos específicos o la ampliación de las librerías genéticas para que de esta manera dichas especies puedan ser detectadas de forma temprana y se puedan tomar las acciones correspondientes para evitar su propagación.

## 3. El monitoreo rutinario en los puertos para detectar y controlar la dispersión de especies exóticas

### 3.1. La detección temprana de especies exóticas e invasoras como estrategia.

La prevención y los esfuerzos para evitar la entrada de nuevas especies exóticas deben ser la base inicial desde donde construir una buena gestión en los puertos para enfrentar las invasiones biológicas. Sin embargo, las estrategias de gestión complementarias como la detección temprana también son necesarias para abordar de forma eficaz este problema. Incluso los esfuerzos más efectivos de prevención no pueden asegurar la ausencia total de nuevos eventos de invasión. En situaciones en las que falla la prevención, un programa de detección temprana puede alertar a las autoridades competentes sobre el establecimiento de una nueva especie, y un programa coordinado de erradicación podría contener o eliminarla antes de que se pudiese propagar a nuevas áreas. En caso de ausencia de programas de detección temprana, la única opción de las autoridades es aceptar a los nuevos invasores y sus diversos impactos económicos y ecológicos.

Hacer frente a una invasión en su fase inicial supone una gran ventaja dado que las poblaciones son más pequeñas y fáciles de manejar (Reaser et al., 2020). Existen varios ejemplos en los que una detección temprana de las especies invasoras ha sido la clave para su erradicación (Simberloff, 2002; Robertson et al., 2017; Champion, 2018). Además, en la mayoría de los casos, una erradicación temprana es menos costosa y preferible a una gestión a largo plazo de la especie y sus impactos (Epanchin-Niell, 2017).

Ante esta situación, se han elaborado distintas estrategias de detección temprana que pueden ser empleadas para hacer frente de una forma más eficaz a las invasiones biológicas. Australia es uno de los países que más invierte en hacer frente a las invasiones biológicas (Bradshaw et al., 2021). Según la Ley de protección del medio ambiente y la biodiversidad del gobierno australiano, una vez detectado un problema como la introducción de una especie exótica, se establece un límite máximo de 90 días para que se adopte un plan de reducción de amenazas. Gracias a esta estrategia y a los fondos dirigidos a la detección temprana de especies, se conocen casos en los que se han podido erradicar por completo especies invasoras, como por ejemplo el mejillón *Mytilopsis sallei*, el cual fue erradicado del puerto de Darwin (Willan et al., 2000).

A nivel europeo, también se han adoptado medidas como el reglamento de ejecución UE 1263/2017 por el que se actualiza la lista de especies exóticas invasoras preocupantes. El objetivo de esta normativa es establecer una lista de especies problemáticas, para darles prioridad a la hora de elaborar planes de detección temprana y medidas rapidas de erradicación de nuevas introducciones (Reglamento de ejecución UE, 1263/2017). A nivel de España, también está en vigor el Real Decreto 216/2019, donde se presenta un catálogo de especies invasoras que incluye las islas Canarias y en el cual se establece que el Ministerio para la Transición Ecológica y las comunidades autónomas, elaborarán coordinadamente estrategias de gestión, control y posible erradicación de especies exóticas invasoras incluidas en el catálogo (Real Decreto 216/2019).

No obstante, a día de hoy no existe un protocolo estandarizado para hacer frente a las nuevas introducciones de especies, y el abanico de estrategias disponibles para la detección temprana de especies va en aumento. Tradicionalmente, la detección de especies exóticas e invasoras se ha llevado a cabo mediante muestreos físicos de especímenes e identificaciones taxonómicas basadas en la morfología de las especies. No obstante, hoy en día existen nuevas tecnologías que emplean herramientas genéticas que facilitan la detección temprana de las especies. El metabarcoding es una de ellas. Esta técnica emplea ADN ambiental, el cual es un recurso excelente que en muchos casos permite detectar especies en muy bajas densidades (Furlan et al., 2019). Esto puede ser muy útil para detectar aquellas especies exóticas recién introducidas en un ecosistema, las cuales presentan generalmente bajas densidades de población y son por tanto, difícilmente detectables mediante métodos convencionales de muestreo (Trebitz et al., 2017). Es en estas condiciones cuando más efectivas son las medidas de actuación contra las invasiones biológicas (Alvarez y Solís, 2018). Debido a ello, el metabarcoding puede servir como un sistema de alerta temprana gracias a la capacidad de detectar especies en bajas densidades, como por ejemplo en el caso de la introducción del mejillón invasor *Arcuatula senhousia* en el Reino Unido, o en el caso del briozoo *Bugula neritina* en Korea (Holman et al., 2019; Kim et al., 2018). Gracias al empleo de técnicas basadas en el ADN ambiental, estas especies exóticas pudieron ser detectadas de forma temprana, permitiendo la puesta en marcha de medidas de actuación para evitar su proliferación descontrolada.

En el caso del puerto de Gijón, estudiado durante el desarrollo de esta tesis, la combinación de técnicas de metabarcoding y muestreos tradicionales ha posibilitado la detección de una gran variedad de especies entre las cuales se incluyen 41 especies no indígenas (Tabla 2). Gracias a estos hallazgos es posible centrar la atención para la gestión portuaria en dichas especies. Una de las vías para gestionar las especies exóticas detectadas mediante técnicas de metabarcoding y confirmar su presencia en la zona es el uso de marcadores genéticos específicos sobre el ADN ambiental. A esto se le deben sumar los muestreos tradicionales en los entornos habitables por las especies objetivo. De esta forma, es posible realizar una detección temprana de las especies con potencial invasor. Además, mediante el empleo de muestreos tradicionales, en caso de confirmar su presencia en el puerto, es posible determinar si los organismos tienen la capacidad de sobrevivir al proceso de introducción al nuevo hábitat o no (lo cual no es posible empleando únicamente el ADN ambiental).

No obstante, los resultados del trabajo realizado en esta tesis también han evidenciado ciertas limitaciones existentes con respecto a las técnicas basadas en el ADN ambiental. En primer lugar, nos encontramos con la ausencia de un proceso o protocolo estandarizado para el tratamiento de los datos de metabarcoding. A la hora de analizar los datos es necesario realizar una serie de procesos de filtrado de calidad para evitar errores a la hora de asignar las especies (Zinger et al., 2019). No obstante, actualmente no existe un método estándar para ello, por lo que las decisiones tomadas a la hora de realizar los análisis bioinformáticos repercuten en los resultados obtenidos, pudiendo afectar a su reproducibilidad (Bailet et al., 2020). De hecho, debido a los estrictos controles de calidad que se emplearon en el tratamiento de los datos obtenidos en el puerto de Gijón, más de un 40% de las secuencias obtenidas inicialmente fueron descartadas, y posteriormente solo un 12% de ellas fueron asignadas con éxito. Esto implica que durante el proceso probablemente puedan haberse producido falsos negativos. A pesar de que es preferible perder información y no generar falsas alarmas (falsos positivos), es importante evitar la pérdida de información que conllevan los falsos negativos. A este problema se le suma el hecho de que las bases de datos genéticas que se emplean para poder identificar las especies mediante el ADN ambiental no están completas, por lo que muchas especies no están representadas (Weigand et al., 2019). Esto implica que dichas especies, que no están contempladas en las bases de datos, no puedan ser identificadas mediante técnicas genéticas. Entre las opciones existentes para poder aumentar la detectabilidad de las especies, están el empleo de réplicas a la hora de realizar los muestreos o la combinación de diferentes marcadores genéticos como el COI, el 18S o el 16S, ya que su eficacia respectiva varía dependiendo de los taxones analizados (Lanzen et al., 2017; Alberdi et al., 2018).

De cara al futuro y a mejorar la eficacia de las técnicas de metabarcoding, es de vital importancia impulsar la actualización de estas bases de datos genéticas, ya que, en la actualidad, muchas especies no son detectadas debido a la falta de datos genéticos. En el mejor de los casos, la cobertura a nivel de especie de las bases de datos en cuanto a macrofauna marina ronda el 40-50% para el COI y el 27-36% para el 18S (Hestetun et al., 2020), los cuales son los genes más estudiados y que más información contienen hoy en día. Esto puede derivar en una mayor representación de las especies comunes, y la subrepresentación de especies menos estudiadas. Es por ello por lo que para que las técnicas basadas en ADN ambiental puedan aumentar su eficacia, es necesario que las bases de datos sean intensamente completadas y actualizadas de manera periódica.

Ante este problema, la presente tesis ha servido para proporcionar una serie de secuencias nuevas pertenecientes a aquellas especies que fueron recogidas durante los muestreos convencionales y que actualmente se encuentran a la disposición del público en el repositorio online de GenBank. De esta forma, y con la colaboración de la comunidad científica, se pretende ampliar las bases de datos genéticas para contribuir a su compleción y así poder garantizar una representación más real de la biodiversidad en futuros estudios basados en técnicas genéticas.

En resumen, partiendo de los resultados de esta tesis, se ha podido comprobar que tanto las técnicas tradicionales como las técnicas genéticas, tienen sus ventajas y desventajas. No obstante, al emplear ambas en un área concreta como es el puerto de Gijón, se han podido detectar gran variedad de especies no nativas, incluyendo algunas que no habían sido previamente descritas en la zona. Estos datos son consistentes con otros estudios que también concluyeron que la combinación de ambos métodos puede facilitar la detección temprana de especies dañinas y promover el desarrollo de planes de actuación para evitar la propagación de especies exóticas e invasoras (Von Ammon et al., 2018; Stat et al., 2019). Por lo tanto, de cara a una gestión eficaz contra las invasiones biológicas, esta tesis sirve para recomendar una estrategia de detección temprana de especies no nativas. Dicha estrategia debería basarse en la realización de muestreos periódicos en los puertos, en los que se combinen técnicas tradicionales y de metabarcoding, para así aumentar la detectabilidad de las especies y poner en marcha planes para evitar su propagación.

### 3.2. ¿Debe ser incluida la presencia de especies exóticas en las evaluaciones ambientales en los puertos?

El uso de índices bióticos se ha vuelto muy relevante a la hora de comunicar y presentar los resultados de las redes de monitoreo o estudios de impacto ambiental de los puertos a los gestores. Estos índices constituyen un método fácil para transmitir los resultados de una manera simple y comprensible. La función de los índices bióticos en las caracterizaciones de biota y las evaluaciones ambientales en los puertos es hoy en día un proceso completamente establecido. En esta tesis doctoral se recomienda una estrategia novedosa para hacer frente a las invasiones biológicas, modificando estos índices para incluir en ellos especies exóticas e invasoras. Como se ha comentado anteriormente, a través de la ejecución de monitoreos periódicos es posible evaluar el estado ambiental de los ecosistemas acuáticos, y de esta manera, se pueden desarrollar estrategias de acción para prevenir un mayor deterioro y pérdida de biodiversidad (Birk et al., 2012; Borja et al., 2010). Actualmente, estos índices pueden ser empleados con datos obtenidos de forma convencional pero también sirven aquellos datos obtenidos mediante técnicas basadas en el ADN ambiental. El uso de índices bióticos adecuados puede suponer una excelente estrategia para alertar sobre la situación de un ecosistema e impulsar las medidas necesarias para su protección.

La mayoría de los índices bióticos se basan en especies de macroinvertebrados a la hora de evaluar el estado ambiental de los ecosistemas acuáticos, debido a que estos contienen familias con distintos niveles de tolerancia a la contaminación (Armitage, 1983). No obstante, estos índices no tienen en cuenta los daños ambientales que pueden provocar las especies invasoras, lo cual constituye un gran inconveniente dado que las especies alóctonas y sus impactos en los ecosistemas marinos son un factor reconocido para la obtención de una buena calidad ambiental de los mares según el Real Decreto 957/2018. Además, las especies invasoras pueden también ser indicadoras de perturbaciones, ya que la contaminación generada mediante las actividades humanas puede eliminar a las especies más sensibles, reduciendo la resistencia del ecosistema y facilitando las invasiones biológicas (Crooks et al., 2011).

En esta tesis se ha podido elaborar un nuevo índice biótico partiendo del AMBI (Azti Marine Biotic Index), que emplea las especies de macroinvertebrados presentes en una zona para evaluar su estado ecológico. Este nuevo índice, al cual se ha nombrado como Blue-gNIS, propone una primera aproximación a un índice biótico que tiene en cuenta a aquellas especies no indígenas e invasoras que estén presentes en el área de estudio. A pesar de que se trata de una propuesta inicial, y deba ser testado en un rango amplio de condiciones ambientales para validarlo, los resultados muestran que el Blue-gNIS tiene correlación con otros índices bióticos aceptados por la comunidad científica. De esta forma, se demuestra la viabilidad de este nuevo índice para realizar valoraciones ambientales en ecosistemas marinos, considerando aquellas especies no indígenas e invasoras que puedan causar daños en el entorno. Este nuevo índice puede suponer una excelente herramienta para comunicar las condiciones ambientales de una forma más completa, de manera que se reflejen los problemas que suponen las invasiones biológicas en los resultados, pudiendo así desencadenar las respuestas necesarias para hacerles frente.

Por lo tanto, en la estrategia que se recomienda en esta tesis, además de emplear métodos de prevención proactivos y de combinar distintas herramientas para la detección temprana de especies, también debería de tenerse en cuenta el Blue-gNIS a la hora de reportar el estado ambiental de un ecosistema.

### 3.3. La basura marina y su papel en las invasiones biológicas.

Para un monitoreo eficaz de nuevas introducciones de especies exóticas e invasoras, es preciso no solo analizar los hábitats recipientes, como pueden ser los puertos en el caso de las invasiones biológicas marinas, sino que también es necesario evaluar la situación de los principales vectores de dispersión de dichas especies. Mediante el monitoreo de las viás de dispersión de especies exóticas e invasoras es posible mejorar la detección temprana y tomar acciones antes de que de lugar el evento de invasión.

En los ecosistemas marinos se han llevado a cabo diversos estudios relacionados con los vectores de dispersión de especies. Mayoritariamente, estos estudios se centran en el monitoreo de aguas de lastre o bioincrustaciones, los cuales suponen el principal vector de dispersión de especie marinas (Bailey, 2015; Meloni et al., 2021). Sin embargo, en los últimos años se ha visto que la basura marina también supone un vector que facilita la dispersión de especies, ya que aporta una superficie a la que los organismos pueden adherirse y desplazarse empleando las corrientes marinas. Este fenómeno puede darse bien con especies autóctonas, pero también con especies exóticas e invasoras (Carlton et al., 2017; Miralles et al., 2018; Mantelatto et al., 2020). Dado que la basura marina es cada vez más abundante en los mares, los problemas que esta genera, ya sean impactos ambientales o por transporte de especies invasoras, también se acentúan. Es por ello por lo que la basura marina también está incluida dentro de los descriptores a considerar a la hora de evaluar el estado ambiental según la directiva UE 845/2017.

Ante esta situación, surge la necesidad de elaborar planes de actuación para reducir la cantidad de basura en los ecosistemas marinos. Para ello se han elaborado convenios internacionales como MARPOL 73/78, que estabecen una serie de reglas para evitar el vertido de basura en el mar por parte de las embarcaciones. A nivel estatal, en la demarcación marina noratlántica, se han desarrollado diversos planes de actuación como el proyecto Puertos Limpios, el proyecto ML-Style en el puerto de Vigo (Boletín Oficial del Estado, 2020) o el Plan de Recepción y Manipulación de Desechos Generados por los Buques y Residuos de Carga en el puerto de Santander (Autoridad Portuaria de Santander, 2015). Todos ellos pretenden implementar áreas para la gestión de residuos y reducir la presencia de basura marina mediante diferentes estrategias.

El puerto de Gijón también está implicado con el problema de la basura marina, habiendo desarrollado propuestas no solo para retirar la basura del mar, sino también para generar concienciación ciudadana tal y como se hizo en las campañas "mójate por un mar sin residuos", dirigido a barcos de pesca, deportivos y de recreación o "basura a mares", dirigido a la ciudadanía general (Autoridad portuaria de Gijón, 2018). Mediante estas campañas, se pretende concienciar a la población sobre el problema ambiental que supone la basura marina. Este tipo de iniciativas son especialmente recomendables si se tiene en cuenta que en España entre un 26% y un 32% de la basura marina proviene del turismo en las playas (Ministerio de Transportes, Movilidad y Agenda Urbana, 2020).

El estudio de las fuentes principales por las que se genera la basura marina permite detectar el origen del problema y de esta forma desarrollar nuevas iniciativas y planes de acción específicos para hacerle frente. La acumulación de basura en el medio, particularmente de plásticos, se atribuye al comportamiento humano, tanto a nivel individual como grupal y/o social (Pahl et al., 2017). En consecuencia, se evidencia que el cambio de comportamiento en la ciudadanía es la medida más desafiante para disminuir la cantidad de basura en el medio marino. De la misma forma, el conocimiento acerca de la función que tiene la basura marina como vector de dispersión de especies dañinas también ofrece una base sobre la cual elaborar planes de actuación efectivos. Además, mediante una transmisión efectiva de estos conocimientos, es posible dar paso a una sociedad mejor informada y concienciada con la protección del medio ambiente y la gestión adecuada de los residuos.

En este contexto, la presente tesis ha servido para poder estudiar el potencial de la basura marina para dispersar especies alrededor del puerto de Gijón. En primer lugar, cabe destacar la gran cantidad de basura encontrada, bien en las playas cercanas al puerto, bien flotando a la deriva, con un total superior a los mil objetos recogidos, lo cual refleja la gran magnitud del problema. La mayoría (más del 60%) de los objetos encontrados eran de plástico, aunque también aparecieron gran cantidad de textiles y objetos de otros materiales.

En esta tesis se han combinado dos estrategias diferentes: por un lado, se han identificado todos aquellos organismos macroscópicos encontrados adheridos a la basura y por otro lado se ha empleado el metabarcoding para detectar el ADN ambiental perteneciente a aquellas especies que pudiesen estar en contacto con la basura, como, por ejemplo, fases larvarias o organismos microscópicos que no puedan ser detectados a simple vista. Los resultados obtenidos señalan el potencial de la basura marina en la dispersión de especies. Esto se debe a que se han encontrado varias especies adheridas a basura flotante, e incluso se ha podido detectar ADN ambiental proveniente de 57 especies marinas (de las cuales 9 no son nativas, y algunas de ellas están catalogadas como invasoras) en diferentes tipos de basura encontrada en playas aledañas a la ciudad de Gijón.

Además, gracias a los muestreos de biota que se llevaron a cabo en el puerto de Gijón, también ha sido posible comparar las comunidades presentes en el puerto con aquellas asociadas a la basura. Los resultados son bastante exclarecedores, puesto que la densidad de especies no nativas encontradas en la basura marina es muy elevada. Es más, se ha visto que, en cuanto a la composición de las comunidades, la basura encontrada en zonas más cercanas al puerto muestra mayor similitud con las comunidades que habitan el puerto en comparación a las especies observadas en la basura recogida en las playas más distantes.

Otra de las observaciones importantes en esta tesis doctoral es que no solo los plásticos suponen un peligro ambiental, sino que otro tipo de basuras que no se suelen tener en consideración como la basura textil, a pesar de no tener tanta flotabilidad ni facilidad de dispersión como el plástico, ha mostrado ser una superficie a la cual ciertas especies tienden a colonizar incluso más que al plástico (Figura 7). Estas especies incluyen algas rojas y briozoos, taxones que contienen especies con capacidades invasoras muy elevadas como *Bugula neritina* y *Asparagopsis armata* entre muchas otras.

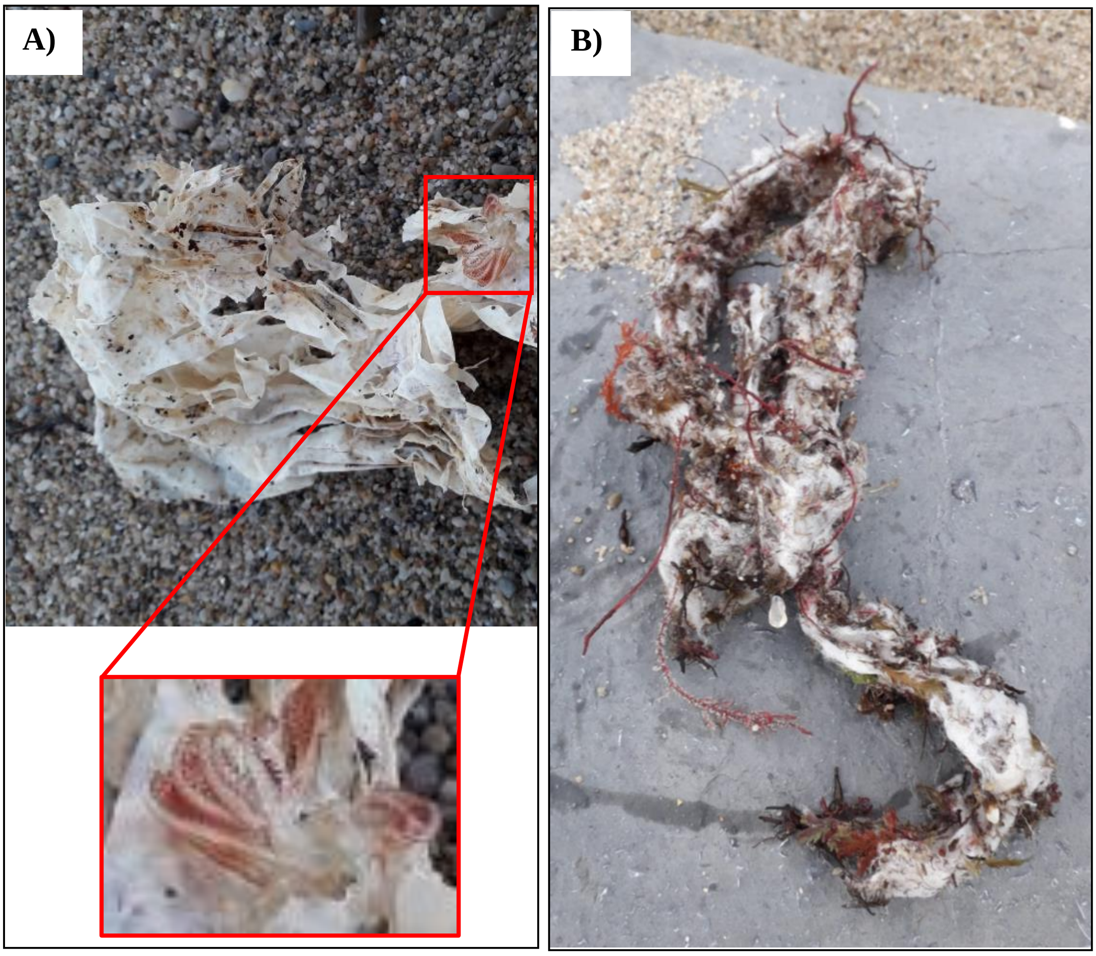



**Figura 7.** Ejemplos de organismos que pueden ser encontrados adheridos a la basura marina. **A)** Restos de un cirrípedo adherido a un plástico. **B)** Fragmentos de algas rojas adheridas a basura textil.

Por lo tanto, los resultados de esta tesis sirven como recordatorio de que la basura marina es un vector que puede facilitar la dispersión de especies y, por ende, favorecer las invasiones biológicas. Es por ello por lo que, además de poner en marcha planes de actuación para disminuir la generación de residuos o para reducir la cantidad de basura en los mares, es necesario concienciar a la ciudadanía sobre la importancia de tratar de forma adecuada los residuos cotidianos. Mediante el deshecho adecuado de la basura, se puede evitar que esta llegue al mar y así prevenir los impactos ambientales que esta pueda generar, como puede ser el favorecimiento de la dispersión de especies exóticas e invasoras a nuevas zonas.

## 4. El papel de la sociedad en la lucha contra las invasiones biológicas

La sociedad juega un papel muy importante en la prevención de las invasiones biológicas. En la mayoría de las ocasiones, la detección temprana de especies no indígenas e invasoras implica tener que realizar monitoreos exhaustivos y cubrir grandes áreas, lo cual muchas veces es económica, o metodológicamente, inviable. Ante esta situación, la ciencia ciudadana puede suponer una clara ventaja y una estrategia eficaz que también puede ser de ayuda para hacer frente a las invasiones biológicas.

Las nuevas tecnologías basadas en el desarrollo de aplicaciones móviles, permiten crear redes de personas voluntarias interconectadas que pueden generar, recolectar y analizar grandes cantidades de datos. Las estrategias de enfrentamiento a las invasiones biológicas pueden aprovecharse y enriquecerse de ello. Se conocen casos en los que la ciudadanía ha colaborado en el seguimiento de especies invasoras como por ejemplo en Estados Unidos, donde gracias a la participación de más de mil voluntarios/as se pudo evaluar la distribución de los cangrejos invasores *Carcinus maenas* y *Hemigrapsus sanguineus* (Delaney et al., 2008). Otro ejemplo más actual, donde la ciencia ciudadana ha resultado ser una herramienta muy eficaz, es el caso de la mantis exótica *Hierodula patellifera* en Francia, cuya distribución pudo ser definida gracias a las observaciones de los/as ciudadanos/as (Moulin, 2020).

No obstante, para que la ciencia ciudadana sea un mecanismo efectivo y viable es necesario que en la sociedad exista interés y concienciación al respecto para que las personas se impliquen y colaboren. Los resultados del estudio social realizado en esta tesis señalan la evidente falta de concienciación existente por parte de los ciudadanos y ciudadanas de Gijón en cuanto al peligro que suponen las especies invasoras. Más de la mitad de las personas encuestadas ignoran el significado de los términos biodiversidad y bioseguridad marina y la mayoría considera que la basura marina es el principal problema ambiental, dejando las invasiones biológicas en un último plano, por debajo de la contaminación por aceite, el biofouling u otros agentes. Los análisis de los resultados de las encuestas a personas que visitaban el puerto muy a menudo parecen indicar que la población basa sus conocimientos en la percepción visual del entorno y no en una percepción cognitiva; Lo que llama la atención y se ve (como la basura marina) es lo que más preocupación genera.

Los datos obtenidos en esta tesis doctoral reflejan la necesidad de concienciar a la población de los problemas que pueden generar las especies invasoras. Los resultados obtenidos en el caso de estudio de Gijón, que concuerdan con los resultados de otros estudios realizados en diferentes localizaciones, indican que el nivel de conocimiento acerca de la peligrosidad de las invasiones biológicas, o la concienciación ambiental, son notablemente bajas en la sociedad (Liu et al., 2014; Báez Gómez, 2016; Seco Méndez, 2018; Kleitou et al., 2019). Esta falta de concienciación puede suponer un lastre para la colaboración entre la sociedad, la comunidad científica y las autoridades responsables a la hora de hacer frente a las especies exóticas e invasoras. Además, se ha visto que un mayor nivel de educación y conocimiento científico puede suponer un mayor apoyo a la investigación científica (Drummond y Fischhoff, 2017), de modo que una sociedad con una baja concienciación ambiental puede limitar el apoyo para la elaboración de proyectos y planes de actuación contra el problema de las invasiones biológicas.

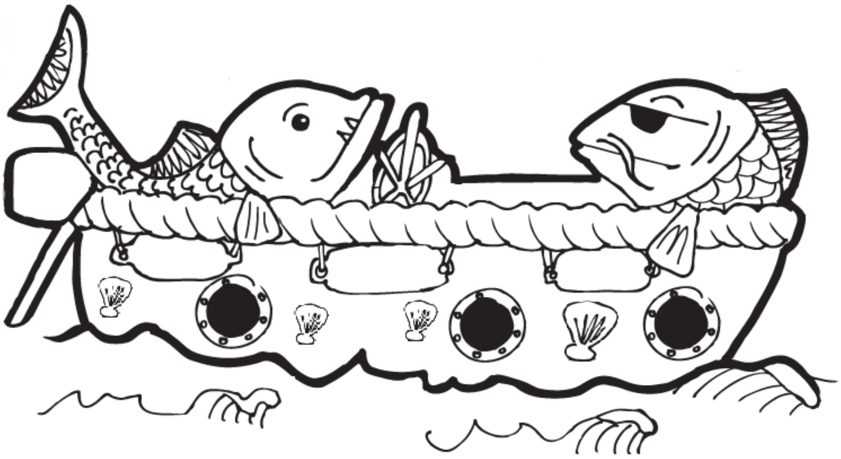
El impulso en la adquisición de conocimientos relacionados con las invasiones biológicas por parte de la sociedad necesita de estudios previos que identifiquen las principales fuentes de información que emplea la sociedad para informarse e instruirse. En el caso de la población de Gijón, si bien se destaca la importancia de la educación como principal fuente de información, cabe mencionar que las redes sociales también son consideradas una de las principales fuentes para adquirir nuevos conocimientos. Sin embargo, se ha demostrado que la información que circula por las redes sociales muchas veces contiene información manipulada, es muy difícil de controlar y puede facilitar información completamente errónea a los/as usuarios/as (Moravec et al., 2018; Shu & Liu, 2019). En el otro extremo se encuentran la literatura y la divulgación científica, las cuales son consideradas como fuentes poco efectivas de transmisión de conocimientos para el ciudadano cómun. Estos resultados son consistentes con otros estudios que reflejan la baja efectividad de las publicaciones científicas a la hora de transmitir conocimientos a la sociedad (Gelcich et al., 2014).

El trabajo desarrollado en esta tesis doctoral, aunque preliminar, refleja la relevancia en promover proyectos educativos que trabajen competencias de concienciación ambiental y traten el problema de las invasiones biológicas. Para ello es recomendable combinar diferentes métodos de comunicación que no excluyan a parte de la población (Spiers et al., 2019). Similarmente, es destacable la necesidad de emplear fuentes fidedignas a la hora de obtener información que nutra fuentes como las redes sociales donde la información puede ser fácilmente manipulada (Naeem et al., 2021). Los proyectos de investigación deberían desarrollar una estrategia de comunicación efectiva en redes sociales (Twitter, Instagram, Facebook), y combinarlo con el uso de estrategias educativas innovadoras, como cursos y charlas divulgativas, para garantizar una transmisión efectiva de los conocimientos a la sociedad (Johnson & Johnson, 2018).

A la hora de poner en marcha este tipo de estrategias educativas es necesario identificar los antecedentes de conciencia social, cultural y ambiental de los posibles participantes. Esta acción puede allanar el camino hacia el éxito de las actividades de concienciación ciudadana, las cuales suponen la base para generar un nivel de preocupación necesario para desencadenar la colaboración ciudadana y hacer frente a las invasiones biológicas de forma exitosa (Encarnação et al., 2021). De hecho, para que este tipo de proyectos sean exitosos, es preciso estudiar de forma previa el contexto existente en cada lugar, ya que un mismo proyecto puede tener diferentes niveles de éxito dentro de una población debido a las características intrínsecas de los individuos (por ejemplo, edad, educación, conciencia ambiental, motivación) y, en consecuencia, también entre diferentes regiones y países (Ganzenvoort & van den Born, 2020; Larson et al., 2020).

Esta tesis establece una base en la que se definen los mecanismos de comunicación más eficaces para concienciar a la población de Gijón. Partiendo de este punto, es posible desarrollar estrategias educativas como cursos y charlas divulgativas para generar conciencia acerca del problema que suponen las invasiones biológicas en el lugar. Se ha visto que este tipo de acciones tienen un efecto positivo en el nivel de concienciación y participación ciudadana (Weeks et al., 2020). En la ciudad de Gijón también se han llevado a cabo actividades educativas contempladas dentro del proyecto Blueports. Dichas actividades incluyen un total de 10 charlas divulgativas presentadas en distintos centros educativos y culturales de la ciudad y una serie de noticias de prensa publicadas en periódicos locales y estatales. Gracias a estas actuaciones, se abren nuevas vías para hacer frente al problema de las invasiones biológicas de forma más cooperativa y eficaz en el futuro.

# Conclusiones



## Conclusiones

**1-** Es posible desarrollar una estrategia portuaria preventiva de reducción de riesgos ante invasiones biológicas basada en el desarrollo de pruebas específicas para la detección temprana de aquellas especies no indígenas con altas probabilidades de invasión. Para ello, en esta tesis doctoral se ha generado una nueva herramienta denominada NIS-ITS (del inglés Non-Indigenous Species InvasionThreat Score) que evalua de forma satisfactoria el riesgo y las probabilidades de invasión de especies no indígenas en ambientes portuarios.

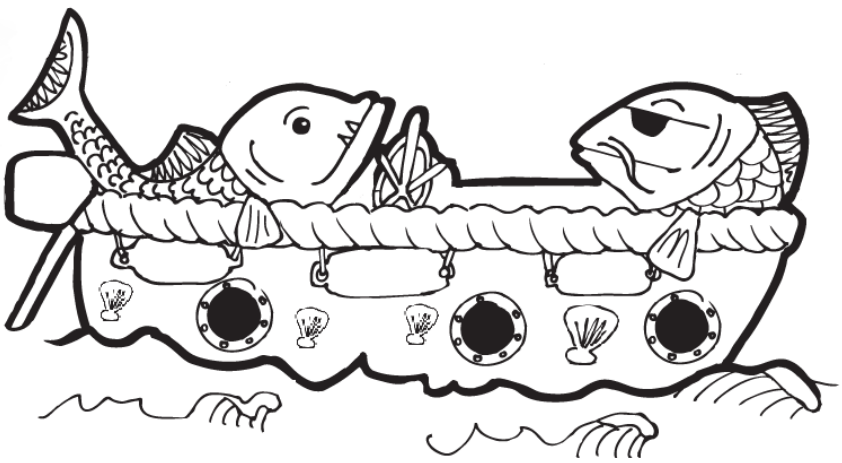
**2-** La implementación de estrategias que incluyan monitoreos periódicos en los puertos puede facilitar la detección temprana de especies no nativas y la puesta en marcha de actuaciones tempranas y efectivas para evitar el establecimiento y proliferación de estas especies y sus consecuentes impactos ambientales y económicos. La combinación de muestreos tradicionales y técnicas de metabarcoding sobre ADN ambiental extraído de agua y sedimentos permite una detección eficiente y temprana. En el puerto de Gijón y en zonas aledañas se ha podido detectar un total de 41 especies exóticas e invasoras.

**3-** Es muy recomendable evaluar el estado ambiental de los ecosistemas teniendo en cuenta los daños ambientales que pueden causar las especies exóticas e invasoras. En esta tesis doctoral se ha desarrollado un nuevo índice biótico Blue-gNIS que considera las especies exóticas e invasoras a la hora de caracterizar los ecosistemas marinos. Incorporar este tipo de índices para llevar a cabo evaluaciones de la calidad del agua podría promover la inclusión, en las políticas nacionales y europeas, de estrategias específicas de prevención y erradicación de especies exóticas en los puertos industriales.

**4-** Los resultados obtenidos en esta tesis doctoral evidencian que la basura marina es una vía relevante de dispersión secundaria de especies no indígenas o incluso de especies dañinas para la salud humana y que podría representar, en si misma, un nuevo hábitat. Se identificaron un total de 717 organismos sobre basura marina y se detectaron 23 NIS, 10 de ellas consideradas invasivas en la zona. Se necesita reforzar las acciones en los puertos industriales y recreativos para mejorar la gestión de este tipo de residuos.

**5-** Las invasiones biológicas mediadas por actividades humanas como las que se realizan en puertos industriales y recreativos, constituyen hoy una amenaza significativa a nivel global para la conservación de la biodiversidad marina. De hecho, el nivel de concienciación, preocupación y participación por parte de los ciudadanos y ciudadanas en ciudades con puertos, como la ciudad de Gijón, parecen ser insuficientes. Es necesario pues, desarrollar métodos eficaces para la transmisión de estos conocimientos, principalmente mediante estrategias educativas, de divulgación en redes sociales y en plataformas de comunicación, participación y concienciación colectiva.

# Conclusions



## Conclusions

**1-** It is possible to develop a preventive port strategy to reduce risks against biological invasions based on the development of specific tests for the early detection of those non-indigenous species with a high probability of invasion. To this end, this doctoral thesis has generated a new tool called NIS-ITS (Non-Indigenous Species Invasion Threat Score) that satisfactorily assesses the risk and probability of invasion of non-indigenous species in port environments.

**2-** The implementation of strategies that include periodic monitoring in ports can facilitate the early detection of non-native species and the implementation of early and effective actions to prevent the establishment and proliferation of these species and their consequent environmental and economic impacts. The combination of traditional sampling and metabarcoding techniques on environmental DNA extracted from water and sediments allows efficient and early detection. In the port of Gijón and in surrounding areas, a total of 41 exotic and invasive species have been detected.

**3-** It is highly recommended to assess the environmental status of ecosystems taking into account the environmental damage that exotic and invasive species can cause. In this doctoral thesis, a new Blue-gNIS biotic index has been developed that considers exotic and invasive species when characterizing marine ecosystems. Incorporating this type of indices to carry out water quality assessments could promote the inclusion, in national and European policies, of specific strategies for the prevention and eradication of exotic species in industrial ports.

**4-** The results obtained in this doctoral thesis show that marine litter is a relevant pathway for secondary dispersal of non-indigenous species or even of species harmful to human health and that it could represent, in itself, a new habitat. A total of 717 organisms were identified on marine litter and 23 NIS were detected, 10 of them considered invasive in the area. It is necessary to reinforce actions in industrial and recreational ports to improve the management of this type of waste.

**5-** Biological invasions mediated by human activities such as those carried out in industrial and recreational ports, constitute a significant threat at a global level for the conservation of marine biodiversity. In fact, the level of awareness, concern and participation by citizens in cities with ports, such as the city of Gijón, seem to be insufficient. It is therefore necessary to develop effective methods for the transmission of this knowledge, mainly through educational strategies, dissemination in social networks and communication, participation and collective awareness platforms.

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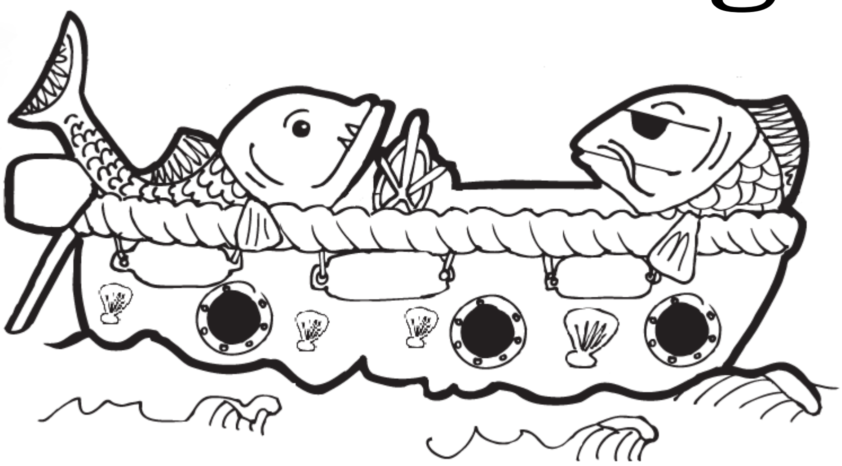

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