Universidad de Oviedo

*Universidá d'Uviéu*

*University of Oviedo*

**Departamento de Bioquímica y Biología Molecular**

**Programa de Doctorado en Biología Molecular y Celular**


# Del puerto a las aguas de lastre: aplicación del "metabarcoding" de ADN para el monitoreo de la biodiversidad introducida por el transporte marítimo

# From port to ballast water: application of DNA metabarcoding for the monitoring of ship-borne biodiversity

**Tesis Doctoral**


Anaïs Rey


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**RESUMEN (en español)**

El transporte marítimo es uno de los vectores más importantes en la introducción y dispersión de especies alóctonas, algas tóxicas y patógenos al rededor del mundo. Miles de organismos, de gran diversidad son transportados diariamente en tanques de agua de lastre o incrustados en el casco de buques y acaban siendo liberados en puertos o en nuevos entornos. Estos puertos a menudo proporcionan hábitats adecuados para el asentamiento de especies y se consideran "puntos calientes" en la introducción de especies alóctonas. El Convenio Internacional para el Control y la Gestión del Agua de Lastre y los Sedimentos de los buques (Convenio BWM) que desde el 2017 está vigente, se adoptó el 2004 con el fin de prevenir, reducir y controlar la introducción de especies alóctonas transportadas por las aguas de lastre. La monitorización de la biodiversidad en las aguas de lastre y en puertos es esencial para el convenio, pero hasta el momento la monitorización se ha basado en la identificación morfológica de organismos, actividad que resulta costosa económicamente y en tiempo. El objetivo general de la tesis es facilitar dicha monitorización explorando la utilidad de las herramientas genéticas como métodos coste-efectivos que podrían integrarse en la investigación y en la gestión de la diversidad de especies asociadas al transporte marítimo. De la evaluación de diversas herramientas genéticas aplicadas a las aguas de lastre y de la investigación en puertos se extrae que el uso del ADN ambiental (ADNa, trazas de ADN liberados en el medio ambiente en forma de células, heces, piel, saliva, moco, ...) y el metabarcoding (asignación taxonómica de individuos de una muestra ambiental basada en sus secuencias de ADN) se distinguen, en esta tesis, como dos posibles catalizadores para proporcionar datos biológicos al Convenio BWM. Los dos casos de estudio demuestran la eficiencia y las limitaciones de estas herramientas para la monitorización de la biodiversidad en las aguas de lastre y en los

puertos. Dichos casos revelan la presencia de especies alóctonas ya documentados con anterioridad, pero además ponen de manifiesto su capacidad para detectar nuevas especies. Es más, esta tesis destaca la importancia de definir protocolos de muestreo inclusivos que consideren la variabilidad espacial y temporal de las comunidades y que se basen en métodos de muestreo adecuados para descubrir todos los taxones presentes en las aguas de lastre y en los puertos. En particular, se resalta que el uso conjunto de ADNa y el metabarcoding sólo pudo detectar un subconjunto de toda la biodiversidad encontrada en los hábitats del puerto, lo que hace pensar que el binomio ADNa metabarcoding podría presentar limitaciones a la hora de convertirse en una posible sustitución de metabarcoding realizada con métodos de muestreo convencionales (por ejemplo, redes de plancton, toma de sedimentos, placas de asentamiento). Sin embargo, el ADNa resulta muy prometedor para el monitoreo de las aguas de lastre de los buques, ya que las características del agua y la gestión asociada (cambio de agua de lastre o tratamiento de agua de lastre) pueden detectarse con este método de muestreo. En general, se concluye que la tesis ha proporcionado nuevos conocimientos sobre las ventajas e inconvenientes de aplicar estas herramientas en la identificación de especies asociadas al transporte marítimo y así proporcionar datos biológicos para el Convenio BWM. Estos nuevos conocimientos incluyen la creación de directrices para realizar estudios de referencia biológica de los puertos con la herramienta ADN metabarcoding y la exploración de la aplicabilidad del ADNa metabarcoding para el monitoreo del agua de lastre.

**RESUMEN (en Inglés)**

Shipping is one of the most important vectors for the introduction and dispersal of Non-Indigenous Species (NIS), harmful algae and pathogens worldwide. Thousands of very diverse species are daily transported in ballast water tanks or through hull fouling, and are released into new environments, including ports. These ports often provide suitable habitats for the settlement of species coming with ship and are considered as “hot-spots” for the introduction of NIS. To prevent, reduce and control further introduction of ballast water-borne NIS, the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM Convention) was conceived in 2004 and is in force since 2017. In this context and in line with this convention, monitoring port and ballast water diversity is essential but has often relied hitherto upon morphological identification of organisms which is costly and time-consuming. The general aim of the thesis was to ease such monitoring by exploring the usefulness of genetic tools, and how these cost-effective methods could be integrated into ship-borne diversity research

and management. Through the evaluation of several genetic tools applied in ballast water and port research, the use of environmental DNA (eDNA, traces of DNA released in the environment in form of cells, feces, skin, saliva, mucus,…) and metabarcoding (taxonomic assignment of individuals from an environmental sample based on their DNA sequences) were emphasized in this thesis as two potential "game-changer" tools to provide biological data for the BWM Convention. Two case studies of their application showed the efficiency and limitations of these tools in monitoring port and ballast water biodiversity, in revealing the presence of previously reported NIS but also their capacity to detect new ones. More importantly, this thesis unveiled the critical importance of defining appropriate sampling protocols, that consider the spatial and temporal variabilities of communities, and rely on adequate sampling methods to uncover all taxa present in ballast water or ports. Particularly, this work discovered that the use of eDNA metabarcoding detected only a subset of all the biodiversity found in the miscellaneous habitats of a port and hence it could have limitations to become a potential substitution of metabarcoding performed on conventional sampling methods (e.g. plankton nets, sediment grab, settlement plates). Yet, eDNA holds great potential for ballast water monitoring and control as ballast water characteristics and application of management (ballast water exchanged or treated) were depicted with this sampling method. Overall, by providing guidelines for DNA metabarcoding-based port biological baseline survey and exploring the applicability of eDNA metabarcoding for ballast water monitoring, the thesis provided new insights into the advantages and drawbacks of implementing such genetic-based species identification to depict ship-borne biodiversity and provide biological data for the BWM Convention.

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**Supervisors:**

Dr. Oihane C.Basurko

Dr. Naiara Rodríguez-Ezpeleta

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## ACRONYMS & ABBREVIATIONS

*16S rRNA*: 16S mitochondrial ribosomal DNA

*18S rRNA:* nuclear 18S small subunit rRNA

*BW:* Ballast Water

*BWE:* Ballast Water Exchange

*BWM Convention:* International Convention for the Control and Management of Ships' Ballast Water and Sediments

*BWTS:* Ballast Water Treatment System

*COI:* mitochondrial cytochrome c oxidase subunit I gene

*DNA:* deoxyribonucleic acid

*eDNA:* environmental DNA

*HAOP:* Harmful Aquatic Organisms and Pathogens

*IAS:* Invasive Alien Species

*IMO:* International Maritime Organization

*LME:* Large Marine Ecoregion

*NGS:* Next-Generation Sequencing

*NICS:* Non-Indigenous and Cryptogenic Species

*NIS:* Non-Indigenous Species

*nMDS*: Non-metric dimensional scaling

*OTU:* Operational Taxonomic Unit

*PBBS:* Port Biological Baseline Survey

*PCA:* Principal Component Analysis

*PCR:* Polymerase Chain Reaction

*RNA:* Ribonucleic acid

*USCG:* United States Coast Guard

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# SUMMARY /

# RESUMEN

Shipping is one of the most important vectors for the introduction and dispersal of Non-Indigenous Species (NIS), harmful algae and pathogens worldwide. Thousands of very diverse species are daily transported in ballast water tanks or through hull fouling, and are released into new environments, including ports. These ports often provide suitable habitats for the settlement of species coming with ship and are considered as "hot-spots" for the introduction of NIS. To prevent, reduce and control further introduction of ballast water-borne NIS, the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM Convention) was conceived in 2004 and is in force since 2017. In this context and in line with this convention, monitoring port and ballast water diversity is essential but has often relied hitherto upon morphological identification of organisms which is costly and time-consuming.

The general aim of the thesis was to ease such monitoring by exploring the usefulness of genetic tools, and how these cost-effective methods could be integrated into ship-borne diversity research and management. Through the evaluation of several genetic tools applied in ballast water and port research, the use of environmental DNA (eDNA, traces of DNA released in the environment in form of cells, feces, skin, saliva, mucus,…) and metabarcoding (taxonomic assignment of individuals from an environmental sample based on their DNA sequences) were emphasized in this thesis as two potential "game-changer" tools to provide biological data for the BWM Convention. Two case studies of their application showed the efficiency and limitations of these tools in monitoring port and ballast water biodiversity, in revealing the presence of previously reported NIS but also their capacity to detect new ones. More importantly, this thesis unveiled the critical importance of defining appropriate sampling protocols, that consider the spatial and temporal variabilities of communities, and rely on adequate sampling methods to uncover all taxa present in ballast water or ports. Particularly, this work discovered that the use of eDNA metabarcoding detected only a subset of all the biodiversity found in the miscellaneous habitats of a port and hence it could have limitations to become a potential substitution of metabarcoding performed on conventional sampling methods (e.g. plankton nets, sediment grab, settlement plates). Yet, eDNA holds great potential for

ballast water monitoring and control as ballast water characteristics and application of management (ballast water exchanged or treated) were depicted with this sampling method.

Overall, by providing guidelines for DNA metabarcoding-based port biological baseline survey and exploring the applicability of eDNA metabarcoding for ballast water monitoring, the thesis provided new insights into the advantages and drawbacks of implementing such genetic-based species identification to depict ship-borne biodiversity and provide biological data for the BWM Convention.

El transporte marítimo es uno de los vectores más importantes en la introducción y dispersión de especies alóctonas, algas tóxicas y patógenos al rededor del mundo. Miles de organismos, de gran diversidad son transportados diariamente en tanques de agua de lastre o incrustados en el casco de buques y acaban siendo liberados en puertos o en nuevos entornos. Estos puertos a menudo proporcionan hábitats adecuados para el asentamiento de especies y se consideran "puntos calientes" en la introducción de especies alóctonas. El Convenio Internacional para el Control y la Gestión del Agua de Lastre y los Sedimentos de los buques (Convenio BWM) que desde el 2017 está vigente, se adoptó el 2004 con el fin de prevenir, reducir y controlar la introducción de especies alóctonas transportadas por las aguas de lastre. La monitorización de la biodiversidad en las aguas de lastre y en puertos es esencial para el convenio, pero hasta el momento la monitorización se ha basado en la identificación morfológica de organismos, actividad que resulta costosa económicamente y en tiempo.

El objetivo general de la tesis es facilitar dicha monitorización explorando la utilidad de las herramientas genéticas como métodos coste-efectivos que podrían integrarse en la investigación y en la gestión de la diversidad de especies asociadas al transporte marítimo. De la evaluación de diversas herramientas genéticas aplicadas a las aguas de lastre y de la investigación en puertos se extrae que el uso del ADN ambiental (ADNa, trazas de ADN liberados en el medio ambiente en forma de células, heces, piel, saliva, moco, ...) y el metabarcoding (asignación taxonómica de individuos de una muestra ambiental basada en sus secuencias de ADN) se distinguen, en esta tesis, como dos posibles catalizadores para proporcionar datos biológicos al Convenio BWM. Los dos casos de estudio demuestran la eficiencia y las limitaciones de estas herramientas para la monitorización de la biodiversidad en las aguas de lastre y en los puertos. Dichos casos revelan la presencia de especies alóctonas ya documentados con anterioridad, pero además ponen de manifiesto su capacidad para detectar nuevas especies. Es más, esta tesis destaca la importancia de definir protocolos de muestreo inclusivos que consideren la variabilidad espacial y temporal de las comunidades y que se basen en métodos de muestreo adecuados para descubrir todos los taxones presentes en las aguas de lastre y en

los puertos. En particular, se resalta que el uso conjunto de ADNa y el metabarcoding sólo pudo detectar un subconjunto de toda la biodiversidad encontrada en los hábitats del puerto, lo que hace pensar que el binomio ADNa metabarcoding podría presentar limitaciones a la hora de convertirse en una posible sustitución de metabarcoding realizada con métodos de muestreo convencionales (por ejemplo, redes de plancton, toma de sedimentos, placas de asentamiento). Sin embargo, el ADNa resulta muy prometedor para el monitoreo de las aguas de lastre de los buques, ya que las características del agua y la gestión asociada (cambio de agua de lastre o tratamiento de agua de lastre) pueden detectarse con este método de muestreo.

En general, se concluye que la tesis ha proporcionado nuevos conocimientos sobre las ventajas e inconvenientes de aplicar estas herramientas en la identificación de especies asociadas al transporte marítimo y así proporcionar datos biológicos para el Convenio BWM. Estos nuevos conocimientos incluyen la creación de directrices para realizar estudios de referencia biológica de los puertos con la herramienta ADN metabarcoding y la exploración de la aplicabilidad del ADNa metabarcoding para el monitoreo del agua de lastre.

# INTRODUCTION

# 1 Maritime shipping and introduction of harmful aquatic organisms and pathogens

## 1.1 Shipping: “a conveyor belt of marine organisms”

The transportation of commodities and people through maritime shipping is playing an undoubted role in the aquatic organisms dispersal around the world (Hulme, 2009; Katsanevakis Stylianos et al., 2014; Minchin et al., 2009; Molnar et al., 2008; Ruiz et al., 2015; Seebens et al., 2013). When describing maritime shipping, the invasion biologist Dr Carton said “we see a conveyor belt of marine organisms wrapping around the world” (Stevens, 1993). The dispersal of organisms via maritime shipping is likely to have started centuries ago. For instance, there are evidences that Romans and Greeks knew about shipworms attached on hull of vessels in the 12th century (Carlton and Hodder, 1995; Ojaveer et al., 2018). Also, Carlton and Hodder (1995) conducted an experimental study on a replica of 16th century sailing vessel, in which they describe the organisms that survived open sea voyages between the harbors attached on the hull. Another example is the introduction of the rockweed *Fucus serratus* and the snail *Littorina littorea* in Atlantic North America that has been linked to the discharge of ballast rock from vessels coming from Europe during the early 19th century (Brawley et al., 2009).

A ship in itself acts as two main vectors for the dispersal of organisms (Figure 1). First, all surfaces of the ship that are exposed to seawater (*e.g.* hull, seachest, propeller, seawater piping systems) are prone to accumulate organisms on the surface, a process termed biofouling (Hewitt et al., 2009). Biofouling communities are usually composed of sessile organisms such as barnacles or algae. Second, ships require to be ballasted to improve the balance, stability and the trim during sea voyages and during maneuvering. The use of any type of ballast loaded inside the ship transported organisms between ports (Hewitt et al., 2009). Ballast is used to increase stability when a ship is empty of cargo and, to compensate for the changing weight induced with fuel consumption during transit and when a ship is loading or discharging cargo (Minchin et al., 2009). Ballast started with the use of rock or sand inside the ship and moved progressively from 1880s toward the use of ambient water to avoid the time-consuming loading of solid materials and also because

water was easily accessible and cheaper (Minchin et al., 2009). Usually uptake of port's water happens during the unloading of the cargo, while the discharge occurs during the cargo loading. The seawater is uptake from the sea-chest, it is pumped through a filter, and transferred to the ballast tanks where it is stored. Sea-chests are equipped with a grating designed to remove large organisms (*e.g.* fishes) but fails to prevent the uptake of small organisms or early life stages of bigger ones (*e.g.* phytoplankton, copepods, larvae of fishes). Inside a ballast water tank, several sub-vectors are present including: the water itself (Gollasch et al., 2002), the sediment at the bottom of the tank (Branstrator et al., 2015) and the internal tank walls through biofouling (Drake et al., 2007). The cargo itself can also act as a potential vector of organisms transfer but is usually associated to terrestrial species such as insects (Rassati et al., 2015).

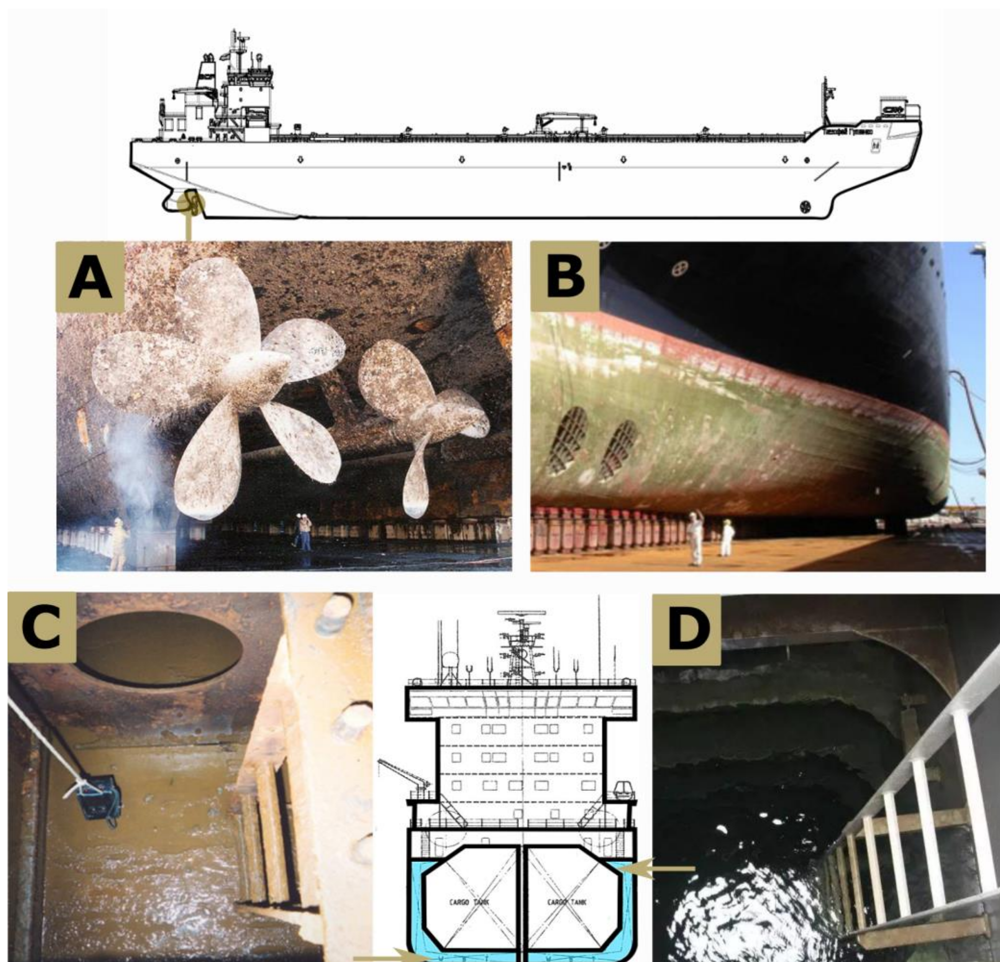


Figure 1. Pictures showing the two main vectors of dispersal of organisms associated with maritime shipping: (A) Biofouling on the propellers, (B) Biofouling on the hull, (C) Ballast sediment and (D) Ballast water

## 1.2 Evolution of maritime shipping increased the dispersal of organisms

During the 19th and 20th centuries, the shipping industry evolved considerably thanks to key innovations including the use of water as ballast, the diesel engine, and the specialization of ships (*e.g.* cargo, bulk carrier) that optimized the transport of specific cargos types (Davidson et al., 2018; Ojaveer et al., 2018). This modernization of shipping played a crucial role in the exponential rise and globalization of maritime trade (Figure 2, Ojaveer et al. (2018)). Such globalization had profound effects on the way of aquatic organisms were dispersed worldwide (Carlton, (1996a). First, the increase in the number and size of ships enlarged the surface area for biofouling organisms to be attached to and expanded the volume of ballast water discharged. Second, higher ship speeds reduced transit length and thereby inflated the number of organisms discharged as more ports could be visited in a shorted time. Also, as transits were shortened, more organisms were able to survive the voyage. Thirdly, the creation of tanks specialized in holding only ballast water avoided the mixing of ballast water with residuals of chemicals or oil present in empty tanks, which derived in improving the quality of the water and thus, increased the survival of organisms. These changes were also associated to the increase in port connectivity around the world, which, altogether, contributed to the dispersal of organisms (Crooks and Suarez, 2006). Indeed, global shipping evolved from "one port to one port travel" to a "hub and spoke" network (Ojaveer et al., 2018). In the "hub and spoke" configuration, cargos are delivered in international hub ports from local and smaller ports before being transported to other hub ports (Peng et al., 2019). As a result, the world's merchant fleet is now associated to a continuous and massive transfer of organisms' assemblages, with up to 10 billion of tons of ballast water translocated each year (David and Gollasch, 2015) and approximately 325 x $10^6$ square meters of ship's hulls wetted surface area available for biofouling (Moser et al., 2016).

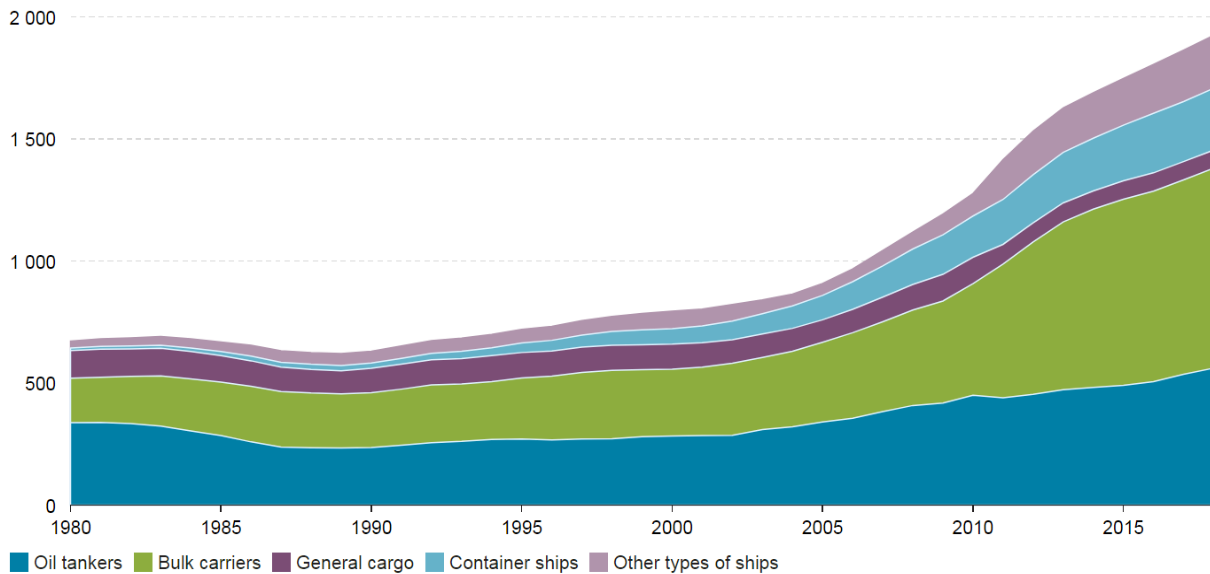


Figure 2. Global merchant fleet by vessel type in millions of dead-weight tons, Source: UNCTADstat

### 1.3 The introduction of harmful aquatic organisms and pathogens with ballast water

The diversity and high density of organisms transferred, and its worldwide impact led maritime shipping to become one of the most prevalent vectors of introduction of Non-Indigenous Species (NIS) and cryptogenic species into new environments (Hewitt et al., 2004; Molnar et al., 2008; Ricciardi, 2016; Ruiz et al., 2015). NIS (synonyms of alien, exotic, non-native, allochthonous) are species introduced into new areas outside of their native ranges due to human-mediated actions, either intentionally (*e.g.* release of fishes for angling) or unintentionally (*e.g.* discharged of ballast water). Contrary to NIS, cryptogenic species have uncertain origins and cannot be described as being native or non-indigenous. Cryptogenic species are very common, and many unrecognized invasions are potentially classified under this terminology (Carlton, 1996b; Darling and Carlton, 2018; David and Gollasch, 2015). One of the first records of a NIS introduced by ballast water was the case of the phytoplankton *Odontella* (*Biddulphia*) *sinensis,* native from Asia and documented in the North Sea in 1903 (Ostenfeld, (1908) in David and Gollasch (2015)). Since then, reports

on introduction of NIS via ballast water have increased in distinct regions of the world, from well-studied areas such as Europe (Tsiamis et al., 2018) and North America ((Davidson et al., 2018)), to less-studied ones such as the Arctic ((Ware et al., 2016)). As an example of this phenomenon, Ruiz et al (2015) estimated that two hundred marine NIS that were introduced in North America (44% of the total) have been initially transferred with shipping.

After the initial released into a new environment, some of the NIS may become established (*i.e.* start to grow and reproduce); others may even become Invasive Alien Species (IAS). IAS are defined by Olenin et al. (2010) as "a subset of established NIS that have spread, are spreading or have demonstrated their potential to spread elsewhere, and have or might have an adverse effect on biodiversity, ecosystem function, socio-economic values and/or human health in invaded regions". The multiple impacts of marine IAS on invaded environments have been widely documented (Mack et al., 2000; Pyšek and Richardson, 2010; Stiger-Pouvreau and Thouzeau, 2015). Consequences of biological invasions include, *inter alia,* impacts on ecological interactions such as competition for resources, including place to settle and spawning grounds (Stiger-Pouvreau and Thouzeau, 2015), predation or grazing (Grosholz et al., 2000) and trophic cascading effects (Levin, 2006) as well as hybridization with native species (Ayres et al., 1999). Besides, the establishment of globally spread NIS into disparate habitats contributes to the generalized biotic homogenization (Clavero and García-Berthou, 2006; Olden, 2006). IAS can also cause serious economic and social impacts on activities and resources related to the invaded environment such as aquaculture, fisheries, tourism and marine infrastructures (Mooney, 2005; Williams and Grosholz, 2008). For example, it has been estimated that the economic losses attributable to invasive species in the United States amount almost $120 billion per year (Pimentel et al., 2005). Another example can be found in North America with the zebra mussel that clogs and invades waterways or water filtration of power plant. The economic cost associated to this species has been estimated to exceed $100 million over the past decades (Strayer, 2008).

Ballast water is also a well-known vector for pathogens and harmful algal species releases (Drake et al., 2007). Human pathogens such as *Vibrio cholera* and *Escherichia coli* have been previously found to survive ballast water conditions due to their strong tolerance to variation of temperature and salinity (McCarthy and Khambaty, 1994; Takahashi et al., 2008). Besides, these pathogens have been recorded with high frequency in ballast water surveys (Burkholder et al., 2007; Ruiz et al., 2000). Furthermore, ballast water contains many phytoplankton species (McCarthy and Crowder, 2000) and among them, some (usually diatoms and dinoflagellates) are forming harmful algal blooms, which can jeopardize sea-life and human health (Burkholder et al., 2007; David and Gollasch, 2015; Hallegraeff and Bolch, 1991). For instance, it was estimated that a single ballast tank contained 300 million viable cysts of the harmful microalgae *Alexandrium tamarense* (Hallegraeff, 2007).

In order to manage and prevent these negative consequences associated to the introduction of NIS and the spread of harmful algal bloom species and pathogens, a set of management actions can be taken. These are commonly divided into four main components: (1) prevention, (2) early detection and rapid response, (3) control and (4) adaptation (Figure 3; Lodge et al. (2006)). *Prevention* focuses on the early steps of the introduction process. It aims at preventing organisms from entering a dispersal vector, and from being transported and released alive. In case this first step fails, the second lever of action is the *early detection* to rapidly identify species introduced in an environment and act before they start reproducing and spreading (Campbell et al., 2007; Davidson et al., 2015; Vander Zanden et al., 2010). *Control* is based on the monitoring of established NIS and aim at managing their ecological and economic impacts, as well as eradicating them, if possible. *Adaptation* is the last management option and consists in changing human behavior and dealing with the cost associated to the consequences of NIS introduction. All these management components are crucial to face and manage the spread of invasion, but experience has shown that the sooner the discovery of a NIS, the higher the probability of a successful intervention is (Vander Zanden et al., 2010). In that sense, the management of the first step of invasions, including prevention and early detection, should be prioritized.

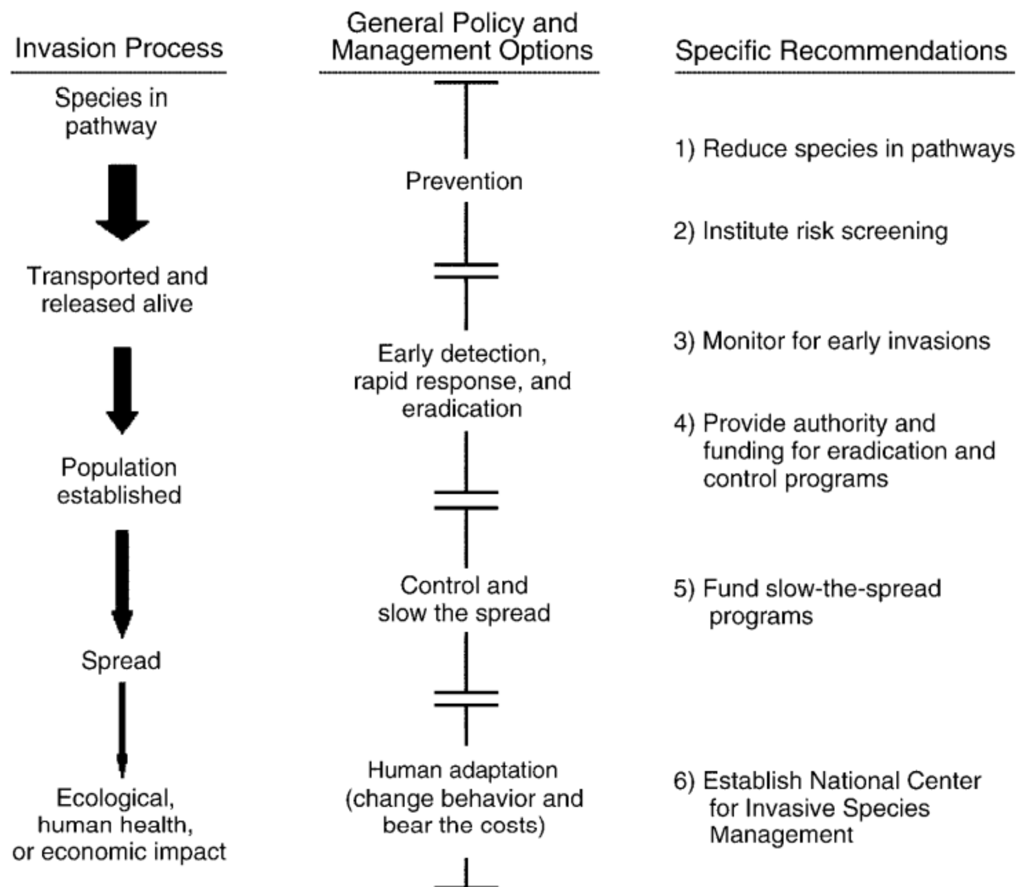


Figure 3. Stages of invasion by Non-Indigenous Species (left column, major policy options (middle column) and major recommendations (right column), from Lodge et al.(2006)

## 1.4 Joint efforts to prevent the spread of organisms with ballast water

Over the years, various national and international regulations have been proposed around the world to minimize the spread of organisms via ballast water (David and Gollasch, 2015). Among those, the two most significant and noteworthy endeavors are undoubtedly those initiated by the International Maritime Organization (IMO) and the United States Coast Guard (USCG). The IMO developed the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM Convention; IMO (2004)). The BWM Convention entered into force in September 2017 (IMO, 2016), involving at the time of writing 79 states, representing more than 81% of the world gross tonnage

(http://www.imo.org/en/About/Conventions/StatusOfConventions/Documents/Status%20-%202019.pdf, last update the 11/02/2019). The main management actions that this framework proposed to prevent further spread of organisms are (1) exchanging coastal/brackish/fresh ballast water with oceanic water, and (2) treating ballast water to inactivate or kill organisms uploaded with ballast. The United States, in contrast, did not sign the BWM Convention but developed instead national regulations regarding ballast water management since 1990 with the USCG and the US Environmental Protection Agency (Verna and Harris, 2016). The USCG, US Environmental Protection Agency and IMO have the same *numerical discharge standards* regarding the maximum number of organisms that can be discharged after the treatment of ballast water (Albert et al., 2013). However, IMO counts the number of "viable" (*i.e.* able to reproduce) organisms whereas the US regulations count the number of "living" (*i.e.* alive) organisms (Blatchley et al., 2018).

# 2 Monitoring ship-borne biodiversity

## 2.1 From ballast to port surveys: great diversity of assemblages

*Assemblages of organisms found in ballast tank:*

Characterizing the biodiversity discharged around the world with ballast water has become an active field of research (Cabrini et al., 2018; Desai et al., 2018; DiBacco et al., 2012; Gollasch et al., 2002; Hutchings, 1992; Ware et al., 2016; Wu et al., 2017). It is clear from this substantial piece of literature that ballast water transported wide range of species assemblages. Ballast tanks are mainly dominated by planktonic species including holo-planktonic species that spends their whole life cycle in the water column but also mero-planktonic species that only have a part of their life cycle in the water column (Hewitt et al., 2009). The associated biota with ballast tank is composed of pathogens, fungi, viruses, bacteria, protists, micro- and macro-algae and zooplankton (see Figure 4 for some examples). Among zooplankton, copepods are the most abundant taxa but other taxa such as barnacles, isopods, mysids, euphausiids, bivalves, gastropods, crabs and fishes are also found in ballast tanks, usually at larval stages (Gollasch et al., 2000, 2002; McCollin et al.,

2007). Importantly, these taxa are not evenly distributed in a ballast tank. For instance, ballast water sediment may contain diapausing eggs of some species that are not present in the water of the same tank (Duggan et al., 2006). Within the water column, vertical migration of zooplankton, patchiness in the distribution of organisms and accumulation of plankton near the tank walls can also result in a heterogenous distribution of organisms. All these factors make representative samples of ballast tank biodiversity difficult to obtain (First et al., 2013; Murphy et al., 2002).

Figure 4. Examples of alive organisms illustrating the broad variety of taxa found in ballast water samples. Photo taken from David & Gollasch (2015).

*Assemblages of organisms found in port:*

Organisms transferred through shipping are primarily taken from and released in ports. Ports comprise a wide range of potential habitats for organisms carried by ships and are hence considered as critical gateways for NIS introduction (Ferrario et al., 2017; Ojaveer et al., 2014). For instance, ports construction such as berths and slipways provide suitable substrates for the settlement of larvae of sessile organisms while soft sediment allows resting stages of zooplankton and dinoflagellates to find appropriate environment (Lehtiniemi et al., 2015).

Ports are considered priority areas for NIS monitoring (Lehtiniemi et al., 2015; Olenin et al., 2016), since once established, NIS can spread from ports into surrounding environments (Keller et al., 2011; Leuven et al., 2009; López-Legentil et al., 2015; Wang et al., 2018). Hence, exhaustive ports monitoring require substantial sampling efforts to collect organism from sediment, water and all the substrates on which biofouling organisms may settle (Hewitt and Martin, 2001). In this perspective, Port Biological Baseline Surveys (PBBS) have been used to standardize port monitoring and provide protocol for port biodiversity inventories as well as for assessment of presence, abundance and distribution of NIS/IAS (see for instance the GloBallast PBBS (2014) and the Helcom/Ospar PBBS (2013)). Specifically, PBBS were developed to inform port authorities about the presence of local and alien taxa and provide them a biological inventory that will serve as baseline data for the BWM Convention (Awad et al., 2014).

PBBS protocols are already developed but differ in scope, scale and exhaustiveness. They range from NIS-targeted detection to whole-community assessments (HELCOM/OSPAR, 2013) and from presence-absence data only to abundance evaluation (Campbell et al., 2007). The two types of PBBS most often used are the CRIMP protocol (Hewitt and Martin, 2001) and the Rapid Assessment Surveys (Minchin, 2007). The CRIMP protocol consists in an exhaustive sampling of all habitats of the ports through various methods (*e.g.* plankton net, sediment grab, divers for visual census and sampling biofouling). In contrast, the Rapid Assessment Surveys is based on *in situ* identification, restricted allocated time and "easy" sampling locations (*e.g.* divers are not used) and

consists in collecting a representative specimen for all types of targeted taxa. This protocol is usually based on a list of target alien species that are not yet present in the port of interest and its surroundings but that are likely to be introduced. Rapid Assessment Survey is particularly useful for management actions as it evaluates the presence and variation of the spatial distribution of "target" species to monitor (Ashton et al., 2006). However, it has also showed some limitations in its capacity to provide comprehensive data by missing the detection of some NIS (Bishop and Hutchings, 2011; Rohde et al., 2017) in particular for small organisms (Kakkonen et al., 2019). Based on both protocols, the Helcom/Ospar developed a new one specifically to answer the requirements of the BWM Convention (HELCOM/OSPAR, 2013)

## 2.2 From morphology to genetic-based species detection

The identification of organism from ships and ports has traditionally relied on the use of morphological taxonomy (Bishop and Hutchings, 2011; Gollasch et al., 2000). Yet, several difficulties are associated to this method including:

(1) The absence of diagnostic morphological traits in damaged and degraded organisms, in development (*e.g.* larvae, egg) or resting stages (*e.g.* spore) which very often led to coarse taxonomic resolution of taxa (*e.g.* "copepods nauplii","bivalvia sp."; DiBacco et al. (2012)).

(2) It can be common for a NIS to be misidentified as a local species or left as "new and undescribed" when organisms from very distinct part of the world are identified by local taxonomist or parataxonomists who lack the full skills of a taxonomist (Bishop and Hutchings, 2011; Campbell et al., 2007; David and Gollasch, 2015; Zenetos et al., 2005). Specifically, for ballast water and ports monitoring, it is difficult to gather taxonomists who have an expertise on the wide range of taxa found in these environments.

(3) It is highly dependent on taxonomic group, which can lead to overlooking small taxa such as protozoa or oomycota in ballast water (Zaiko et al., 2015b).

(4) It is labor intensive, costly and often slow to get the results when many or large samples need to be identified (Pfrender et al., 2010).

The described limitations likely led to underestimating the biodiversity of NIS introduced with shipping, as most of taxa not identified to species level were categorized as cryptogenic (for example DiBacco et al. (2012)) or left undescribed. Resolving species identity is crucial to define an invasion status. Hence, to circumvent some of the limitations of morphological taxonomy identification, DNA-based species detection has been developed and increasingly applied to NIS monitoring since 1980 (Figure 5).

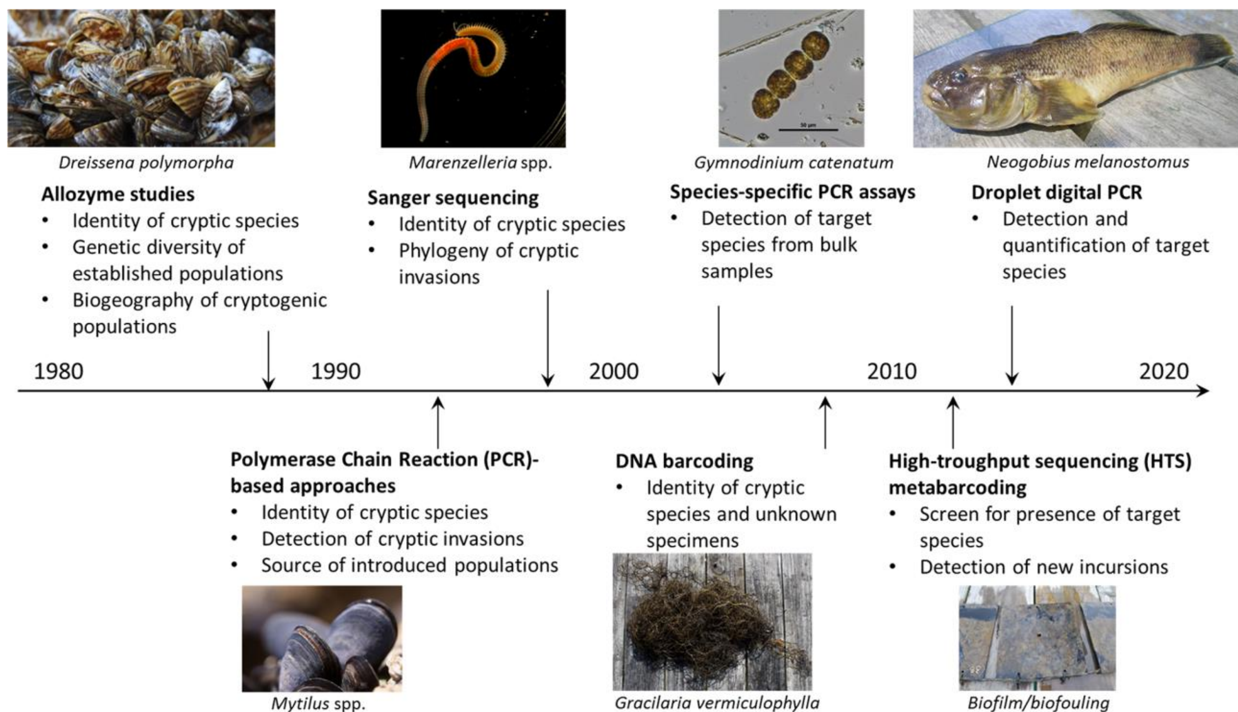


Figure 5. Timeline of molecular methods applications to marine bioinvasions research and surveillance, with images visualizing examples of species or biological matrices to which the method was applied in the context of bioinvasions, from Ojaveer et al (2018).

At first, DNA-based methods were used to distinguish between cryptic species (two or more species morphologically indistinguishable classified as one species, McDonald and Koehn (1988)) and identify the source of introduced population (Ward and Andrew, 1995). Species-specific assays were also developed to detect target species from an environmental sample such as water or sediment. Species-specific assays are based on the design of short fragments called *primers* that bind to a specific region of the genome of the species of interest only. The development of these approaches has played a pivotal role in the detection of many harmful species in ballast water (see for instance for *Vibrio cholerae* Fykse et al.(2012)) and port (see for instance for *Styela clava* Gillum et al. (2014)). However, although target species detection is of vital importance for the early detection and monitoring of targeted harmful species, the detection of non-target species was not possible until the development of the so-called DNA barcoding in the early 2000s (Hebert et al., 2003a). In the DNA barcoding, a short-standardized DNA fragment called *barcode,* is extracted from a single organism, amplified usually with group-specific or "universal" primers during Polymerase Chain Reaction (PCR), and then sequenced. The resulting sequence is then compared to sequences available in public reference databases such as the BOLD database (www.barcodinglife.org). It is worth mentioning that DNA barcoding is based on one major assumption namely that intraspecific sequence polymorphism is lower than interspecific divergence. This is referred to as barcoding gap (Meyer and Paulay, 2005). DNA barcoding has been applied to detect the biodiversity both in ports (Miralles et al., 2016) and in ballast water (Briski et al., 2011). In particular, there are several examples of DNA barcoding used in combination with morphological taxonomy to characterize ballast water taxa and benefit from the advantages of both methods (Briski et al., 2014, 2015; Ware et al., 2016). In these studies, morphology was used to count alive and dead organisms of each taxa and barcoding served for species identification. Yet, because the whole laboratory process is performed on one specimen at a time, DNA barcoding is also labor-intensive. This impedes the use of DNA barcoding for large monitoring such as screening taxa in many ships coming to a port or performing exhaustive Port Biological Baseline Surveys.

## 2.3 DNA metabarcoding: a game-changing approach for ship-borne biodiversity surveillance

The recent development of Next-Generation Sequencing (NGS, also called High-Throughput Sequencing) technologies opened the door to perform DNA barcoding simultaneously on all organisms present in an environmental sample (Shokralla et al., 2012). This technique has been named *DNA metabarcoding* (Taberlet et al., 2012) and can provide several hundred thousand to ten millions sequenced reads in parallel. The process involves five main steps, as described in Figure 6. In Step #1 samples of a targeted environment are collected. In Step #2 the samples are pre-processed for DNA extraction (*e.g.* filtration of water, homogenization of bulk organisms). Step #3 consists in the preparation of samples for the sequencing with NGS platforms (*e.g.* Illumina Miseq). This step includes 3 additional sub-steps. First, DNA is extracted and then the target DNA region is amplified by PCR. Second, unique nucleotide sequences (called "indexes") specific to each sample are added using PCR so that each sample can be discriminate from the others. Thirdly, samples are pooled together and send to sequencing. In Step #4 the sequences retrieved from each sample are taxonomically identified by the use of bioinformatics analysis. This step generally includes the removal of primers and bad quality sequences, the alignment of sequences against a reference alignment database to retain only sequences that align inside the barcode region, the removal of chimeras (hybrid sequences), the clustering of sequences into Operational Taxonomic Unit (OTU), the removal of OTUs with a single sequence and finally, the taxonomic assignment of OTUs against database. In Step #5, data are interpreted to understand community patterns.

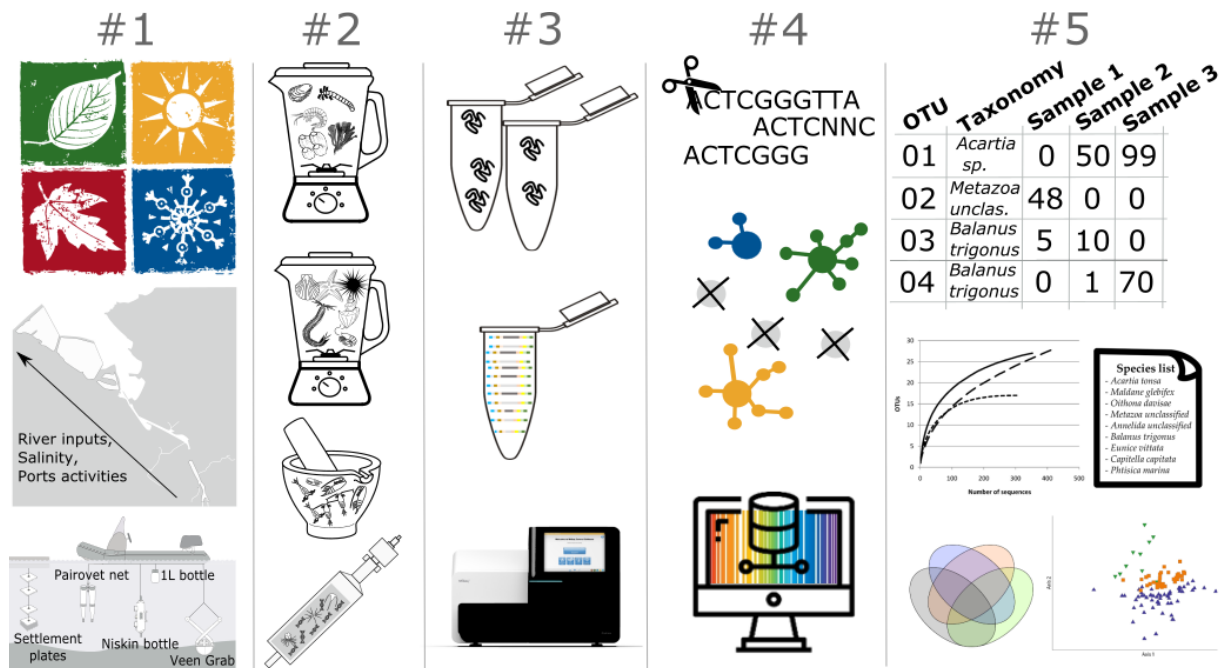


Figure 6. Schematic representation of the five main steps of metabarcoding exemplified for port monitoring surveys

The DNA metabarcoding enables the screening of many samples in a relatively short amount of time, which outstrips the ability of morphological-based taxonomy for biodiversity monitoring (Aylagas et al., 2018; Ji et al., 2013). Metabarcoding has been applied in a broad range of environments, from terrestrial (Ji et al., 2013), to freshwater (Elbrecht et al., 2017b) and marine ones (Chain et al., 2016). It is also increasingly acknowledged as valuable tool to detect NIS from a community (Brown et al., 2016) and perform ecological status assessment (Aylagas et al., 2018). DNA metabarcoding is also viewed as a promising tool to screen ship-borne biodiversity (Comtet et al., 2015; Darling and Frederick, 2017). The applications and developments of the technique in ballast water (Darling et al., 2018; Lohan et al., 2015; Lymperopoulou and Dobbs, 2017; Zaiko et al., 2015b) and ports (Brown et al., 2016; Grey et al., 2018; Koziol et al., 2018; Lacoursière-Roussel et al., 2018) are rocketing. With this rise of metabarcoding-based monitoring applications, developing and standardizing protocols to optimize biodiversity recovery has become an increasing necessity. Hitherto, most of the works in this direction has focused on post-sampling steps, spanning from samples pre-processing to bioinformatic

pipelines (steps #2 to #5 in Figure 6; see for instance Aylagas (2017) and Elbrecht (2017)), including *inter alia*:

*(1) DNA extraction protocols:*

While home-made DNA extraction protocols have been shown optimal in some cases (Corell and Rodríguez-Ezpeleta, 2014), their standardization remain limited. Hence, they are not recommended for monitoring in constrast to commercial kits, which have fixed protocols and do not contain toxic substances. For sediment samples or bulk organisms retrieved from sediment and biofouling samples, the use of MoBio PowerSoil or PowerMax Soil kit has been generally recommended as the optimal kit (Ammon et al., 2018; Aylagas et al., 2016b; Lanzén et al., 2017; Lekang et al., 2015). For zooplankton and filtered water samples, Qiagen's DNeasy Blood & Tissue Kit usually outperforms other protocols and is commonly used (Clarke et al., 2017; Hinlo et al., 2017; Jeunen et al., 2019; Spens et al., 2017). Also, the development of automated DNA/RNA/proteins extraction processing (QIAcube) encourages the use of Qiagens' DNeasy Blood & Tissue Kit for biodiversity monitoring, which includes usually hundreds of samples to process.

*(2) Trial of "universal" primers:*

A rapid evolution of metabarcoding consisted in moving from a single barcode to a multi-barcode approach, taking advantage of each barcode specificity to provide the most exhaustive view possible of the sampled community (Clarke et al., 2017; Drummond et al., 2015; Grey et al., 2018; Günther et al., 2018). The most commonly used barcodes are the mitochondrial cytochrome c oxidase subunit I gene (COI) for metazoan, usually amplified with the degenerated primers developed by Leray et al (2013) for marine taxa, and the nuclear 18S small subunit rRNA (18S rRNA) for eukaryotes, with common primers such as those developed by Amaral-Zettler (2009) or Zhan et al (2014). It is also worth mentioning the 16S mitochondrial ribosomal DNA (16S rRNA) that is currently being developed for aquatic invasive invertebrates (Klymus et al., 2017).

*(3) The development of bioinformatic pipelines:*

The use of open and free standardized pipelines for data processing, from raw sequences cleaning and filtering to taxonomical assignment of sequences, is critical to standardize

bioinformatic analyses and foster comparability. Examples of such pipelines include for example MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP) and QIIME (http://qiime.org/).

While the optimization of the metabarcoding process led to an increase in standardization and reliability of the technique, few studies so far have attempted to evaluate the impact of the sampling method, seasonality and locality on the recovered biodiversity (see step #1 of Figure 6). More specifically, how sampling protocol might affect the identification of some taxa of interest for ship-borne biodiversity monitoring remains to be better understood. Indeed, as explained above, conventional protocols for PBBS or ballast water monitoring have been developed for morphological taxonomy and understanding if these protocols are appropriate for metabarcoding monitoring or, on the contrary, need adjustments, is critical before using this promising technique.

## 2.4 Environmental DNA: cost-effective and non-intrusive sampling method

Conventional sampling methods (*e.g.* plankton net, sediment grab, settlement plates) used for marine monitoring are being superseded by the development of the environmental DNA ("eDNA"). eDNA is defined as any traces of DNA released in the environment (*e.g.* water or sediment) in form of cells, feces, skin, saliva, mucus, blood, gametes, *etc* (Shaw et al., 2017). The use of eDNA is non-intrusive and greatly reduces cost and time associated to sampling (Borrell et al., 2017). Indeed sampling of a community with eDNA only requires the collection of a little volume of water (from few ml to few liters, Rees et al (2014)) or few grams of sediment (Turner et al., 2015) in comparison to conventional sampling methods where more time (and consequently more resources) is spend for the sampling (Borrell et al., 2017). Also, when comparing with conventional sampling methods, eDNA has been shown to be very efficient for detecting rare, elusive or invasive species (Jerde et al., 2011; Thomsen et al., 2012). Hence, the possibility of using

eDNA in studies targeting eukaryote taxa, with a focus on macro-organisms, resulted in rocketing of publications per year (Figure 7a). As for metabarcoding, the on-going optimization of the eDNA workflow, specifically for capturing eDNA and extracting the DNA, remains a priority in eDNA-related research (Figure 7a). Recently, the combination of open cellulose nitrate filters, or enclosed filters such as Sterivex™ filtration system filters, with Qiagen's DNeasy Blood & Tissue extraction Kit has been recommended for aquatic eDNA monitoring (Djurhuus et al., 2017; Hinlo et al., 2017; Jeunen et al., 2019; Spens et al., 2017). One advantage of enclosed filter is the reduced contamination risk, which happened to be a common issue in eDNA-studies with the risk of introducing false-positive detection (Deiner et al., 2017; Goldberg et al., 2016).

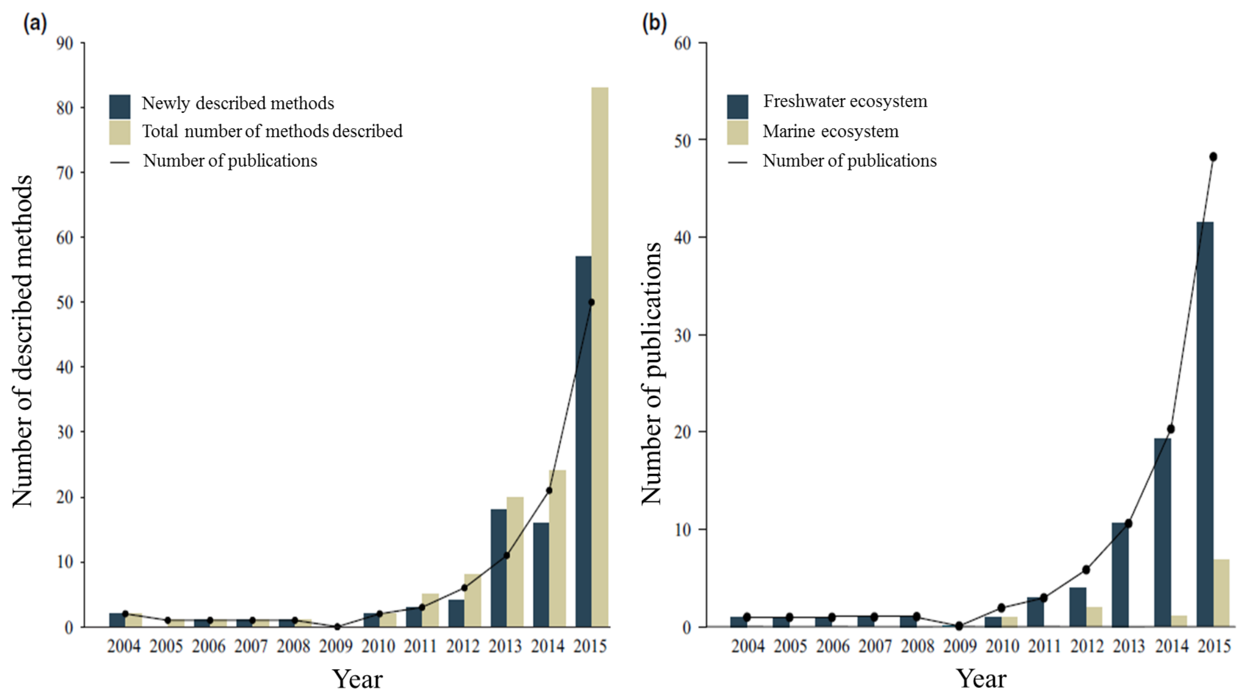


Figure 7. Meta-analysis of aquatic eDNA literature displaying (a) the total number of described methods and (b) the proportion of aquatic eDNA research performed in freshwater and marine ecosystems, from Jeunen et al. (2019)

To date, eDNA has been more developed for freshwater than marine environment (Figure 7b) but the number of studies targeting the marine realm is quickly increasing (see for instance Lacoursière-Roussel et al. (2018), Jeunen et al. (2018, 2019), Borrell et al. (2017), Günther et al (2018)). It was perhaps due to the initial perception that species detection with eDNA from seawater can be more challenging because of the large volume and the dynamic nature (tidal and current) of the ocean that might rapidly dilutes and disperses eDNA from its initial source (Díaz-Ferguson and Moyer, 2014). Several evidences in coastal and offshore systems encouragingly support the ability of eDNA to represent local and contemporary communities (Collins et al., 2018; Jeunen et al., 2018; O'Donnell et al., 2017; Port et al., 2016; Yamamoto et al., 2017). It has been suggested that eDNA is transported over small distances only (within 100-200 m, Jeunen et al. (2018)) and detects species present or recently present (in the last 50 hours approximately, Collins et al. (2018)).

Many studies assessed the reliability of eDNA to monitor aquatic monitoring in comparison with conventional surveys (see among others Deiner et al. (2016) for freshwater macroinvertebrate with kicknet sampling; Kelly et al. (2017) for marine epibenthic macroinvertebrates with benthic sled; Port et al. (2016) for marine fishes with scuba diving). In general, studies tend to conclude that eDNA can be used as a complementary method, different species/taxa being retrieved with different methods, but highlight that eDNA can also detect a high diversity of taxa and increases the resolution of taxonomic identification in comparison with other methods (Deiner et al., 2017). Consequently, implementing filtered water-based eDNA metabarcoding into ship-borne biodiversity monitoring has recently started and primally focused on ports environment (Borrell et al., 2017; Grey et al., 2018; Koziol et al., 2018; Lacoursière-Roussel et al., 2018). Nonetheless, the advantages and drawbacks of eDNA sampling in comparison with conventional sampling methods have only been scarcely studied (see however Koziol et al. (2018) for ports) and warrant further research. In particular, such comprehensive comparisons are lacking for ballast environments.

Overall, genetic tools overcome some of the main limitations of morphological identification and have been described as a promising revolution in the assessment and management of species diversity (Fonseca et al., 2010; Wood et al., 2013). In particular, DNA metabarcoding has been proposed as an appealing and cost-effective approach to perform biodiversity monitoring surveys and early detection of NIS (Comtet et al., 2015; Xiong et al., 2016). Yet, application of genetic tools for ballast water research and management requires further testing to understand how these tools could be integrated into regular monitoring programs (Comtet et al., 2015).

# OBJECTIVES

*General objectives:*

The **general objectives** of the thesis are (1) to evaluate the potential of genetic tools to provide valuable biological data for monitoring ship-borne biodiversity, with a particular focus on DNA metabarcoding, and (2) to provide guidelines to ease the implementation of DNA metabarcoding for ports and ballast water monitoring.

*Secondary objectives:*

To answer these two general objectives, **three secondary objectives** have been developed, each associated to a specific chapter of this thesis and detailed in Table 1.

Table 1. Description of the secondary objectives of the thesis

| Secondary objectives | Specific aims | Methods | Related chapters |
|---|---|---|---|
| *Identify how **genetic tools** could be integrated into the preventive measures associated with the BWM Convention* | ○ Define what are the **most promising genetic tools for ballast water monitoring** and address their associated advantages and limitations<br>○ Propose which genetic tools could be included in the different guidelines of the BWM Convention requiring **biological data** | ○ Exhaustive literature review of **studies applying genetic tools to ballast water and ports related research**<br>○ Scrutinizing all **articles, regulations and associated guidelines** of the BWM Convention | **I** |
| *Evaluate the potential of **environmental DNA metabarcoding** to become an easy sampling method to screen **ballast water biodiversity*** | ○ **Characterize the ballast water community retrieved with eDNA metabarcoding** and compare its accuracy with conventional ballast water monitoring based on morphological identification<br>○ Assess if eDNA metabarcoding can provide reliable information to evaluate the **efficiency of any type of ballast water management applied** | ○ Sampling of ballast water from **11 vessels entering the Chesapeake Bay (USA)** and comparison with morphological taxonomy<br>○ Comparison of communities of vessels with **treated and exchanged ballast water** | **II** |

| | | | |
|---|---|---|---|
| | ○ Explore the effects of **sampling procedures** on ballast water communities | ○ Comparison of communities retrieved from different **ballast water sampling points** on board of vessel and from an experimental ballast tank of 300m³ where port's water was held for 2 days | |
| *Design a cost-effective and standardized protocol for **Port Biological Baseline Survey based on DNA metabarcoding** and propose **guidelines** to ease its integration into ports monitoring for the BWM Convention* | ○ Evaluate the importance of using different **conventional sampling methods** to maximize the recovery of biodiversity and assess the reliability of **environmental DNA** to characterize port communities<br>○ Define if **sampling seasonality** affects port communities, and if yes, evaluate which seasons allow to recover the maximum biodiversity to provide the most cost-effective protocol<br>○ Define if **sampling locality** in an estuary has an impact on the port biodiversity retrieved and if sites inside the estuary are more prone to the settlement of NIS that sites located outside | ○ Comparison of biodiversity detected with **zooplankton nets, water, sediment grab** and **settlement plates** into the port of Bilbao (Spain)<br>○ Comparison of **Autumn, Winter, Spring** and **Summer** communities of the port of Bilbao over one year<br>○ Comparison of four sites chosen into the port of Bilbao with **three sites located in the outer part of the estuary and one site located inside the estuary** | **III** |

# CHAPTER I

# The challenges and promises of genetic approaches for ballast water management

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**Abstract:**

Ballast water is a main vector of introduction of Harmful Aquatic Organisms and Pathogens, which includes Non-Indigenous Species. Numerous and diversified organisms are transferred daily from a donor to a recipient port. Developed to prevent these introduction events, the International Convention for the Control and Management of Ships' Ballast Water and Sediments will enter into force in 2017. This international convention is asking for the monitoring of Harmful Aquatic Organisms and Pathogens. In this review, we highlight the urgent need to develop cost-effective methods to: (1) perform the biological analyses required by the convention; and (2) assess the effectiveness of two main ballast water management strategies, *i.e.* the ballast water exchange and the use of ballast water treatment systems. We have compiled the biological analyses required by the convention, and performed a comprehensive evaluation of the potential and challenges of the use of genetic tools in this context. Following an overview of the studies applying genetic tools to ballast water related research, we present metabarcoding as a relevant approach for early detection of Harmful Aquatic Organisms and Pathogens in general and for ballast water monitoring and port risk assessment in particular. Nonetheless, before implementation of genetic tools in the context of the Ballast Water Management Convention, benchmarked tests against traditional methods should be performed, and standard, reproducible and easy to apply protocols should be developed.

**Keywords:**

ballast water management convention; non-indigenous species; early detection; genetic methods; metabarcoding

# 1 Introduction

Ballast water discharges are recognized as critical sources of pathogens, harmful algae blooms and Non-Indigenous Species (NIS) introduction (Aguirre-Macedo et al., 2008; Drake and Lodge, 2004; Hallegraeff, 2007; Molnar et al., 2008). To prevent potential environmental, human health and socioeconomic impacts of these introductions, the International Convention for the Control and Management of Ships' Ballast Water and Sediments (referred after as "BWM Convention") was adopted in February 2004 by the International Maritime Organization (IMO, 2004). The BWM Convention will enter into force in September 2017 as the required ratification by at least 30 States representing 35% of world merchant shipping tonnage has finally been reached (IMO, 2016). One of the many challenges of the BWM Convention is ballast water monitoring in commercial ports, *i.e.* screening the whole biodiversity discharged from ballast water to guarantee the prevention and control of Harmful Aquatic Organisms and Pathogens (HAOP) which includes NIS, in recipient ports, which are also monitored for presence of HAOP. Traditionally, ballast water biological inventories, which should preferably be done to the lowest taxonomic level, have relied upon morphological identification, which is costly, time-consuming (Ji et al., 2013) and requires a high level of taxonomy expertise, skill that is becoming rare (Agnarsson and Kuntner, 2007). Besides, one of the most crucial issues associated with this traditional approach is the difficulty to identify early developmental stages (*e.g.* larvae and eggs), broken organisms or morphologically indistinguishable species; all are common in ballast water (Gollasch et al., 2002). Thus, alternative fast, cost-effective, accurate and broadly applicable methods need to be developed in order to improve ballast water monitoring (Lehtiniemi et al., 2015). Genetic methods overcome some of the main limitations of morphological identification and have been described as a challenging revolution in the assessment and management of species diversity (Fonseca et al., 2010; Wood et al., 2013). Specially, numerous reviews highlight the promises of these tools for studying marine biological invasions processes (Rius et al., 2015; Viard et al., 2016), including early detection (Bott, 2015; Comtet et al., 2015) and provide recommendations regarding the associated technical challenges and possible solutions of using High-Throughput Sequencing technologies in such context (Xiong et al., 2016). As a

result, a great number of ballast water related studies already use the advantages of genetic tools to describe the in-tank biodiversity of ballast water (*e.g.* eukaryotes, (Zaiko et al., 2015b) and viruses, (Kim et al., 2015)) and sediments (*e.g.* diapausing eggs of invertebrates, (Briski et al., 2011) as well as to provide helpful biological data for assessing the efficiency of ballast water managements (Briski et al., 2015; Hess-Erga et al., 2010). The rapid development of genetic tools applied to ballast water management have prompted the need for detailed assessments of their relevance and synthetic views of the available tools, as emphasized by Darling and Frederick (Darling and Frederick, 2017). While their work provides a comprehensive analysis of the genetic tools for ballast water monitoring in a general context, here, we give a thorough assessment and analyze the suitability of genetic tools to provide data for the BWM Convention and their feasibility in real conditions. For that aim, we meticulously studied the BWM Convention to detect opportunities where genetic tools could provide biological data while evaluating and discussing their great promise as valuable alternatives or additions by early detecting Harmful Aquatic Organisms and Pathogens introduced via ballast water.

## 2 The preventing actions of the BWM Convention and their required biological analyses

The BWM Convention is based on regulations defining the specific legal preventive actions to manage the introduction of Harmful Aquatic Organisms and Pathogens (HAOP), and on Guidelines providing technical guidance for all stakeholders to help the implementation of these regulations (Table 2).

Table 2. Regulations and associated Guidelines of the BWM Convention; shaded grey indicates Guidelines where genetic tools could provide relevant biological data

| Regulations (R) | Guidelines (G) |
|---|---|
| **Section A: General provisions** | |
| R. A1: Definitions | |
| R. A2: General applicability | |
| R. A3: Exceptions | |
| R. A4: Exemptions | G7: Guidelines for Ballast Water Risk Assessment |
| R. A5: Equivalent Compliance | G3: Guidelines for Ballast Water Management Equivalent Compliance |
| **Section B: Ballast Water Management and control requirements for ships** | |
| R. B1: Ballast Water Management Plan | G4: Guidelines for Ballast Water Management and the Development of Ballast Water Management Plans |
| R. B2: Ballast Water Record Book | |
| R. B3: Ballast Water Management for ships | G5: Guidelines for Ballast Water Reception Facilities |
| R. B4: Ballast Water Exchange | G14: Guidelines on Designation of Areas for Ballast Water Exchange<br>G6: Guidelines for Ballast Water Exchange |
| R. B5: Sediments Management for Ships | G1: Guidelines for Sediment Reception Facilities<br>G12: Guidelines on Design and Construction to Facilitate Sediment Control on Ships |
| R. B6: Duties of officers and crew | |
| **Section C: Special requirements in certain areas** | |
| R. C1: Additional measures<br>R. C2: Warning concerning Ballast Water uptake in certain areas and related flag state measures<br>R. C3: Communication of information | G13: Guidelines for Additional Measures regarding Ballast Water Management including Emergency Situations |
| **Section D: Standard for Ballast Water Management** | |
| R. D1: Ballast Water Exchange standard | G2: Guidelines on Ballast Water Sampling<br>G6: Guidelines for Ballast Water Exchange<br>G11: Guidelines for Ballast Water Exchange Design and Construction Standards |
| R. D2: Ballast Water performance standard | G2: Guidelines on Ballast Water Sampling |
| R. D3: Approval requirements for Ballast Water Management Systems | G8: Guidelines for Approval of Ballast Water Management Systems<br>G9: Procedure for Approval of Ballast Water Management Systems that Make Use of Active Substance |
| R. D4: Prototype of Ballast Water Treatment Technologies | G10: Guidelines for Approval and Oversight of Prototype Ballast Water Treatment Technology Programs |
| R. D5: Review of standards by the organization | |
| **Section E: Survey and certification requirements for Ballast Water Management** | |
| R. E1: Surveys | |
| R. E2: Issuance or Endorsement of a certificate | |
| R. E3: Issuance or Endorsement of a certificate by another party | |
| R. E4: Form of the certificate | |

| R. E5: Duration and validity of the certificate | |
|---|---|
| **Article** | **Guidelines** |
| Article 9: Inspection of ships | G15: Guidelines for Port State Control under the BWM Convention |

To understand the common requirements for ballast water monitoring and facilitate the detection of opportunities where genetic tools could provide relevant biological data for the BWM convention, all preventive actions are grouped in four main categories (Figure 8) and further described below. To prevent new introduction of HAOP, biological analyses (in red in Figure 8) are required and the nature of the analyses and method used will be different depending on the targeted action.

- Actions related to "Testing the compliance with the BWM Convention requirements": Ships will be first inspected for compliance with the BWM Convention by the port state control through an administrative control of the documentations required on board (*i.e.* ballast water record book) (King and Tamburri, 2010; Wright and Welschmeyer, 2015); then, if evidences of non-appropriate management are found, ballast water may be sampled for a first and quick indicative biological analysis, and for a more detailed one if additional support for non-compliance is found. The biological methods to assess the compliance must meet certain criteria to be applicable (David and Gollasch, 2015; IMO, 2015); for example, they shall be designed to take into account organismal minimum size, abundance and viability but also to be fast, applicable onboard, and usable by a non-specialist (David and Gollasch, 2015; Wright and Welschmeyer, 2015). Regarding the type of analysis and organisms, the method can be qualitative, semi-quantitative, and quantitative. For now, the recommended methods for detailed analysis are visual counting including mobility test for zooplankton, counting chamber with epifluorescence microscopy as well as machine counts coupled with viability stains for phytoplankton, and grow of bacterial colonies for indicator microbes (David and Gollasch, 2015).

- Actions related to "Development of Ballast Water Management strategies": A major concern of the BWM Convention is to test and approve the efficacy of the two mains ballast water management actions, which are the interim Ballast Water Exchange (BWE) in open sea, at least from 200 nautical miles from the nearest land and at 200 meter depth, and the installation of on-board Ballast Water Treatment Systems (BWTS) such as filtration combined with chemical (*e.g.* chlorination, use of biocides) or physical treatments (*e.g.* UV radiation, deoxygenation) (David and Gollasch, 2015; Stehouwer et al., 2015; Tsolaki and Diamadopoulos, 2010). When the Ballast Water Exchange is not possible because the distance and depth requirements cannot be met, alternative area to do the exchange needs to be designate and biological assessment of the area should be performed to check the presence of Harmful Aquatic Organisms and Pathogens (HAOP). To test the performance of these measures, Regulations D-1 and D-2 standards (IMO, 2004) were set; the former by exchanging ballast water in a rate enough that guarantees "almost" clean waters, and the later by limiting the amount of viable individuals (zooplankton and phytoplankton) and concentration of indicator microbes that can be discharged (Albert et al., 2013). To assess the compliance with Regulation D-2, Ballast Water Treatment Systems must be rigorously tested before approval with similar biological methods as the one used "in real" for testing the compliance of a vessel. During the land-based and ship-board tests for approval, biological methods are used to detect, enumerate, identify viable organisms, and these methods need to take into account the organisms' rarity in treated water.
- Actions related to "Risk assessment for granting exemptions": Shipping companies will likely seek exemptions from applying the BWM Convention to avoid the extra time and investment required for BWE and BWTS. Regulation A-4 states that granting exemptions for 5 years is possible if the process follows Guidelines G7, which provides advice regarding scientifically robust risk assessments (IMO, 2007). Three risk assessment approaches have been outlined based on comparison of environmental factors such as temperature and salinity or distribution of HAOP, to assess the likelihood of survival of a transferred species between recipient and

donor regions: the environmental matching, the species biogeographical, and the species-specific approaches (IMO, 2007). Biological methods are required for doing such risk assessments and are based on comprehensive port baseline surveys and identification of target species (HELCOM/OSPAR, 2013).

- Actions related to "Additional measures for warning concerns": The BWM Convention encourages monitoring ballast water uptake zones, especially in areas known to contain populations of HAOP such as harmful algae bloom species near sewage outfalls (IMO, 2004). Also, parties may develop a higher level of protection against species introduction if they prove the nature of their concern (*i.e.* the potential consequences of the introduction of harmful organisms in the concerned area) and detail the additional measures required. If identification of species is needed, it must be done following a scientific risk assessment at least to the same level of rigor as in Guidelines G7.

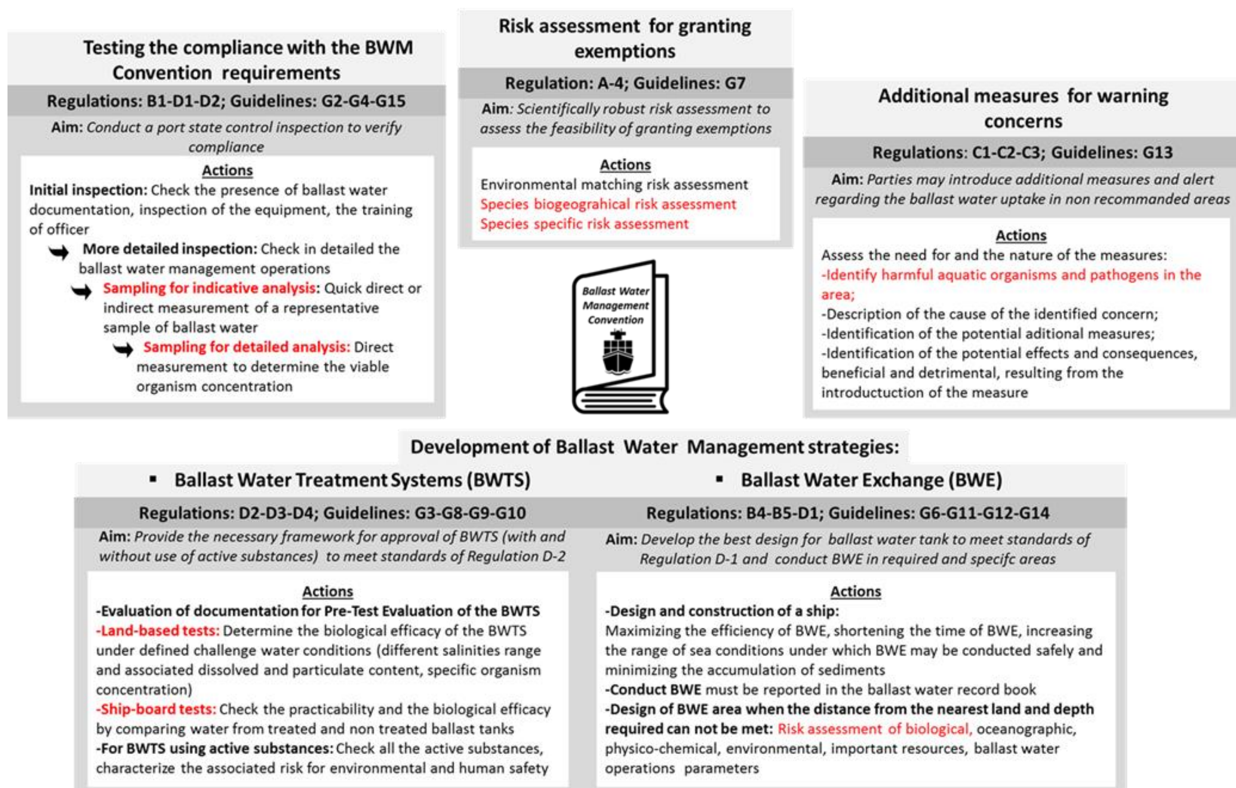


Figure 8. Highlights of the main actions with associated Regulations and Guidelines proposed by the BWM Convention grouped in four categories to prevent Harmful Aquatic Organisms and Pathogens (HAOP) introduction. The actions requiring biological analyses are shown in red.

## 3 Overview of the genetic tools and their application in ballast water management

Genetic tools have been increasingly developed to screen ballast water biodiversity and, tightly related, also for monitoring commercials ports (Table 3). In general, genetic tools provide more accurate and cost-effective taxonomic identification compared to a visual taxonomy approach at all life stages such as eggs, spores, larvae, resting stages, juveniles and broken/incomplete adults; this is particularly relevant for Non-Indigenous Species (NIS) planktonic life-history stages (Darling et al., 2008; Harvey et al., 2009; Mountfort et al., 2012), benthic invertebrates and resting stages from ballast tanks sediments (Briski et al., 2010, 2011). Besides, genetic tools proved to be highly sensitive to early detect organisms in very low abundance (Pochon et al., 2013; Zhan et al., 2013). The workflow of the genetic-based studies includes (1) sampling for target species, (2) extraction of the molecule of interest (DNA or RNA), (3) amplification of a particular region of the genome or transcriptome and analysis of the amplified product(s).

(1) Genetic analyses have been performed on all types of samples retrieved from ballast tanks or ports such as sorted individual specimens (Miralles et al., 2016), mixed specimens (*e.g.* zooplankton Brown et al. (2016), filtered water (Zaiko et al., 2015b) or sediment (Briski et al., 2011). From filtered water or sediment, it is possible to extract environmental DNA (eDNA) which refers to the genetic material released in the surrounding environment in form of feces, saliva, tissue, or simply free DNA (Lodge et al., 2012). Analyzed eDNA isolated from several liters of water seems very promising as it allows early identification of taxa present in low abundances such as recently introduced NIS, which are not detectable by traditional means (Lodge et al., 2012; Valentini et al., 2016).

(2) Genetic tools target mostly DNA as a molecule for species identification because it is stable, long-lived and can be easily preserved (Pereira et al., 2008). Yet, DNA is limited when detecting living organisms is required, so RNA is usually targeted instead (Cenciarini-Borde et al., 2009; Doblin et al., 2007).

(3) Most genetic tools described in Table 2 are based on the Polymerase Chain Reaction (PCR). This technique utilizes primers (short DNA fragments that bind to a specific region

of the genome) to amplify a region of interest. Primers can be species-specific(Patil et al., 2005), target a given taxonomic group (Stehouwer et al., 2013a) or even the whole diversity (Zaiko et al., 2015b). In the conventional PCR, the amplified product is monitored at the end of the reaction, and can be either analyzed by electrophoresis to check presence/absence or to differentiate among closely related species (*e.g.* DGGE; see (Darling and Blum, 2007), or sequenced. In the Quantitative PCR (qPCR), the amplified product is measured in real time during the PCR allowing to quantify the copy number of the targeted gene (Nathan et al., 2014; Wood et al., 2013). Amplicons from single species are generally obtained using non-specific primers and directly sequenced by Sanger in a process named barcoding (Hebert et al., 2003a). Mix of species can be amplified using species-specific primers to look for a given species and sequenced by Sanger or amplified using more generic primers to amplify several species at the same time and sequenced on High-Throughput Sequencing (HTS) platforms (Shokralla et al., 2012) in a process called metabarcoding (Taberlet et al., 2012), which has been described as a powerful tool to detect invasive species (Comtet et al., 2015; Xiong et al., 2016). At the end of the process, comparison of the sequences to a reference database such as BOLD (www.barcodinglife.org) to assign barcodes to species or taxonomic groups is required. The choice of the DNA barcode depends on the research question (*e.g.* species detection level or broad biodiversity assessment) and the taxonomic group of interest. Traditionally, the mitochondrial cytochrome c oxidase subunit I gene (COI) is used for metazoans (Hebert et al., 2003b), the 18S RNA gene for protists (Forster et al., 2016) and the 16S RNA for bacteria (Muyzer et al., 1993). The metabarcoding approach has been widely used in ballast water biodiversity monitoring to screen zooplankton (Ghabooli et al., 2016) and phytoplankton (Steichen and Quigg, 2015). Also, interestingly, the development of HTS is opening opportunities to assess the biodiversity of usually under looked microorganisms such as bacteria, virus (Kim et al., 2015; Ma et al., 2009; Xu et al., 2011) and eukaryotic microorganisms also called protists (Lohan et al., 2015; Pagenkopp Lohan et al., 2017; Steichen et al., 2014). Many protists are toxic and pathogen species and are considered to be very abundant in ballast water, which underlines the importance of detecting them

with a passive surveillance genetic approach such as developed in Lohan and collaborators (2015).

Table 3. Examples of studies applying genetic tools to ballast water management

| Mole-cule | Techniques | Target gene(s) | Target organisms | Objectives | References |
|---|---|---|---|---|---|
| DNA<br><br>RNA | qPCR<br><br>NASBA | groEL and tcpA | *Vibrio cholerae* | To develop molecular methods for rapid monitoring of *V. cholerae* in ballast water according to the detection levels set by the IMO (1CFU/100 ml) | (Fykse et al., 2012) |
| DNA | qPCR | 18S and ctxA | *Vibrio cholerae* and *Potamocorbula amurensis* | To test the efficiency of qPCR for detecting *V. cholerae* and *P.amurensis* in sediments, biofilm, benthic assemblage and seawater | (Mountfort et al., 2012) |
| DNA | qPCR<br><br>Metabarcoding (Cloning) | 18S, 16S, LSU, 23S, myc, hly, himA, lpaH, hsp, ctxA, vvhA | Harmful microalgae, cyanobacteria, Bacteria | To determine the tank variability resulting from ballast age, season, region, vessel characteristics, and exchange status | (Burkholder et al., 2007) |
| DNA<br>RNA | qPCR<br>Species-specific sequencing | 16S and mcyE | Toxic cyanobacteria: *Microcystis sp* and *Anabaena sp* | To investigate harmful microalgae dynamics (viability and toxin production) in ballast water and to provide data for port to-port risk assessment | (Doblin et al., 2007) |
| DNA | Species-specific sequencing | SSU and LSU rDNA | Harmful algae: *Gymnodinium catenatum* | To specifically detect *G. catenatum* in cultures, in heterogeneous ballast water and environmental samples | (Patil et al., 2005) |
| DNA | Species-specific sequencing<br>Metabarcoding (Cloning) | 18S, ITS, 5.8S, 28S | Nonnative bivalves, crustaceans and algae | To assess the presence of targeted species in ballast water and ocean environmental samples | (Harvey et al., 2009) |
| DNA | Metabarcoding (Cloning) | 16S | Cultivable bacteria | To explore for the presence of novel micro-organisms species in ballast water | (Xu et al., 2011) |
| DNA | RFLP | COI | *Carcinus. maenas* and *C. aestuarii* | To detect *C. maenas* and *C. aestuarii* larvae in environmental samples | (Darling et al., 2008) |
| DNA | RFLP<br>Metabarcoding (Cloning) | 16S | Planktonic bacterial community | To compare bacterial community structure of ballast water to the local seawater; and to use this study as background information to risk assessment | (Ma et al., 2009) |
| DNA | DGGE<br>Species-specific sequencing | COI and 18S | Asterias larvae | To specifically detect *A. amurensis* larvae in ballast water samples | (Deagle et al., 2003) |
| DNA | DGGE<br>Barcoding | 16S | Heterotrophic bacterial community | To study survival and succession of heterotrophic bacteria after disinfection by ultraviolet irradiation or ozonation of seawater | (Hess-Erga et al., 2010) |

| | | | | | |
|---|---|---|---|---|---|
| DNA | DGGE Barcoding | 16S | Phytoplankton | To compare different analytical techniques for phytoplankton counting and identification | (Stehouwer et al., 2013a) |
| DNA | DGGE Barcoding | 16S | Microbial community | To study the environmental impact of acetate compounds of Peracleans@Ocean Ballast Water Treatment System on microbial dynamic | (Stehouwer et al., 2013b) |
| DNA | DGGE Barcoding | 18S | Eukaryotic microorganisms and dinoflagellates and diatoms | To examine the eukaryotic microorganism diversity being discharged into a port | (Steichen et al., 2014) |
| DNA | DGGE Barcoding | 18S | Dinoflagellates and diatoms | To test the viability of phytoplankton transiting via ballast water | (Steichen and Quigg, 2015) |
| DNA | Barcoding | COI and 16S | Invertebrate dormant stages | To assess the effect of the saltwater flushing regulations on the density and diversity of invertebrate dormant stages in ballast sediment | (Briski et al., 2010) |
| DNA | Barcoding | COI and 16S | Diapausing eggs | To test the accuracy of DNA barcoding as a tool for species-level identification of diapausing eggs of invertebrates found in ballast sediment | (Briski et al., 2011) |
| DNA | Barcoding | COI and 16S | Adult macro-invertebrates | To explore the presence and species diversity of adult macroinvertebrates transported by transoceanic and coastal vessels | (Briski et al., 2012) |
| DNA | Barcoding | 16S | *Pseudomonas sp* and *Vibrio sp* | To evaluate a rapid and cost-effective method for monitoring bacteria in ballast water | (Emami et al., 2012) |
| DNA | Barcoding | COI and 16S | Zooplankton | To compare the efficiency of ballast water exchange plus ballast water treatment versus ballast water treatment alone | (Briski et al., 2015) |
| DNA | Barcoding | COI and 16S | Mollusks | To investigate exotic mollusks in 3 ports of different intensities of maritime traffic in the Cantabrian Sea | (Pejovic et al., 2016) |
| DNA | Barcoding | COI | Zooplankton | To examine the genetic information of the known NIS *Acartia tonsa* in estuaries including the commercial port of Bilbao and to discover any novel invasive species | (Albaina et al., 2016) |
| DNA | Barcoding | 18 and COI | Invertebrates | To inventory the fouling invertebrate communities from 8 ports in Northern Spain | (Miralles et al., 2016) |
| DNA | Barcoding | COI, 12S and 16S | Zooplankton | To identify coastal NIS present in the non-exchanged ballast water: assessment of the effectiveness of ballast water exchange | (Ware et al., 2016) |
| DNA | Metabarcoding | 16S | Microbial community | To determine the effectiveness of NaOH treatment to reduce | (Fujimoto et al., 2014) |

| | | | | | |
|---|---|---|---|---|---|
| | | | | microbial community structure in ballast water | |
| DNA | Metabarcoding | COI | Mollusk *Peringia ulvae* | To detect mollusk species that could have survived in ballast water | (Ardura et al., 2015a) |
| DNA | Metabarcoding | 16S | Viral community | To investigate the composition and taxonomic diversity of viruses in ballast water | (Kim et al., 2015) |
| DNA | Metabarcoding | V4 and V9 SSU | Protists | To identify microbial eukaryotes present in ballast water | (Lohan et al., 2015) |
| DNA | Metabarcoding | COI | All organisms | To assess the performance of metabarcoding for species detection in ballast water and assess consistency of different sequencing platforms; and possible biases due to DNA decay and/or degradation in ballast water | (Zaiko et al., 2015a) |
| DNA | Metabarcoding | RuBisCo and COI | All organisms | To assess the applicability of metabarcoding for detection of organisms in ballast waters during en-route survey | (Zaiko et al., 2015b) |
| DNA | Metabarcoding | 18S | Zooplankton | To detect NIS with the application of metabarcoding in 16 Canadian ports | (Brown et al., 2016; Chain et al., 2016) |
| DNA | Metabarcoding | 18S | Zooplankton | To assess community changes in zooplankton transiting in ballast water | (Ghabooli et al., 2016) |
| DNA | Metabarcoding | 18S | Eukaryotic from early biofouling communities | To assess the potential of metabarcoding to detect the community structure of biofouling eukaryotic assemblages and the patterns of initial succession in ports | (Zaiko et al., 2016) |
| DNA | Metabarcoding | V4 SSU | Protists | To compare diversity and community composition of protists in ballast water among and between ports | Pagenkopp Lohan et al., 2017 |
| DNA | Metabarcoding | 16S | Bacteria | To analyze diversity and community composition of bacterial assemblages in ballast water of commercial ships following voyages in the North Atlantic Ocean. | (Lymperopoulou and Dobbs, 2017) |
| DNA | Metabarcoding | 16S | Bacteria | To characterize changes in bacterial assemblages of ballast water and assess the effects of ballast water exchange, duration time of the voyage as well as season sampling | (Johansson et al., 2017) |

# 4 Genetic data for helping the implementation of the BWM Convention

The potential of the most promising genetic tools for performing biological analyses required by the Guidelines of the Convention are exposed in Table 4, and are further described in sections 4.1 to 4.2. This analysis comes from crossing the biological analyses required for each Guidelines and the review of the studies presented in Table 3, where a genetic approach has been used to supply biological data relevant for BWM Convention.

Table 4. Main genetic techniques with their related characteristics for ballast and port water monitoring and BWM Convention's Guidelines where they could be integrated (see also Table 2)

| | **Monitoring application (and associated guidelines)** | | | |
|---|---|---|---|---|
| | **For ports and specific areas** | | **For ballast water discharges or in tank** | |
| | **Biological baseline survey (G7 and G14)** | **Target species identification (G7, G13 and G14)** | **Assessing viable community (G2, G8, G10 and G15)** | **Target species identification (G2, G8, G10 and G15)** |
| **Genetic method proposed** | **Metabarcoding** | **qPCR** | **Metabarcoding** | **qPCR** |
| ***Key requirements of the BWM Convention:*** | | | | |
| *Estimation of viability* | Not required | | RNA or DNA coupled with propidium monoazide: need experimental studies (*e.g.* mock communities) | |
| *Estimation of abundance* | Low: relative abundance, still limited | Yes | Low: relative abundance, still limited | Yes |
| *Species level detection* | Moderate to high | High | Moderate to high | High |
| *Low abundance detection* | Moderate to high | High | Moderate to high | High |
| *Portability* | Not required | Not required | Portables machines: PCR, sequencer | Portable qPCR: need further development |
| *Time-Consumption per analysis* | Weeks with traditional sequencing platform but could be reduced to hours with portable devices (*e.g.* MinION) | Hours | Weeks with traditional sequencing platform but could be reduced to hours with portable devices (*e.g.* MinION) | Hours |
| *Cost: operating costs, consumable, personnel* | Low for high number of samples processed | Low | Low for high number of samples processed | Low |
| *Standardization* | High potential: need developed protocol | High: need developed species-specific primers for all targeted species | High potential: need developed protocol | High: need developed species-specific primers for all targeted species |

### 4.1 Compliance inspection: Guidelines G2, G8, G10 and G15

Testing the compliance with the discharge standards of Regulation D-2 does not require species identification (except for indicator microbes) and only relies on the number of viable organisms. Nonetheless, the Article 6 of the BWM Convention asks to each Party to promote relevant information on the effectiveness of ballast water management obtained from any monitoring program (IMO, 2004). In this context, identification of organisms discharged is beneficial to assess the efficiency of the ballast water management measures and thus reduce the events of introduction of Harmful Aquatic Organisms and Pathogens, at the same time, early detect introduction of a potential NIS.

#### 4.1.1 Determine viability of organisms

Viability is the key criteria to assess if a vessel will comply with Regulation D-2 and the universal method to detect only viable organisms is yet to be developed. Nonetheless, genetic tools hold great potential if their targets are linked with viability such as using RNA or if the technique used prevent the amplification of DNA from dead cells such as with the application of propidium monoazide (PMA) (Fujimoto et al., 2014). Doblin et al (2007) shows the potential of RNA analyses to reveal that harmful algae species could synthesis active toxin after 11 days in a ballast water tank. To assess the living community discharged with ballast water targeting RNA instead of DNA for metabarcoding has been proposed as an appealing solution (Visco et al., 2015; Zaiko et al., 2015a, 2015b) since studies already successfully developed protocols based on RNA metabarcoding to screen living protists (Pawlowski et al., 2014; Visco et al., 2015). Yet, RNA is very difficult to preserve, and thus working with RNA is more challenging when compared to DNA. Nonetheless, several studies have proposed optimized protocols to extract high quality RNA (Asai et al., 2015; Zhang et al., 2013). Concerning the use of propidium monoazide, it seems well defined for microbes as Fujimito et al (2014) used it to show alkali ballast water treatment efficacy to reduce alive microbial diversity. Nonetheless, its potential remains unclear for multicellular organisms such as for zooplankton tested in compliance (Lance and Carr, 2012; Zetsche and Meysman, 2012) and further research is needed.

#### 4.1.2 Test the efficacy of ballast water management

Genetic methods have been successfully used to provide biological data on the effects of ballast water management by identifying the community found in the tanks or at the discharge line after the Ballast Water Exchange (Briski et al., 2010, 2015; Ware et al., 2016), by assessing the efficacy of Ballast Water Treatment Systems to reduce bacteria and phytoplankton diversity (Fujimoto et al., 2014; Stehouwer et al., 2013b, 2013b) and by identifying the composition of "recolonizer" organisms after the application of a treatment (Hess-Erga et al., 2010). A future interesting direction will be to develop RNA metabarcoding (see 4.1.1) to provide a better depiction of "the living organisms" after the application of the Ballast Water Treatment System.

#### 4.1.3 Assess the representativeness of sampling to inspect compliance

Sampling for compliance testing requires that samples must be representative of the whole ballast water discharged (IMO, 2008). The large volumes of water, tank characteristics (shape, size and number) and heterogeneous distribution along with low density of organisms within tanks make very challenging to develop the best representative approach for compliance sampling and research is still going on (Basurko and Mesbahi, 2011; Carney et al., 2013; Hernandez et al., 2017). To our knowledge, genetic tools have never been applied to such use yet. Metabarcoding could be very useful to assess if there is a significant difference in the communities inferred using alternative sampling approaches. Indeed, the volume, number and frequency of samples, and the availability to sample from the discharged lines or directly from the tank are factors that need to be tested and optimized to get an accurate representation of the discharged community.

### 4.2 Target species detection and biological monitoring: Guidelines G7, G13 and G14

#### 4.2.1 Risk assessment for granting exemptions

HELCOM/OSPAR Guidelines have been developed in line with the Guidelines G7 to provide advice on risk assessment procedures for granting exemptions from the port survey sampling to the decision support and administrative decisions (HELCOM/OSPAR, 2013). The biological methods proposed are based on considerable sampling effort of all

types of organisms to detect Non-Indigenous Species (NIS) and native harmful species and on checking the presence of potential pathogens. The analysis of NIS is done with a visual taxonomic identification approach. The port of Rotterdam, for example, has conducted such survey and 32 NIS were detected over 225 species identified to the species level recorded in more than 250 samples taken from 118 different locations (Gittenberger et al., 2014). The species accumulation curves indicated that more sampling would have resulted in scoring more species for several habitats of the port. So, despite such important sampling effort, the entire diversity of the habitats was not recovered. Genetic methods, by enhancing detection sensitivity, increasing specificity of target identification and reducing monitoring time and costs, hold great promise over traditional methods (Darling and Mahon, 2011) and has been proposed as a cost-effective method in this context (Comtet et al., 2015; Lehtiniemi et al., 2015).. Genetic-based background information to assess the risk associated with Harmful Aquatic Organisms and Pathogens in ports are numerous. Examples are (Ma et al., 2009) for pathogens, (Miralles et al., 2016; Pejovic et al., 2016) for invasive mollusks, (Doblin et al., 2007) for harmful algae species, (Brown et al., 2016) for zooplankton, (Hirst and Bott, 2016) for fouling organisms and zooplankton, (Pochon et al., 2015; Zaiko et al., 2016) for early fouling organisms. One very interesting study in Canadian ports shows that metabarcoding detected 24 NIS species of zooplankton of which 11 were firstly reported in this regions (Brown et al., 2016). Also, the combination of sampling artificial settlement plates with DNA metabarcoding based species detection seems very promising to early detect potential invasive species in ports where species are at very low densities and morphologically undistinguishable early life stages occur (Pochon et al., 2015).

#### 4.2.2 Specific ballast water uptake zone

As described in section 2. The preventing actions of the BWM Convention and their required biological analyses in Actions related to “Development of Ballast Water Management Strategies” and “Additional measures for warning concerns”, biological monitoring is required when there is a need to design alternative ballast water exchange areas and when a Party shall warn about the uptake of ballast water in certain zones. In both cases, metabarcoding appear as sensitive methods to perform such biological

monitoring as they allow to detect sewage contamination environment by identifying sewage-related bio-indicator microbial species (Tan et al., 2015). Also, qPCR has been demonstrated to play a major role in identifying harmful algae bloom species, understanding their ecology as well as facilitating the management of their outbreaks (Antonella and Luca, 2013).

# 5 Future investments for the use of genetic tools in ballast water management

## 5.1 Standardization and further development of genetic methods

Implementation of genetic methods for regular application in ballast water monitoring requires benchmarking against traditional methods and standardization of procedures (Aylagas et al., 2016a; Aylagas and Rodríguez-Ezpeleta, 2016). Each step, from sampling to taxonomic assignment, can be performed using alternative protocols and the choice of the most appropriate will depend on i) type of sample, *e.g.* water, sediment, fouling, ii) taxonomic group tackled, *e.g.* bacteria, protists, metazoa, iii) pursued aim, *e.g.* to obtain a comprehensive species inventory or look for a specific species, iv) and other specific conditions such as the need to detect only living organisms. It is thus crucial that reproducible protocols are defined, tested and broadly communicated so that results obtained can be comparable and usable for regular monitoring purposes (Cristescu, 2014). Also, to validate protocols, one key element is the use of artificial communities to i) develop rigorous laboratory workflows (Port et al., 2016) and standard bioinformatic pipelines (Brown et al., 2015; Flynn et al., 2015) and to ii) assess the sensitivity of genetic tools such as metabarcoding to detect invasive species in complex environmental samples (Pochon et al., 2013). Also, one important technical shortcoming of DNA metabarcoding is the PCR bias (amplification success is not equal for all taxa present in a sample) which can lead to false negatives and impede estimating taxa abundances based on sequences (Elbrecht and Leese, 2015). To overcome PCR bias, community genome sequencing (metagenomic) could be applied (Taberlet et al., 2012). Although this approach shows promising results regarding their use to species identification and estimate relative abundance (Gómez-Rodríguez et al., 2015; Srivathsan et al., 2015; Tang et al., 2014, 2015),

it is much more expensive and requires more complicated data analyses that rely on the availability of large numbers of complete genomes in databases, which is currently only existing for bacteria.

### 5.2 Improvement of reference databases

Both DNA barcoding and metabarcoding rely on the availability of a well populated and curated reference database that associates DNA sequences with species names (*e.g.* BOLD www.barcodinglife.org, Silva www.arb-silva.de). Unfortunately, such databases are far from being complete (Aylagas et al., 2014; Comtet et al., 2015) and are usually biased towards certain organismal groups (Briski et al., 2016). Recently, significant effort has been devoted to increase reference databases, and the number of sequences, especially from eukaryotes, has notably increased during the past few years (Briski et al., 2016). Additionally, the AquaNIS database (www.corpi.ku.lt/databases/aquanis) which constitutes the reference information system on aquatic non-indigenous and cryptogenic species, is also gathering molecular information among all the information recorded for a species and has undertaken a promising step forward action to integrate molecular data into the management of NIS. Also, the metabarcoding research is moving forward to the use of a multiple marker approach (*e.g.* COI and 18S) instead of a single marker which help providing a better catalogue of biodiversity (Cowart et al., 2015; Marcelino and Verbruggen, 2016; Zaiko et al., 2015b). This approach is thus, strongly encouraged for future studies assessing the ballast water discharged community.

### 5.3 Increasing practicability of genetic tools in the field

The BWM Convention requires fast, portable and user-friendly sampling and analysis protocols which appears difficult to achieve with genetic tools because most require specialized equipment and training. Nonetheless, several alternatives have been designed and could be considered for detecting species in ballast water samples such as combining "minutes"-DNA/RNA extraction techniques with portable DNA/RNA amplification devices such as portable PCR or Loop-mediated isothermal amplification (LAMP) machine (Agrawal et al., 2007; Lee, 2017). LAMP is commonly used for food safety testing

for pathogens and fungi as it has a very short reaction time, only requires a heating block to perform the amplification and holds high sensitivity for species detection (Niessen et al., 2013). Also, in the context of passive surveillance (*i.e.* screening the biodiversity) of ballast water, the development of the Oxford Nanopore Technologies® MinION device, a miniaturized and portable real-time high-throughput sequencer, shows great promises to reduce the time of samples processing and facilitate *in situ* monitoring for environmental research (Edwards et al., 2016; Mitsuhashi et al., 2017; Ramgren et al., 2015). Mitsuhashi et al (2017) shows the possibility of rapid sequencing and bacterial composition identification within 2 hours. Nonetheless, the in-field preparation of samples (homogenization, DNA/RNA extraction and library preparation) as well as the bioinformatic analyses still needs optimized protocols and further adjustments to be used routinely in ballast water monitoring.

### 5.4 Exploring alternative sampling strategies

eDNA appears very promising source from which to infer biodiversity that reduces sampling effort especially in a context of port baseline survey or targeted species identification where exhaustive sampling is usually required. The field of sampling eDNA for detecting invasive species is of growing interest as numerous studies show its great potential (Ardura et al., 2015b; Jerde et al., 2011; Simmons et al., 2015), and eDNA should be a focal point of research efforts to apply its use in ports monitoring in a near future. Yet, eDNA degradation appears to be influenced by environmental conditions and the approximate time range can go from less than one day to a couple of weeks (Barnes et al., 2014; Thomsen et al., 2012) challenging it use as a proxy for the living organisms present in an environment such as a ballast water tank. Future work is needed to disentangle the complex relationship between eDNA persistence in ballast tank and the actual presence of organisms, especially in a context of ballast water sampling for compliance. But, interestingly, monitoring eDNA in ballast water can detect species that survive the harsh conditions of a ballast water tank by showing an increase in proportion of DNA signal during the journey (Ardura et al., 2015a; Zaiko et al., 2015b). These studies show the potential of eDNA sample to monitor ballast water biodiversity to potentially detect trend

in species that could need a special attention to be monitored in case of discharge of ballast water into a port.

### 5.5 Efforts to integrate of genetic methods into monitoring programs

Despite the potential of genetic tools for ballast water monitoring and management, the application of these tools by stakeholders remains still experimental, as it has associated challenges (Bucklin et al., 2016; Darling, 2015; Darling and Mahon, 2011; Kelly et al., 2014; Wood et al., 2013). Only very few countries such as New Zealand or Australia started to integrate genetic tools to early detect invasive species and harmful algal blooms species (Hirst and Bott, 2016; Wood et al., 2013). One major issue is the difficult communication between researchers and stakeholders, which makes the former unaware of the real problems and the latter unknowledgeable of the potential of genetic tools (Darling, 2015). To provide an effective integration of genetic methods into monitoring programs, geneticists, environmental monitoring agencies and ballast water policy stakeholders (*e.g.* Port State Control) should together define standardized processes so that genetic methods can provide reliable data for decision making (*e.g.* interdisciplinary theoretical and hands-on workshops are encouraged).

# 6 Conclusions and future directions

The analyze of the research studies using genetic tools for ballast water monitoring exposed the great potentials of such tools to be applied from the risk assessment to the early detection and monitoring of Non-Indigenous Species and so, provide valuable information at all stages of the invasion process (Figure 9).

Given their potential in terms of accuracy and cost-effectiveness along with their fast-growing evolution, genetic tools, with an emphasize on metabarcoding, should definitely continue to be developed for ballast water monitoring. DNA metabarcoding and qPCR standardized protocols need to be created for performing respectively, port baseline surveys and target species detection required in the BWM Convention in Guidelines G7, G13 and G14. Nonetheless, genetic tools are facing technical challenges associated to the techniques itself (*e.g.* lack of completed reference database, standardized protocols) or specific to ballast water monitoring. Indeed today, genetic tools do not fulfill all the key criteria of the BWM Convention such as portability and rapidity of results or viability assessment. Yet, extensive experimental research is needed to assess the potential of RNA to detect the viable community discharged via ballast water (*e.g.* use of artificial communities), as well as testing the feasibility for on-board genetic analyses. Also, the evaluation of the number and minimum dimension of organisms discharged via ballast water remains very limited with genetic tools alone and needs to be combined with other appropriate tools. But we are confident that the worldwide burning development of DNA/RNA-based environmental monitoring is leading to better refinement of these tools for qualitative assessment of biodiversity discharged via ballast water.

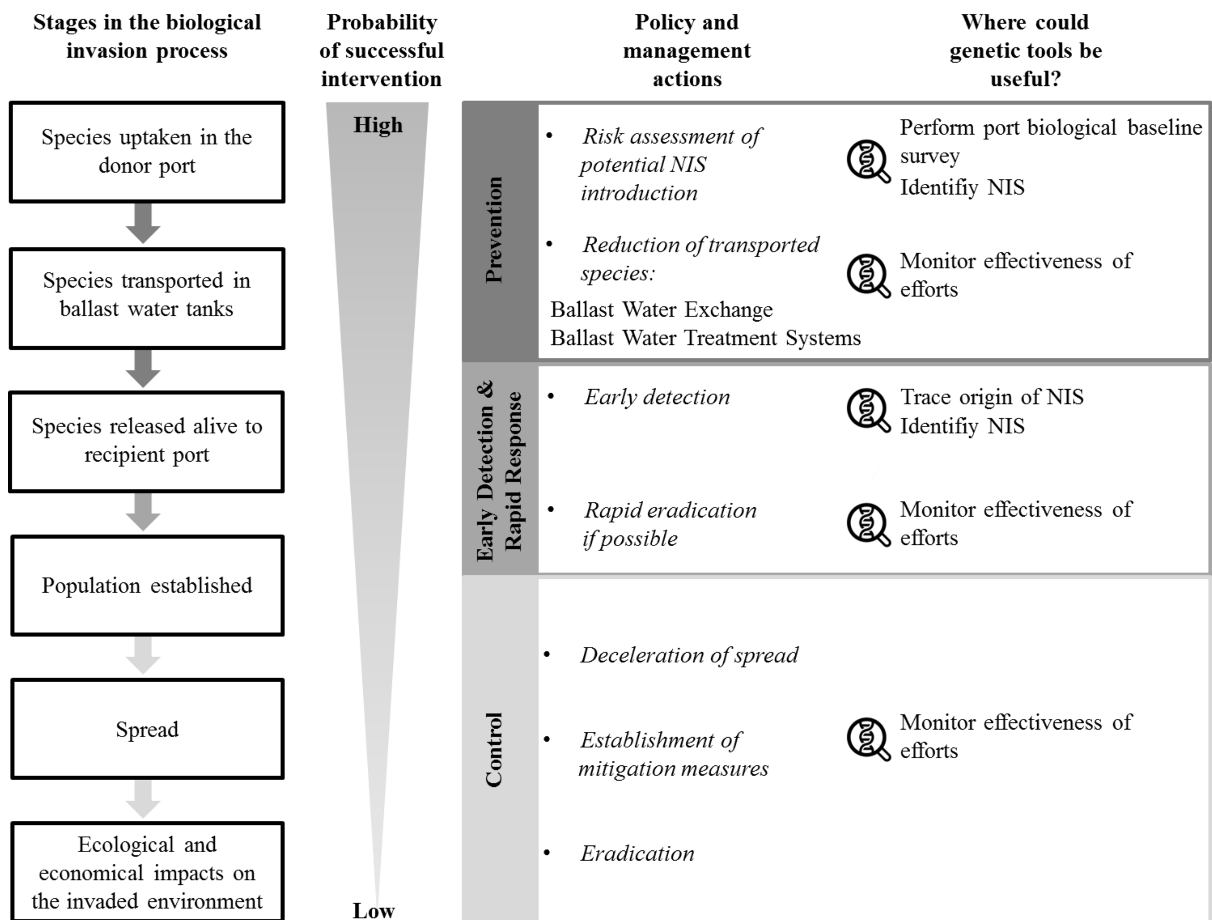


Figure 9. Stages of the ballast water induced invasion process with the corresponding management actions (adapted from Lodge et al., 2006) and usefulness of potential genetic tools

## 7 Acknowledgments

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# CHAPTER II

# Environmental DNA metabarcoding: a promising tool for ballast water monitoring

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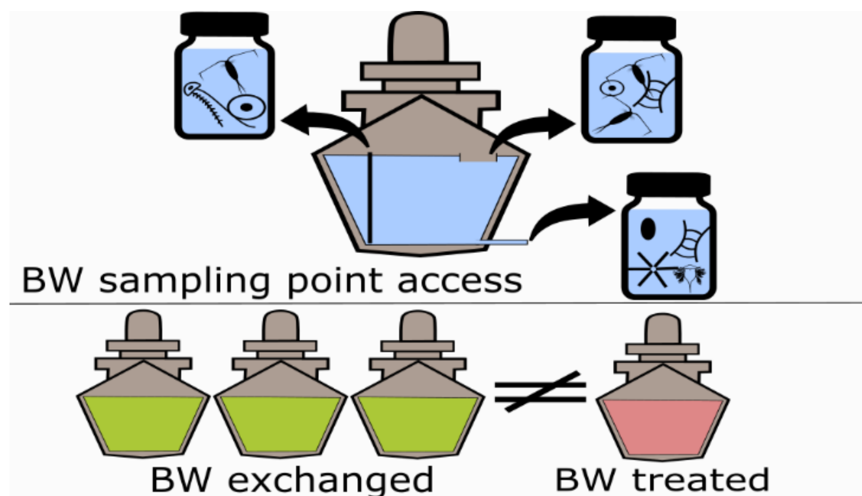
**Abstract:**

Non-indigenous species are introduced worldwide with ballast water (BW). To prevent further introductions, oceanic BW exchange and BW treatment systems are utilised, but their efficiency needs to be monitored. To that aim, characterizing BW communities is essential but usually relies on exhaustive sampling and morphological taxonomic identification, which does not always allow a fine taxonomic resolution. The use of environmental DNA (eDNA) metabarcoding holds great promise to circumvent the drawbacks of BW conventional sampling. We evaluated its potential by 1) assessing whether impact of BW management type could be identified, 2) analyzing the influence of BW sampling points on communities, 3) comparing eDNA efficiency in taxa identification with conventional monitoring and 4) identifying non-indigenous taxa. BW were sampled from 11 vessels arriving to the Chesapeake Bay (USA). BW sampling points impacted the communities, advocating that sampling should be performed from the same point to ensure comparability. BW treated communities were distinct from BW exchange communities and signals of source port and of ocean exchange were observed. More diversity was identified with eDNA than with morphological taxonomy and the non-

indigenous copepod *Oithona davisae*, not reported before in the Chesapeake Bay was detected. Overall, this study highlights for the first time the potential of eDNA metabarcoding for BW monitoring, but more comprehensive sampling will be needed to optimize the approach.

**Graphical abstract:**




**Keywords:**

environmental DNA; metabarcoding; ballast water; ballast water management; ballast water exchange; ballast water treatment system; ballast water sampling point; non-indigenous species

# 1 Introduction

Merchant shipping is estimated to transport about 3500 million tons of ballast water (BW) per year (Endresen et al., 2004), providing an opportunity for thousands of species to be transported daily between bio-geographic regions as BW associated biota (Carlton, 1999). Consequently, BW has been described as one of the main vectors of Non-Indigenous Species (NIS) dispersal worldwide (Davidson et al., 2018; Ruiz et al., 2015; Seebens et al., 2013). Developed for preventing further introductions, the currently in force International Convention for the Control and Management of Ships' Ballast Water and Sediments (IMO, 2004) recommends two main options for BW management: 1) BW exchange, *i.e.* replacing coastal/brackish/fresh BW with oceanic BW or 2) BW treatment, *i.e.* killing organisms collected during BW uptake with a designated, approved treatment technology (IMO, 2004).

Determining the diversity and condition of the biota discharged after BW exchange or treatment is essential to assess their efficiency in reducing transfer of NIS and, therefore, BW monitoring is an ongoing and active line of research (Briski et al., 2015; Carney et al., 2017; Darling et al., 2018; Simard et al., 2011; Ware et al., 2014). In traditional surveys, BW taxa are identified using morphological taxonomy (DiBacco et al., 2012; Gollasch et al., 2000; Simard et al., 2011). However, this technique has limitations as the organisms found in BW cover a wide range of taxa, can be degraded and damaged, and are often present at early development stages (Ware et al., 2014). These limitations frequently translate into only acquiring a coarse level taxonomic classification, such as phyla or class (Carney et al., 2017). Additionally, BW monitoring requires the use of sampling gear, such as plankton net (Carney et al., 2017; Chu et al., 1997; Darling et al., 2018), which necessitates time to set up and to collect samples. Adequate time for these processes can be limited on board commercial vessels as they attempt to spend minimal time in port and generally begin discharging BW immediately upon docking.

There has been a growing interest in applying metabarcoding, the simultaneous identification of taxa present in a complex environmental sample (Taberlet et al., 2012), for BW monitoring to circumvent the limitations of morphological taxonomy and better

characterize communities (Brinkmeyer, 2016; Darling et al., 2018; Gerhard and Gunsch, 2019; Johansson et al., 2017; Kim et al., 2015; Lymperopoulou and Dobbs, 2017; Ng et al., 2015; Pagenkopp Lohan et al., 2017). Nevertheless, most of these studies have relied on sampling large volumes of BW (*e.g.* 100L) to recover planktonic organisms (Zaiko et al., 2015b), which is unrealistic for routine BW monitoring, or have focused only on microorganisms (Brinkmeyer, 2016; Gerhard and Gunsch, 2019; Kim et al., 2015; Lymperopoulou and Dobbs, 2017; Pagenkopp Lohan et al., 2016, 2017). With the recently introduced environmental DNA (eDNA) metabarcoding method (Deiner et al., 2017), it is now possible to recover signals of multicellular organisms from filtered water through the recovery of traces of DNA released in the environment in the form of cells, feces, skin, saliva and mucus, etc. (Shaw et al., 2017). Yet, although eDNA is burgeoning as a cost-effective method to uncover biodiversity, to our knowledge, no study has fully explored its potential for BW monitoring.

Hence, the aim of this study is to assess the potential of eDNA metabarcoding as an easy tool for BW monitoring. For this purpose, we have characterized the eukaryote communities found in only three liters of BW collected from eleven vessels entering Baltimore and Norfolk ports (USA). First, we evaluated whether different BW sampling points influenced the communities retrieved from eDNA by sampling ballast tanks of commercial vessels and using an experimental tank. Secondly, we tested whether the effect of common BW characteristics (*i.e.* BW age, type of BW management, location of BW source) on BW communities could be identified with eDNA metabarcoding. Thirdly, we assessed the capacity of eDNA to identify zooplankton taxa through comparisons with plankton samples identified by morphological taxonomy. Fourthly, we identified taxa with high propagule pressure that were discharged into the two studied ports to evaluate the potential for introduction of NIS.

# 2 Materials and methods

## 2.1 Sampling collection and processing

Ballast water (BW) samples (n=39) were collected from 11 bulk cargo vessels arriving to Chesapeake Bay between mid-October and early December 2017 (Figure 10). Vessels were sampled at two coal terminals in the Port of Baltimore (CNX Marine Terminal and Curtis Bay) and three terminals in the Port of Hampton Roads Norfolk (Kinder Morgan, Norfolk Southern, DTA). For each BW sample, the history of the ballast tank sampled was recorded, including the source location and date of uptake, the type and date of management measure (BW exchange or BW treatment) applied, and the location of the BW exchange (Table S1). Triplicates of 1 L BW samples were collected from each vessel using methods adjusted for the different BW sampling points. BW samples were collected from one of three different sampling points: i) the manhole - obtained by lowering a bleach-cleaned plastic bailer one meter below the surface, ii) the sounding pipe - water was pumped to the deck using a bleach-cleaned hose connected to a hand pump, and iii) the discharge line – accessed via the vessel “testing valve” (Figure S1). Each sample was poured into a bleach-cleaned 1 L bottle. Immediately after collection (30-45min), samples were filtered through 0.45 μm mesh size nucleopore Whatman filters (VWR International, Atlanta, GA, USA) placed with single-use sterile forceps on a single-use sterile filter apparatus (Fisher Scientific, Inc., Pittsburgh, PA, USA) using a vacuum pump. Filtration negative controls (1 L of distilled water) were included during the filtration process. Filters were stored at −80°C until DNA extraction.

For three vessels (V3, V5, V7), zooplankton samples were collected for morphological taxonomic identification. A plankton net (30 cm-diameter conical net, 35 μm mesh size) was used to collect approximately 2 $m^3$ per tank (± 0.2 $m^3$) by completing multiple vertical tows from the bottom of the tank. Each sample was preserved in formaldehyde. All organisms were morphologically identified to the lowest taxonomic level possible.

Baltimore port water held in a 300 m$^3$ tank for two days in a BW treatment system test facility was sampled through the tank (in-tank sampling) and through the discharge line (in-line sampling; Figure S2). In-tank sampling was conducted via the manhole using bleached bailers to sample 4 x 1 L samples one meter below the surface, 1 L sample at mid-depth, and 1 L sample at the bottom. In-line sequential sampling was conducted by collecting 1 L samples after discharge of 90, 150 and 210 m$^3$ to have the most accurate representation of the discharge event (Bailey and Rajakaruna, 2017). In-line integrated sampling was conducted by collecting 3 x 1 L samples from a cistern that collected 90 L of water (aerated to prevent settling and ensure homogenization of the sample) throughout the discharge sequence.

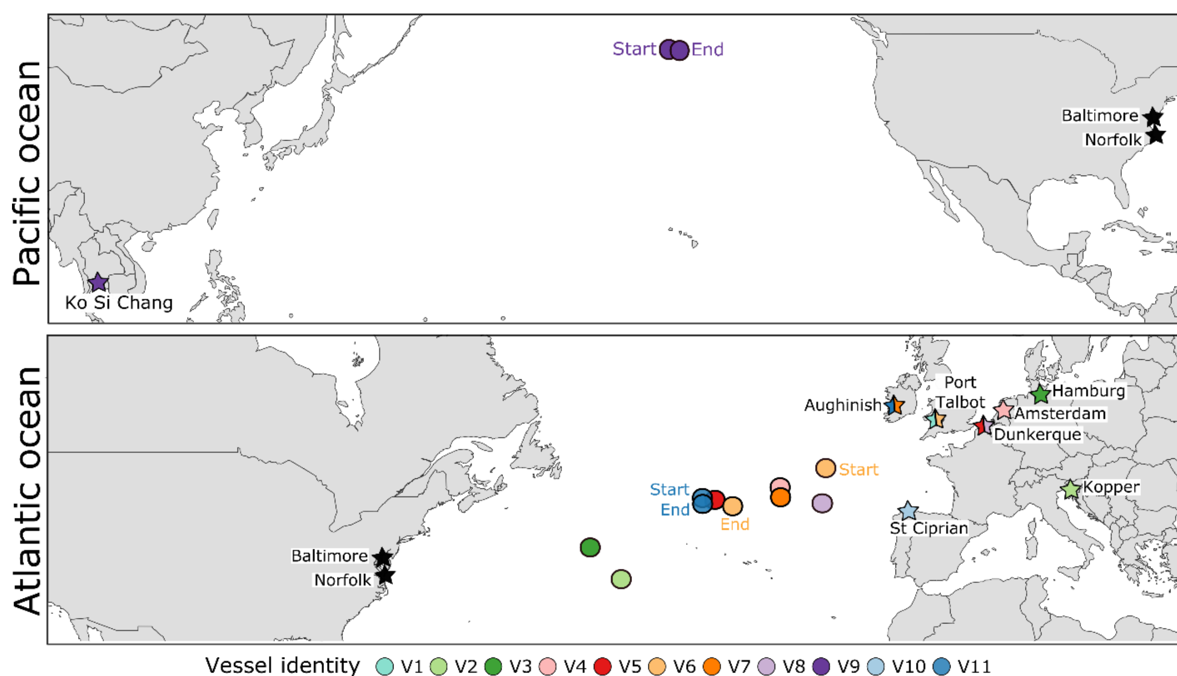



Figure 10. Location of BW uptake and exchange. For each vessel (represented by one color), port source (colored stars) and BW exchange location (circles) when performed are indicated. Only vessels V1 and V10 did not perform BW exchange. Black stars represent the two destination ports. Start and end are the locations of the beginning and end of the BW exchange performed with empty-refill method (with the flow-through BW exchange method, the exchange is done in one location only).

## 2.2 DNA extraction, library preparation and sequencing

Genomic DNA was extracted from each filter with the DNeasy blood & tissue kit (QIAGEN) following the protocol for filtered samples without preservation buffer (Spens et al., 2017). For each set of extractions, a negative control (*i.e.* empty tube) was added and processed as the other samples. DNA concentration was measured with the Quant-iT dsDNA HS assay kit using a Qubit 2.0 Fluorometer (Life Technologies), purity was inferred from 260/280 and 260/230 absorbance ratios with the ND-1000 Nanodrop (Thermo Scientific), and integrity was assessed by electrophoresis on a 0.7% (w/v) agarose gel. The following primer pairs were used: mlCOIintF/dgHCO2198 (COI primers) targeting a 313 bp fragment of the cytochrome oxidase I (COI) gene (Leray et al., 2013) and the Uni18S/Uni18SR (18S primers) targeting a fragment (400 to 600 bp) of the hypervariable V4 region of the 18S rRNA gene (Zhan et al., 2013, 2014). For both primer sets, PCR amplifications were performed in two rounds. For the first PCR, 1 µl of genomic DNA (5 ng/µl) was added to a mix consisting in 5 µl of 1X Phusion High-Fidelity PCR Master Mix (Thermo Scientific), 0.2 µl of each primer (0.2 µM) and 3.6 µl of MilliQ water. For the COI primers, PCR conditions consisted on an initial 3 min denaturation step at 98 °C; followed by 35 cycles of 10 s at 98 °C, 30 s at 46 °C and 45 s at 72 °C and finally 5 min at 72 °C. For the 18S primers, PCR conditions consisted of an initial 3 min denaturation step at 98 °C; followed by 25 cycles of 10 s at 98 °C, 30 s at 50 °C and 45 s at 72 °C and finally 10 min at 72 °C. Negative controls were included within each set of PCRs. Once purified using AMPure XP beads (Beckman Coulter), the PCR products were used as a template for the generation of the dual-indexed amplicons in a second PCR round following the "16S Metagenomic Sequence Library Preparation" protocol (Illumina) using the Nextera XT Index Kit (Illumina). Multiplexed PCR products were purified a second time using the AMPure XP beads, quantified using Quant-iT dsDNA HS assay kit and a Qubit 2.0 Fluorometer, then normalized and pooled at equal concentrations and sequenced using the 2 x 300 paired-end MiSeq (Illumina). Reads were demultiplexed based on their barcode sequences.

### 2.3 Raw read preprocessing, clustering and taxonomic assignment

Forward and reverse primers were removed using Cutadapt (Martin, 2011) using the anchored 5′ adapter and for paired end reads options for both barcodes. For the COI sequences, forward and reverse reads were merged using PEAR (Zhang et al., 2014) with a minimum overlap of 217 bp (Aylagas and Rodríguez-Ezpeleta, 2016). For the 18S sequences, forward and reverse reads were merged with a minimum overlap of 49 bp and the forward and reverse non-merged reads were trimmed respectively at 230 and 201 bp (below this threshold, bases quality score was decreasing) and pasted introducing a N in between (Jeraldo et al., 2014). Merged reads with average Phred quality score above 20 were retained using Trimmomatic (Bolger et al., 2014). Sequences with no ambiguous bases were aligned to BOLD (https://www.boldsystems.org) or SILVA (https://www.arb-silva.de/documentation/release-132/) for COI and 18S barcodes respectively using Mothur (Schloss et al., 2009) and only sequences aligning to the barcode region were kept. Chimeras were detected with the *de novo* mode of UCHIME (Edgar et al., 2011) and removed. The remaining reads were clustered into OTUs using Swarm with defaut settings (Mahé et al., 2014) and singleton OTUs were removed. To account for contamination, we subtracted the number of reads of each OTU present in the negative control samples from each sample where these OTUs were present and the resulting number became the total number of sequences of this OTU in each biological sample, following Nguyen et al. (2015) and used in environmental DNA studies (Grey et al., 2018; Port et al., 2016). The remaining OTUs were taxonomically assigned following the Wang method (Wang et al., 2007) using BOLD (accessed in May 2018) and PR2 (release 4.10.0) for COI and 18S barcodes respectively. Samples with less than 100 reads (n=1) after quality filtering were removed from subsequent analyses. Taxonomic assignment was further tested with maximum Likelihood phylogenies. Sequences belonging to the same order to the assigned species were aligned with MAFFT (Katoh and Standley, 2013) and used for building trees using the unpartitioned GTRCAT model as implemented in RAxML (Stamatakis, 2014) assessing branch support by a 100 replicate bootstrap analysis.

### 2.4 Community analyses

All community analyses were performed on Hellinger-transformed OTU abundances (square root of relative abundance; (Legendre and Gallagher, 2001)). Statistical analyses were conducted using RStudio (Team, 2015) with phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen et al., 2013) and dendextend (Galili, 2015) libraries. Non-metric dimensional scaling (nMDS) was conducted using Euclidean distances among all BW sample pairs. For samples taken from the manhole, redundancy analyses (RDA) were performed to test the relationship between community variation and BW characteristics (*i.e.* geographic coordinates of BW uptake source port and of BW exchange, BW age after exchange and BW temperature). Significance was tested using 999 permutations. Variance partitioning (Borcard et al., 1992) was conducted to assess whether the different sets of BW characteristics explained similar fractions of community variation. Linear regression was performed to test and quantify the relationship between BW age after exchange and Shannon index. The impact of sampling points (in-tank *versus* in-line sampling) on community from the experimental tank was firstly identified with hierarchical clustering on Euclidean distances using the Ward clustering algorithm (R function 'hclust' with option Ward D.2), and then tested for significance using a permutational multivariate analysis of variance (PERMANOVA) after checking for multivariate homogeneity of group dispersions (betadisper).

## 3 Results

### 3.1 Overview of ballast water communities

None of the samples collected from vessel V1 could be amplified with either barcode and two samples from V10 and two samples from V8 were not amplified or resulted in less than 100 reads with 18S. The rest of the samples were successfully amplified for both barcodes (Table S2). Overall, after quality filtering, a total of 1,491,281 and 2,462,059 reads with an average of 41,424 and 76,939 reads per sample were retained respectively for COI and 18S (Table S2). For 18S, a large fraction of the reads (93 %) was assigned to phylum, whereas for COI, only 9 % were assigned to phylum. This difference

was probably due to a large presence of non-metazoan taxa (Figure S3), which are underrepresented in the BOLD database. The communities obtained with COI and 18S are different (Figure 11). Oomycota, Arthropoda and Cnidaria dominated the communities in number of reads with COI whereas Dinoflagellata, Ciliophora and Arthropoda were the dominant phyla with 18S. Among these abundant phyla, Arthropoda, Cnidaria and Ciliophora were always identified in the vessels where morphological taxonomy on zooplankton was performed (Figure S4). More zooplankton phyla were retrieved with eDNA than by morphological identification and, except for one vessel, all phyla identified with morphology were also found with eDNA. Larvae, copepod nauplii and eggs were common in samples identified with morphology.

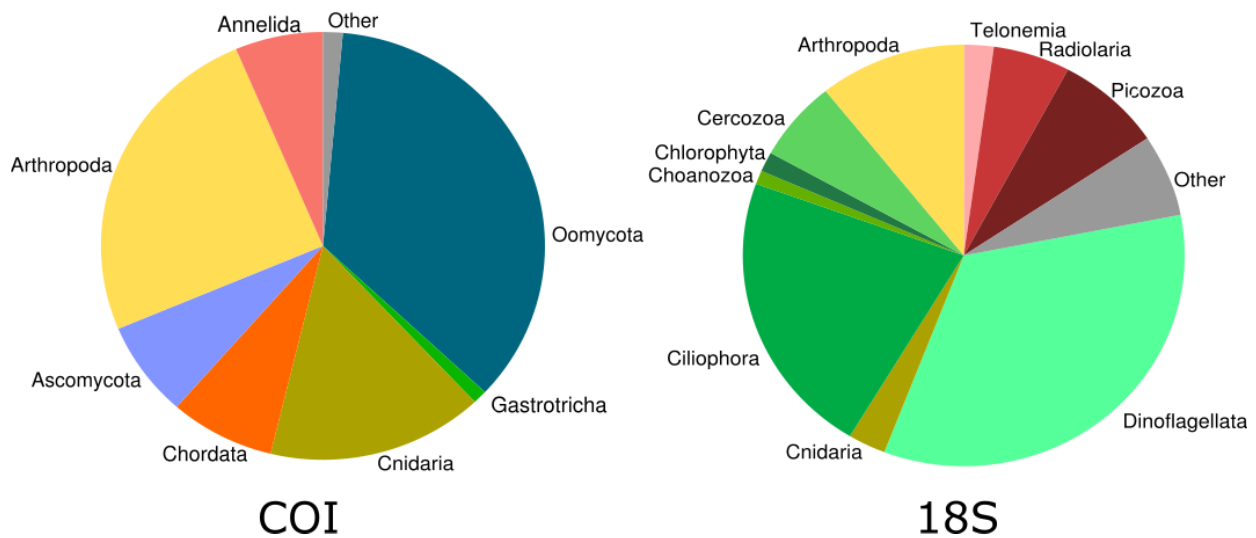



Figure 11. Relative abundance of reads classified to at least the Phylum level for all BW samples retrieved with both barcodes. Only Phyla representing more than 1% of the total abundance are shown.

### 3.2 Variability in ballast water communities among vessels

Important variability in taxonomic composition was observed among vessels with both barcodes (Figure 12), and whereas replicates were very similar, samples from the same

vessel but collected at different sampling points resulted in different communities (Figure 12 and 13). Manhole samples from vessels performing BW exchange in the North Atlantic Ocean (V2 to V8) group together separated from manhole samples from V9, which performed BW exchange in the North Pacific Ocean (Figure 13). Interestingly, manhole samples from V8 are more similar to manhole samples from other vessels of the North Atlantic than to sounding pipe samples from the same vessels. This can be explained by the presence of Oomycota (COI) and Dinoflagellata (18S) in the manhole, which are also generally abundant in samples of other vessels taken from manhole and are less present in V8 samples taken at the sounding pipe. Also, Ascomycota taxa (COI and 18S) were present in great abundance in the sounding pipe and were rare in samples taken at the manhole from V8 or other vessels. Communities of vessel V10, operating in the Atlantic but with BW treatment instead of BW exchange, were different to the other Atlantic vessels and mainly dominated by Oomycota (COI) and Ciliophora (18S). Also, in this vessel, different sampling points provided different communities: samples from the sounding pipe of V10 were characterized by the chrysophyte *Pedospumella sinomuralis* and the ciliophoran *Miamiensis sp.* whereas the discharge line samples were characterized by the phytoplankton *Stephanodiscus sp.* and *Picomonas judraskeda* for COI and 18S respectively. Within the same vessel, communities retrieved at the sounding pipe were less rich and diverse than those retrieved from either the manhole or the discharge line (Figure S5).

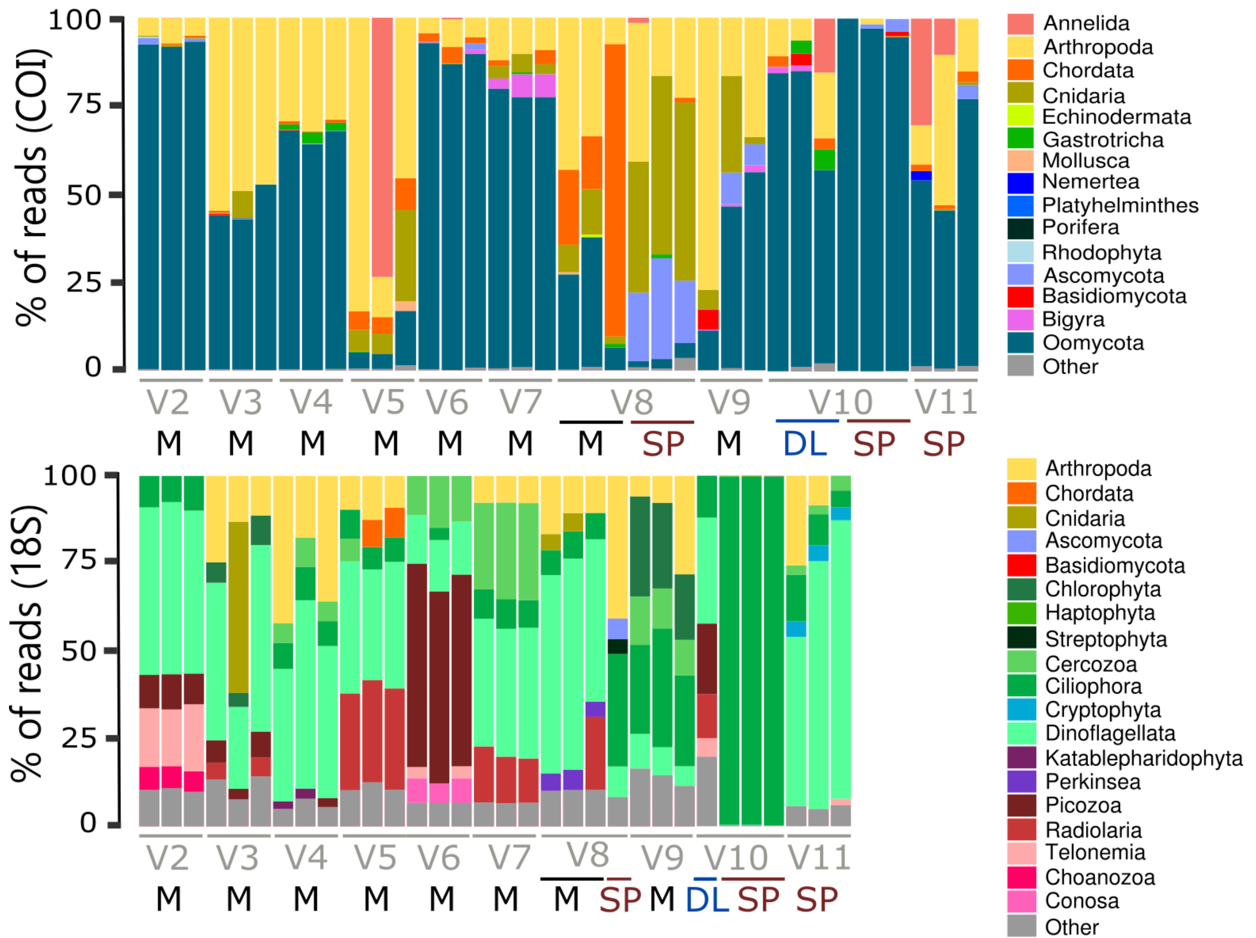



Figure 12. Relative abundance of reads classified to at least Phylum level for the 5 most abundant Phyla per barcode, per replicate of each vessel sampled via manhole (M), discharge line (DL) or sounding pipe (SP). Top panel is for COI and bottom panel is for 18S barcode.

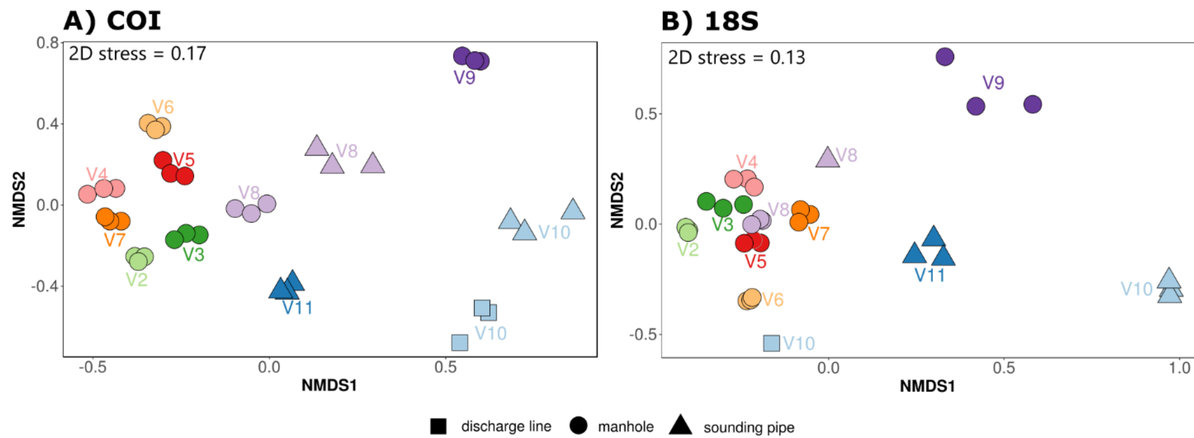



Figure 13. Nonmetric dimensional scaling plots in two dimensions of Hellinger-transformed OTU abundances for all BW samples for COI (A) and 18S (B). Samples of each vessel are differently colored, and the vessel identity is written on the plot. Symbols represent the type of sampling point.

### 3.3 Impact of sampling point in the experimental tank communities

The experimental tank samples collected from the manhole were generally distinct from the ones collected at the discharge line (Figure 14). Indeed, we found significant differences in terms of composition and structure between the communities of the experimental tank retrieved through the manhole and through the discharge line (COI: betadisper P=0.87; permanova $R^2$=0.56, P=0.002 and 18S: betadisper P=0.87; permanova $R^2$=0.37, P=0.003). In this experiment, the main difference in taxa composition between the two sampling points was explained by variation in the abundance of *Acartia tonsa* OTUs for both barcodes (Figure 14). *Acartia tonsa* dominated samples collected through the manhole but did not dominate samples taken at the discharge line. For 18S, the abundance of OTUs identified as Rotifera taxa and *Gymnodinium sp.* also varied between sampling points and were more abundant at the discharge line than from the manhole. Alpha diversity was significantly different between sampling points for both barcodes (COI: Wilcoxon-Mann-Whitney test based on Shannon diversity index, P= 0.002; 18S: Student t-test based on Shannon diversity, P= 0.033), but contrasting patterns were observed. With COI, samples taken at the discharge line were more diverse and richer (mean of Shannon

index = 3.61) than manhole samples (mean of Shannon index = 1.43), whereas for 18S less difference between sampling points was observed (mean of Shannon Index for in-line = 2.89 and for in-tank = 3.03).

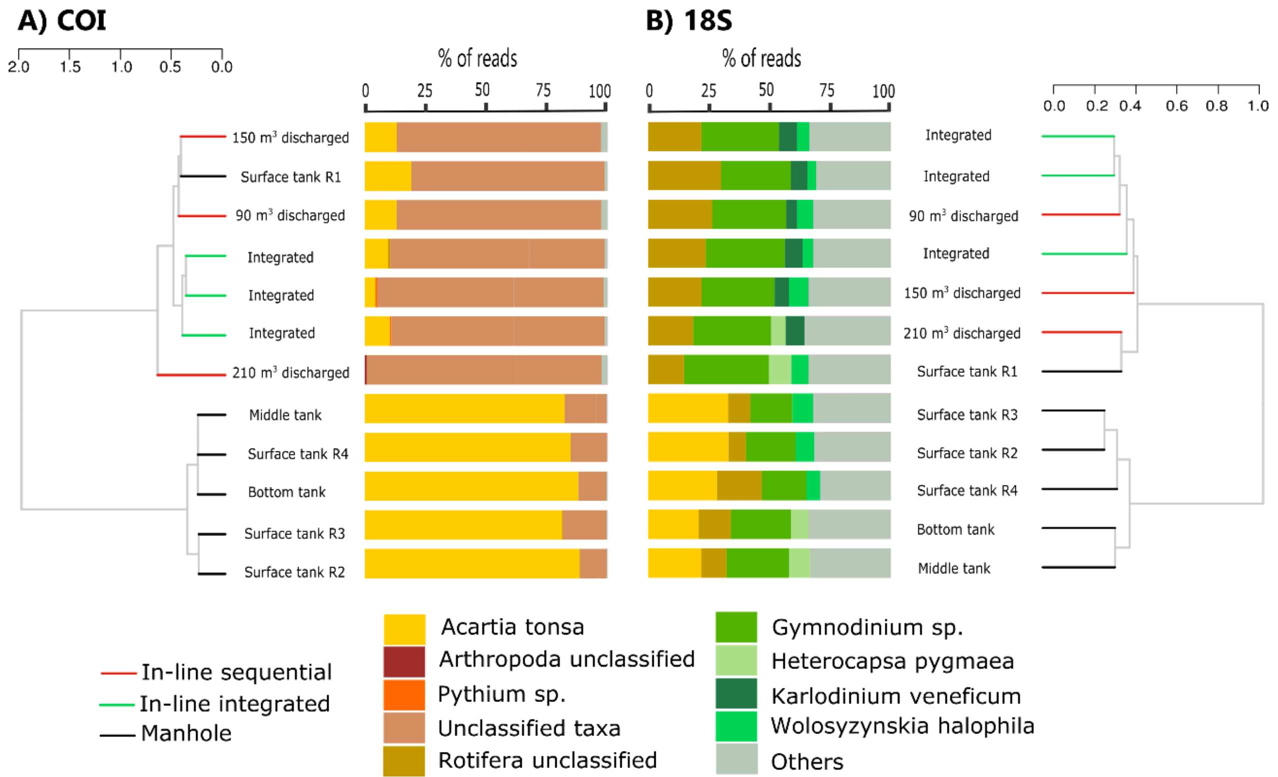



Figure 14. Hierarchical cluster analyses of Hellinger-transformed OTU abundances for the samples of the experimental tank taken at the discharge line (in-line integrated and in-line sequential) and from the manhole; followed by bars representing the taxonomic composition of each sample for COI (A) and 18S (B). Each bar is the relative abundance of reads classified to a taxon where we represented only the 5 most abundant taxa per barcode.

## 3.4 Effects of ballast water exchange location, source port location and ballast water ages

Among vessels which exchanged BW, we explored the impact of location of BW exchange, location of BW source port, BW temperature and BW age (*i.e.* age since exchange). All explained a significant proportion of community variation (Table 5). For both barcodes, more than 50% of community variation was explained when taking all these explanatory variables into account. The effect of location of BW exchange was stronger than the three other explanatory factors and explained 23% of community variation for COI and 27% for 18S. Nonetheless, the structure and composition of communities still exhibited a significant signature of source port location, the latter explaining 12 and 15% of community variation for COI and 18S respectively. BW age also appeared as an important factor governing community composition and we observed that older BW was less rich and diverse than younger BW (Figure S6). This negative relationship holds when the outlier V9 is omitted for COI ($R^2$=0.72, P=9.93e-07) but not for 18S ($R^2$=0.14, P=0.0906). Temperature also had a significant influence but of minor extent compared to the other factors (9% for COI and 8% for 18S). Overall, each set of explanatory variables explained different fractions of community variation (Figure S7).

Table 5. Effect of BW characteristics on communities tested and quantified using redundancy analysis[a]

| Barcode | Explanatory variables | D.f. | Adjusted $R^2$ | Pvalue |
|---|---|---|---|---|
| **COI** | BWE location | 2 | 0.234 | 0.001 |
| | BW source location | 1 | 0.121 | 0.001 |
| | BW age | 1 | 0.127 | 0.001 |
| | Temperature | 1 | 0.092 | 0.002 |
| | All | 5 | 0.511 | 0.001 |
| **18S** | BWE location | 2 | 0.270 | 0.001 |
| | BW source location | 1 | 0.149 | 0.001 |
| | BW age | 1 | 0.149 | 0.001 |
| | Temperature | 1 | 0.082 | 0.003 |
| | All | 5 | 0.541 | 0.001 |

[a] BWE location: coordinates of BW exchange location, BW source location: coordinates of BW uptake in source port, BW age: days since the BW exchange was performed, Temperature: Temperature of the BW at the sampling event, D.f.: degrees of freedom

### 3.5 Detection of zooplankton taxa with high propagule pressure

We found 30 zooplankton species present in at least 5 vessels for one of the barcodes (Table S3). Among these species, the majority (22) were cosmopolitan or semi-cosmopolitan and were not considered as potential taxa of interest due to their global distribution. Interestingly, among these species, five species inhabit coastal, estuarine or brackish environment, suggesting that these species may have not been removed after the BW exchange in open sea. The remaining eight species were not cosmopolitan and were distributed in restricted regions of the world, which did not include the Chesapeake Bay. Among these species, only *Oithona davisae* was described as a non-indigenous species in AquaNIS (AquaNIS. Editorial board., 2015), Nemesis (Fofonoff et al., 2019) or WiRMS (Ahyong et al., 2019) databases. The phylogenetic placement of the OTU identified as *Oithona davisae* in the study with other sequences of this species retrieved from BOLD database supports the taxonomic assignment (Figure S8).

## 4 Discussion

### 4.1 BW communities are different in function of sampling points

Our study shows for the first time that BW collected from the same tank using different sampling points results in different communities, and that samples collected from the sounding pipe are less rich and diverse compared to manhole and discharge line samples. In-tank sampling via the sounding pipe is often used due its practicability on-board in comparison to accessing the manhole (Gollasch et al., 2003; Mitchell et al., 2014). However, its structure (either perforated along the pipe meaning sampling across the whole tank, or only open at the bottom) may alter the representativeness of this sampling method (Pazouki et al., 2009; Taylor and Rigby, 2001). Indeed, it was observed that samples taken at the sounding pipe only retrieved between 0 and 60% of the plankton retrieved from net samples taken from the manhole of the same tank (David and Gollasch, 2015; Sutton et al., 1998). Despite being based on a limited number of vessels, our results

confirm previous observations that highlight the low representativeness of sounding pipe sampling compared to manhole sampling.

Although BW monitoring is currently performed through in-tank sampling, either via manholes or sounding pipes (Darling et al., 2018; Kim et al., 2015; Lymperopoulou and Dobbs, 2017), in-line sampling (from the discharge line) is considered less biased (Bailey and Rajakaruna, 2017). In-line sampling allows the continued sampling of entrained organisms throughout the entire tank discharge, whereas in-tank sampling may be affected by the patchiness of organisms inside the tank (Murphy et al., 2002; Rajakaruna et al., 2018; Sutherland et al., 2001). The experimental tank results presented here confirm this observation as manhole samples were dominated by the copepod *Acartia tonsa,* whereas in-line samples were not, suggesting that the manhole sampling may have collected "patches" of *A. tonsa.* This may have increased the detectability of this species with eDNA. Overall, significant differences in community composition were observed between the two sampling points for both barcodes. Manhole samples were significantly less rich and diverse than in-line samples, although this was only detected with COI. Altogether, our results on actual BW samples and in the experimental tank stress the need for careful consideration of sampling methodology when comparing BW communities of vessels sampled via different points.

### 4.2 BW characteristics are retrieved with eDNA metabarcoding

This study demonstrates that eDNA metabarcoding can differentiate vessels based on their BW characteristics, encouraging its future use for ballast water monitoring. We recovered signals of both BW exchange and source port location, the former being responsible for the most important fraction of community variation. Dinoflagellata and Arthropoda (mainly copepods) dominated exchanged BW samples, which is consistent with previous findings associating these groups to exchange in open sea (Briski et al., 2013; Darling et al., 2018; Hallegraeff, 2015; Paolucci et al., 2017). Source port location was also an important factor for explaining community variation, which is congruent with the

detection of few species inhabiting coastal, estuary or brackish environments in the taxa observed in at least 5 vessels. It is acknowledged that BW exchange is not 100% efficient in removing coastal taxa (Briski et al., 2012; Chan et al., 2015; Simard et al., 2011; Ware et al., 2016), and our results confirmed previous observations that metabarcoding can track signals of source ports even after BW exchange was performed (Darling et al., 2018; Lymperopoulou and Dobbs, 2017).

We also observed that BW age influenced, to some degree, the alpha diversity. Indeed, the negative relationship observed was affected by the outlier vessel V9, which was carrying older BW sourced in another ocean. However, the relationship remained strongly significant for COI (not for 18S) when this vessel was excluded, and only vessels carrying BW from the same ocean region were considered. Overall, among vessels sampled through the manhole that had performed BW exchange, vessels V6 and V9 had the oldest BW (19 and 55 days respectively), which coincided with the smallest Shannon indices. A negative relationship between diversity and BW age has been previously observed for phytoplankton (Burkholder et al., 2007) and zooplankton (Ghabooli et al., 2016). Particularly Burkholder et al. (2007) observed no culturable phytoplankton after 33 days. Interestingly, V9 was characterized by a chrysophyte species for which cyst formation is a key feature of the group in order to survive harsh conditions (Findenig et al., 2010), stressing that some species can endure long voyages. Nonetheless, the difference in community structure and alpha diversity for V9 in comparison to other samples might not be solely explained by its old BW, but also because it is the only vessel coming from another ocean region (for source port and BW exchange). Although these different factors are confounded for this vessel, this highlights again the power of eDNA metabarcoding to differentiate BW with distinct characteristics.

As BW exchange will be soon phased out as a management method and replaced by the use of BW treatment systems to meet the D2-standard (IMO, 2017), the development of methods to assess the efficiency of BW treatment systems on communities is logically gaining more attention (Briski et al., 2015; Fujimoto et al., 2014; Paolucci et al., 2017). The use of metabarcoding has been proposed as a major tool to characterize the difference between communities (Fujimoto et al., 2014; Johansson et al., 2017; Rey et al., 2018). Using metabarcoding, Fujimoto et al. (2014) have shown that BW treatment systems can reduce the diversity of microorganisms. Here, although we had only 2 vessels with treated BW, we also highlight an important effect of the BW treatment system. Indeed, DNA amplification failed for vessel V1, which was treated with filtration and chlorination. Whereas, for V10 (treated with filtration and electro-catalysis oxidation process) DNA amplification was successful, for some samples, but among the amplified samples, some resulted in low number of reads. This difference observed between V1 and V10 could suggest a discrepancy in efficiency of BW treatment system, but this would require further sampling to determine whether it is indicative of a more general tendency. Also, the samples of V10 were found to be markedly different from samples originating from vessels without BW treatment system. This could suggest the possibility of differentiating vessels implementing different BW management techniques (exchange or treated) using eDNA metabarcoding. Finally, V10 was characterized by the phytoplankton *Stephanodiscus sp.*, which belongs to the order Thalassiosirales. Taxa of Thalassiosirales have been observed to regrow after treatment with UV light (Liebich et al., 2012) and are also known to form resting stages in sediment (Grigorszky et al., 2017; Shikata et al., 2008). Further studies will be needed to assess the efficiency of treatment systems to prevent regrowth of phytoplankton as it is an important phenomenon (García-Garay et al., 2018; Grob and Pollet, 2016).

## 4.3 eDNA metabarcoding has potential to detect BW taxa

Our BW monitoring based on eDNA metabarcoding detected the non-indigenous copepod *Oithona davisae*, thought to be native to Japan and Korea and known to have spread with BW (Fofonoff et al., 2019). The confidence in taxonomic assignment was strong as it was found with both barcodes, phylogenetic placement for COI and morphological taxonomy. This copepod has invaded several locations in Europe, including the North Sea (Cornils and Wend-Heckmann, 2015), and we found it particularly abundant in the two vessels coming from this area (Dunkerque). This species inhabits coastal and estuarine habitats (Ferrari and Orsi, 1984), suggesting that BW exchange was not entirely efficient in removing coastal organisms, as described in the above section. To our knowledge, *O. davisae* has not yet been introduced in the Chesapeake Bay (Fofonoff et al., 2019). However, European ports are the main source region of BW for bulk carriers entering in the Chesapeake Bay (Carney et al., 2017), which suggests that *O. davisae* may have a high propagule pressure in this bay, increasing its chance of colonization. Hence, future monitoring should be particularly cautious toward this species. In addition, seven other species not already detected in the Chesapeake Bay were found in at least 5 vessels. However, while for *O. davisae* the confidence in taxonomic assignment was strong, we cannot be as confident for these seven species. With the exception of the polychaeta *Mysta picta,* common in European coastal waters (main source of BW in the study), the remaining species are restricted to other specific areas in the world, suggesting that either they have been introduced in European waters and remain yet unreported or the taxonomic assignment is inaccurate. These records therefore demand further confirmation.

When comparing zooplankton diversity retrieved by eDNA metabarcoding with morphological identification, we observed that i) abundant phyla identified with eDNA were also retrieved with taxonomy and ii) more phyla were detected with eDNA metabarcoding than with morphology. The identification based on morphology also showed common characteristics of BW composed of larvae, nauplii and eggs, and due to the difficulty in identifying these groups using morphological traits, these taxa remained

assigned to coarse taxonomic level (Carney et al., 2017; Chu et al., 1997; Ware et al., 2016; Wonham et al., 2001). This highlights the tremendous potential of eDNA metabarcoding to characterize BW taxa at a finer taxonomic level (genus or species), which is essential to detect taxa of concern, in comparison to traditional monitoring. Importantly, our study shows the advantage of this technique to detect taxa spreading globally with BW, but that are overlooked with traditional sampling methods. For example, Oomycota were detected in every vessel, as was also observed in another BW eDNA study (Zaiko et al., 2015b). More than 60 % of the known species belonging to this Phylum are plant pathogens (Choi et al., 2015; Thines and Kamoun, 2010), among which pathogens such as *Albugo candida*, *Phytophthora sp.* and *Pythium sp.* were found in our survey. Nonetheless, with eDNA metabarcoding it is not possible to distinguish between live and dead organisms, which limits its use as a compliance tool for enforcing IMO and USCG discharge regulations where viability is a key component (Darling et al., 2017; Rey et al., 2018). Emerging studies considered the use of eRNA in complement to eDNA to better depict the "live" biodiversity in a similar environment , the bilge water of boats (Pochon et al., 2017; Zaiko et al., 2018), but experimental analyses still need to be developed to validate this hypothesis.

Finally, in this study we used two barcodes (COI and 18S) that are usually chosen to study eukaryotes, and showed that both detected distinct taxa, not only in terms of species but also at the phyla level. Thereby, we highlight their complementarity and further emphasize the need for coupling barcodes for invasive species detection (Ammon et al., 2018; Borrell et al., 2017; Grey et al., 2018). Our study contributes to a growing number of BW monitoring studies using filtered water with examples spanning from bacteria (Brinkmeyer, 2016; Lymperopoulou and Dobbs, 2017; Ng et al., 2015) and viruses (Kim et al., 2015), to eukaryotes (Pagenkopp Lohan et al., 2016, 2017; Steichen et al., 2014; Zaiko et al., 2015b). Collectively, these studies highlight the capacity of filtered water sampling to become a very cost-effective approach for BW monitoring, especially when considering the use of multiple barcodes as developed in other environments (Drummond et al., 2015; Stat et al., 2017). From a single BW sample, it could be possible to target the whole biodiversity and hence better characterize the discharged communities into ports.

## 5 Acknowledgments

We thank the crew of the 11 vessels and their agents (Blue water shipping company, Capes Shipping Agency and T.Parker Host) at the ports of Baltimore and Hampton Roads for authorizing vessel access. In addition, we thank Kinder Morgan Pier IX and DTA terminals for permitting our access. We thank Fred Dobbs for laboratory facilities, Iñaki Mendibil and Elisabete Bilbao for laboratory work, Kevin Mitchell for helping with ballast water sampling and the Maritime Environmental Resource Center team for their support with the experimental tank sampling. This manuscript is a result of the Aquainvad-ED (AQUAtic INVADers: Early Detection, Control and Management - www.aquainvad-ed.com) project funded by the European Union (Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement no 642197). K. Pagenkopp Lohan is funded as a Robert and Arlene Kogod Secretarial Scholar. This manuscript is contribution X [upon acceptance] from the Marine Research Division of AZTI.

# 6 Supplementary figures

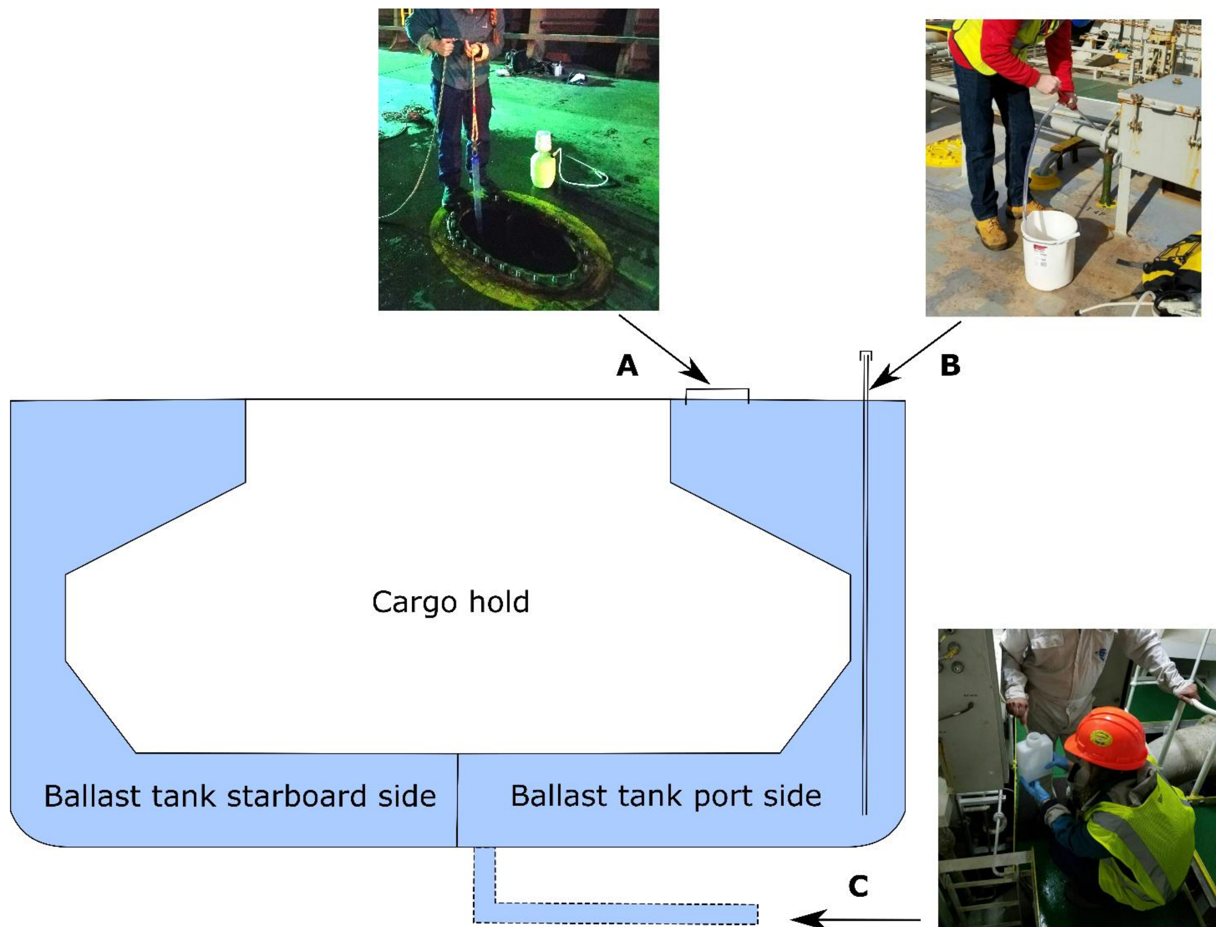



Figure S1. Schematic representation of the three sampling points used in the study to sample ballast water from tank of bulk cargo vessels. Sampling through manhole is represented in A, through sounding pipe is represented in B and at the discharge line in C.

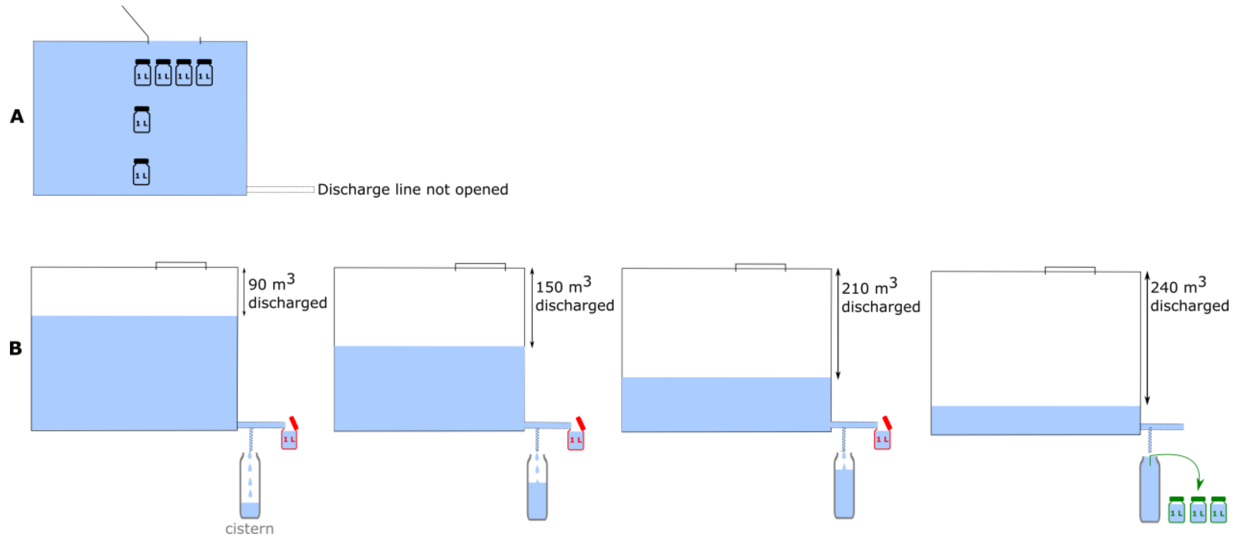



Figure S2. Schematic representation of the sampling in the experimental tank. In-tank sampling with each 1 L sample represented by a black bottle (A), in-line sequential sampling with each 1 L sample represented by a red bottle and in-line integrated sampling with each 1 L sample represented by a green bottle taken from the 90 L cistern at the end of the discharge (B).

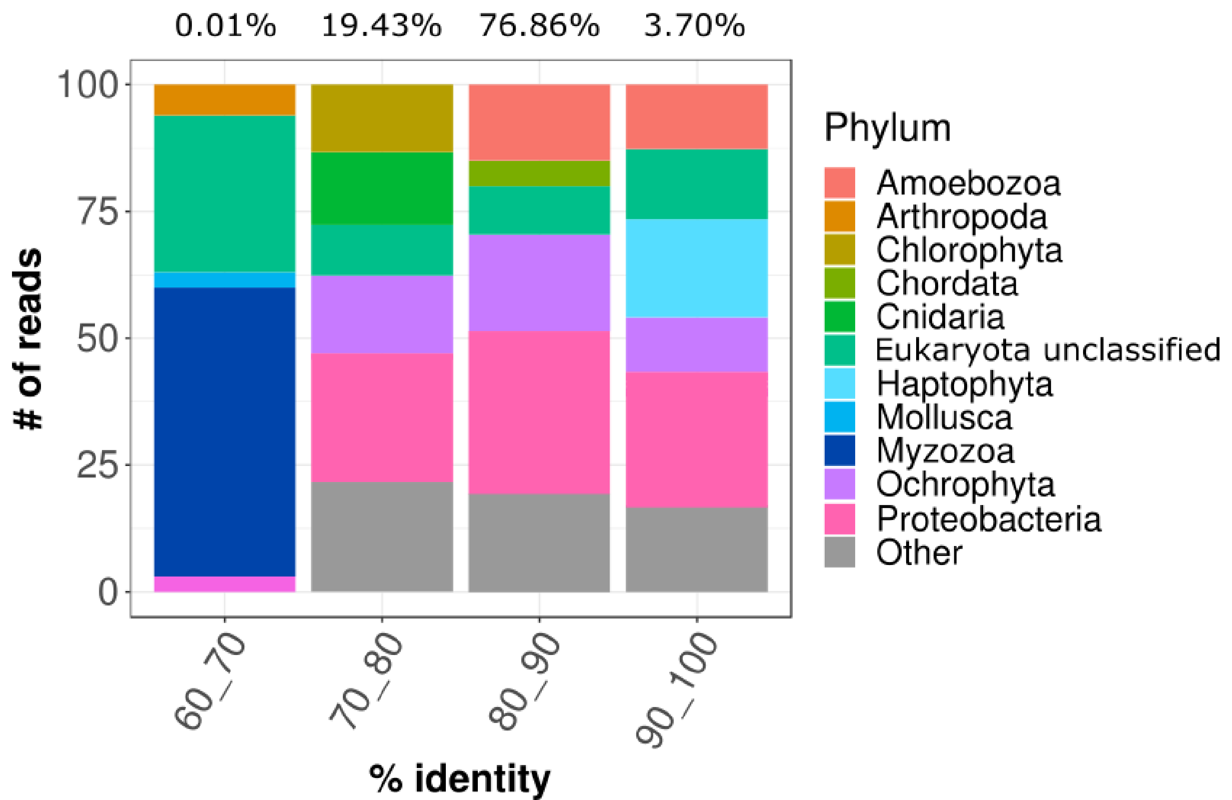



Figure S3. Relative abundance of filtered water reads assigned to each phylum for four percent identity ranges when blasting against NCBI the unclassified reads (*i.e.* reads not classified below the phylum level) found with COI barcode. The 5 most abundant phyla per percent identity range are represented, the remaining being grouped under “other”. Above each bar is indicated the percentage of reads for each percent identity range.

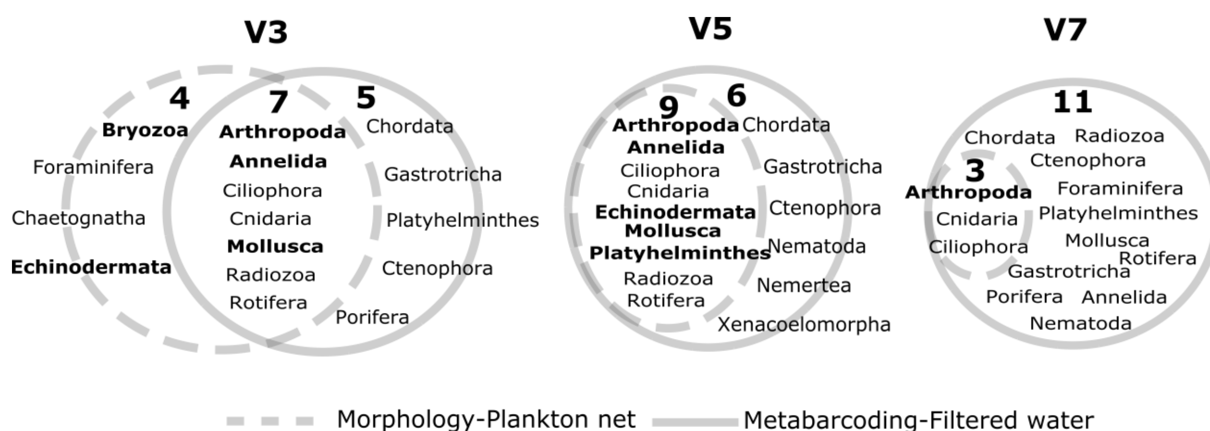



Figure S4. Comparison of taxonomic composition obtained from eDNA metabarcoding of filtered water samples and morphological taxonomy of plankton net samples for vessels V3, V5 and V7. Venn diagrams represent the number of phyla unique and shared between both methods. Phyla found at larval, nauplii or egg stages with morphological taxonomy are in bold.

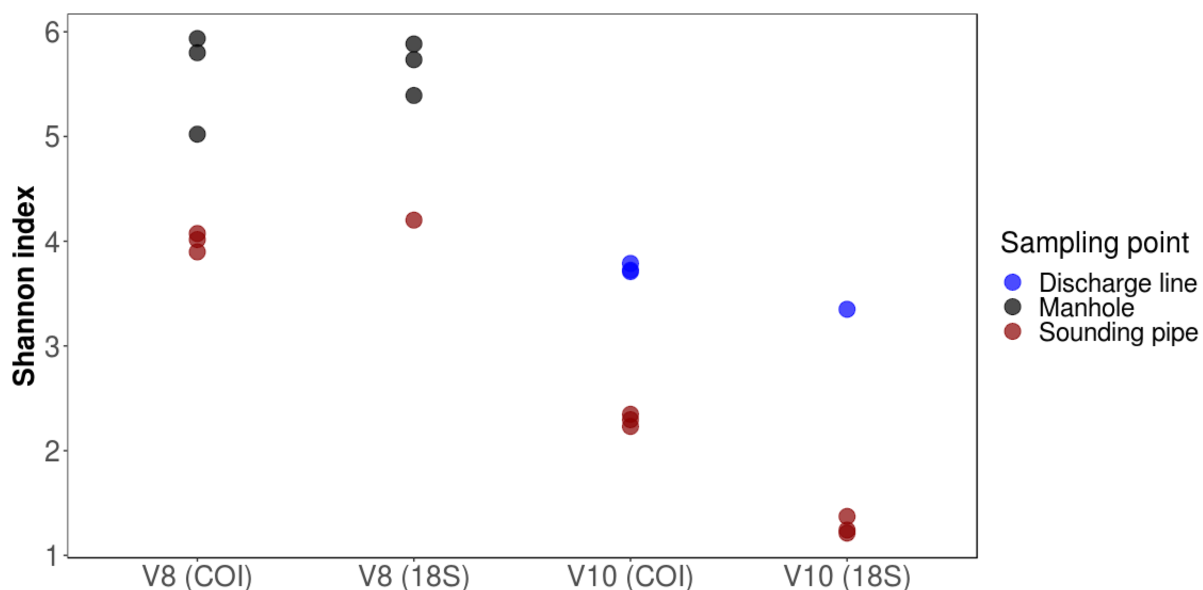



Figure S5. Shannon index for the samples taken from two sampling points for vessels V8 and V10 with COI and 18S.

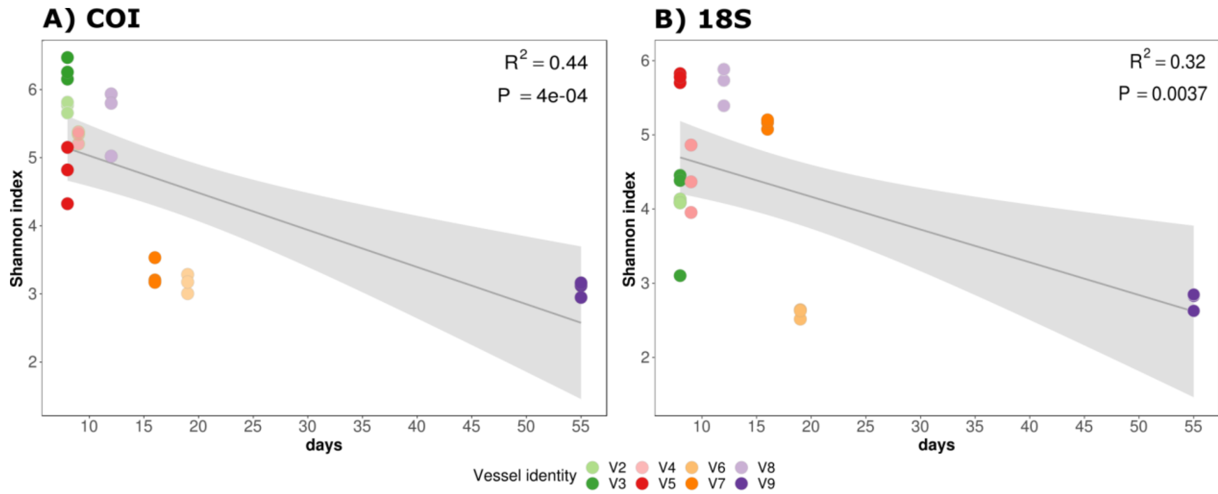



Figure S6. Linear regressions of Shannon index against BW age after BW exchange for COI (A) and 18S (B). $R^2$ is the coefficient of determination and P is the P-value associated to the test.

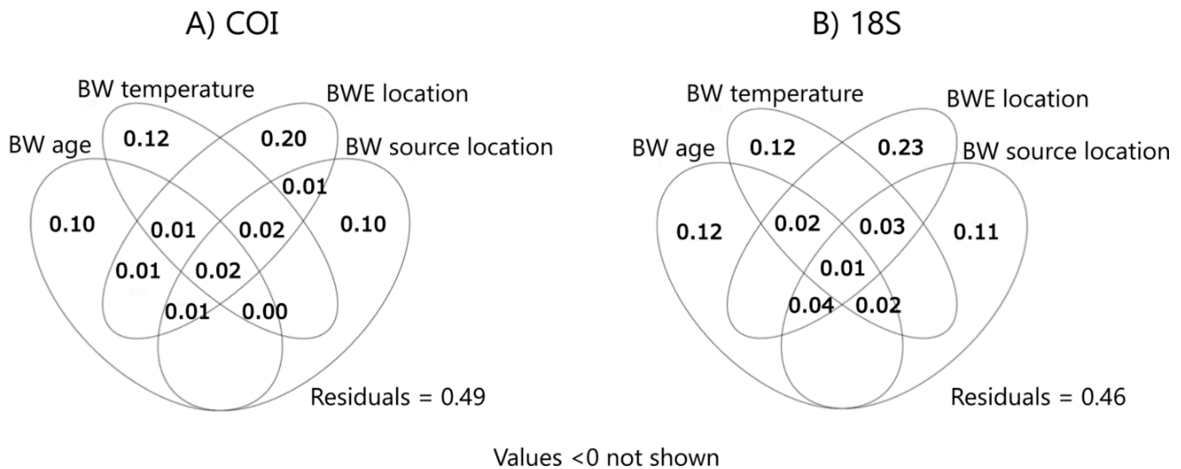



Figure S7. Venn diagrams representing the variance partitioning among the 4 sets of explanatory variables for COI (A) and 18S (B).

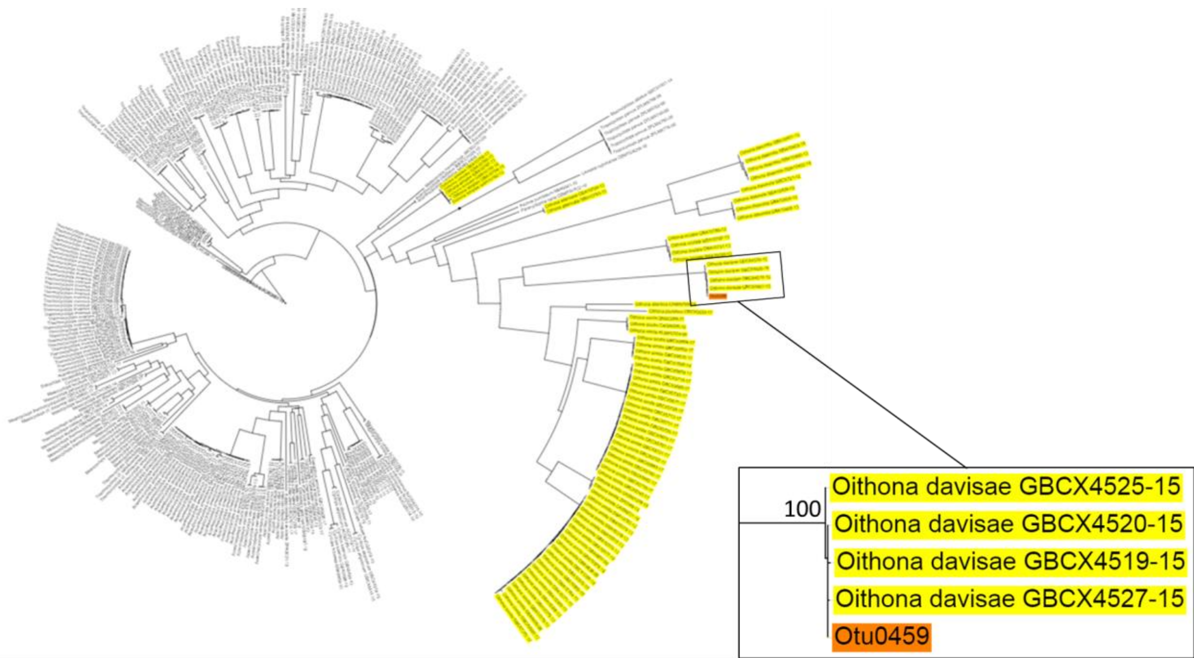



Figure S8. Phylogenetic tree obtained with the representative sequence of the OTU identified in the study as *Oithona davisae* (OTU0459 in orange) and all sequences of Cyclopoida order in BOLD database. Sequences from *Oithona* genus are in yellow.

# 7 Supplementary tables

Table S1. Sampling information of the 11 vessels sampled in the study

| Sample name | Sampling date | Ballast Water history | | | | |
|---|---|---|---|---|---|---|
| | | Salinity (ppt) | Tempe -rature (Cº) | Source port | BW Management (BWE=BW exchange; BWTS=BW treatment) | Time after BW Management (days) |
| V1 | 12/10/2017 | 32.7 | 20.5 | Port Talbot (United Kingdom) | BWTS (filtration and chlorination) | 12 |
| V2 | 13/10/2017 | 37.6 | 23.8 | Koper (Slovenia) | BWE (Flow-Through) | 8 |
| V3 | 13/10/2017 | 36.8 | 23.6 | Hamburg (Germany) | BWE (Flow-Through) | 8 |
| V4 | 14/10/2017 | 36.7 | 24.3 | Amsterdam (Netherlands) | BWE (Flow-Through) | 9 |
| V5 | 14/10/2017 | 36.2 | 22.9 | Dunkerque (France) | BWE (Flow-Through) | 8 |
| V6 | 16/10/2017 | 36.2 | 23.6 | Port Talbot (United Kingdom) | BWE (Empty-Refill) | 19 |
| V7 | 16/10/2017 | 36.9 | 22.8 | Aughinish (Ireland) | BWE (Flow-Through) | 16 |
| V8 | 22/11/2017 | na | na | Dunkerque (France) | BWE (Flow-Through) | 12 |
| V9 | 28/11/2017 | 33 | 14.3 | Ko Si Chang (Thailand) | BWE (Empty-Refill) | 55 |
| V10 | 29/11/2017 | na | na | San Ciprian (Spain) | BWTS (filtration and electro-catalysis oxidation process) | 23 |
| V11 | 02/12/2017 | 36.9 | 10.4 | Aughinish (Ireland) | BWE (Empty-Refill) | 22 |

Table S2. Sample information and number of sequences remaining after each pre-processing step. "na" under reads indicates that DNA amplification failed.

| Sample name | Barcode | Sample type | Sampling point | Vessel identity | Replicate | # raw reads | # reads after primer removal | # reads after paired-end merging | # reads after quality filtering | # reads after aligning to the amplicon region | # reads after chimera removal | # reads after removal of singleton OTU | # reads after negative control filtering |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **V1-R1** | COI | vessel | manhole | V1 | 1 | na | na | na | na | na | na | na | na |
| **V1-R2** | COI | vessel | manhole | V1 | 2 | na | na | na | na | na | na | na | na |
| **V1-R3** | COI | vessel | manhole | V1 | 3 | na | na | na | na | na | na | na | na |
| **V2-R1** | COI | vessel | manhole | V2 | 1 | 68297 | 67201 | 41480 | 41480 | 36685 | 31326 | 29108 | 28836 |
| **V2-R2** | COI | vessel | manhole | V2 | 2 | 51016 | 50253 | 35568 | 35568 | 31712 | 26771 | 25058 | 24753 |
| **V2-R3** | COI | vessel | manhole | V2 | 3 | 70479 | 69669 | 47325 | 47325 | 41689 | 36048 | 33543 | 33160 |
| **V3-R1** | COI | vessel | manhole | V3 | 1 | 79225 | 78542 | 57365 | 57365 | 51632 | 46903 | 44804 | 44426 |
| **V3-R2** | COI | vessel | manhole | V3 | 2 | 51719 | 51293 | 39453 | 39453 | 35803 | 28644 | 25577 | 25329 |
| **V3-R3** | COI | vessel | manhole | V3 | 3 | 79093 | 78474 | 59271 | 59271 | 54856 | 47780 | 45006 | 44488 |
| **V4-R1** | COI | vessel | manhole | V4 | 1 | 69289 | 68728 | 62074 | 62074 | 38860 | 30383 | 27730 | 26667 |
| **V4-R2** | COI | vessel | manhole | V4 | 2 | 75060 | 74510 | 64311 | 64311 | 39401 | 34409 | 32352 | 31164 |
| **V4-R3** | COI | vessel | manhole | V4 | 3 | 62646 | 62084 | 55125 | 55125 | 35464 | 28823 | 26686 | 25703 |
| **V5-R1** | COI | vessel | manhole | V5 | 1 | 79226 | 78500 | 71292 | 71292 | 67598 | 59906 | 56323 | 55529 |
| **V5-R2** | COI | vessel | manhole | V5 | 2 | 81191 | 80340 | 71566 | 71566 | 66996 | 58533 | 55319 | 54367 |
| **V5-R3** | COI | vessel | manhole | V5 | 3 | 73882 | 73031 | 60406 | 60406 | 55108 | 49959 | 47613 | 46494 |
| **V6-R1** | COI | vessel | manhole | V6 | 1 | 73957 | 73253 | 59278 | 59278 | 48346 | 46386 | 44843 | 44441 |
| **V6-R2** | COI | vessel | manhole | V6 | 2 | 70550 | 69819 | 59322 | 59322 | 45773 | 42359 | 40681 | 40117 |
| **V6-R3** | COI | vessel | manhole | V6 | 3 | 68342 | 67752 | 58364 | 58364 | 45632 | 42666 | 41037 | 40521 |
| **V7-R1** | COI | vessel | manhole | V7 | 1 | 73191 | 72636 | 54818 | 54818 | 47879 | 45254 | 43743 | 42753 |
| **V7-R2** | COI | vessel | manhole | V7 | 2 | 75209 | 74644 | 61142 | 61142 | 53034 | 49188 | 47415 | 46513 |
| **V7-R3** | COI | vessel | manhole | V7 | 3 | 71896 | 71419 | 57608 | 57608 | 50140 | 47075 | 45447 | 44585 |
| **V8-M-R1** | COI | vessel | manhole | V8 | 1 | 51126 | 50723 | 34248 | 34248 | 28027 | 24839 | 22896 | 21826 |
| **V8-M-R2** | COI | vessel | manhole | V8 | 2 | 90144 | 89501 | 58084 | 58084 | 45975 | 43798 | 41749 | 40636 |
| **V8-M-R3** | COI | vessel | manhole | V8 | 3 | 76044 | 75538 | 52489 | 52489 | 44371 | 41625 | 39433 | 38121 |
| **V8-SP-R1** | COI | vessel | sounding pipe | V8 | 1 | 82890 | 82298 | 71443 | 71443 | 63389 | 59914 | 57954 | 56490 |
| **V8-SP-R2** | COI | vessel | sounding pipe | V8 | 2 | 137743 | 136673 | 110636 | 110636 | 98778 | 95875 | 93420 | 92127 |
| **V8-SP-R3** | COI | vessel | sounding pipe | V8 | 3 | 111705 | 110842 | 85765 | 85765 | 77436 | 74200 | 72163 | 71305 |
| **V9-R1** | COI | vessel | manhole | V9 | 1 | 85383 | 84682 | 76880 | 76880 | 73465 | 73330 | 71402 | 70558 |
| **V9-R2** | COI | vessel | manhole | V9 | 2 | 87153 | 86356 | 77149 | 77149 | 71974 | 71723 | 69628 | 69194 |
| **V9-R3** | COI | vessel | manhole | V9 | 3 | 97205 | 96473 | 85492 | 85492 | 81594 | 81271 | 79289 | 77970 |
| **V10-DL-R1** | COI | vessel | discharge line | V10 | 1 | 67712 | 67234 | 53349 | 53349 | 45973 | 44688 | 43464 | 36607 |
| **V10-DL-R2** | COI | vessel | discharge line | V10 | 2 | 88593 | 87429 | 69631 | 69631 | 53681 | 52650 | 51040 | 43067 |
| **V10-DL-R3** | COI | vessel | discharge line | V10 | 3 | 106925 | 106112 | 89070 | 89070 | 75173 | 73317 | 71432 | 60391 |
| **V10-SP-R1** | COI | vessel | sounding pipe | V10 | 1 | 53315 | 52581 | 13914 | 13914 | 390 | 387 | 360 | 269 |
| **V10-SP-R2** | COI | vessel | sounding pipe | V10 | 2 | 57936 | 57116 | 16467 | 16467 | 762 | 755 | 705 | 486 |
| **V10-SP-R3** | COI | vessel | sounding pipe | V10 | 3 | 62569 | 61794 | 12532 | 12532 | 865 | 821 | 764 | 585 |
| **V11-SP-R1** | COI | vessel | sounding pipe | V11 | 1 | 80853 | 80298 | 63787 | 63787 | 54498 | 51666 | 49843 | 43994 |
| **V11-SP-R2** | COI | vessel | sounding pipe | V11 | 2 | 60654 | 60203 | 44320 | 44320 | 40187 | 37851 | 35168 | 31475 |
| **V11-SP-R3** | COI | vessel | sounding pipe | V11 | 3 | 71248 | 70610 | 49274 | 49274 | 45694 | 41268 | 39086 | 36334 |
| **B-eDNA_1** | COI | experimental tank | manhole-surface | na | 1 | 52302 | 51876 | 46607 | 46607 | 35723 | 33968 | 32819 | 21646 |
| **B-eDNA_2** | COI | experimental tank | manhole-surface | na | 2 | 55418 | 54962 | 52280 | 52280 | 46862 | 45574 | 44500 | 34233 |
| **B-eDNA_3** | COI | experimental tank | manhole-surface | na | 3 | 49059 | 48679 | 45567 | 45567 | 40465 | 39381 | 38383 | 27588 |
| **B-eDNA_4** | COI | experimental tank | manhole-surface | na | 4 | 59287 | 58893 | 55504 | 55504 | 47185 | 46313 | 45221 | 34990 |
| **B-eDNA_5** | COI | experimental tank | manhole-middle depth | na | 1 | 41678 | 41332 | 39510 | 39510 | 33606 | 32900 | 32041 | 23108 |
| **B-eDNA_6** | COI | experimental tank | manhole-bottom | na | 1 | 47688 | 47188 | 45031 | 45031 | 37632 | 36523 | 35383 | 25089 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **B-eDNA_7** | COI | experimental tank | discharge line-sequential-90$m^3$ | na | 1 | 53631 | 53214 | 47153 | 47153 | 27868 | 27044 | 26312 | 15179 |
| **B-eDNA_8** | COI | experimental tank | discharge line-sequential-150$m^3$ | na | 1 | 51533 | 51059 | 45146 | 45146 | 28239 | 27318 | 26536 | 15513 |
| **B-eDNA_9** | COI | experimental tank | discharge line-sequential-210$m^3$ | na | 1 | 42345 | 41977 | 37334 | 37334 | 22661 | 21910 | 21258 | 11238 |
| **B-eDNA_10** | COI | experimental tank | discharge line-integrated | na | 1 | 64611 | 64088 | 57883 | 57883 | 37075 | 35468 | 34287 | 22953 |
| **B-eDNA_11** | COI | experimental tank | discharge line-integrated | na | 2 | 54567 | 54185 | 49874 | 49874 | 28203 | 27121 | 26293 | 15124 |
| **B-eDNA_12** | COI | experimental tank | discharge line-integrated | na | 3 | 53969 | 53534 | 47856 | 47856 | 31909 | 30513 | 29449 | 18142 |
| **C1** | COI | Negative control of filtration | na | na | 1 | 10928 | 10749 | 10560 | 10560 | 2350 | 2346 | 2256 | 0 |
| **C2** | COI | Negative control of filtration | na | na | 2 | 8101 | 7886 | 7646 | 7646 | 3954 | 3951 | 3784 | 0 |
| **C4** | COI | Negative control of filtration | na | na | 3 | 44224 | 43627 | 33698 | 33698 | 21322 | 20928 | 20177 | 0 |
| **C5** | COI | Negative control of filtration | na | na | 4 | 16741 | 16495 | 16415 | 16415 | 2805 | 2804 | 2664 | 0 |
| **C6** | COI | Negative control of filtration | na | na | 5 | 13071 | 12798 | 12773 | 12773 | 8636 | 8636 | 8363 | 0 |
| **C7** | COI | Negative control of filtration | na | na | 6 | 39838 | 39385 | 37279 | 37279 | 29387 | 29381 | 28606 | 0 |
| **Cext_nº2** | COI | Negative control of extraction | na | na | 1 | 1647 | 1588 | 1578 | 1578 | 1192 | 1191 | 1147 | 0 |
| **Cext_nº3** | COI | Negative control of extraction | na | na | 2 | 9425 | 9243 | 9043 | 9043 | 8104 | 8104 | 7794 | 0 |
| **Cext_nº4** | COI | Negative control of extraction | na | na | 3 | 16690 | 16454 | 15521 | 15521 | 10134 | 10053 | 9658 | 0 |
| **Cext_nº5** | COI | Negative control of extraction | na | na | 4 | 7421 | 7198 | 6706 | 6706 | 5106 | 5033 | 4937 | 0 |
| **Ctr_PCR** | COI | Negative control of PCR | na | na | 1 | 2358 | 1992 | 1949 | 1949 | 1865 | 1865 | 1787 | 0 |
| **V1-R1** | 18S | vessel | manhole | V1 | 1 | na | na | na | na | na | na | na | na |
| **V1-R2** | 18S | vessel | manhole | V1 | 2 | na | na | na | na | na | na | na | na |
| **V1-R3** | 18S | vessel | manhole | V1 | 3 | na | na | na | na | na | na | na | na |
| **V2-R1** | 18S | vessel | manhole | V2 | 1 | 104255 | 99915 | 95821 | 95821 | 89607 | 75621 | 60985 | 60287 |
| **V2-R2** | 18S | vessel | manhole | V2 | 2 | 103728 | 98595 | 93970 | 93970 | 86314 | 71307 | 58006 | 57445 |
| **V2-R3** | 18S | vessel | manhole | V2 | 3 | 162012 | 159394 | 154004 | 154004 | 148556 | 124613 | 104923 | 104246 |
| **V3-R1** | 18S | vessel | manhole | V3 | 1 | 216410 | 213412 | 211480 | 211479 | 209439 | 205771 | 177118 | 176432 |
| **V3-R2** | 18S | vessel | manhole | V3 | 2 | 130649 | 128947 | 128285 | 128285 | 126119 | 110079 | 91925 | 91642 |
| **V3-R3** | 18S | vessel | manhole | V3 | 3 | 26255 | 25766 | 25316 | 25316 | 24922 | 24465 | 20460 | 19871 |
| **V4-R1** | 18S | vessel | manhole | V4 | 1 | 89936 | 88356 | 87575 | 87575 | 85535 | 69687 | 54411 | 54069 |
| **V4-R2** | 18S | vessel | manhole | V4 | 2 | 134758 | 132565 | 131507 | 131506 | 128728 | 102714 | 82228 | 81704 |
| **V4-R3** | 18S | vessel | manhole | V4 | 3 | 145657 | 143235 | 142420 | 142420 | 138354 | 107369 | 87002 | 86642 |
| **V5-R1** | 18S | vessel | manhole | V5 | 1 | 110416 | 108604 | 107962 | 107962 | 103522 | 78515 | 59085 | 58318 |
| **V5-R2** | 18S | vessel | manhole | V5 | 2 | 121400 | 119427 | 118741 | 118741 | 114746 | 89861 | 68096 | 67463 |
| **V5-R3** | 18S | vessel | manhole | V5 | 3 | 122204 | 120217 | 119576 | 119576 | 115126 | 89982 | 68520 | 67801 |
| **V6-R1** | 18S | vessel | manhole | V6 | 1 | 127287 | 122409 | 120586 | 120585 | 113890 | 101650 | 84104 | 83739 |
| **V6-R2** | 18S | vessel | manhole | V6 | 2 | 100903 | 98028 | 96512 | 96512 | 92332 | 82011 | 66947 | 66480 |
| **V6-R3** | 18S | vessel | manhole | V6 | 3 | 113260 | 110381 | 108495 | 108495 | 105333 | 97429 | 79534 | 79046 |
| **V7-R1** | 18S | vessel | manhole | V7 | 1 | 126274 | 123509 | 121429 | 121429 | 118116 | 114850 | 97225 | 96245 |
| **V7-R2** | 18S | vessel | manhole | V7 | 2 | 142000 | 138466 | 137109 | 137109 | 132131 | 115263 | 95930 | 95140 |
| **V7-R3** | 18S | vessel | manhole | V7 | 3 | 94147 | 91997 | 91083 | 91083 | 87955 | 74139 | 59829 | 58929 |
| **V8-M-R1** | 18S | vessel | manhole | V8 | 1 | 118621 | 116581 | 115796 | 115796 | 112342 | 85726 | 66597 | 65586 |
| **V8-M-R2** | 18S | vessel | manhole | V8 | 2 | 130878 | 128518 | 127695 | 127695 | 123444 | 92578 | 71270 | 70450 |
| **V8-M-R3** | 18S | vessel | manhole | V8 | 3 | 129092 | 126945 | 125929 | 125929 | 122895 | 104194 | 84150 | 83469 |
| **V8-SP-R1** | 18S | vessel | sounding pipe | V8 | 1 | 139853 | 137741 | 133579 | 133579 | 130511 | 111914 | 88948 | 88298 |
| **V8-SP-R2** | 18S | vessel | sounding pipe | V8 | 2 | 404 | 366 | 288 | 288 | 279 | 266 | 94 | 79 |
| **V8-SP-R3** | 18S | vessel | sounding pipe | V8 | 3 | na | na | na | na | na | na | na | na |
| **V9-R1** | 18S | vessel | manhole | V9 | 1 | 64753 | 58797 | 51493 | 51493 | 45436 | 45340 | 40025 | 38936 |
| **V9-R2** | 18S | vessel | manhole | V9 | 2 | 96067 | 85450 | 74219 | 74218 | 63964 | 63065 | 54607 | 53240 |
| **V9-R3** | 18S | vessel | manhole | V9 | 3 | 95806 | 88834 | 80693 | 80693 | 73174 | 72409 | 62149 | 60536 |
| **V10-DL-R1** | 18S | vessel | discharge line | V10 | 1 | na | na | na | na | na | na | na | na |
| **V10-DL-R2** | 18S | vessel | discharge line | V10 | 2 | 43712 | 43102 | 42837 | 42837 | 42728 | 39864 | 33527 | 31384 |
| **V10-DL-R3** | 18S | vessel | discharge line | V10 | 3 | na | na | na | na | na | na | na | na |
| **V10-SP-R1** | 18S | vessel | sounding pipe | V10 | 1 | 157477 | 153742 | 152168 | 152168 | 148431 | 144772 | 125661 | 125295 |
| **V10-SP-R2** | 18S | vessel | sounding pipe | V10 | 2 | 130523 | 128120 | 126732 | 126731 | 124541 | 122306 | 105146 | 104854 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **V10-SP-R3** | 18S | vessel | sounding pipe | V10 | 3 | 140205 | 137553 | 136477 | 136477 | 134014 | 128339 | 111287 | 111136 |
| **V11-SP-R1** | 18S | vessel | sounding pipe | V11 | 1 | 62283 | 61603 | 59521 | 59521 | 57039 | 44357 | 35683 | 33738 |
| **V11-SP-R2** | 18S | vessel | sounding pipe | V11 | 2 | 105357 | 103821 | 102611 | 102610 | 99193 | 79523 | 65729 | 62814 |
| **V11-SP-R3** | 18S | vessel | sounding pipe | V11 | 3 | 175515 | 173300 | 168924 | 168924 | 164948 | 150900 | 129770 | 126824 |
| **B-eDNA_1** | 18S | experimental tank | manhole-surface | na | 1 | 142722 | 141157 | 140378 | 140378 | 139579 | 109479 | 90618 | 85876 |
| **B-eDNA_2** | 18S | experimental tank | manhole-surface | na | 2 | 164714 | 162540 | 161354 | 161354 | 160526 | 132538 | 108936 | 103745 |
| **B-eDNA_3** | 18S | experimental tank | manhole-surface | na | 3 | 151877 | 150150 | 149376 | 149376 | 148489 | 115716 | 95900 | 91108 |
| **B-eDNA_4** | 18S | experimental tank | manhole-surface | na | 4 | 113908 | 112719 | 112198 | 112198 | 111518 | 88193 | 73094 | 68364 |
| **B-eDNA_5** | 18S | experimental tank | manhole-middle depth | na | 1 | 175155 | 172873 | 171994 | 171994 | 171272 | 152982 | 129834 | 125010 |
| **B-eDNA_6** | 18S | experimental tank | manhole-bottom | na | 1 | 134597 | 133000 | 132134 | 132134 | 131478 | 114975 | 94653 | 89848 |
| **B-eDNA_7** | 18S | experimental tank | discharge line-sequential-90$m^3$ | na | 1 | 158454 | 156578 | 155901 | 155901 | 155426 | 129901 | 109301 | 104405 |
| **B-eDNA_8** | 18S | experimental tank | discharge line-sequential-150$m^3$ | na | 1 | 159749 | 157830 | 157200 | 157200 | 156684 | 121489 | 101619 | 96585 |
| **B-eDNA_9** | 18S | experimental tank | discharge line-sequential-210$m^3$ | na | 1 | 149213 | 147464 | 146791 | 146791 | 146246 | 116876 | 97125 | 92267 |
| **B-eDNA_10** | 18S | experimental tank | discharge line-integrated | na | 1 | 190055 | 187801 | 187201 | 187201 | 186533 | 136291 | 113590 | 108205 |
| **B-eDNA_11** | 18S | experimental tank | discharge line-integrated | na | 2 | 220795 | 218331 | 217782 | 217782 | 217123 | 175957 | 150251 | 145244 |
| **B-eDNA_12** | 18S | experimental tank | discharge line-integrated | na | 3 | 212283 | 209755 | 208997 | 208997 | 208247 | 170214 | 142433 | 137375 |
| **C1** | 18S | Negative control of filtration | na | na | 1 | 1217 | 1170 | 1127 | 1127 | 1106 | 1085 | 746 | 0 |
| **C2** | 18S | Negative control of filtration | na | na | 2 | 1351 | 959 | 891 | 891 | 662 | 625 | 458 | 0 |
| **C4** | 18S | Negative control of filtration | na | na | 3 | 688 | 660 | 624 | 624 | 617 | 610 | 434 | 0 |
| **C5** | 18S | Negative control of filtration | na | na | 4 | 2917 | 2854 | 2823 | 2823 | 2793 | 2777 | 2294 | 0 |
| **C6** | 18S | Negative control of filtration | na | na | 5 | 1490 | 1439 | 1391 | 1391 | 1355 | 1031 | 679 | 0 |
| **C7** | 18S | Negative control of filtration | na | na | 6 | 575 | 544 | 509 | 509 | 498 | 388 | 239 | 0 |
| **Cext_nº2** | 18S | Negative control of extraction | na | na | 1 | 347 | 329 | 293 | 293 | 281 | 278 | 171 | 0 |
| **Cext_nº3** | 18S | Negative control of extraction | na | na | 2 | 949 | 886 | 823 | 823 | 816 | 729 | 521 | 0 |
| **Cext_nº4** | 18S | Negative control of extraction | na | na | 3 | 848 | 800 | 774 | 774 | 767 | 764 | 598 | 0 |
| **Cext_nº5** | 18S | Negative control of extraction | na | na | 4 | 7823 | 7704 | 7674 | 7674 | 7664 | 5872 | 4840 | 0 |
| **Ctr_PCR** | 18S | Negative control of PCR | na | na | 1 | 588 | 556 | 548 | 548 | 541 | 479 | 355 | 0 |

Table S3. List of zooplankton species identified with at least one of 2 barcodes in at least 5 of the vessels

| Species | Phyla | # vessels per barcode | Distribution | Habitat | References | Taxa of concern |
|---|---|---|---|---|---|---|
| ***Acartia (Acanthacartia) tonsa*** | Arthropoda | 10 (18S) - 7 (COI) | Cosmopolitan | Neritic and brackish | (Razouls et al., 2005) | No |
| ***Acartia (Acartiura) clausi*** | Arthropoda | 5 (18S) - 0 (COI) | Cosmopolitan | Neritic and brackish | (Razouls et al., 2005) | No |
| ***Eteone heteropoda*** | Annelida | 0 (18S) - 5 (COI) | New England to Gulf of Mexico | Neritic | (WoRMS Editorial Board, 2019) | No |
| ***Limecola balthica*** | Mollusca | 6 (18S) - 0 (COI) | North West Atlantic, Notrh Sea, Baltic Sea | Neritic and esturine | (WoRMS Editorial Board, 2019) | No |
| ***Mytilus edulis*** | Mollusca | 5 (18S) - 2 (COI) | North Atlantic from Arctic Ocean to South Carolina | Neritic, estuarine and oceanic | (CABI, 2019a; WoRMS Editorial Board, 2019) | No |
| ***Streblospio benedicti*** | Annelida | 0 (18S) - 5 (COI) | North East Atlantic, Hawai, West Coast of North America and Europe | Neritic and esturine | (Fofonoff et al., 2019) | No |
| ***Oikopleura (Vexillaria) dioica*** | Chordata | 6 (18S) - 0 (COI) | Semi-cosmopolitan in temperate to tropical waters | Neritic but rarely found in open sea | (Palomares, M.L.D and Pauly, 2018; WoRMS Editorial Board, 2019) | No |
| ***Hippopodius hippopus*** | Cnidaria | 7 (18S) - 0 (COI) | Circum-(sub)tropical | Neritic and oceanic | (Palomares, M.L.D and Pauly, 2018; WoRMS Editorial Board, 2019) | No |
| ***Liriope tetraphylla*** | Cnidaria | 7 (18S) - 0 (COI) | Circum-(sub)tropical | Neritic and oceanic | (Palomares, M.L.D and Pauly, 2018; WoRMS Editorial Board, 2019) | No |
| ***Lucicutia flavicornis*** | Arthropoda | 5 (18S) - 1 (COI) | Cosmopolitan | Neritic and oceanic | (Razouls et al., 2005) | No |
| ***Nannocalanus minor*** | Arthropoda | 0 (18S) - 5 (COI) | Cosmopolitan | Neritic and oceanic | (Razouls et al., 2005) | No |
| ***Nanomia bijuga*** | Cnidaria | 6 (18S) - 2 (COI) | Cosmopolitan | Neritic and oceanic | (WoRMS Editorial Board, 2019) | No |
| ***Aglaura hemistoma*** | Cnidaria | 5 (18S) - 3 (COI) | Circum-(sub)tropical | Neritic and oceanic | (Palomares, M.L.D and Pauly, 2018; WoRMS Editorial Board, 2019) | No |
| ***Clausocalanus furcatus*** | Arthropoda | 7 (18S) - 4 (COI) | Cosmopolitan | Neritic and oceanic | (Mazzocchi and Paffenhöfer, 1998; Razouls et al., 2005) | No |
| ***Doliolum denticulatum*** | Chordata | 5 (18S) - 0 (COI) | Semi-cosmopolitan in temperate to tropical waters | Neritic and oceanic | (Palomares, M.L.D and Pauly, 2018; WoRMS | No |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | Editorial Board, 2019) | |
| ***Oithona similis*** | Arthropoda | 6 (18S) - 0 (COI) | Cosmopolitan | Neritic and oceanic | (Razouls et al., 2005) | No |
| ***Paracalanus parvus parvus*** | Arthropoda | 8 (18S) - 1 (COI) | Cosmopolitan | Neritic and oceanic | (Razouls et al., 2005) | No |
| ***Pelagia noctiluca*** | Cnidaria | 5 (18S) - 2 (COI) | Semi-cosmopolitan in temperate to tropical waters | Neritic, esturine and oceanic | (Palomares, M.L.D and Pauly, 2018; WoRMS Editorial Board, 2019) | No |
| ***Sphaeronectes gracilis*** | Cnidaria | 0 (18S) - 7 (COI) | Cosmopolitan | Neritic and oceanic | (Doyle et al., 2008; WoRMS Editorial Board, 2019) | No |
| ***Sulculeolaria quadrivalvis*** | Cnidaria | 7 (18S) - 0 (COI) | Semi-cosmopolitan in temperate to tropical waters | Neritic and oceanic | (WoRMS Editorial Board, 2019) | No |
| ***Undinula vulgaris*** | Arthropoda | 8 (18S) - 1 (COI) | Cosmopolitan | Neritic and oceanic | (Razouls et al., 2005) | No |
| ***Delibus nudus*** | Arthropoda | 5 (18S) - 1 (COI) | Cosmopolitan | Oceanic | (Razouls et al., 2005) | No |
| ***Bestiolina similis*** | Arthropoda | 6 (18S) - 0 (COI) | South West Atlantic, Indian Ocean, Pacific Ocean | Neritic and estuarine | (Razouls et al., 2005) | Yes |
| ***Calocalanus minutus*** | Arthropoda | 6 (18S) - 0 (COI) | Namibia, Guinea Basin, Indian Ocean and New Zealand | Neritic and oceanic | (Razouls et al., 2005) | Yes |
| ***Cordagalma ordinatum*** | Cnidaria | 5 (18S) - 0 (COI) | Gulf of Mexico, Lanzarote Island,Mediteranean sea | Neritic and oceanic | (Razouls et al., 2005; WoRMS Editorial Board, 2019) | Yes |
| ***Diplastrella megastellata*** | Porifera | 6 (18S) - 0 (COI) | Eastern Caribbean | Brackish | (Palomares, M.L.D and Pauly, 2018; WoRMS Editorial Board, 2019) | Yes |
| ***Lepidodermella squamata*** | Gastrotricha | 0 (18S) - 6 (COI) | Spain and Portugal | Freshwater | (WoRMS Editorial Board, 2019) | Yes |
| ***Mysta picta*** | Annelida | 5 (18S) - 0 (COI) | Europe | Neritic | (WoRMS Editorial Board, 2019) | Yes |
| ***Oithona davisae*** | Arthropoda | 7 (18S) - 3 (COI) | California (USA), Europe, Japan and Korea | Estuarine | (Fofonoff et al., 2019) | Yes |
| ***Polydora haswelli*** | Annelida | 6 (18S) - 0 (COI) | New-Zealand and Australia | Neritic | (Read, 2010; WoRMS Editorial Board, 2019) | Yes |

# CHAPTER III

# Guidelines and considerations for metabarcoding-based port baseline biodiversity surveys: towards improved marine non-indigenous species monitoring

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**Abstract:**

Monitoring introduction and spread of non-indigenous species via maritime transport requires performing port biological baseline surveys. Yet, the comprehensiveness of these surveys is often compromised by the large number of habitats present in a port, the seasonal variability of the inhabiting communities and the time-consuming morphological approach used for taxonomic classification. Metabarcoding represents a promising alternative for rapid comprehensive port biological baseline surveys, but, before this technique can be routinely applied in this context, standardized protocols should be developed. We applied metabarcoding using two alternative barcodes (based on the Cytochrome Oxidase I or the 18S ribosomal RNA gene) to about two hundred port samples collected i) from diverse habitats (water column – including environmental DNA and zooplankton, sediment and fouling structures), ii) at different sites within the port (from inner to outer estuary), and iii) during the four seasons of the year. Comparing the biodiversity metrics and taxonomic composition derived from each sample group, we investigated the impact of the different strategies for metabarcoding-based port biodiversity baseline surveys. Each sampling method resulted in a distinct community profile and water samples alone did not produce comprehensive macroorganismal biodiversity to substitute traditional sampling. Sampling at different seasons and locations resulted in higher observed biodiversity, but nestedness analyses suggested that sampling could be reduced to two seasons. Metabarcoding also allowed to detect previously

recorded non-indigenous species as well as to reveal presence of new ones, even if in low abundance. We provide the first comprehensive evaluation of metabarcoding for port biodiversity baseline surveys from which we have derived guidelines for how, when and where samples should be collected. These guidelines are key to develop the cost-effective and standardizable approach needed for monitoring non-invasive species introduction to ports via ballast water and hull fouling, particularly relevant with the recent entry into force of the International Convention for the Control and Management of Ships' Ballast Water and Sediments.

**Key-words:**

ballast water; environmental DNA; environmental monitoring; invasive species; metabarcoding; non-indigenous species; port biological baseline surveys

# 1 Introduction

Globalization has led to an increased maritime transportation, with more and larger ships transferring species into ports via ballast water and hull fouling (Seebens et al., 2013). Among the thousands species arriving daily (Carlton, 1999), some are Non-Indigenous Species (NIS) and can become invasive, unbalancing the equilibrium of the recipient ecosystem (Bax et al., 2003). Thus, being important gateways for introduction of NIS, ports need to be monitored for supporting legal frameworks aiming at controlling biological invasions (Lehtiniemi et al., 2015).

One of these legal frameworks is the International Convention for the Control and Management of Ships' Ballast Water and Sediments (IMO, 2004), currently in force. This convention requires that ships treat their ballast water before its release to port, unless they show that the risk of transferring NIS between the donor and recipient ports is limited (David et al., 2013). Such risk assessment relies on the combination of port environmental (temperature and salinity) and biological information. The obtention of biological information is done by cataloguing biodiversity through Port Biological Baseline Surveys (PBBS). Given the diverse range of habitats (such as soft sediment, water column, or exposed and sheltered artificial structures) allowing presence of distinct organismal groups (such as benthic macrofauna, fouling organisms or planktonic organisms), comprehensive PBBS require collecting samples using various methods (Kraus et al., 2018) and at different spatial and temporal scales (Lehtiniemi et al., 2015).

Global initiatives have been initiated to standardize sampling for PBBS (Awad et al., 2014; HELCOM/OSPAR, 2013; Kraus et al., 2018), and are based on morphological taxonomic identification of the species found. Yet, this method lacks accuracy for identification of larvae and eggs, developmental stages at which many NIS are transported (Gittenberger et al., 2014), and relies on taxonomists who are often specialized on the local biota, but who have limited knowledge of alien taxa (Pyšek et al., 2013) and/or on specific taxonomic groups coexisting in a port (Bishop and Hutchings, 2011). Moreover morphological identification is time-consuming (Mandelik et al., 2010), especially when considering the several tens of samples required to characterize a port. These limitations

translate into PBBS being completed several years after sample collection (Bott, 2015), reducing the effectiveness of prevention of NIS introduction and spread control strategies.

Metabarcoding (Taberlet et al., 2012), the simultaneous identification of taxa present in a complex environmental sample based on a conserved DNA fragment, is revolutionizing traditional biodiversity monitoring (Creer et al., 2016; Elbrecht et al., 2017b), and could represent the cost-effective and possible to standardize alternative required for PBBS (Lehtiniemi et al., 2015). Metabarcoding can be applied to DNA extracted from bulk samples (*e.g.* zooplankton) or to environmental DNA (eDNA), defined as traces of DNA released in the environment in form of cells, feces, skin, saliva, mucus, etc. (Shaw et al., 2017). Metabarcoding has been described as a promising tool for NIS monitoring (Comtet et al., 2015) and has been applied to port zooplankton (Brown et al., 2016) and eDNA samples (Borrell et al., 2017; Grey et al., 2018; Lacoursière-Roussel et al., 2018). Yet, no previous study has developed a standardized sampling and data analysis protocol that allows comparable comprehensive overviews of ports' biodiversity considering different substrates and spatial and temporal variability.

In order to provide guidelines for performing standardized, comprehensive, accurate and cost-effective metabarcoding-based PBBS, we evaluated the effect of i) using various sampling methods to capture biodiversity found in different substrates, ii) sampling at different sites from outer to inner estuary, iii) sampling during different seasons of the year, and iv) using alternative genetic markers.

## 2 Materials and methods

### 2.1 Sampling

Zooplankton, sediment, fouling and water samples were collected from the port of Bilbao in autumn 2016, winter 2017, spring 2017, and late summer 2017 at four sites (Figures 15 and S9). Sites 1, 2 and 3 depict the outer parts of the port with busy berths and are characterized by high water depth (10-30 m) and salinity (>30 PSU); site 4 depicts the inner, less busy part of the port and is characterized by low water depth (6-9 m) and lower salinity (>20 PSU). Zooplankton, sediment and fouling sampling was based on the protocol designed by Helcom/Ospar (HELCOM/OSPAR, 2013). Zooplankton samples

were collected in vertical tows using Pairovet nets (mesh sizes of 60 and 280 μm) at three points per site, mixing the collected material to have one sample per mesh size per site. The collected zooplankton was grinded with a mortar until no integer organism could be appreciated and was stored in 96% ethanol at -20 °C. Sediment samples were collected by sieving material collected from a Van Veen grab (0.07–0.1 $m^2$) with a 1 mm mesh size sieve. Retained material was processed following Aylagas et al. (2016b), and retrieved benthic macroinvertebrates were stored in 96% ethanol at -20 °C. Fouling samples were collected by placing 15 x 15 cm polyvinyl chloride plates at 1, 3 and 7 m depth in each site. Plates were deployed in winter and spring and recovered in spring and late summer respectively (see Table S4). At recovery, plates were placed in individual sterile plastic bags, soaked in 96% ethanol and thoroughly scrapped with a scalpel to retrieve fouling organisms attached to it. The detached organisms were then homogenized with a blender and stored in 96% ethanol at -20 °C. At each sampling site, 1 L surface water sample was collected from each sampling point with a bottle. Samples from the same site were combined into a single sample by site. In sites 1 and 3, three additional samples were taken at maximum depth using a Niskin bottle and combined. Each 3 L combined water sample was filtered using a 0.45 μm Sterivex filter unit (Merck Chemicals & Life Science), which was stored at -80 °C until further use. Additionally, surface water samples from three sampling sites from the ports of Vigo and A Coruña, located in Galicia, Northwestern Spain, were collected in March 2017 and filtered as described above.

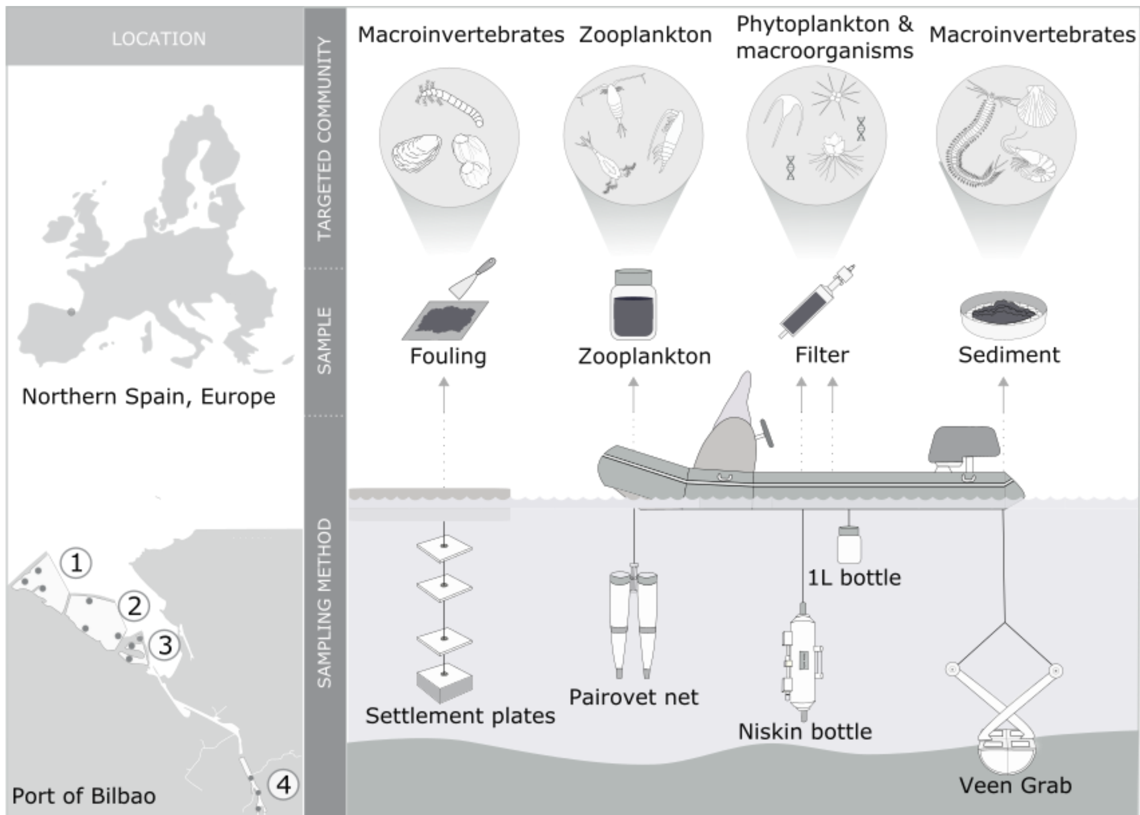


Figure 15. Sampling protocol depicting sampling location (Port of Bilbao), sites (1, 2, 3 and 4) and points per site (dots); and illustrating the sampling methods used and the targeted biological communities.

## 2.2 DNA extraction, library preparation and sequencing

Total genomic DNA was isolated from zooplankton samples with the DNeasy blood & tissue kit (QIAGEN), benthic macroinvertebrate and fouling organism samples with, respectively, PowerMax and PowerSoil DNA Isolation Kits (MOBIO) replacing the initial bead-beating step by an overnight incubation at 56°C with proteinase K (0.4 mg/ml), and filter samples with the DNeasy blood & tissue kit (QIAGEN) following the "SX filters without preservation buffer" developed by Spens et al. (2017). Negative controls were included in each batch of DNA extraction and followed the whole process. DNA concentration was measured with the Quant-iT dsDNA HS assay kit (Thermo Scientific) using a Qubit 2.0 Fluorometer (Life Technologies), purity was inferred from 260/280 and 260/230 absorbance ratios with the ND-1000 Nanodrop (Thermo Scientific), and integrity

was assessed by electrophoresis in 0.7% agarose. Two primer pairs were used: mlCOIintF/dgHCO2198 ("COI primers"), targeting a 313 bp fragment of the cytochrome oxidase I (COI) gene (Leray et al., 2013) and 1389F/1510R ("18S primers"), targeting a variable length fragment (87 to 186 bp) of the V9 hypervariable region of the 18S rRNA gene (Amaral-Zettler et al., 2009). DNA extracted from zooplankton, fouling organism and filter samples were amplified with both primer pairs, whereas DNA from sediment was only amplified with the "COI primers". PCR amplifications were performed in two rounds. For the first PCR, 1 µl of genomic DNA (5 ng/µl) was added to a mix consisting in 5 µl of 1X Phusion Master Mix (Thermo Scientific), 0.2 µl of each primer (0.2 µM) and 3.6 µl of MilliQ water. For the "18S primers", PCR conditions consisted on an initial 3 min denaturation step at 98 °C; followed by 25 cycles of 10 s at 98 °C, 30 s at 57 °C and 30 s at 72 °C and finally 10 min at 72 °C. For the "COI primers", PCR conditions consisted on an initial 3 min denaturation step at 98 °C; followed by 35 cycles of 10 s at 98 °C, 30 s at 46 °C and 45 s at 72 °C and finally 5 min at 72 °C. Negative controls were included within each set of PCRs. For each DNA extract, three PCR amplifications were performed and pooled. Once purified using AMPure XP beads (Beckman Coulter), the mixed PCR products were used as template for the generation of dual-indexed amplicons in a second PCR round following the "16S Metagenomic Sequence Library Preparation" protocol (Illumina) using the Nextera XT Index Kit (Illumina). Multiplexed PCR products were purified again using the AMPure XP beads, quantified using Quant-iT dsDNA HS assay kit and a Qubit 2.0 Fluorometer (Life Technologies), normalized to equal concentration and sequenced using the 2 x 300 paired-end MiSeq (Illumina). Reads were demultiplexed based on their barcode sequences.

### 2.3 Raw read preprocessing, clustering and taxonomic assignment

After quality checking of demultiplexed paired-end reads with FASTQC (Andrews, 2010), forward and reverse primers were removed using Cutadapt (Martin, 2011) with the "anchored 5′ adapter" and for "paired end reads" options and with the "linked adapter" option for COI and 18S respectively. Forward and reverse reads were merged using PEAR

(Zhang et al., 2014) with a minimum sequence overlap of 217 bp and maximum amplicon length of 313 bp and of 80 and 190 bp for COI and 18S barcodes respectively. Merged reads with average Phred quality score lower than 20 were removed with Trimmomatic (Bolger et al., 2014). Using Mothur (Schloss et al., 2009), sequences without ambiguous bases were aligned to BOLD (https://www.boldsystems.org) or SILVA (https://www.arb-silva.de/documentation/release-132/) for COI and 18S respectively, and only those covering the barcode region were kept. Chimeras, detected using *de novo* mode of UCHIME (Edgar et al., 2011) , were removed, and remaining reads were clustered into OTUs using Swarm 2.2.1 with defaut settings (Mahé et al., 2014). "Singleton" OTUs, composed by a single read, were removed, and the remaining OTUs were taxonomically assigned using the Naïve Bayes Classifier (Wang et al., 2007) using BOLD (accessed in May 2018) or PR2 (release 4.10.0) databases for COI and 18S respectively.

### 2.4 Community analyses

Apart from the complete dataset, we created two subsets: a PBBS-targeted taxa dataset, including only reads classified to the class level and excluding those matching to non-targeted groups for PBBS such as Mammalia, Aves, Insecta, Collembola, Arachnida and all classes of Fungi; and a NICS dataset, including only reads matching either the Non-Indigenous and Cryptogenic Species (NICS) previously detected in the port of Bilbao (Adarraga and Martínez, 2011, 2012; Butrón et al., 2011; Martínez and Adarraga, 2006; Tajadura et al., 2016; Zorita et al., 2013) or the AquaNIS database (AquaNIS. Editorial board., 2015). Most analyses were conducted using RStudio (Team, 2015) with *vegan* (Oksanen et al., 2013), *adespatial* (Dray et al., 2018) and *indicspecies* (De Cáceres and Legendre, 2009) libraries. Indicator species analyses (Dufrene and Legendre, 1997) were performed on 1) the PBBS targeted taxa dataset to identify indicator taxa of each sampling method and 2) the NICS dataset to identify non-indigenous indicator taxa of each sampling site. These analyses were based on the IndVal index calculated as the product of the degree of specificity (measuring the uniqueness to a sampling method or site) and the degree of fidelity (measuring the frequency of occurrence within a sampling method or

site) of an OTU to a given sampling condition. Statistical significance of associations was assessed by performing 10,000 permutations. The effects of season and locality on PBBS targeted taxa communities were tested for significance using a permutational multivariate analysis of variance (PERMANOVA) after checking for multivariate homogeneity of group dispersions (betadisper). PERMANOVA and betadisper were performed on OTU abundance (Hellinger distance) and presence/absence (Jaccard dissimilarities). The contribution of replacement (changes in OTU identity) and nestedness (richness differences where one sample is a subset of a richer sample) to beta diversity of PBBS targeted taxa between seasons and between sites was computed using the relativized nestedness index of Podani & Schmera (2011) based on Jaccard dissimilarities matrix. For each sampling method, replacement and nestedness were calculated between pairwise comparisons of 1) samples belonging to the same site but sampled at different seasons (season variation of beta diversity), and 2) samples belonging to the same season but sampled at different sites (spatial variation of beta diversity). The mean proportion of nestedness and replacement contribution to beta-diversity between sites and between seasons was then calculated.

# 3 Results

## 3.1 Communities retrieved by each sampling method and genetic marker

A total of 5,718,639 and 7,055,675 COI and 18S barcode reads were kept for analysis (Table S4). The reads corresponding to the 192 samples collected at the port of Bilbao resulted in 40,318 and 20,473 OTUs for COI and 18S respectively. For all sampling methods, rarefaction curves of OTUs against reads approached saturation, indicating enough sequencing effort (Figures 16A and S10). The majority of the OTUs, 89% for COI and 73% for 18S, were unique to one sampling method (Figure 16B), and, for both barcodes, but particularly for 18S, filtered water resulted in higher OTU richness and unique OTUs than other sampling methods (Figures 16A, B and S10). Different sampling methods retrieved distinct biological communities as observed with the PCA (Figure 16C)

and confirmed with PERMANOVA analyses (COI: $R^2$=0.28, p-value= 9.99e-05; 18S: $R^2$=0.30, p-value=9.99e-05).

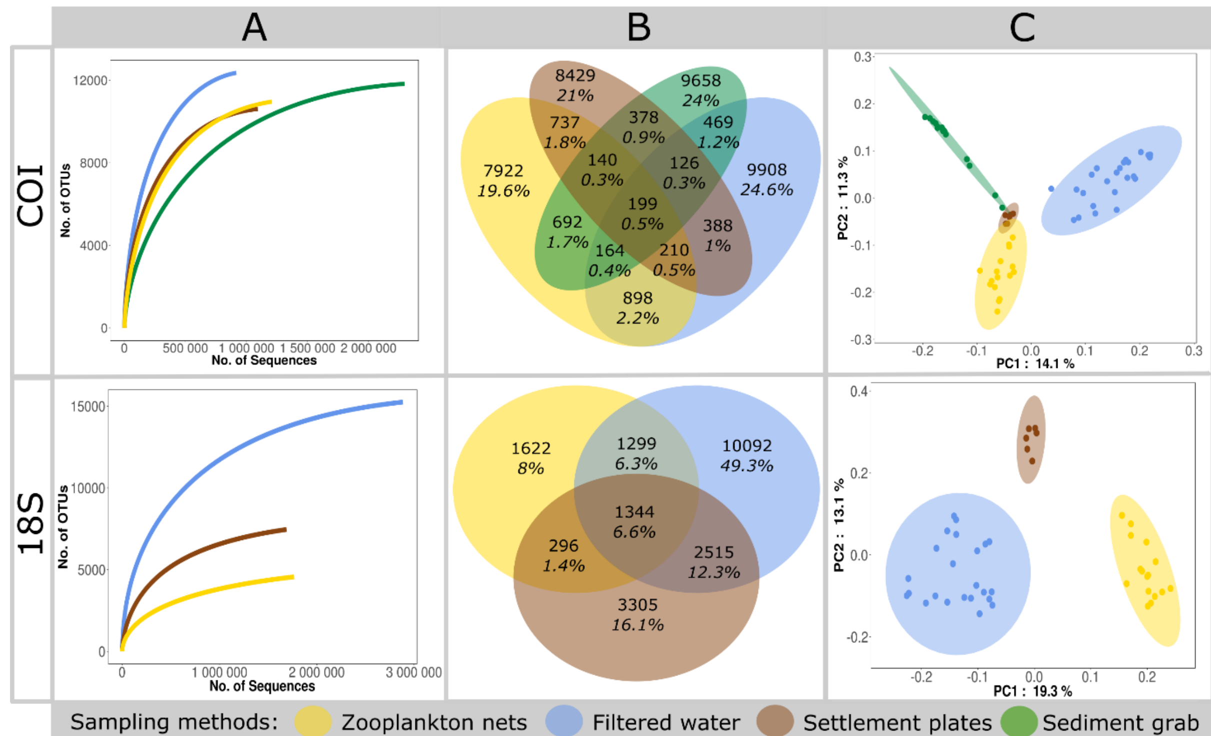


Figure 16. Overall description of community detected per barcode. A: OTU accumulation curves per sampling method. B: Venn diagrams of the number and percentage of OTUs shared between sampling methods. C: Principal Component Analyses of the Hellinger-transformed abundances of OTUs. Sample scores are displayed in scaling 1 with ellipses representing the 95% confidence dispersion of each sampling method.

The percentage of reads assigned to species or higher taxonomic levels differed between sampling methods and barcodes. The majority of the reads were assigned to species level for zooplankton nets (COI: 84% and 18S: 75%), settlement plates (COI: 69% and 18S: 69%) and sediment grabs (COI: 64%); for filtered water, although 51% of reads were assigned to species level with 18S, only 8% could be assigned to a species with COI (Figure 17A). Filtered water analyzed with COI had also the largest proportion of reads that could not be assigned to phylum (87%). This is possibly due to the amplification of non-metazoan taxa, which are underrepresented in the BOLD database (Figure S11), which was confirmed with BLAST searches against GenBank (data not shown).

Both barcodes detected a wide range of eukaryotic groups (Figure 17B). With COI, the greatest majority of reads belonged to metazoans, with Arthropoda dominating all sampling methods except sediment grabs, dominated by Annelida. With 18S, a more diverse spectrum of taxa was retrieved, including phytoplankton and macroalgae; as expected, the number of reads assigned to these phyla in zooplankton nets and settlement plates was very low in comparison to filtered water.

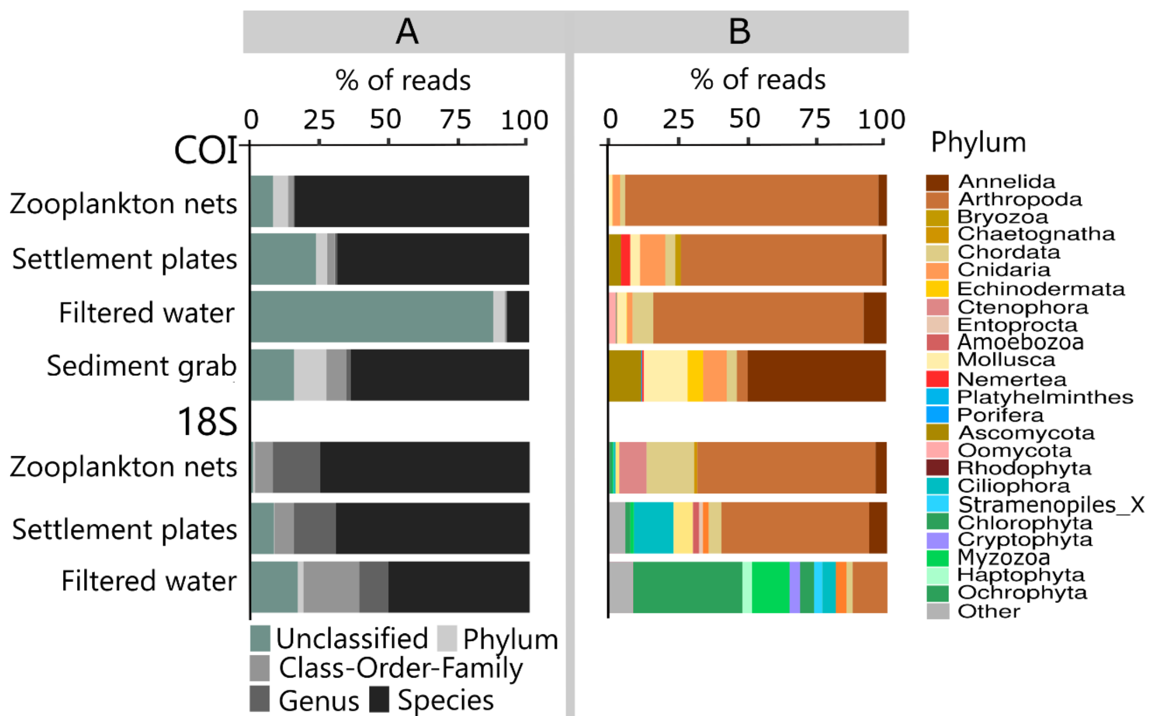


Figure 17. Taxonomic assignment per barcode and per sampling method. A: Percentage of reads assigned to each taxonomic level. B: Relative abundance of reads classified to at least Phylum level for the 10 most abundant phyla.

### 3.2 Distribution of port biological baseline survey targeted taxa

A total of 16,828 and 9,091 OTUs were kept as PBBS targeted taxa for COI and 18S respectively. From those, indicator analysis identified respectively 2,600 (15%) and 1,700 (19%) OTUs significantly associated to one of the sampling methods. Settlement plates were associated with 1,268 (COI) and 808 (18S) OTUs, zooplankton nets with 1,001 (COI) and 315 (18S) OTUs, and sediment grabs with 238 (COI) OTUs. Only 12 OTUs (9 of which

were metazoans) were indicators of filtered water with COI while 580 (3 of which were metazoans), were associated to this sampling method with 18S. We observed expected strong associations of some taxa to one particular sampling method: fouling bivalves (Ostreoida, Mytilida) with settlement plates, copepods (Calanoida, Cyclopoida) with zooplankton nets and sea urchins (Spatangoida) with sediment grabs (Figure 18). Yet, less obvious associations were also observed such as barnacles (Sessilia) and two polychaeta orders (Spionida, Phyllodocida) with zooplankton nets or dinoflagellates (Peridiniales and Gymnodiniales) with settlement plates, illustrating the advantages of a complementary sampling approach to recover the diversity of these taxonomic groups.

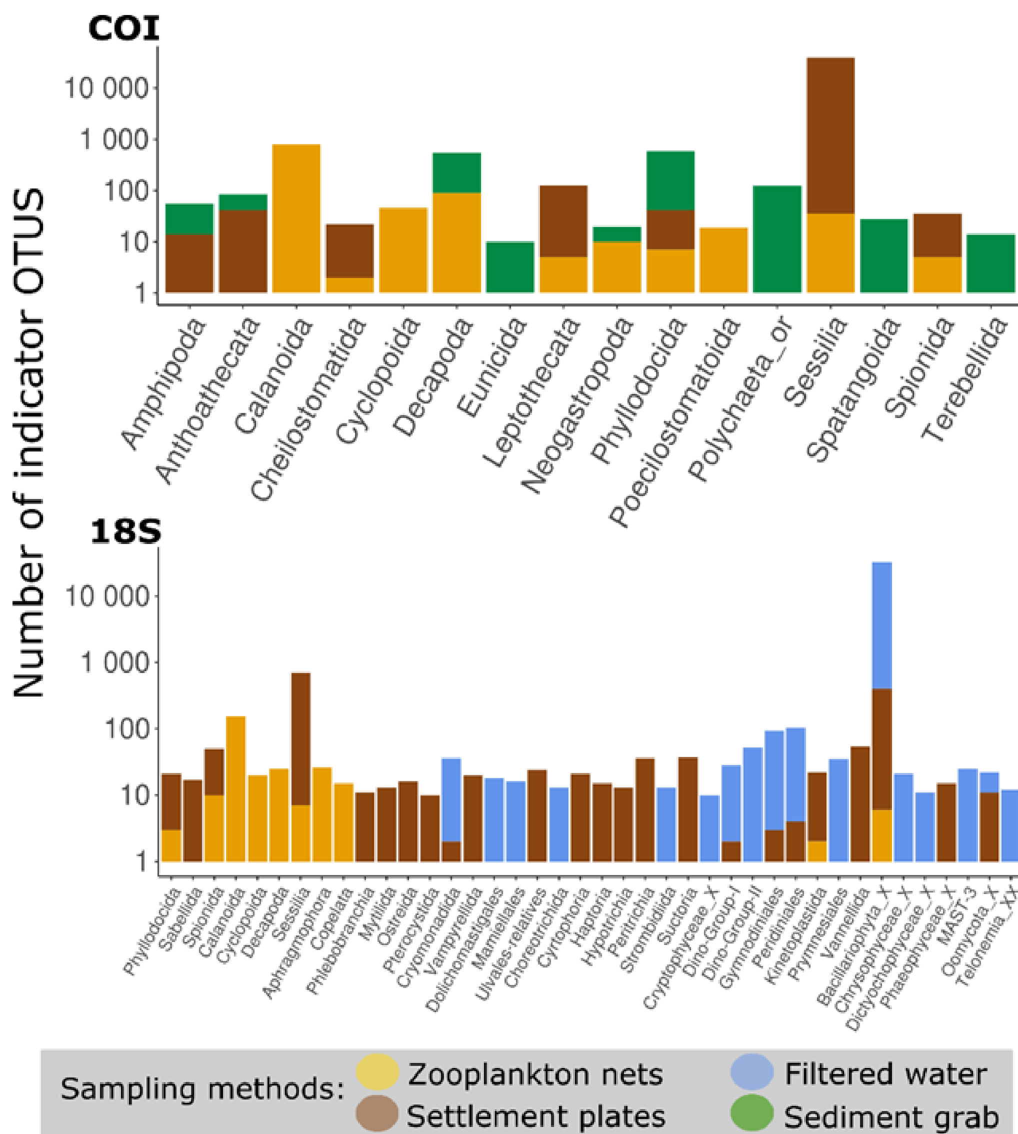


Figure 18. Distribution of indicator OTUs associated to each sampling method at the order level (in log scale) for COI and 18S. Only orders with at least 10 indicator OTUs are shown.

The need for complementarity of sampling methods was further confirmed by PCA on PBBS target taxa (Figure 19). For both barcodes, expected taxonomic differences were found between the sampling methods: sediment grabs were characterized by the polychaeta *Maldane glebifex* (Figure 19B), zooplankton nets were distinguished by several copepods (Figure 19B, D) and settlement plates, by the encrusting *Semibalanus balanoides* (Figure 19D). Filtered water communities were characterized differently according to each barcode. For COI, filtered water samples were not differentiated as a distinct group and were in general close to those retrieved with zooplankton samples collected at the same season (Figure 19A). In contrast, for 18S, filtered water was different due to the presence of the phytoplankton species *Phaeocystis globosa* (Figure 19D); yet, when targeting only metazoan taxa, the patterns observed with COI were similarly to the ones retrieved with 18S (Figure S12A). Concerning filtered samples, when looking only at metazoan taxa, the proportion of OTUs detected was largely different compared to the other methods (Figure S13). For instance, Decapoda orders had a low OTU diversity with filtered water (COI: 0,7%; 18S: 7%), whereas more OTU diversity could be detected for Calanoida (COI: 8%; 18S: 35%), Sabellida (COI: 15%; 18S: 28%) and Leptothecata (COI: 13%; 18S: 58%). In general, filtered water recovered the smallest metazoan OTU diversity.

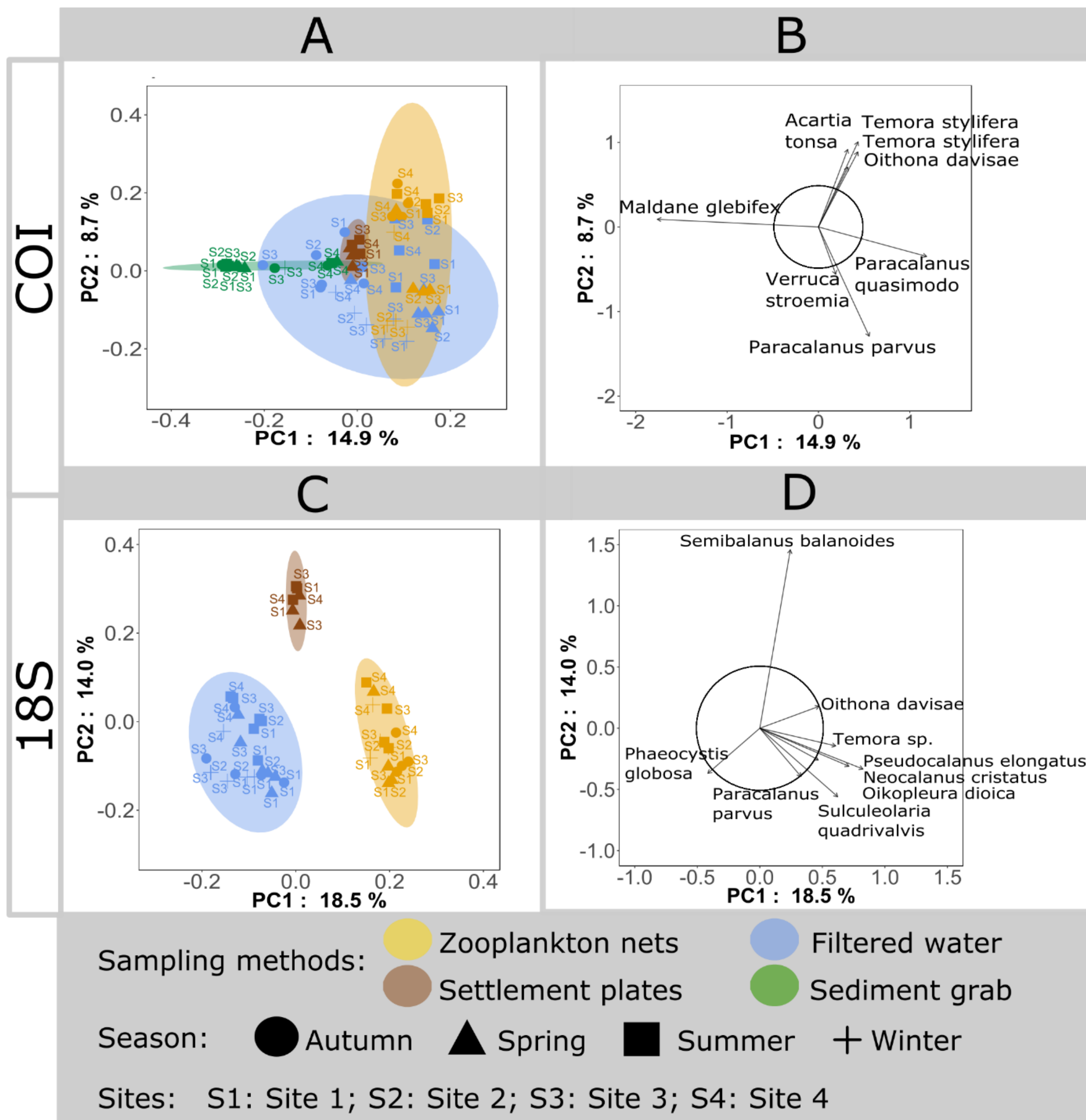


Figure 19. Principal Component Analyses of Hellinger-transformed abundances of OTUs included in the *PBBS targeted taxa dataset* for COI (A-B) and 18S (C-D). A & C: Samples scores in scaling 1 with ellipses representing the 95% confidence dispersion of each sampling method. B & D: OTU scores in scaling 1 with the circle of equilibrium contribution. Only OTUs whose projected length in these two principal component axes exceeding the value of equilibrium contribution are represented.

### 3.3 Influence of sampling seasonality and locality on detected biodiversity

Seasonal variations contributed significantly to differences in community composition of zooplankton nets and filtered water but not of sediment grabs (Table S5). Total OTU richness and unique OTU richness varied between seasons for all sampling methods (Figure 20A, B). Spring and late summer were generally richer than autumn and winter, excepted for filtered water communities depicted with 18S. For sediment grabs and zooplankton nets with 18S, a strong proportion of OTUs found in autumn and winter were a subset of OTUs retrieved in late summer and spring. Indeed, the nestedness component of sediment grab assemblages represented between 60 to 70% of total compositional variations among these pairs of seasons (Figure 20D). For zooplankton net assemblages, 63 and 72% of nestedness was observed between late summer and spring and between late summer and autumn respectively, suggesting that late summer recovered a majority of spring and autumn diversity (Figure 20D). Thus, the combination of spring and late summer for sediment grabs retrieved 85% of the total OTU richness over the four seasons, while for zooplankton nets, in late summer alone 71% was retrieved (Figure 20C, E). For filtered water and settlement plates, seasonal community changes were driven by both OTU replacement and nestedness, with similar relative contributions (Figure 20D). The seasonal influence observed in intra-port samples was also observed between ports (Figure S14). The differences in communities between Bilbao, A Coruña and Vigo (ports belonging to the same ecoregion but separated by over 500 km) during the same season was generally smaller than that between seasons in the same port, indicating that communities were driven by seasonality rather than location (Figure S14 and Table S6). This pattern was more pronounced with 18S than with COI.

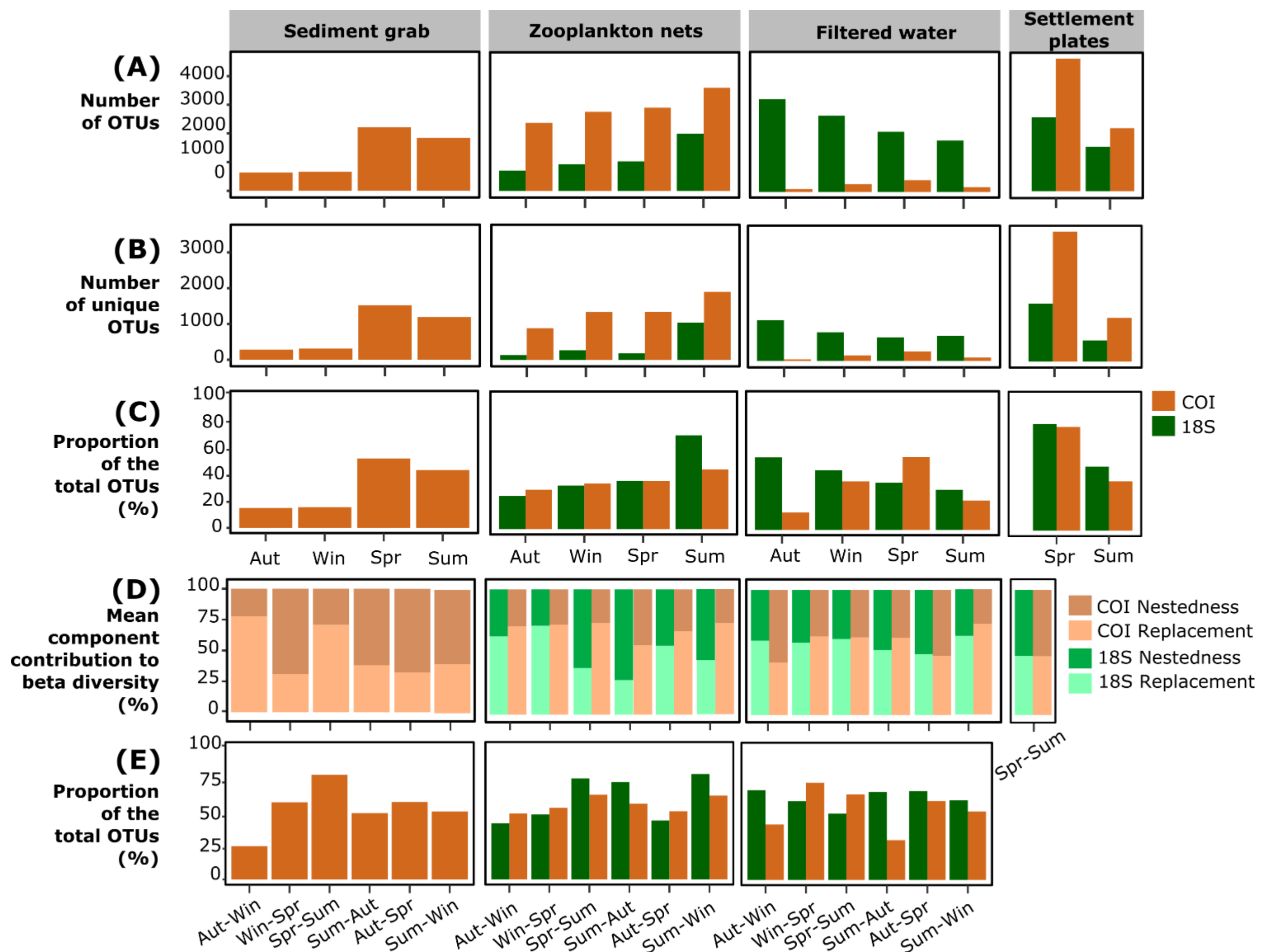


Figure 20. Seasonal variation of alpha and beta diversity for each sampling method with COI and 18S. A: Total OTU richness recovered at each season. B: OTU richness unique to each season. C: Proportion of the total OTU richness detected with one season. D: Decomposition of between-season beta diversity into replacement and nestedness components. E: Proportion of the total OTU richness detected with two seasons.

Locality within the Bilbao port appeared to impact on benthic assemblages (Table S5), since sites outside the estuary (sites 1 to 3) were different from site 4 inside the estuary; mainly, the polychaete *Maldane glebifex* was less abundant in site 4 (Figure 19B). Regarding zooplankton nets, site 4 was the main driver of difference in communities as, when not considered, no significant differences between sites were observed (Table S5). Each site harbored a similar proportion of the total OTU richness found by each sampling method (Figure 21C). This proportion did not exceed 60%. Indeed, OTU replacement contributed more to community variation among sites than nestedness, especially with sediment grab and settlement plates (Figure 21D). OTU replacement was more important when

comparing sites from outside the estuary (sites 1, 2 and S) with site 4 inside the estuary, while it contributed less to community variation among sites 1,2 and 3. This is congruent with site 4 having generally more unique OTUs than the other sites (Figure 21B). An exception was observed for filtered water with COI, where site 4 had the lowest OTU richness and unique OTU richness, and where nestedness contributed more to the variation in community composition in comparison to sites 1 and 2 (Figure 21A, B, D).

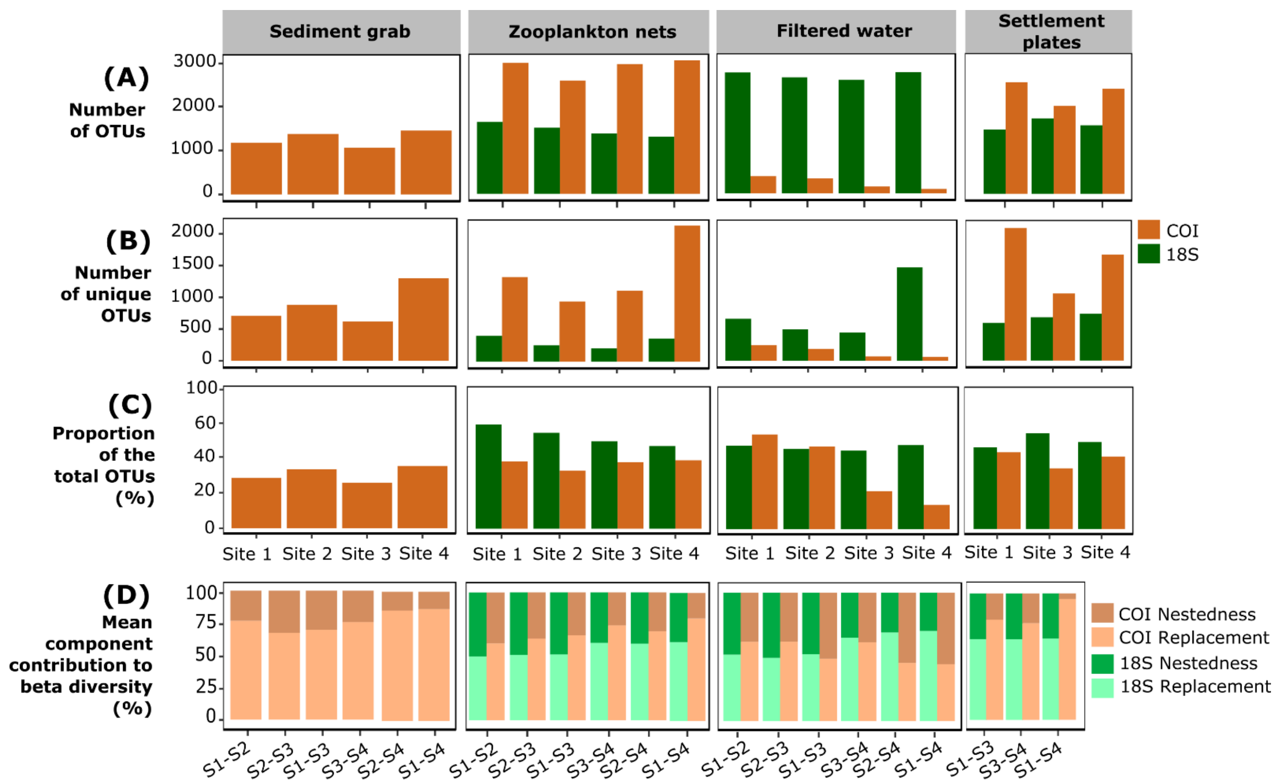


Figure 21. Spatial variation of alpha and beta diversity for each sampling method with COI and 18S. A: Total OTU richness recovered at each site. B: OTU richness unique to each site. C: Proportion of the total OTU richness detected with one site. D: Decomposition of between-site beta diversity into replacement and nestedness components

### 3.4 Detection of Non-Indigenous and Cryptogenic Species (NICS)

Our port baseline biodiversity survey detected 79 NICS, among which 29 were previously recorded in the port Bilbao (Table S7). Most of the other species (43) were previously detected NICS inside the port's Large Marine Ecoregion (LME) but, for 7 of them (*Ammothea hilgendorfi, Bugulina fulva, Grandidierella japonica, Melita nitida, Neodexiospira brasiliensis, Pseudochattonella verruculosa, Tubificoides pseudogaster*), it was, to our knowledge, the first report in the LME. The indicator species analysis performed on non-indigenous OTU revealed that site 4 was associated to the highest non-indigenous and cryptogenic diversity with, for COI, 603 OTU indicators, and for 18S, 24 OTU indicators. These OTUs belonged to 10 NICS with *Oithona davisae*, *Acartia tonsa* and *Allita succinea*, found by both barcodes, *Ficopomatus enigmaticus,* with 18S and *Amphibalanus eburneus*, *Grandidierella japonica*, *Polydora cornuta*, *Austrominius modestus*, *Monocorophium acherusicum*, *Xenostrobus securis,* with COI. Regarding the three other sites, for COI, site 1 was associated to one *Clytia hemisphaerica* OTU and to one *Balanus trigonus* OTU, site 2, to three *Clytia hemisphaerica* and four *Mytilus edulis* OTUs and site 3, to one *Balanus trigonus* OTU. For 18S, only site 1 was associated to one OTU, assigned to *Gymnodinium aureolum.*

## 4 Discussion

In order to be comprehensive, port baseline surveys need to have an extensive spatial and temporal coverage so that most biodiversity is captured by the collected samples. Unlike morphological taxonomic identification, metabarcoding allows the analysis of hundreds of samples is a cost-effective manner (Aylagas et al., 2018), enabling for comprehensive PBBS. Metabarcoding-based PBBS can follow a taxonomy dependent or independent approach. The first approach requires comparing the sequences against reference databases for taxonomic assignment, whose accuracy strongly depends on the completeness of the reference database; this reference database is particularly relevant for detection of NICS. The second approach consists on inferring diversity metrics from sequences to compare across samples for which the development of standardized procedures is mandatory so that comparisons are possible. Here we have focused on developing such procedures by evaluating alternative strategies.

## 4.1 A comprehensive metabarcoding-based port baseline survey should rely on a combination of sampling methods

Past attempts on using metabarcoding for port monitoring have relied on a unique sampling method (Borrell et al., 2017; Brown et al., 2016; Grey et al., 2018; Lacoursière-Roussel et al., 2018; Zaiko et al., 2016) and all have neglected sediment. Here, in order to develop a comprehensive port sampling protocol, we have adapted the HELCOM/OSPAR guidelines to collect zooplankton, fouling, and sediment samples for genetic analyses. The metabarcoding analysis of the collected samples has shown that each sampling method recovered a distinct subset of the port community, and that, despite some taxa being expectedly associated to a given sampling method, the total diversity, even within specific taxonomic groups, was only recovered by combining different methods.

## 4.2 eDNA cannot be used as an alternative to conventional sampling methods for macroorganism detection

As an attempt to find a sampling method that would capture a large fraction of the diversity with limited effort, we also explored metabarcoding on eDNA extracted from filtered water samples. Recent applications of this technique have shown its potential to recover spatial and temporal variation of macroorganismal communities (Bista et al., 2017; O'Donnell et al., 2017), including detection of non-indigenous taxa (Klymus et al., 2017). Consequently, recent studies have considered eDNA for port surveys (Borrell et al., 2017; Grey et al., 2018; Lacoursière-Roussel et al., 2018); yet, none of them has evaluated the macroorganismal biodiversity assessments obtained with eDNA in comparison to that obtained by other more conventional sampling methods. As suggested by other studies (Djurhuus et al. (2018) and Macher et al. (2018)), our analyses show that, compared to conventional sampling methods, eDNA recovered only a subset of the metazoan diversity and did not provide additional information on targeted groups. This suggests that, for PBBS, eDNA for macroorganismal detection does not provide additional benefits compared to conventional sampling for PBBS taxa detection. Yet, additional tests using

group specific markers (*e.g.* 12S rRNA for chordates) could provide more insights into the benefits of including eDNA for PBBS. Importantly, these tests will be possible with little additional effort because filtered water still needs to be collected for assessing phytoplankton diversity.

### 4.3 A comprehensive metabarcoding-based port baseline survey should include spatiotemporal sampling

While it is expected that increased temporal coverage will retrieve more taxa, the costs associated to extensive sampling demands an evaluation of the benefits gained by sampling at each season. Indeed, the HELCOM/OSPAR protocol limits sampling to late summer for sediment and fouling, and to spring and late summer for plankton. Here we show that for sediment and fouling, spring sampling provides higher diversity than late summer. Although sampling in late summer could be appropriate for morphological taxonomy because of the more abundance of adult individuals, for metabarcoding, sampling in spring is preferable because during this season i) sizes of organisms are less variable and thus metabarcoding is less likely to under detect small organisms (Elbrecht et al., 2017a) and ii) species diversity is at its maxima due to being a high recruitment period with abundant organisms at early life stages (Bijleveld et al., 2018). For zooplankton diversity, our results show that the HELCOM/OSPAR approach of sampling in spring and late summer produces the highest diversity. However, the data obtained from filtered water were inconclusive. Although spring was the most diverse season for COI, this was not the case for 18S, and, overall, other combinations of seasons appeared to perform better. Previous studies have already shown that seasonal variations are important considerations for eDNA studies (Lacoursière-Roussel et al., 2018), but further characterizations are needed over multiple years to design an adequate protocol for maximizing biodiversity recovery with eDNA. Our study also highlights the influence of sampling site in recovered biodiversity, although only one of the four sites sampled, the less saline one located at the inner part of the estuary, showed a significantly different community composition.

Concerning spatial sampling, we observed no significant differences between sites when using eDNA, except between the only site located in the inner estuary, and the others and only when using zooplankton, and significant differences overall when using sediment grabs. The proportion of the total OTUs found in a single site was low, suggesting that spatially comprehensive sampling is crucial to recover the port´s biodiversity. Interestingly, site 4 was not only the most different from all four sites but was also the one that recorded the largest number of NIS. In coherence with our findings, it has been observed that brackish environments favors NIS settlement (Zorita et al., 2013) because these species usually support wider range of salinity (Cardeccia et al., 2018). Thus, samples for port monitoring should include those with a wide range of abiotic conditions and covering priority sampling, such as highly active ship berths, potential reservoirs of newly arrived NIS (Hewitt and Martin, 2001).

### 4.4 Metabarcoding provides valuable information on Non-Indigenous Species

Our metabarcoding port biodiversity baseline survey detects species previously recorded in the port of Bilbao and species known to be present in the port´s large marine ecoregion. Importantly, it also detects presence of seven species for which no records exist so far in this ecoregion, highlighting the potential of metabarcoding for early NICS detection. Nonetheless, not all NICS species previously recorded in Bilbao were found by our analyses. This might be due to these species not being present in the port at the time of our survey, to database incompleteness or to biases of the metabarcoding process such as differential DNA extraction, primer non-specificity, etc (Xiong et al., 2016). Database incompleteness and primer bias could also explain why 35 and 42% of NICS were uniquely detected with COI and 18S respectively and stresses the need of increasing reference databases and of using multiple universal primers for species detection (Grey et al., 2018). Importantly, our study confirmed that metabarcoding can detect species occurring at low abundance (Pochon et al., 2013) as we found the non-indigenous amphipod *Melita nitida*

with only 18 reads while it took intensive surveys in 2013, 2014 and 2016 in 3 distinct sampling regions of the Bay of Biscay to record 76 individuals (Gouillieux et al., 2016).

## 5 Conclusions

Based on the comparative analysis of more than two hundred samples assessing the use of alternative sampling methods, and of sampling at different seasons of the year and at different port locations, we have demonstrated the suitability of metabarcoding for port biodiversity surveys and NIS monitoring and settled the guidelines for future studies. We show that i) combining two pairs of universal primers provides a more holistic view of the port biodiversity, ii) a combination of sampling methods is necessary to recover the different taxonomic groups, iii) environmental DNA cannot replace traditional sampling; vi) sampling should take place in spring and late summer preferably and v) spatial coverage should cover the port's salinity gradient. These guidelines and considerations are particularly relevant due to the recent entry into force of the International Convention for the Control and Management of Ships' Ballast Water and Sediments.

## 6 Acknowledgments

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## 7 Supplementary figures

Figure S9. Picture of the port depicting location of each site sampled. A: Examples of the different types of structures at each site. B: Fouling communities settled on plates after 3 months. Picture frames are colored according to the site they were taken at.

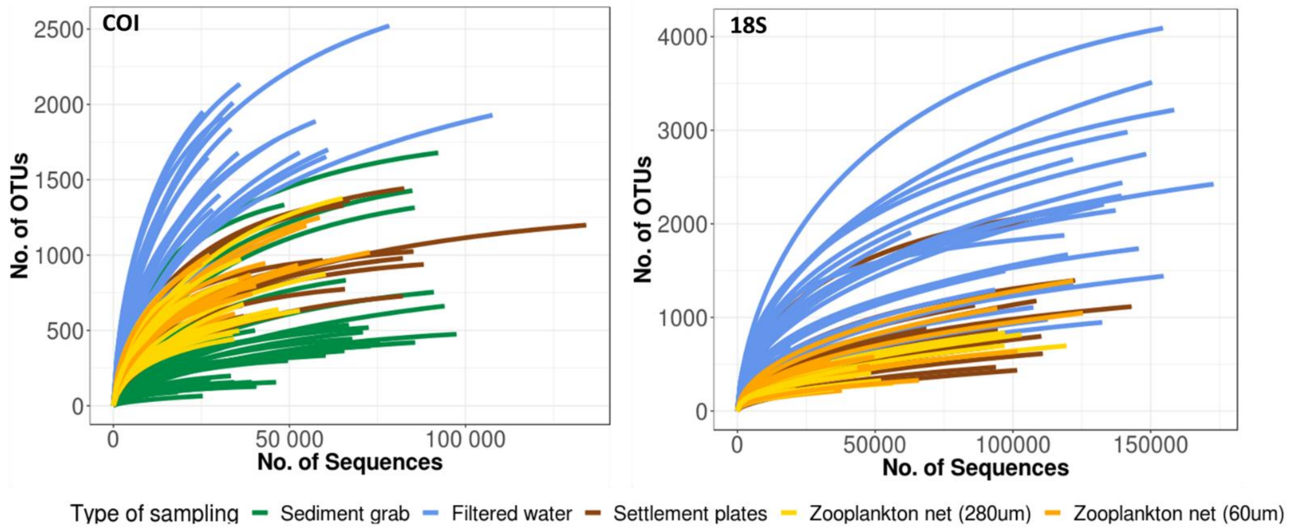


Figure S10. OTU accumulation curves per sample for COI and 18S barcodes

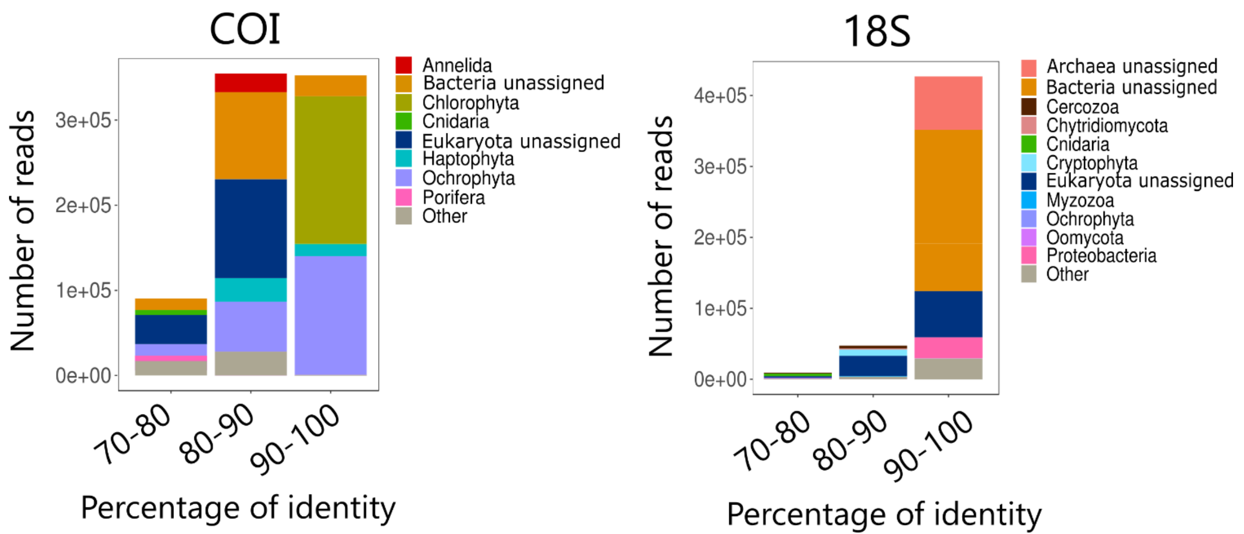


Figure S11. Number of filtered water reads assigned to each phylum for three percent identity ranges when blasting the unclassified reads (*i.e.* reads not classified below the phylum level) against NCBI. The 5 most abundant phyla per percent identity range are represented, the remaining being grouped under “other”.

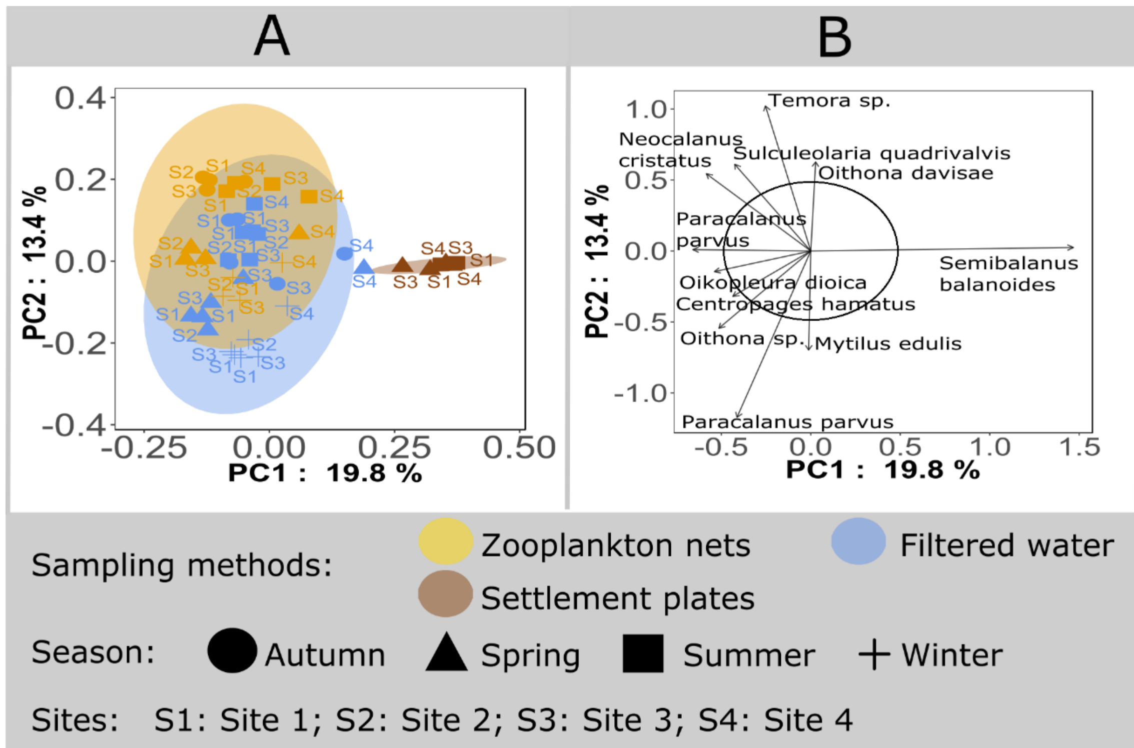


Figure S12. Principal Component Analyses of the Hellinger-transformed abundances of OTU included in the PBBS targeted metazoan taxa dataset for 18S. A: Samples scores in scaling 1 with ellipses representing the 95% confidence dispersion of each sampling method. B: OTU scores in scaling 1 with the circle of equilibrium contribution. Only OTUs whose projected length in these two principal component axes exceeding the value of equilibrium contribution are represented.

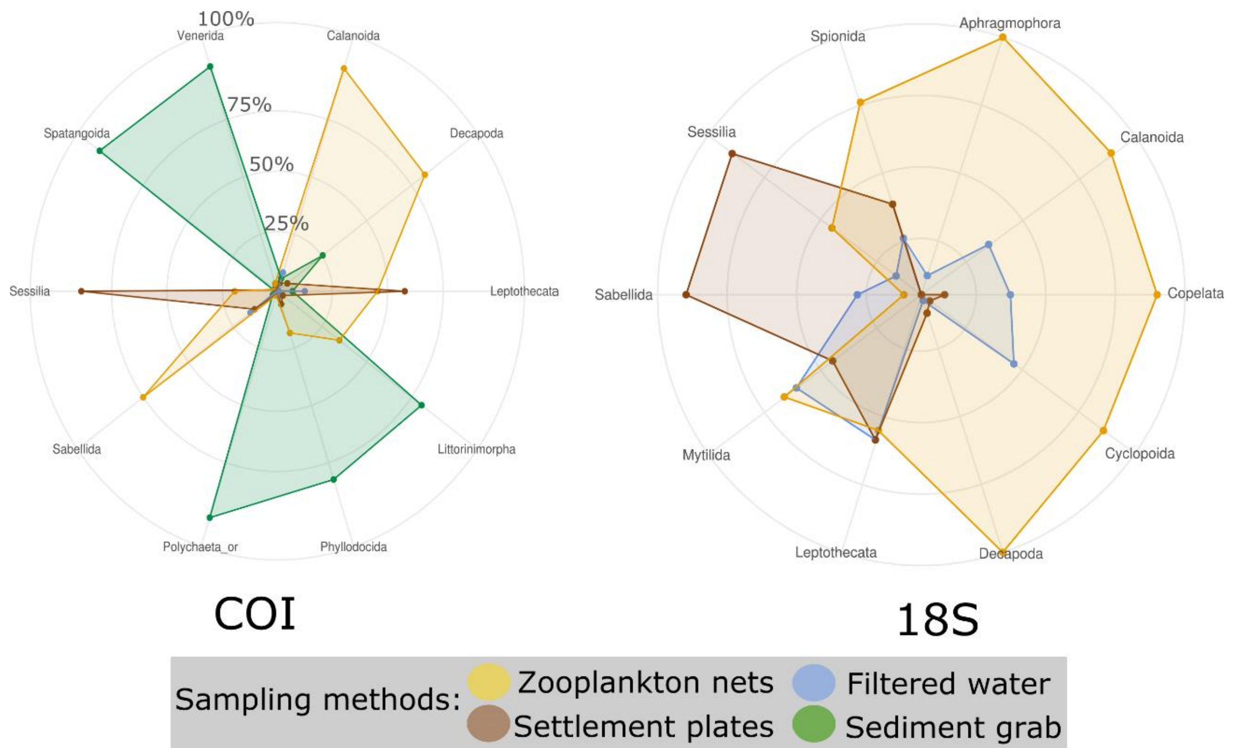


Figure S13. Percentage of the total OTU richness detected by each sampling method for the 10 most diverse metazoan orders of the PBBS targeted taxa dataset for each barcode.

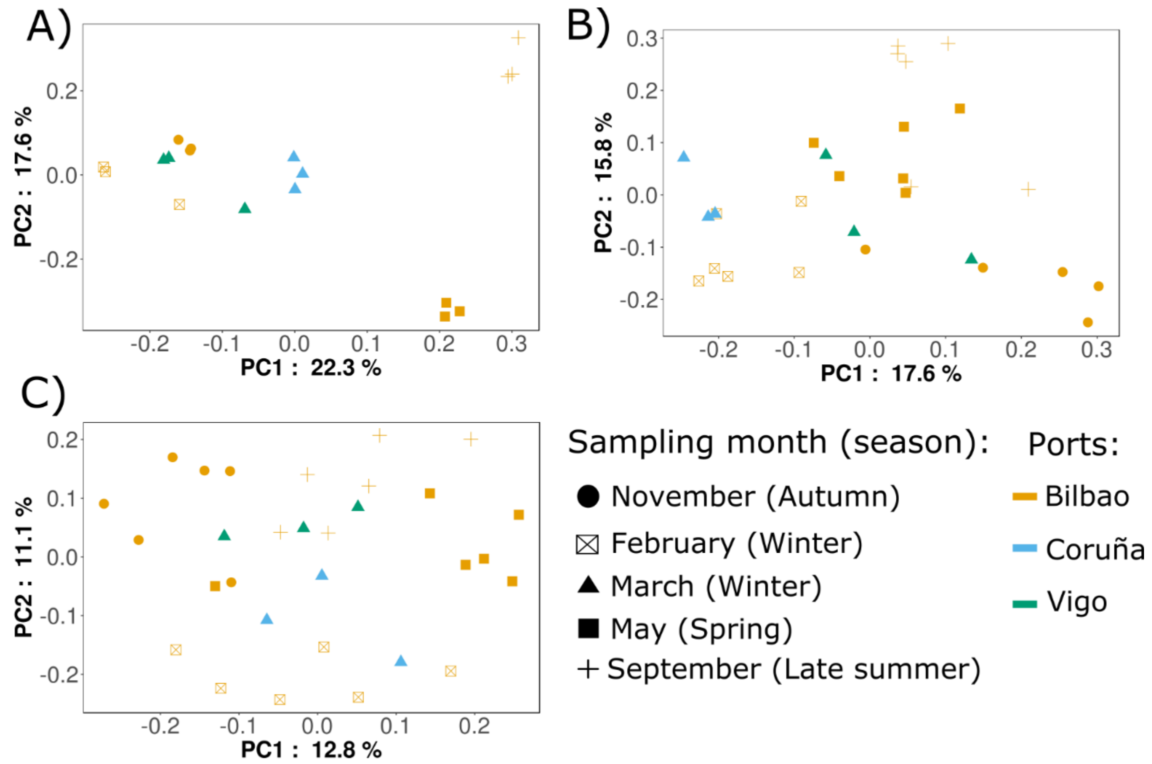


Figure S14. Principal Component Analyses (PCA) of the Hellinger-transformed abundances of OTUs included in the PBBS targeted taxa dataset of Vigo, A Coruña and Bilbao filtered water samples. Sample scores are displayed in scaling 1. A: PCA performed with 18S. B: PCA performed with 18S targeting only metazoan. C: PCA performed with COI.

# 8 Supplementary tables

Table S4. Sample information and number of sequences remaining after each pre-processing step. Note that sediment grabs and settlement plates recovered in the first week of October (labelled with an asterisk) are grouped with the "late summer" samples in the text; this is because, although sampling was planned in late September, bad meteorological conditions delayed the sampling for 10 days.

| Sample ID | Barcode | Sample type | Port | Depth | Sampling date (day/month/year) | Site | Replicate | # raw reads | # reads after primer removal | # reads after paired-end merging | # reads after quality filtering | # reads after nonaligned removal | # reads after chimera removal | # reads after singleton OTU removal |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **COI W01** | COI | filtered water | Bilbao | surface | 22/11/2016 | site 2 | 1 | 31778 | 31291 | 29495 | 29495 | 29406 | 27253 | 24277 |
| **COI W02** | COI | filtered water | Bilbao | surface | 22/11/2016 | site 3 | 1 | 39265 | 38581 | 36117 | 36117 | 36052 | 33630 | 30221 |
| **COI W03** | COI | filtered water | Bilbao | surface | 22/11/2016 | site 4 | 1 | 40691 | 39952 | 7551 | 7551 | 7468 | 7257 | 6476 |
| **COI W04** | COI | filtered water | Bilbao | bottom | 22/11/2016 | site 1 | 1 | 46432 | 45530 | 42036 | 42036 | 41898 | 38340 | 34655 |
| **COI W05** | COI | filtered water | Bilbao | bottom | 22/11/2016 | site 3 | 1 | 48876 | 48042 | 44234 | 44234 | 43965 | 39650 | 35952 |
| **COI W06** | COI | filtered water | Bilbao | surface | 03/02/2017 | site 1 | 1 | 51695 | 50720 | 43101 | 43101 | 42948 | 37566 | 33997 |
| **COI W07** | COI | filtered water | Bilbao | surface | 03/02/2017 | site 2 | 1 | 45931 | 44755 | 39443 | 39443 | 39282 | 36247 | 33434 |
| **COI W08** | COI | filtered water | Bilbao | surface | 03/02/2017 | site 3 | 1 | 36893 | 36325 | 30898 | 30898 | 30801 | 28944 | 26826 |
| **COI W09** | COI | filtered water | Bilbao | surface | 03/02/2017 | site 4 | 1 | 39568 | 38854 | 8377 | 8377 | 8128 | 7868 | 7098 |
| **COI W10** | COI | filtered water | Bilbao | bottom | 03/02/2017 | site 1 | 1 | 49050 | 48225 | 39525 | 39525 | 39272 | 34280 | 30918 |
| **COI W11** | COI | filtered water | Bilbao | bottom | 03/02/2017 | site 3 | 1 | 40098 | 39391 | 32466 | 32466 | 32293 | 28331 | 25502 |
| **COI W12** | COI | filtered water | A Coruña | surface | 22/03/2017 | site 1 | 1 | 62036 | 60537 | 32103 | 32103 | 31223 | 30053 | 27831 |
| **COI W13** | COI | filtered water | Bilbao | surface | 22/11/2016 | site 1 | 1 | 44746 | 43989 | 41784 | 41784 | 41664 | 39155 | 35496 |
| **COI W15** | COI | filtered water | Bilbao | surface | 31/05/2017 | site 1 | 1 | 69927 | 68366 | 60020 | 60020 | 59858 | 56948 | 52897 |
| **COI W16** | COI | filtered water | Bilbao | surface | 31/05/2017 | site 2 | 1 | 80837 | 79761 | 68935 | 68935 | 68213 | 65129 | 60438 |
| **COI W17** | COI | filtered water | Bilbao | surface | 31/05/2017 | site 3 | 1 | 41673 | 40822 | 32222 | 32222 | 32076 | 30195 | 28199 |
| **COI W18** | COI | filtered water | Bilbao | surface | 31/05/2017 | site 4 | 1 | 56885 | 56301 | 16874 | 16874 | 16745 | 16083 | 14935 |
| **COI W19** | COI | filtered water | Bilbao | bottom | 31/05/2017 | site 1 | 1 | 74522 | 73562 | 68857 | 68857 | 68633 | 65466 | 60992 |
| **COI W20** | COI | filtered water | Bilbao | bottom | 31/05/2017 | site 3 | 1 | 60165 | 59553 | 52623 | 52623 | 52471 | 49654 | 46077 |
| **COI W21** | COI | filtered water | Bilbao | surface | 05/09/2017 | site 1 | 1 | 81573 | 80638 | 69396 | 69396 | 68672 | 61801 | 57458 |
| **COI W22** | COI | filtered water | Bilbao | surface | 05/09/2017 | site 2 | 1 | 77006 | 76094 | 55950 | 55950 | 21303 | 19753 | 18398 |
| **COI W23** | COI | filtered water | Bilbao | surface | 05/09/2017 | site 3 | 1 | 82201 | 81045 | 47265 | 47265 | 46670 | 44508 | 41788 |
| **COI W24** | COI | filtered water | Bilbao | surface | 05/09/2017 | site 4 | 1 | 60620 | 60075 | 27504 | 27504 | 26988 | 25621 | 24136 |
| **COI W25** | COI | filtered water | A Coruña | surface | 22/03/2017 | site 2 | 1 | 54498 | 53616 | 35677 | 35677 | 35419 | 34060 | 32042 |
| **COI W26** | COI | filtered water | A Coruña | surface | 22/03/2017 | site 3 | 1 | 51630 | 51040 | 36659 | 36659 | 36470 | 34364 | 31628 |
| **COI W27** | COI | filtered water | Vigo | surface | 13/03/2017 | site 1 | 1 | 45098 | 44341 | 34555 | 34555 | 34504 | 31536 | 29348 |
| **COI W28** | COI | filtered water | Vigo | surface | 13/03/2017 | site 2 | 1 | 54052 | 53065 | 50096 | 50096 | 49261 | 48400 | 44830 |
| **COI W29** | COI | filtered water | Vigo | surface | 13/03/2017 | site 3 | 1 | 62377 | 61623 | 50636 | 50636 | 50356 | 47294 | 43896 |
| **COI W30** | COI | filtered water | Bilbao | bottom | 05/09/2017 | site 1 | 1 | 100345 | 99330 | 91182 | 91182 | 90585 | 83566 | 78355 |
| **COI W31** | COI | filtered water | Bilbao | bottom | 05/09/2017 | site 3 | 1 | 128427 | 126986 | 117786 | 117786 | 117391 | 113863 | 107656 |
| **B01** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 1 | 1 | 36493 | 35692 | 34252 | 34252 | 15804 | 15540 | 14806 |
| **B02** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 1 | 2 | 43012 | 41995 | 37201 | 37201 | 36571 | 36212 | 34782 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **B03** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 1 | 3 | 29374 | 28796 | 27926 | 27926 | 27855 | 26156 | 24645 |
| **B04** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 2 | 1 | 38286 | 37575 | 35837 | 35837 | 35742 | 34849 | 33386 |
| **B05** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 2 | 2 | 34207 | 33072 | 30750 | 30750 | 28947 | 28621 | 27104 |
| **B06** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 2 | 3 | 25205 | 24268 | 23389 | 23389 | 23289 | 22778 | 21599 |
| **B07** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 3 | 1 | 52491 | 51745 | 48711 | 48711 | 48084 | 48001 | 46189 |
| **B08** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 3 | 2 | 45142 | 43000 | 38373 | 38373 | 37703 | 37489 | 36090 |
| **B09** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 3 | 3 | 31974 | 30843 | 29662 | 29662 | 29502 | 29240 | 28092 |
| **B10** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 4 | 1 | 46438 | 45722 | 44154 | 44154 | 43214 | 42811 | 40604 |
| **B11** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 4 | 2 | 25920 | 25530 | 24655 | 24655 | 12165 | 12123 | 11511 |
| **B12** | COI | sediment grab | Bilbao | bottom | 20/12/2016 | site 4 | 3 | 38941 | 37978 | 36482 | 36482 | 36425 | 36339 | 34601 |
| **B13** | COI | sediment grab | Bilbao | bottom | 27/02/2017 | site 1 | 1 | 54226 | 49500 | 42867 | 42867 | 42607 | 42447 | 40635 |
| **B14** | COI | sediment grab | Bilbao | bottom | 27/02/2017 | site 1 | 2 | 50443 | 47957 | 41232 | 41232 | 41041 | 40975 | 39295 |
| **B15** | COI | sediment grab | Bilbao | bottom | 27/02/2017 | site 1 | 3 | 19360 | 18845 | 13107 | 13107 | 11226 | 11099 | 10500 |
| **B16** | COI | sediment grab | Bilbao | bottom | 27/02/2017 | site 2 | 1 | 22100 | 21112 | 19339 | 19339 | 19266 | 19040 | 18304 |
| **B17** | COI | sediment grab | Bilbao | bottom | 27/02/2017 | site 2 | 2 | 42278 | 41350 | 36426 | 36426 | 36358 | 36039 | 34419 |
| **B18** | COI | sediment grab | Bilbao | bottom | 27/02/2017 | site 2 | 3 | 17913 | 17559 | 17061 | 17061 | 9769 | 9526 | 9061 |
| **B19** | COI | sediment grab | Bilbao | bottom | 27/02/2017 | site 3 | 1 | 54040 | 46479 | 26367 | 26367 | 26323 | 26314 | 25371 |
| **B20** | COI | sediment grab | Bilbao | bottom | 27/02/2017 | site 3 | 2 | 36804 | 35641 | 32899 | 32899 | 32826 | 30859 | 29353 |
| **B21** | COI | sediment grab | Bilbao | bottom | 27/02/2017 | site 3 | 3 | 32119 | 30961 | 27573 | 27573 | 27224 | 27026 | 25972 |
| **B22** | COI | sediment grab | Bilbao | bottom | 03/02/2017 | site 4 | 1 | 32145 | 30517 | 26630 | 26630 | 18274 | 17920 | 17126 |
| **B23** | COI | sediment grab | Bilbao | bottom | 03/02/2017 | site 4 | 2 | 31322 | 30714 | 28847 | 28847 | 28751 | 28652 | 27612 |
| **B24** | COI | sediment grab | Bilbao | bottom | 03/02/2017 | site 4 | 3 | 30156 | 28410 | 24451 | 24451 | 24321 | 23978 | 22768 |
| **COIB 25** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 1 | 1 | 91717 | 89304 | 84857 | 84857 | 64845 | 64257 | 60320 |
| **COIB 26** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 1 | 2 | 109492 | 107474 | 106805 | 106805 | 103434 | 102239 | 97454 |
| **COIB 27** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 1 | 3 | 80984 | 79293 | 68276 | 68276 | 44576 | 43613 | 40241 |
| **COIB 28** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 2 | 1 | 102907 | 101388 | 93349 | 93349 | 90585 | 90071 | 85710 |
| **COIB 29** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 2 | 2 | 76830 | 75703 | 73909 | 73909 | 73479 | 72646 | 67870 |
| **COIB 30** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 2 | 3 | 77884 | 76865 | 74139 | 74139 | 73829 | 72772 | 69183 |
| **COIB 31** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 3 | 1 | 82121 | 80935 | 79550 | 79550 | 79090 | 78737 | 72528 |
| **COIB 32** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 3 | 2 | 103992 | 102384 | 100279 | 100279 | 80871 | 80230 | 75691 |
| **COIB 33** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 3 | 3 | 105460 | 104323 | 102994 | 102994 | 101043 | 99718 | 94070 |
| **COIB 34** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 4 | 1 | 112028 | 110473 | 104945 | 104945 | 103440 | 98466 | 92261 |
| **COIB 35** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 4 | 2 | 97456 | 96268 | 94722 | 94722 | 92543 | 91921 | 85606 |
| **COIB 36** | COI | sediment grab | Bilbao | bottom | 01/06/2017 | site 4 | 3 | 101578 | 100180 | 98328 | 98328 | 96204 | 91685 | 84947 |
| **COIB 37** | COI | sediment grab | Bilbao | bottom | 04/10/2017* | site 1 | 1 | 75321 | 73480 | 72395 | 72395 | 53123 | 52555 | 49604 |
| **COIB 38** | COI | sediment grab | Bilbao | bottom | 04/10/2017* | site 1 | 2 | 77083 | 73161 | 70897 | 70897 | 70185 | 69296 | 65629 |
| **COIB 39** | COI | sediment grab | Bilbao | bottom | 04/10/2017* | site 1 | 3 | 68123 | 67141 | 49709 | 49709 | 48971 | 48197 | 44108 |
| **COIB 40** | COI | sediment grab | Bilbao | bottom | 04/10/2017* | site 2 | 1 | 115044 | 112979 | 111580 | 111580 | 99213 | 96522 | 91025 |
| **COIB 41** | COI | sediment grab | Bilbao | bottom | 04/10/2017* | site 2 | 2 | 91015 | 87724 | 79827 | 79827 | 78418 | 77643 | 73017 |
| **COIB 42** | COI | sediment grab | Bilbao | bottom | 04/10/2017* | site 2 | 3 | 80526 | 79280 | 78000 | 78000 | 77837 | 75563 | 70885 |
| **COIB 43** | COI | sediment grab | Bilbao | bottom | 04/10/2017* | site 3 | 1 | 78492 | 77379 | 76149 | 76149 | 42070 | 41643 | 38065 |
| **COIB 44** | COI | sediment grab | Bilbao | bottom | 04/10/2017* | site 3 | 2 | 77397 | 75746 | 74146 | 74146 | 73331 | 71437 | 66908 |
| **COIB 45** | COI | sediment grab | Bilbao | bottom | 04/10/2017* | site 3 | 3 | 42359 | 41443 | 40175 | 40175 | 36023 | 35500 | 32752 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **COIB 46** | COI | sediment grab | Bilbao | bottom | 04/10/ 2017* | site 4 | 1 | 83168 | 81828 | 79206 | 79206 | 73402 | 71389 | 66028 |
| **COIB 47** | COI | sediment grab | Bilbao | bottom | 04/10/ 2017* | site 4 | 2 | 79472 | 78302 | 74878 | 74878 | 73599 | 71470 | 66869 |
| **COIB 48** | COI | sediment grab | Bilbao | bottom | 04/10/ 2017* | site 4 | 3 | 70208 | 68455 | 61788 | 61788 | 54137 | 52480 | 48555 |
| **COIP VC01** | COI | settlement plates | Bilbao | 1m | 27/06/ 2017 | site 1 | 1 | 85134 | 84243 | 83453 | 83453 | 83304 | 71283 | 65353 |
| **COIP VC02** | COI | settlement plates | Bilbao | 3m | 27/06/ 2017 | site 1 | 1 | 100539 | 99215 | 96421 | 96421 | 95491 | 93730 | 88153 |
| **COIP VC03** | COI | settlement plates | Bilbao | 7m | 27/06/ 2017 | site 1 | 1 | 95052 | 93342 | 92113 | 92113 | 91900 | 87598 | 82228 |
| **COIP VC14** | COI | settlement plates | Bilbao | 1m | 27/06/ 2017 | site 3 | 1 | 99834 | 97478 | 66467 | 66467 | 65705 | 63271 | 59445 |
| **COIP VC15** | COI | settlement plates | Bilbao | 3m | 27/06/ 2017 | site 3 | 1 | 88047 | 86628 | 75145 | 75145 | 74994 | 72172 | 67115 |
| **COIP VC16** | COI | settlement plates | Bilbao | 7m | 27/06/ 2017 | site 3 | 1 | 100915 | 99051 | 96893 | 96893 | 94214 | 91043 | 85223 |
| **COIP VC17** | COI | settlement plates | Bilbao | 1m | 27/06/ 2017 | site 4 | 1 | 150591 | 148618 | 143863 | 143863 | 143266 | 141954 | 134296 |
| **COIP VC18** | COI | settlement plates | Bilbao | 3m | 27/06/ 2017 | site 4 | 1 | 94730 | 93368 | 91495 | 91495 | 91324 | 88646 | 82627 |
| **COIP VC19** | COI | settlement plates | Bilbao | 7m | 27/06/ 2017 | site 4 | 1 | 92957 | 91627 | 91352 | 91352 | 89763 | 86237 | 82207 |
| **COIP VC28** | COI | settlement plates | Bilbao | 1m | 04/10/ 2017* | site 1 | 1 | 72143 | 70904 | 64376 | 64376 | 32715 | 31746 | 29181 |
| **COIP VC29** | COI | settlement plates | Bilbao | 3m | 04/10/ 2017* | site 1 | 1 | 53001 | 51942 | 51289 | 51289 | 51220 | 48404 | 44535 |
| **COIP VC30** | COI | settlement plates | Bilbao | 7m | 04/10/ 2017* | site 1 | 1 | 44359 | 43581 | 42953 | 42953 | 42088 | 38848 | 36501 |
| **COIP VC37** | COI | settlement plates | Bilbao | 1m | 04/10/ 2017* | site 3 | 1 | 64929 | 63894 | 45400 | 45400 | 44406 | 42279 | 39706 |
| **COIP VC38** | COI | settlement plates | Bilbao | 3m | 04/10/ 2017* | site 3 | 1 | 42316 | 41760 | 40774 | 40774 | 40651 | 39942 | 37108 |
| **COIP VC39** | COI | settlement plates | Bilbao | 7m | 04/10/ 2017* | site 3 | 1 | 73037 | 71825 | 70449 | 70449 | 70054 | 69372 | 65805 |
| **COIP VC41** | COI | settlement plates | Bilbao | 3m | 04/10/ 2017* | site 4 | 1 | 51549 | 50767 | 50195 | 50195 | 49844 | 47640 | 44788 |
| **COIP VC42** | COI | settlement plates | Bilbao | 7m | 04/10/ 2017* | site 4 | 1 | 54630 | 53884 | 52570 | 52570 | 52398 | 51852 | 48198 |
| **COIZ 26** | COI | zooplankton net (280 µm) | Bilbao | water column | 05/09/ 2017 | site 1 | 1 | 56513 | 55050 | 54021 | 54021 | 53377 | 51013 | 72858 |
| **COIZ 28** | COI | zooplankton net (280 µm) | Bilbao | water column | 05/09/ 2017 | site 2 | 1 | 62452 | 61237 | 60174 | 60174 | 57337 | 56383 | 46913 |
| **COIZ 30** | COI | zooplankton net (280 µm) | Bilbao | water column | 05/09/ 2017 | site 3 | 1 | 69231 | 67760 | 66330 | 66330 | 65642 | 63722 | 52478 |
| **COIZ 32** | COI | zooplankton net (280 µm) | Bilbao | water column | 05/09/ 2017 | site 4 | 1 | 81349 | 80328 | 75528 | 75528 | 75172 | 75137 | 52979 |
| **Z02A** | COI | zooplankton net (280 µm) | Bilbao | water column | 22/11/ 2016 | site 1 | 1 | 40893 | 40531 | 39764 | 39764 | 38643 | 38390 | 54818 |
| **Z04A** | COI | zooplankton net (280 µm) | Bilbao | water column | 22/11/ 2016 | site 2 | 1 | 39521 | 39074 | 38130 | 38130 | 37748 | 37457 | 60324 |
| **Z06A** | COI | zooplankton net (280 µm) | Bilbao | water column | 22/11/ 2016 | site 3 | 1 | 23872 | 23549 | 22908 | 22908 | 22402 | 22322 | 58606 |
| **Z08A** | COI | zooplankton net (280 µm) | Bilbao | water column | 22/11/ 2016 | site 4 | 1 | 43546 | 43202 | 42460 | 42460 | 41146 | 40893 | 65093 |
| **Z10A** | COI | zooplankton net (280 µm) | Bilbao | water column | 03/02/ 2017 | site 1 | 1 | 37677 | 37305 | 36840 | 36840 | 36716 | 35958 | 40559 |
| **Z12A** | COI | zooplankton net (280 µm) | Bilbao | water column | 03/02/ 2017 | site 2 | 1 | 37951 | 37564 | 36717 | 36717 | 36630 | 36246 | 35755 |
| **Z14A** | COI | zooplankton net (280 µm) | Bilbao | water column | 03/02/ 2017 | site 3 | 1 | 28614 | 28202 | 27517 | 27517 | 27433 | 27123 | 33806 |
| **Z16A** | COI | zooplankton net (280 µm) | Bilbao | water column | 03/02/ 2017 | site 4 | 1 | 42491 | 42149 | 41376 | 41376 | 41339 | 41038 | 39492 |
| **Z18A** | COI | zooplankton net (280 µm) | Bilbao | water column | 31/05/ 2017 | site 1 | 1 | 36918 | 36533 | 30606 | 30606 | 30140 | 29276 | 18195 |
| **Z20A** | COI | zooplankton net (280 µm) | Bilbao | water column | 31/05/ 2017 | site 2 | 1 | 31607 | 31243 | 26001 | 26001 | 25652 | 25055 | 34544 |
| **Z22A** | COI | zooplankton net (280 µm) | Bilbao | water column | 31/05/ 2017 | site 3 | 1 | 29304 | 29018 | 26092 | 26092 | 25905 | 25228 | 37019 |
| **Z24A** | COI | zooplankton net (280 µm) | Bilbao | water column | 31/05/ 2017 | site 4 | 1 | 42635 | 42236 | 41318 | 41318 | 41236 | 40992 | 36786 |
| **COIZ 25** | COI | zooplankton net (60 µm) | Bilbao | water column | 05/09/ 2017 | site 1 | 1 | 86368 | 85564 | 79973 | 79973 | 77273 | 76888 | 34349 |
| **COIZ 27** | COI | zooplankton net (60 µm) | Bilbao | water column | 05/09/ 2017 | site 2 | 1 | 61130 | 60078 | 58739 | 58739 | 56998 | 55811 | 33929 |
| **COIZ 29** | COI | zooplankton net (60 µm) | Bilbao | water column | 05/09/ 2017 | site 3 | 1 | 63009 | 61771 | 60722 | 60722 | 60250 | 58777 | 33423 |
| **COIZ 31** | COI | zooplankton net (60 µm) | Bilbao | water column | 05/09/ 2017 | site 4 | 1 | 72011 | 70453 | 67052 | 67052 | 66948 | 66682 | 43190 |
| **Z01A** | COI | zooplankton net (60 µm) | Bilbao | water column | 22/11/ 2016 | site 1 | 1 | 47554 | 47005 | 45309 | 45309 | 44046 | 43574 | 25110 |
| **Z05A** | COI | zooplankton net (60 µm) | Bilbao | water column | 22/11/ 2016 | site 3 | 1 | 43554 | 43132 | 42351 | 42351 | 42165 | 41843 | 39090 |
| **Z07A** | COI | zooplankton net (60 µm) | Bilbao | water column | 22/11/ 2016 | site 4 | 1 | 38312 | 38044 | 37653 | 37653 | 37623 | 37555 | 35392 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Z09A** | COI | zooplankton net (60 µm) | Bilbao | water column | 03/02/ 2017 | site 1 | 1 | 40710 | 40354 | 39865 | 39865 | 39460 | 38866 | 27452 |
| **Z11A** | COI | zooplankton net (60 µm) | Bilbao | water column | 03/02/ 2017 | site 2 | 1 | 36790 | 36440 | 35891 | 35891 | 35657 | 35177 | 25043 |
| **Z13A** | COI | zooplankton net (60 µm) | Bilbao | water column | 03/02/ 2017 | site 3 | 1 | 47959 | 47503 | 47005 | 47005 | 46862 | 45632 | 29902 |
| **Z15A** | COI | zooplankton net (60 µm) | Bilbao | water column | 03/02/ 2017 | site 4 | 1 | 44259 | 43876 | 43252 | 43252 | 43194 | 43069 | 21837 |
| **Z17A** | COI | zooplankton net (60 µm) | Bilbao | water column | 31/05/ 2017 | site 1 | 1 | 37020 | 36657 | 32598 | 32598 | 32229 | 31683 | 25080 |
| **Z19A** | COI | zooplankton net (60 µm) | Bilbao | water column | 31/05/ 2017 | site 2 | 1 | 37991 | 37652 | 34037 | 34037 | 33634 | 33169 | 22113 |
| **Z21A** | COI | zooplankton net (60 µm) | Bilbao | water column | 31/05/ 2017 | site 3 | 1 | 35512 | 35194 | 28849 | 28849 | 28646 | 28139 | 34879 |
| **Z23A** | COI | zooplankton net (60 µm) | Bilbao | water column | 31/05/ 2017 | site 4 | 1 | 38518 | 38166 | 37626 | 37626 | 37599 | 37451 | 36249 |
| **COI W32** | COI | filtered water (extraction negative control) | na | na | na | na | 1 | 3479 | 3117 | 169 | 169 | 166 | 164 | 132 |
| **COI W14** | COI | filtered water (extraction negative control) | na | na | na | na | 2 | 163 | 91 | 69 | 69 | 67 | 66 | 49 |
| **COIC tr** | COI | filtered water (PCR negative control) | na | na | na | na | 1 | 811 | 228 | 215 | 215 | 213 | 213 | 178 |
| **18SP VC01** | 18S | settlement plates | Bilbao | 1m | 27/06/ 2017 | site 1 | 1 | 123041 | 114368 | 114354 | 114354 | 109034 | 109017 | 108565 |
| **18SP VC02** | 18S | settlement plates | Bilbao | 3m | 27/06/ 2017 | site 1 | 1 | 78447 | 72550 | 72538 | 72538 | 68992 | 68956 | 68607 |
| **18SP VC03** | 18S | settlement plates | Bilbao | 7m | 27/06/ 2017 | site 1 | 1 | 60778 | 55546 | 55536 | 55536 | 54686 | 54629 | 54375 |
| **18SP VC14** | 18S | settlement plates | Bilbao | 1m | 27/06/ 2017 | site 3 | 1 | 145381 | 136801 | 136757 | 136757 | 129806 | 129796 | 128858 |
| **18SP VC15** | 18S | settlement plates | Bilbao | 3m | 27/06/ 2017 | site 3 | 1 | 95286 | 89386 | 89363 | 89363 | 86783 | 86766 | 86226 |
| **18SP VC16** | 18S | settlement plates | Bilbao | 7m | 27/06/ 2017 | site 3 | 1 | 80079 | 73552 | 73542 | 73542 | 72423 | 72379 | 71834 |
| **18SP VC17** | 18S | settlement plates | Bilbao | 1m | 27/06/ 2017 | site 4 | 1 | 129830 | 121250 | 121223 | 121223 | 117756 | 117745 | 116990 |
| **18SP VC18** | 18S | settlement plates | Bilbao | 3m | 27/06/ 2017 | site 4 | 1 | 101684 | 95396 | 95374 | 95374 | 93138 | 93122 | 92699 |
| **18SP VC19** | 18S | settlement plates | Bilbao | 7m | 27/06/ 2017 | site 4 | 1 | 103742 | 95369 | 95364 | 95364 | 92095 | 91983 | 91230 |
| **18SP VC28** | 18S | settlement plates | Bilbao | 1m | 04/10/ 2017* | site 1 | 1 | 157551 | 146503 | 146460 | 146460 | 143976 | 143879 | 143073 |
| **18SP VC29** | 18S | settlement plates | Bilbao | 3m | 04/10/ 2017* | site 1 | 1 | 123901 | 114741 | 114735 | 114735 | 111304 | 111294 | 110864 |
| **18SP VC30** | 18S | settlement plates | Bilbao | 7m | 04/10/ 2017* | site 1 | 1 | 102975 | 95971 | 95966 | 95966 | 94201 | 94166 | 93865 |
| **18SP VC37** | 18S | settlement plates | Bilbao | 1m | 04/10/ 2017* | site 3 | 1 | 133517 | 126613 | 126591 | 126591 | 123331 | 123298 | 122588 |
| **18SP VC38** | 18S | settlement plates | Bilbao | 3m | 04/10/ 2017* | site 3 | 1 | 105133 | 97121 | 97107 | 97107 | 95348 | 95086 | 94472 |
| **18SP VC39** | 18S | settlement plates | Bilbao | 7m | 04/10/ 2017* | site 3 | 1 | 111897 | 103978 | 103976 | 103976 | 101987 | 101908 | 101553 |
| **18SP VC41** | 18S | settlement plates | Bilbao | 3m | 04/10/ 2017* | site 4 | 1 | 103154 | 95596 | 95587 | 95587 | 93584 | 93192 | 92766 |
| **18SP VC42** | 18S | settlement plates | Bilbao | 7m | 04/10/ 2017* | site 4 | 1 | 124552 | 112761 | 112747 | 112747 | 111691 | 111257 | 110340 |
| **18SW 01** | 18S | filtered water | Bilbao | surface | 22/11/ 2016 | site 2 | 1 | 135513 | 127580 | 127552 | 127552 | 123342 | 123191 | 121889 |
| **18SW 02** | 18S | filtered water | Bilbao | surface | 22/11/ 2016 | site 3 | 1 | 166710 | 156214 | 156165 | 156165 | 152813 | 152672 | 150559 |
| **18SW 03** | 18S | filtered water | Bilbao | surface | 22/11/ 2016 | site 4 | 1 | 169264 | 160261 | 160195 | 160195 | 156733 | 156668 | 154495 |
| **18SW 04** | 18S | filtered water | Bilbao | bottom | 22/11/ 2016 | site 1 | 1 | 130338 | 122981 | 122936 | 122936 | 119618 | 119603 | 118705 |
| **18SW 06** | 18S | filtered water | Bilbao | surface | 03/02/ 2017 | site 1 | 1 | 168156 | 158881 | 158833 | 158833 | 134650 | 134453 | 133008 |
| **18SW 07** | 18S | filtered water | Bilbao | surface | 03/02/ 2017 | site 2 | 1 | 153522 | 144650 | 144601 | 144601 | 141390 | 141234 | 139762 |
| **18SW 08** | 18S | filtered water | Bilbao | surface | 03/02/ 2017 | site 3 | 1 | 72513 | 65390 | 65374 | 65374 | 63875 | 63787 | 62971 |
| **18SW 09** | 18S | filtered water | Bilbao | surface | 03/02/ 2017 | site 4 | 1 | 176785 | 166863 | 166841 | 166841 | 160743 | 160678 | 158594 |
| **18SW 10** | 18S | filtered water | Bilbao | bottom | 03/02/ 2017 | site 1 | 1 | 39670 | 22069 | 22045 | 22045 | 19869 | 19869 | 19433 |
| **18SW 11** | 18S | filtered water | Bilbao | bottom | 03/02/ 2017 | site 3 | 1 | 163562 | 152885 | 152823 | 152823 | 138592 | 138556 | 137323 |
| **18SW 12** | 18S | filtered water | A Coruña | surface | 22/03/ 2017 | site 1 | 1 | 142600 | 134795 | 134774 | 134774 | 86045 | 85983 | 85139 |
| **18SW 13** | 18S | filtered water | Bilbao | surface | 22/11/ 2016 | site 1 | 1 | 161269 | 152433 | 152395 | 152395 | 150389 | 150119 | 148458 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **18SW15** | 18S | filtered water | Bilbao | surface | 31/05/2017 | site 1 | 1 | 130753 | 124635 | 124614 | 124614 | 95162 | 94568 | 93579 |
| **18SW16** | 18S | filtered water | Bilbao | surface | 31/05/2017 | site 2 | 1 | 150067 | 140251 | 140157 | 140157 | 109333 | 108740 | 107433 |
| **18SW17** | 18S | filtered water | Bilbao | surface | 31/05/2017 | site 3 | 1 | 138331 | 131569 | 131544 | 131544 | 125239 | 123155 | 121885 |
| **18SW18** | 18S | filtered water | Bilbao | surface | 31/05/2017 | site 4 | 1 | 193079 | 177884 | 177836 | 177836 | 175487 | 175261 | 172864 |
| **18SW19** | 18S | filtered water | Bilbao | bottom | 31/05/2017 | site 1 | 1 | 199122 | 188039 | 187942 | 187942 | 141626 | 141338 | 139502 |
| **18SW20** | 18S | filtered water | Bilbao | bottom | 31/05/2017 | site 3 | 1 | 131180 | 125133 | 125114 | 125114 | 98839 | 98092 | 97235 |
| **18SW21** | 18S | filtered water | Bilbao | surface | 05/09/2017 | site 1 | 1 | 160997 | 150667 | 150619 | 150619 | 148336 | 147549 | 145691 |
| **18SW22** | 18S | filtered water | Bilbao | surface | 05/09/2017 | site 2 | 1 | 169008 | 159037 | 159014 | 159014 | 156945 | 156357 | 154725 |
| **18SW23** | 18S | filtered water | Bilbao | surface | 05/09/2017 | site 3 | 1 | 125704 | 117946 | 117937 | 117937 | 117496 | 113985 | 112661 |
| **18SW24** | 18S | filtered water | Bilbao | surface | 05/09/2017 | site 4 | 1 | 145936 | 137923 | 137912 | 137912 | 137472 | 133719 | 132432 |
| **18SW25** | 18S | filtered water | A Coruña | surface | 22/03/2017 | site 2 | 1 | 177256 | 165771 | 165738 | 165738 | 137226 | 137111 | 135698 |
| **18SW26** | 18S | filtered water | A Coruña | surface | 22/03/2017 | site 3 | 1 | 174510 | 163866 | 163836 | 163836 | 140913 | 140759 | 139259 |
| **18SW27** | 18S | filtered water | Vigo | surface | 13/03/2017 | site 1 | 1 | 133990 | 124010 | 123963 | 123963 | 119933 | 119773 | 118421 |
| **18SW28** | 18S | filtered water | Vigo | surface | 13/03/2017 | site 2 | 1 | 149793 | 139808 | 139792 | 139792 | 119383 | 119245 | 117915 |
| **18SW29** | 18S | filtered water | Vigo | surface | 13/03/2017 | site 3 | 1 | 139203 | 130624 | 130586 | 130586 | 121174 | 120981 | 119215 |
| **18SW30** | 18S | filtered water | Bilbao | bottom | 05/09/2017 | site 1 | 1 | 158556 | 148412 | 148133 | 148133 | 144176 | 143728 | 141639 |
| **18SW31** | 18S | filtered water | Bilbao | bottom | 05/09/2017 | site 3 | 1 | 132628 | 123767 | 123759 | 123759 | 122961 | 121389 | 119994 |
| **18SZ25** | 18S | zooplankton net (60 µm) | Bilbao | water column | 05/09/2017 | site 1 | 1 | 147197 | 137017 | 136857 | 136857 | 123960 | 123511 | 121971 |
| **18SZ26** | 18S | zooplankton net (280 µm) | Bilbao | water column | 05/09/2017 | site 1 | 1 | 126885 | 118546 | 118531 | 118531 | 104893 | 104302 | 103173 |
| **18SZ27** | 18S | zooplankton net (60 µm) | Bilbao | water column | 05/09/2017 | site 2 | 1 | 109496 | 100920 | 100885 | 100885 | 95828 | 95509 | 94368 |
| **18SZ28** | 18S | zooplankton net (280 µm) | Bilbao | water column | 05/09/2017 | site 2 | 1 | 139925 | 131083 | 131067 | 131067 | 120936 | 120603 | 119469 |
| **18SZ29** | 18S | zooplankton net (60 µm) | Bilbao | water column | 05/09/2017 | site 3 | 1 | 144972 | 134980 | 134959 | 134959 | 126774 | 126464 | 125431 |
| **18SZ30** | 18S | zooplankton net (280 µm) | Bilbao | water column | 05/09/2017 | site 3 | 1 | 123031 | 115826 | 115812 | 115812 | 98166 | 97837 | 96986 |
| **18SZ31** | 18S | zooplankton net (60 µm) | Bilbao | water column | 05/09/2017 | site 4 | 1 | 111717 | 104574 | 104561 | 104561 | 102696 | 102342 | 101644 |
| **18SZ32** | 18S | zooplankton net (280 µm) | Bilbao | water column | 05/09/2017 | site 4 | 1 | 114319 | 106507 | 106497 | 106497 | 98096 | 97907 | 97147 |
| **Z01D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 22/11/2016 | site 1 | 1 | 54103 | 52839 | 52837 | 52837 | 43740 | 43460 | 43325 |
| **Z02D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 22/11/2016 | site 1 | 1 | 80572 | 78575 | 78571 | 78571 | 57291 | 56780 | 56584 |
| **Z03D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 22/11/2016 | site 2 | 1 | 72464 | 70735 | 70718 | 70718 | 48609 | 48241 | 48081 |
| **Z04D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 22/11/2016 | site 2 | 1 | 40804 | 39862 | 39861 | 39861 | 30370 | 30018 | 29932 |
| **Z05D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 22/11/2016 | site 3 | 1 | 64179 | 62656 | 62654 | 62654 | 23539 | 23461 | 23392 |
| **Z06D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 22/11/2016 | site 3 | 1 | 48966 | 47862 | 47859 | 47859 | 16179 | 16103 | 16049 |
| **Z07D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 22/11/2016 | site 4 | 1 | 45630 | 44359 | 44356 | 44356 | 38116 | 37973 | 37903 |
| **Z08D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 22/11/2016 | site 4 | 1 | 64440 | 62884 | 62881 | 62881 | 35452 | 35129 | 35045 |
| **Z09D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 03/02/2017 | site 1 | 1 | 65744 | 64088 | 64077 | 64077 | 50466 | 49936 | 49755 |
| **Z10D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 03/02/2017 | site 1 | 1 | 50234 | 49118 | 49112 | 49112 | 39177 | 38733 | 38631 |
| **Z11D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 03/02/2017 | site 2 | 1 | 47823 | 46673 | 46671 | 46671 | 29030 | 28813 | 28702 |
| **Z12D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 03/02/2017 | site 2 | 1 | 39279 | 38234 | 38229 | 38229 | 21425 | 21167 | 21099 |
| **Z13D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 03/02/2017 | site 3 | 1 | 64380 | 62855 | 62851 | 62851 | 24116 | 24054 | 23970 |
| **Z14D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 03/02/2017 | site 3 | 1 | 74376 | 72631 | 72629 | 72629 | 19180 | 19118 | 19060 |
| **Z15D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 03/02/2017 | site 4 | 1 | 74614 | 72487 | 72481 | 72481 | 60752 | 60406 | 60282 |
| **Z16D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 03/02/2017 | site 4 | 1 | 94402 | 92295 | 92287 | 92287 | 52355 | 52288 | 52200 |
| **Z17D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 31/05/2017 | site 1 | 1 | 85035 | 83080 | 83042 | 83042 | 46539 | 46199 | 46075 |
| **Z18D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 31/05/2017 | site 1 | 1 | 44722 | 43681 | 43679 | 43679 | 26433 | 26122 | 26029 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Z19D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 31/05/2017 | site 2 | 1 | 71073 | 68987 | 68948 | 68948 | 34167 | 33999 | 33881 |
| **Z20D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 31/05/2017 | site 2 | 1 | 84134 | 81974 | 81970 | 81970 | 49093 | 48710 | 48553 |
| **Z21D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 31/05/2017 | site 3 | 1 | 92830 | 90613 | 90507 | 90507 | 33615 | 33506 | 33411 |
| **Z22D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 31/05/2017 | site 3 | 1 | 68448 | 66667 | 66665 | 66665 | 24926 | 24815 | 24751 |
| **Z23D** | 18S | zooplankton net (60 µm) | Bilbao | water column | 31/05/2017 | site 4 | 1 | 75573 | 73284 | 73227 | 73227 | 66321 | 65997 | 65880 |
| **Z24D** | 18S | zooplankton net (280 µm) | Bilbao | water column | 31/05/2017 | site 4 | 1 | 96181 | 94030 | 94024 | 94024 | 43702 | 43608 | 43507 |
| **18SW 14** | 18S | filtered water (extraction negative control) | na | na | na | na | 1 | 2755 | 2229 | 2221 | 2221 | 2039 | 2039 | 1972 |
| **18SW 32R** | 18S | filtered water (extraction negative control) | na | na | na | na | 2 | 359 | 164 | 164 | 164 | 163 | 163 | 160 |

Table S5. Results of the PERMANOVA test for the effects of seasonality and locality for the port of Bilbao. Values represent the degree of freedom (Df), the test value (F value), the amount of explained variation (R2). In significance column, "*" indicates significant and "ns" non-significant at P value= 0.05 respectively. An asterisk in the F column indicates group dispersions are significantly non-homogeneous at P value=0.05.

| Sampling variable | Sampling type | Barcode | Metric | Df | F | $R^2$ | Significance |
|---|---|---|---|---|---|---|---|
| Season | Sediment grab | COI | Abundance | 3 | 0.54 | 0.12 | ns |
| | | | Presence/Absence | 3 | 1.05 | 0.21 | ns |
| | Zooplankton nets | COI | Abundance | 3 | 35.5 | 0.47 | * |
| | | | Presence/Absence | 3 | 16.3 | 0.29 | * |
| | | 18S | Abundance | 3 | 45.7 | 0.53 | * |
| | | | Presence/Absence | 3 | 31.1 | 0.44 | * |
| | Filtered water | COI | Abundance | 3 | 1.8 | 0.31 | * |
| | | | Presence/Absence | 3 | 1.4 | 0.26 | * |
| | | 18S | Abundance | 3 | 4.9 | 0.55 | * |
| | | | Presence/Absence | 3 | 2.3 | 0.36 | * |
| Sites (all sites) | Sediment grab | COI | Abundance | 3 | 4.81 | 0.55 | * |
| | | | Presence/Absence | 3 | 1.41 | 0.26 | * |
| | Zooplankton nets | COI | Abundance | 3 | 20.6 | 0.34 | * |
| | | | Presence/Absence | 3 | 14.5 * | 0.27 | * |
| | | 18S | Abundance | 3 | 19.7 | 0.33 | * |
| | | | Presence/Absence | 3 | 0.9 | 0.18 | ns |
| | Filtered water | COI | Abundance | 3 | 1.1 | 0.21 | ns |
| | | | Presence/Absence | 3 | 1.1 | 0.21 | ns |
| | | 18S | Abundance | 3 | 1 | 0.2 | ns |
| | | | Presence/Absence | 3 | 1.2 | 0.24 | ns |
| Sites (sites 1,2 & 3) | Sediment grab | COI | Abundance | 2 | 3.9 | 0.46 | * |
| | | | Presence/Absence | 2 | 1.19 | 0.21 | * |
| | Zooplankton nets | COI | Abundance | 3 | 0.4 | 0.08 | ns |
| | | | Presence/Absence | 2 | 0.8 | 0.15 | ns |
| | | 18S | Abundance | 2 | 0.5 | 0.09 | ns |
| | | | Presence/Absence | 2 | 0.6 | 0.12 | ns |
| | Filtered water | COI | Abundance | 2 | 0.8 | 0.15 | ns |
| | | | Presence/Absence | 2 | 0.9 | 0.17 | ns |
| | | 18S | Abundance | 2 | 0.5 | 0.1 | ns |
| | | | Presence/Absence | 2 | 0.7 | 0.13 | ns |

Table S6. Results of the PERMANOVA test for the effects of seasonality and port locality for the filtered water samples from the 3 ports (Bilbao, A Coruña, Vigo). Tests are based on Hellinger-transformed abundances of OTUs. Values represent the degree of freedom (Df), the test value (F value), the amount of explained variation (R2). In significance column, “*” indicates significant and "ns" non-significant at P value= 0.05 respectively. An asterisk in the F column indicates group dispersions are significantly non-homogeneous at P value=0.05.

| Sampling variable | Dataset | Barcode | Df | F | $R^2$ | Significance |
|---|---|---|---|---|---|---|
| Port | PBBS targeted taxa | COI | 2 | 1.56* | 0.10 | * |
| | PBBS targeted taxa | 18S | 2 | 2.18* | 0.22 | * |
| | PBBS targeted taxa metazoan only | | 2 | 1.44* | 0.10 | ns |
| Season | PBBS targeted taxa | COI | 3 | 2.37 | 0.21 | * |
| | PBBS targeted taxa | 18S | 3 | 4.54* | 0.49 | * |
| | PBBS targeted taxa metazoan only | | 3 | 3.63 | 0.30 | * |

Table S7. List of non-indigenous and cryptogenic species found in the port of Bilbao (Large Marine Ecoregion (LME) numbers are 22 for North Sea, 23 for Baltic sea, 24 for Celtic Biscay Shelf, 26 for Mediterranean Sea and A1 for Macaronesia)

| Species | Number of COI reads | Number of 18S reads | Previously detected in Bilbao | Previously detected in the Bilbao LME (if not, details of introduction event in other LMEs are provided) | Status | References |
|---|---|---|---|---|---|---|
| ***Bugulina fulva*** | 101 | 11 | no | no but present in 22, 24 and A1 | cryptogenic | (Fofonoff et al., 2019; Porter et al., 2017) |
| ***Pseudochattonella verruculosa*** | 0 | 7486 | no | no but present in LMEs 22 and 23 | introduced | (Katsanevakis Stylianos et al., 2014) |
| ***Tubificoides pseudogaster*** | 21 | 0 | no | no but present in LMEs 22 and 23 | cryptogenic | (Kvist and ErsÉus, 2018) |
| ***Melita nitida*** | 18 | 0 | no | no but present in LMEs 22, 23 and 24 | Introduced | (Gouillieux et al., 2016) |
| ***Grandidierella japonica*** | 15081 | 18 | no | no but present in LMEs 23,24,25 and 26 | Introduced | (Droual et al., 2017) |
| ***Ammothea hilgendorfi*** | 0 | 3 | no | no, but present in LMEs 22 and 24 | introduced | (AquaNIS. Editorial board., 2015) |
| ***Neodexiospira brasiliensis*** | 0 | 37396 | no | no, but present in LMEs 22 and 24 | introduced | (AquaNIS. Editorial board., 2015) |
| ***Boccardia proboscidea*** | 0 | 3 | yes | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Sabella spallanzanii*** | 0 | 7 | yes | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Styela plicata*** | 0 | 3 | yes | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Colpomenia peregrina*** | 0 | 13 | yes | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Ficopomatus enigmaticus*** | 0 | 1114 | yes | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Pseudo-nitzschia australis*** | 0 | 544 | yes | yes | cryptogenic | (Butrón et al., 2011) |
| ***Heterosigma akashiwo*** | 0 | 3793 | yes | yes | cryptogenic | (Butrón et al., 2011) |
| ***Fibrocapsa japonica*** | 0 | 6 | yes | yes | introduced | (Butrón et al., 2011) |
| ***Prorocentrum cordatum*** | 0 | 691 | yes | yes | introduced | (Butrón et al., 2011) |
| ***Antithamnionella spirographidis*** | 0 | 175 | yes | yes | introduced | (Martínez and Adarraga, 2006) |
| ***Asparagopsis armata*** | 110 | 48 | yes | yes | introduced | (Martínez and Adarraga, 2006) |
| ***Bonnemaisonia hamifera*** | 13 | 22 | yes | yes | introduced | (Martínez and Adarraga, 2006) |
| ***Magallana gigas*** | 15738 | 103 | yes | yes | introduced | (Martínez and Adarraga, 2006) |
| ***Spongoclonium caribaeum*** | 0 | 6 | yes | yes | introduced | (Martínez and Adarraga, 2006) |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***Xenostrobus securis*** | 40 | 0 | yes | yes | introduced | (Martínez and Adarraga, 2006) |
| ***Mytilus galloprovincialis*** | 68 | 0 | yes | yes | cryptogenic | (Tajadura et al., 2016) |
| ***Obelia geniculata*** | 0 | 11 | yes | yes | cryptogenic | (Tajadura et al., 2016) |
| ***Orthopyxis integra*** | 0 | 4 | yes | yes | cryptogenic | (Tajadura et al., 2016) |
| ***Amphibalanus amphitrite*** | 38916 | 0 | yes | yes | introduced | (Tajadura et al., 2016) |
| ***Austrominius modestus*** | 210728 | 0 | yes | yes | introduced | (Tajadura et al., 2016) |
| ***Clytia hemisphaerica*** | 14751 | 4715 | yes | yes | introduced | (Tajadura et al., 2016) |
| ***Oithona davisae*** | 84594 | 192440 | yes | yes | introduced | (Uriarte et al., 2016) |
| ***Pseudodiaptomus marinus*** | 2706 | 0 | yes | yes | introduced | (Uriarte et al., 2016) |
| ***Acartia Acanthacartia tonsa*** | 189833 | 31333 | yes | yes | introduced | (Uriarte et al., 2016) |
| ***Balanus trigonus*** | 78865 | 0 | yes | yes | introduced | (Zorita et al., 2013) |
| ***Monocorophium acherusicum*** | 196 | 0 | yes | yes | introduced | (Zorita et al., 2013) |
| ***Monocorophium sextonae*** | 209 | 0 | yes | yes | introduced | (Zorita et al., 2013) |
| ***Petricolaria pholadiformis*** | 2 | 0 | yes | yes | introduced | (Zorita et al., 2013) |
| ***Ruditapes philippinarum*** | 4419 | 83 | yes | yes | introduced | (Zorita et al., 2013) |
| ***Polydora websteri*** | 29 | 1114 | no | yes | introduced | (Agencia Vasca del Agua, 2014) |
| ***Ostrea stentina*** | 5650 | 0 | no | yes | cryptogenic | (AquaNIS. Editorial board., 2015) |
| ***Bugula neritina*** | 11085 | 5394 | no | yes | cryptogenic | (AquaNIS. Editorial board., 2015) |
| ***Amphibalanus eburneus*** | 28606 | 0 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Amphibalanus improvisus*** | 32 | 0 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Blackfordia virginica*** | 124 | 2 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Botryllus schlosseri*** | 26336 | 0 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Crepidula fornicata*** | 4 | 0 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Dasysiphonia japonica*** | 5 | 0 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Gymnodinium microreticulatum*** | 0 | 2 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Hemigrapsus penicillatus*** | 214 | 0 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Microcosmus squamiger*** | 68 | 482 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Ostreopsis siamensis*** | 0 | 6 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Paracaprella pusilla*** | 12 | 0 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Polydora cornuta*** | 32777 | 0 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Tenellia adspersa*** | 13 | 0 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Watersipora subtorquata*** | 232 | 1396 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***Cordylophora caspia*** | 0 | 514 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Corella eumyota*** | 0 | 3191 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Mercenaria mercenaria*** | 0 | 43 | no | yes | introduced | (AquaNIS. Editorial board., 2015) |
| ***Hemigrapsus takanoi*** | 19185 | 0 | no | yes | Introduced | (CABI, 2019b) |
| ***Monocorophium insidiosum*** | 71 | 0 | no | yes | cryptogenic | (Esquete et al., 2011) |
| ***Phallusia mammillata*** | 2 | 38 | no | yes | cryptogenic | (Goulletquer, 2016) |
| ***Mytilicola intestinalis*** | 33 | 22 | no | yes | Introduced | (Goulletquer, 2016) |
| ***Pseudopfiesteria shumwayae*** | 0 | 7 | no | yes | cryptogenic | (Guiry and Guiry, 2019) |
| ***Leathesia marina*** | 0 | 43 | no | yes | cryptogenic | (Guiry and Guiry, 2019) |
| ***Pachygrapsus marmoratus*** | 21 | 0 | no | yes | Introduced | http://www.nonnativespecies.org/ |
| ***Terebella lapidaria*** | 3614 | 0 | no | yes | cryptogenic | (Miralles et al., 2016) |
| ***Sternaspis scutata*** | 14475 | 370 | no | yes | cryptogenic | (Moreira and Troncoso, 2007) |
| ***Gymnodinium aureolum*** | 0 | 14163 | no | yes | cryptogenic | (OBIS, 2019) |
| ***Chaetoceros rostratus*** | 0 | 170 | no | yes | Introduced | (OBIS, 2019) |
| ***Coscinodiscus wailesii*** | 0 | 596 | no | yes | Introduced | (OBIS, 2019) |
| ***Diadumene lineata*** | 0 | 4 | no | yes | introduced | (OBIS, 2019; WoRMS Editorial Board, 2019) |
| ***Diplosoma listerianum*** | 91 | 0 | no | yes | cryptogenic | (Pérez-Portela et al., 2013) |
| ***Pfiesteria piscicida*** | 0 | 5 | no | yes | cryptogenic | (Rublee et al., 2005) |
| ***Stephanopyxis palmeriana*** | 0 | 104 | no | yes | cryptogenic | (Urrutia and Casamitjana, 1981) |
| ***Electra pilosa*** | 10 | 40 | no | yes | cryptogenic | (WoRMS Editorial Board, 2019) |
| ***Gymnodinium catenatum*** | 0 | 87 | no | yes | cryptogenic | (WoRMS Editorial Board, 2019) |
| ***Protomonostroma undulatum*** | 0 | 164 | no | yes | introduced | (WoRMS Editorial Board, 2019) |
| ***Crassostrea virginica*** | 0 | 67793 | no | yes | introduced | (WoRMS Editorial Board, 2019) |
| ***Magallana angulata*** | 17 | 0 | no | yes | introduced | (WoRMS Editorial Board, 2019) |
| ***Karlodinium veneficum*** | 0 | 2847 | no | yes | cryptogenic | (Zhang et al., 2008) |
| ***Caulacanthus ustulatus*** | 9 | 0 | no | yes | cryptogenic | (Zuccarello et al., 2002) |

# GENERAL

# DISCUSSION

The burgeoning number of studies relying on genetic tools to detect harmful organisms (including harmful algae blooms, non-indigenous, cryptogenic and invasive species) and pathogens from ships and ports prompts the need to assess their applicability to monitoring actions taken to prevent further ship-borne introduction. This last part of the thesis gives an overview of new insights we have gained into the use of genetic tools to provide reliable data for ship-borne biodiversity monitoring. In particular, we discuss about the potential of using environmental DNA to perform rapid port and ballast water surveys, challenges of using DNA metabarcoding in species detection and potential ways forwards to improve it. We finish by discussing future applications for DNA metabarcoding for ship-borne biodiversity monitoring.

## 1 Feedback on the potential of eDNA for ship-borne biodiversity monitoring

Based on recommendations of Chapter I, we assessed the potential of using eDNA as the most rapid and cost-effective method to perform baseline surveys of biodiversity in ballast water (Chapter II) and port (Chapter III). Three main findings about eDNA were observed in this thesis.

First, we confirmed the current observations from the literature that eDNA metabarcoding from water can be used to retrieve patterns of community variation (*e.g.* temporal variation in freshwater macroinvertebrates communities with Bista et al. (2017), spatial variation of coastal taxa with Jeunen et al. (2018)). In our case, we showed that eDNA is a reliable technique to depict patterns of plankton communities. Indeed, in Chapter III, it was proved that eDNA samples were closer to plankton net communities than to communities collected with other sampling methods. Besides, eDNA also exhibited similar seasonal patterns than plankton communities. In Chapter II, impact of ballast water management was retrieved with eDNA indicating its capacity to depict plankton responses. These outcomes strongly encourage the development of eDNA as cost-effective sampling method allowing researchers to characterize and depict communities' changes and responses, with application such as understanding the impacts of ballast water management on the communities discharged into ports. In this respect, an

appealing perspective may be to use eDNA coupled to machine learning algorithms to find biomarkers that could allow to differentiate between ballast water that performed ocean exchange from those that were treated with different types of treatment systems. Such approaches are under development and have been for example tested with bacteria by comparing port, ballast and ocean waters (Gerhard and Gunsch, 2019). A logical follow-up of Chapter II would be to develop similar approaches focused on finding markers associated to each type of ballast water management to compare their efficiency.

Second, in Chapter II eDNA outperformed morphological identification of ballast water planktonic taxa by retrieving a wider diversity in general. This was also reported by Slijkerman et al. (2017) who found that in the ballast water of three vessels only 20 species were identified to the species level with morphology whereas 66 species were identified with eDNA. Yet, when comparison was done with plankton net instead of with eDNA sampling, and subsequently analyzed with metabarcoding (Chapter III), eDNA identified only a subset of the port's taxa retrieved by plankton net samples. While such comparison between plankton net and eDNA metabarcoding remains to be further tested for ballast water, eDNA remains as a promising candidate for ballast water monitoring in comparison to plankton net for two main reasons. (1) We observed that time, space and accessibility on board can be very limited, which can make complicated the use of plankton net. (2) In a near future all vessels in international routes will have to treat their ballast water instead of performing oceanic exchange (IMO, 2017). Treated water will contain organisms in low abundance and more volumes will need to be sampled to find enough organisms (Frazier et al., 2013). In such cases, plankton net will require many tows or big volume of water in comparison to eDNA sampling. Yet, results from the experimental tank in Chapter II suggested that eDNA detected patchiness of organisms, which can have an impact on the retrieved community. More in-depth studies are therefore needed to understand if the eDNA signals are homogenously distributed inside the tank or if sampling at different tank's depth will be needed.

Third, we observed that eDNA was able to detect dominant taxa but had a limited capacity to recover all metazoan taxa present in the diverse habitats of the port (Chapter

III). Hence, we concluded that used alone, eDNA may underestimate metazoan diversity, as suggested by Koziol et al. (2018). Koziol et al. (2018) found that water eDNA detected only 34 %–38 % of the overall eukaryotic families found across all substrates (including sediment, settlement plates and zooplankton nets). Many factors can influence the amount of eDNA released by organisms in the environment but more importantly the amount of eDNA present in the environment in not homogenous among species and even among life stages of a same species (Barnes and Turner, 2016; Goldberg et al., 2016). This phenomenon might limit the use of eDNA to detect all species from a multiple habitat environment. For instance, the potential limitation of DNA shedding rates for invertebrates with exoskeleton may impede their detection from eDNA. Indeed, we found in Chapter III that 82 % (COI) and 100 % (18S) of the total Decapoda OTUs diversity retrieved in the study was detected in plankton nets (proving their organismal presence in the water column) whereas only 0.7 % (COI) and 7 % (18S) was recorded with eDNA. Decapoda present an exoskeleton since the larval stage (Gao et al., 2017) which may diminish the release of DNA in water and prevent their detection with eDNA (Walsh et al., 2018). However, this limitation remains scarcely studied in the literature and warrants further research (Cristescu and Hebert, 2018; Tréguier et al., 2014), specifically for ship-borne taxa monitoring as invertebrates are common NIS (Chapters II & III, Levings et al. (2004); Walsh et al., (2018)). Also, in this thesis, most of reads obtained with COI were assigned to Bacteria or phytoplankton taxa. This is a common phenomenon with COI (Borrell et al., 2017; Kelly et al., 2017; Stat et al., 2017). Hence, the combination of eDNA which by principle recovers all taxa present in the water (targeted or not by conventional sampling for PBBS such as Bacteria) with "universal" primers can lead to the preferential stochastic amplification of some taxa, which might limit the detection of taxa targeted by conventional sampling methods. For instance, Macher et al. (2018) performed comparisons between bulk-sample metabarcoding and eDNA metabarcoding for benthic macroinvertebrates in river. They concluded that using bulk-sample would be more reliable than eDNA to recover benthic macroinvertebrates taxa because eDNA sampling captures a wider biodiversity of organisms, which then outcompetes target-taxon DNA during PCR and sequencing. Developing group specific primers to ensure representative

amplification across all taxa will be one way forward to optimize eDNA method for whole port's community detection (Berry et al., 2019; Clarke et al., 2017; Jeunen et al., 2019; Klymus et al., 2017). As an example, Jeunen et al. (2019) showed that using two group specific primers with 16S, one for fish and for crustacean, 42 taxa were identified and among these taxa, only four were retrieved with "universal" COI primers.

## 2 Room for improvement of Non-Indigenous and Cryptogenic Species detection with DNA metabarcoding

In this thesis, we showed that DNA metabarcoding could be a promising tool to confirm the presence of NICS, with example in both the port of Bilbao and in ballast water (*e.g. Acartia tonsa*; Chapters II & III). We also highlighted its great potential to early detect potentially recent or overlooked species introduction, *e.g. Melitida nitida* in Bilbao (Chapter III) or *Oithona davisae* in ballast water discharged into the Chesapeake Bay (Chapter II). Yet, we noticed that not all NICS recorded with previous monitoring in the port of Bilbao (Chapter III) were detected with DNA metabarcoding. Also, taxonomic assignment confidence was relatively low for some species present in ballast water (Chapter II). The following sections discuss the challenges associated to species detection with DNA metabarcoding and propose potential ways to overcome some of its limitations.

### 2.1 Increase the barcoding effort locally to fill global databases

One of the main limitations that DNA metabarcoding is usually facing for the detection of species is linked to the completeness of genetic reference databases (Cristescu, 2014; Stefanni et al., 2018; Wangensteen and Turon, 2017). The incompleteness of databases inherently leads to overlooking the NICS for which no barcodes are available. Encouragingly, on the 68 NICS known to be present in the port of Bilbao (Chapter III), 70 % had a barcode in BOLD or PR2, the two databases used in this thesis. This pattern is congruent with the general trend discussed in Chapter I regarding the improvement of reference databases completeness. It is also in line with the current state of barcoding efforts observed for aquatic NICS at the European level (Figure 22). Despite these positive signs, barcoding effort must be continued to complete reference database for NICS, especially as current coverage is highly heterogeneous among phyla. For instance, 70 % of

Platyhelminthes NICS are not yet barcoded whereas only 12 % of Chordata NICS are not. In the case of Platyhelminthes, the lack of barcodes could be due to technical problems rather than lack in barcoding effort. Indeed, it was observed for this phylum that PCR amplification failed with classical COI primers used in barcoding due to high nucleotide variation at primers sites. However, the recent development of degenerated primers designed specifically for this phylum (Van Steenkiste et al., 2015) could increase amplification success, allowing more barcodes to be added in a near future.

In this respect, priority should be given to barcoding rare NICS, either defined as established in only one Large Marine Ecoregion (LME), or as present in low abundance inside a LME. An increased availability of barcodes for rare NICS would allow to monitor and track the spread of these species early on rather than just barcoding (and hence monitoring) them only when they have become abundant. This recommendation is particularly important as most of the NICS not yet barcoded (78%) are only present in one LME according to the data of the AquaNIS database used to create Figure 22. For this reason, we advocate that 1) join efforts are needed within each LME between taxonomists and molecular biologists (Kvist, 2013), and 2) that future port surveys allocate part of their funds to complete databases through barcoding efforts, as already implemented in pest-monitoring programs (Dias et al., 2017). Furthermore, it has been proposed that relying on local genetic reference databases could enhance species detection and remove potential wrong assignment (Deiner et al., 2017). Successful application of the use of local databases includes for example the use of a COI reference database for the North Sea metazoan by Knebelsberger et al. (2014). However, while this is very useful for identifying local biodiversity or to follow the dispersal of expected or important NICS in an area (Brown et al., 2016), this approach may have limited use in a context of early detecting NICS introduced via shipping. Indeed, the use of local reference database may impede the recovery of species introduced from a very distinct part of the world. By increasing barcoding effort locally and uploading these data on global curated databases such as BOLD for COI it will confidently increase the detection of NICS whatever the region of port or ballast water monitored.

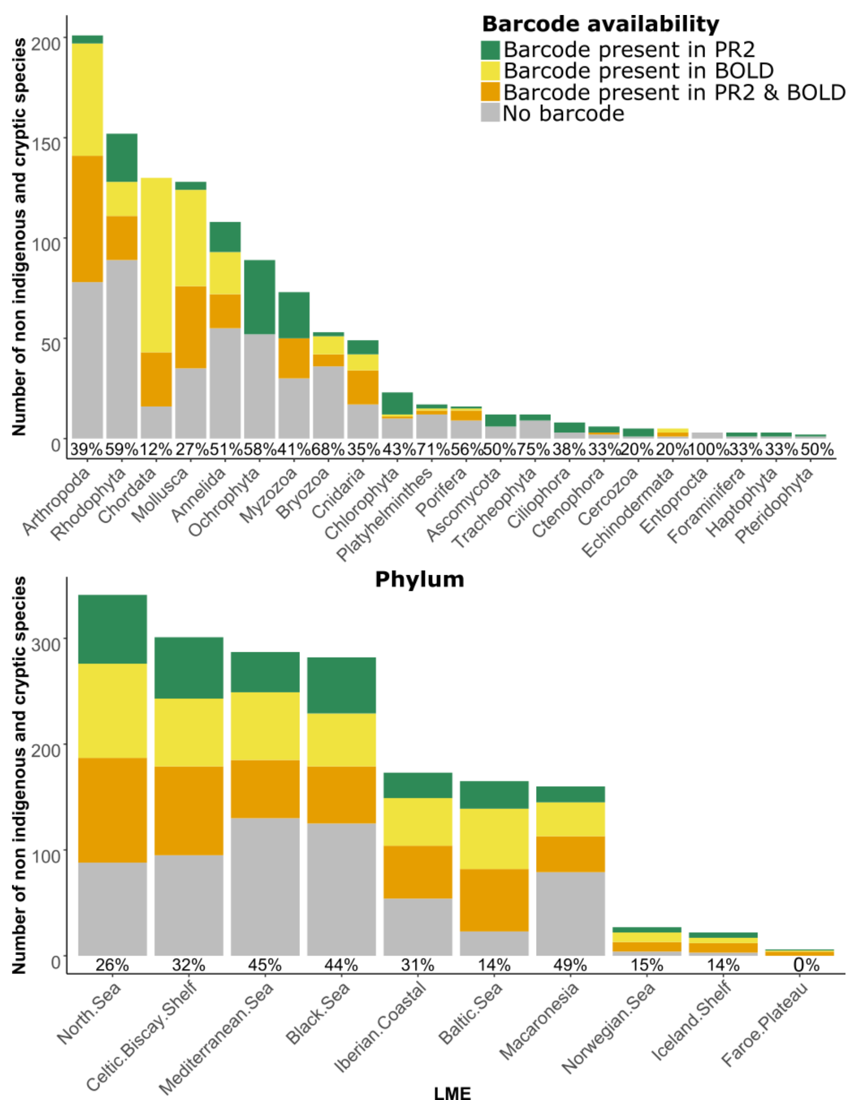


Figure 22. Barcode availability in PR2 and BOLD genetic reference databases for all Non-Indigenous and Cryptogenic Species (NICS) present in Europe presented by phylum and by Large Marine Ecoregion (LME). The list of NICS was retrieved from the AquaNIS database (http://www.corpi.ku.lt/databases/index.php/aquanis). Number below each bar represent the percentage of NICS with no barcode in each category.

## 2.2 Rely on a multiple barcode approach

Many NICS found in this thesis were detected with only one barcode (either COI or 18S), highlighting the need for combining several barcodes to increase species detectability. Indeed, not all NICS have been barcoded with all barcodes (Figure 22). Also,

biases can occur during PCR amplification, which may lead to favoring the amplification of one taxon over another (Deiner et al., 2017; Elbrecht and Leese, 2015). The choice of which barcode region, which primers and which databases are used inherently impacts species detection and must be carefully defined. We chose to focus in the thesis on COI for metazoan and on 18S for eukaryotes, based on the curated databases BOLD and PR2 for COI and 18S respectively. It should be noted that PR2 is not specifically designed for metazoan but was chosen, as in other studies (Ammon et al., 2018), to have a database that goes down to species level for phytoplankton and metazoan. Indeed, the main other curated database for 18S, SILVA, often does not provide species level detection. We did not consider NCBI, even if more sequences are probably present, as it does not require voucher species to prove species identification and sequences are not comprehensively verified, which can lead to more common ambiguous or incorrect annotation (Ammon et al., 2018; Djurhuus et al., 2018; Leray et al., 2018; Vilgalys, 2003). Recently, a curated classification algorithm for COI sequences uploaded in NCBI was developed and should be further tested to enhance species detection by allowing more confidence in the certainty of the assignment (Heller et al., 2018).

Using "universal" primers is tempting to provide a more comprehensive view of the community (Elbrecht et al., 2017b; Wangensteen et al., 2018a) and detect potentially a broader range of NICS (Borrell et al., 2017; Grey et al., 2018). However, each "universal" primers will not detect all taxa present in an environment (Deagle et al., 2014; Jeunen et al., 2018). As mentioned in the discussion about environmental DNA, group-specific primers could be an alternative. Because of these limitations, the field of metabarcoding is consequently moving towards species detection via targeted multi-marker, as we performed in the thesis, to take the advantage of each barcode or primers and increase taxonomic breadth (Drummond et al., 2015; Günther et al., 2018; Kelly et al., 2017; Stat et al., 2017). However, it is worth mentioning that as more primers are used, the cost-efficiency of metabarcoding as a monitoring tool also decreases (Jeunen et al., 2018). Given this trade-off between biodiversity detection and cost-efficiency, the choice of how many and which primers to use needs to be carefully considered and this will be an important area of future research to standardize and optimize metabarcoding-based monitoring.

## 2.3 Provide a level of confidence in taxonomic assignment

When detecting a new introduction of NICS in a particular environment, the degree of certainty about the identification must be reliable. Indeed, false positives (*i.e.* identifications of species not present in the area) are particularly problematic in the context of the early detection of NICS as they may trigger the development of unnecessary management actions (Comtet et al., 2015; Darling and Mahon, 2011; Xiong et al., 2016). In Chapter III, we provided a list of NICS detected in the port of Bilbao with the DNA metabarcoding approach, but we acknowledge that we did not investigate the confidence in their taxonomic assignments. Some of these species were previously reported in the port which strenghten the confidence we may have in their taxonomic assignment. For other species not previously observed in the port such as *Grandidierella japonica* or *Melitida nitida* however, the accuracy of their taxonomic assignment requires further investigation. Consequently, the next step before providing this list of species to the Bilbao Port Authority for the granting exemption procedure of the BWM Convention, will be to assess the level of confidence for each newly detected species. Also, we showed in Chapter II that the identity of some species detected in ballast water, remained doubtful. These species were almost all identified with only 18S and their distribution did not match with the source ports of ballast waters. Similarly to our case, Stefanni et al. (2018) considered several taxa assigned with 18S were considered as "plausible misidentifications". Hence, taxonomic assignment based solely on 18S must be taken with caution due to its poor taxonomic resolution (Clarke et al., 2017; Wangensteen et al., 2018b).

By combining independent and different approaches, we were able to confidently identify the non-indigenous copepod *Oithona davisae* in Chapter II. Indeed, we found this species with two barcodes, COI and 18S, as well as with morphological identification and confirmed its potential distribution in the source port of the ballast water. The combination of multiple approaches is increasingly developed for NICS detection (Deiner et al., 2017; Grey et al., 2018; Stefanni et al., 2018) and is recommended before informing managers and starting preventive actions. Several ways forward may improve the certainty in taxonomic assignment including working with local taxonomists to check the list of

species found with metabarcoding to detect potential misidentification and considering the distribution and ecology of the species in case of dubious detection. The use of phylogenic trees could also be another independent approach to assess the strength of taxonomic confidence and ensure adequate assignment (see Chapter II for *Oithona davisae* but also Darling et al. (2018) and Hawlitscheck et al. (2018)). However, this approach is dependent on the availability in genetic databases of closely related species (*e.g.* from the same genus or family) along with the number of sequences of the species considered. Finally, species-specific assays like quantitative PCR could be used to confirm the presence of a NICS. In any case, it should be recommended to provide a level of certainty for each record of new introduction of NICS.

## 3 The next steps for the future integration of genetic tools into ship-borne biodiversity monitoring

We previously discussed the different challenges associated to the use of metabarcoding for providing baseline species lists for ports and ballast water, and how to enhance its sensitivity to detect NICS. While more work is still needed to optimize NICS detection, another important parameter to think about is its applicability for current preventive actions such as the BWM Convention. We showed the potential of DNA metabarcoding to perform PBBS in Chapter III and of eDNA metabarcoding for fast and detailed analysis of ballast water communities in Chapter II. However, we argue here that there are still some adjustments and evaluations to be done before their routine implementation.

### 3.1 DNA metabarcoding for PBBS performed for the BWM Convention: the case of exemptions

We highlighted in Chapter I that DNA metabarcoding could become a cost-effective and standardized method to perform PBBS which were developed to support the granting exemptions process under regulation A4 of the BWM Convention. An exemption can only be granted if a scientific risk assessment, based on Guidelines G7 of the BWM Convention, proves that there is no risk of introducing new harmful organisms between

the ports of the specific route. To that aim, the Helcom/Ospar protocol was developed to provide a standardize sampling and analysis framework in the countries in which these conventions apply (HELCOM/OSPAR, 2013). In the objective of using DNA metabarcoding to perform PBBS for exemption procedures, we adapted in Chapter III the Helcom/Ospar protocol for the processing of samples with genetic analyses. The guidelines proposed in Chapter III could be integrated in the Helcom/Ospar protocol without critical changes to the original protocol. Indeed, the Helcom/Ospar protocol is asking for sampling benthic and fouling organisms during late summer and zoo- and phyto- plankton during both spring and late summer. We recommend including spring and late summer for all type of communities when using DNA metabarcoding to take advantage of its efficiency in detecting early stages of organisms during spring which are difficulty identified with morphological taxonomy. The biological inventory performed with DNA metabarcoding in the port of Bilbao may timely serve the Port Authority of Bilbao if a ship owner is asking an exemption between Bilbao and another port.

While the Helcom/Ospar protocol asks for the identification of all species present in a port, the risk assessment used for exemption is mostly based on evaluating the presence of 70 important NICS for the Helcom/Ospar region, called "Target Species". One major question asked by the risk assessment is "Are target species equally common in both ports?". This question can be answered through abundance, biomass or occurrence data. A major challenge of metabarcoding is its limitation to accurately estimate the number of organisms or biomass for each species present in a sample based on the number of reads of the species (Elbrecht and Leese, 2015; Saitoh et al., 2016). Yet, previous PBBS relying on taxonomy recommended aborting the abundance measurement as being too time-consuming, costly and not enough standardized (Gittenberger et al., 2014). This is exemplified in the website gathering all data for exemptions on Helcom/Ospar where abundance is only sporadically recorded (http://jointbwmexemptions.org/ballast_water_RA/apex/f?p=104:12). Hence, Gittenberger et al. (2014) propose the use of occurrence data (the percentage of species presence among samples) instead for providing a cost-effective measure of species spreading into the port. Such data could be also supplied by metabarcoding. In agreement with these authors, we

recommend the use of occurrence data to facilitate the implementation and benefits from the advantages of metabarcoding for the Helcom/Ospar procedure of exemption (*e.g.* cost-effectiveness, standardization).

### 3.2 The applicability of metabarcoding for monitoring the impact of ballast water management: evaluation of viability

We showed in Chapter II the potential of using eDNA for ballast water monitoring. However, we did not consider in this chapter the assessment of viability, which is yet a focal point of ballast water monitoring (Chapter I, Darling and Frederik (2017)). Specifically, in the context of the BWM Convention it is crucial to develop tools which can determine the viability of organisms to provide relevant information on the efficiency of ballast water management. While for now metabarcoding is limited in providing a number of viable organisms to assess if a vessel complies with the discharge standards of Regulation D-2, it could be used to compare the efficiency of the different ballast water treatment systems. Indeed, metabarcoding could be developed to detect which taxa are more likely to not be removed or killed and which taxa can regrowth after the application of a particular type of ballast water treatment system. For instance, we observed in Chapter II that samples of ballast water treated were characterized by a phytoplankton taxon of Thalassiosirales which were also observed by Liebich et al. (2012) as regrowth taxa after the treatment of ballast water. Yet, we were not able to assure with eDNA metabarcoding that this taxon was alive or dead at the time of the sampling. We already highlighted the use of eRNA instead of eDNA as a potential solution because RNA is considered as a better proxy of organismal activity than DNA as it degrades more rapidly in the environment (Li et al., 2017; Xu et al., 2017; Zaiko et al., 2018). The comparison of eDNA vs eRNA in bilge water of recreational boats by Pochon et al. (2017) showed that each approach detected distinct communities, with potential legacy DNA from fungi. However, it did not clearly show if eRNA was indeed better suited to detect alive or recently alive organisms in comparison to eDNA. Pochon et al. (2017) concluded that using only OTUs found with both eDNA and eRNA could improve the detection of the living proportion of a community. Hitherto, eRNA has been usually used instead of eDNA in

sediments, as it is known that relic of DNA can accumulate in sediment (Dowle et al., 2016; Pawlowski et al., 2014). In marine water however, eDNA persistence seems to be quite short (few days, Collins et al. (2018)) so that the advantages of using eRNA in water may be less straightforward. Nonetheless, an appealing perspective of this thesis would be to assess if eRNA is really the future of metabarcoding for ballast water by building artificial communities where the exact state (alive, just killed, recently dead, dead for a long time) of organisms could be controlled. In these mock communities, all organisms of one species should be represented in only one state to test the hypothesis that organisms from a species dead for a long time will still be detected with eDNA and not with eRNA (Figure 23).

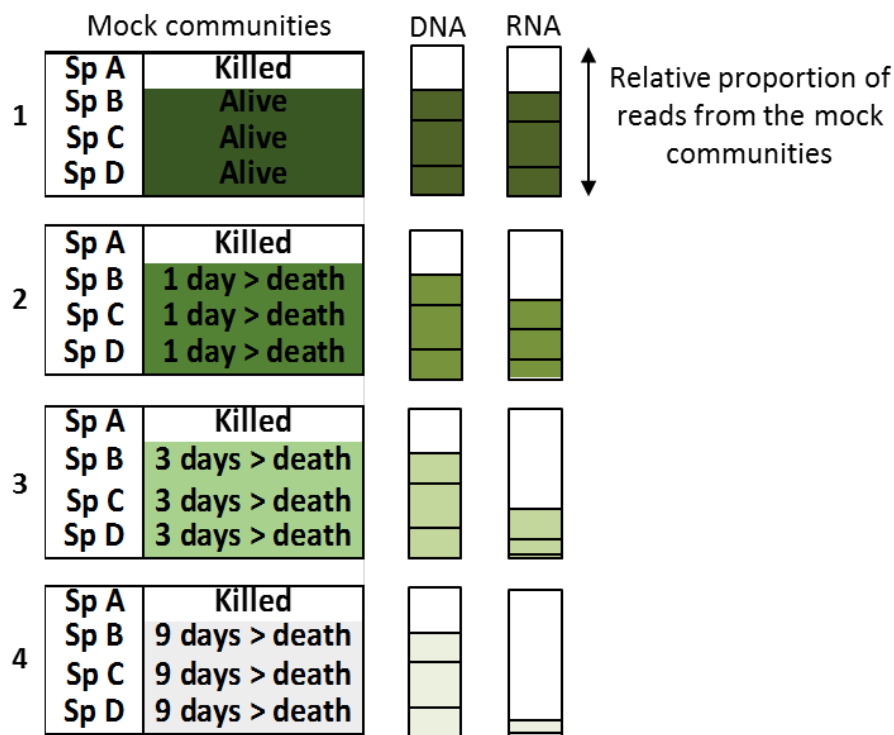


Figure 23. Illustration of the hypothesis of the differences which could be observed between community composition of mock communities with different states of life based on DNA and on RNA. In this example, there is 4 mock communities with the exact same species and number of organisms per species but in each mock, we will change the state of life for some species and for one species the state will always be the same ("killed"). State "killed" means that the species will be added just after killing it to have a baseline. Mock community 1 will be with alive organisms, mock community 2 with organisms recently dead (for a day), mock community 3 with organisms dead for 3 days and mock community 4 with organisms dead for 9 days. The relative proportion of reads of each species in the mock community will be compared between eDNA and eRNA. We hypothesized that eRNA will difficultly be detected for species dead for a long time in contrary to eDNA.

# 4 An integrative approach to better monitor Non-Indigenous and Cryptogenic Species

We showed in the thesis that performing PBBS is essential to provide baseline biodiversity data and detect new NICS introduced in the environment (Chapter III). One step forward to improve the management of NICS and allow reliable risk-assessment would be to also integrate the surveillance of potential introduction vectors (as for instance Chapter II) into monitoring schemes. For this purpose, the integration of maritime traffic data (*e.g.* frequency of traffic, volume of ballast water discharged) and environmental parameters (*e.g.* salinity and temperature) could allow to prioritize the sampling of vessels coming from a port with high propagule pressure and with similar environmental conditions. Maritime traffic data have for example been used to predict and quantify the probability of new invasions with ballast water in specific area such as Alaska (Verna et al., 2016) but also worldwide (Seebens et al., 2013, 2016).

In the case of Bilbao, and to complement Chapter III, we performed an analysis of ballast water discharge patterns, following the methodology presented by David et al. (2012, 2018), to identify which ports Bilbao had the strongest connection with. The Port Authority of Bilbao provided data on daily maritime traffic from 2007 to 2017 which allowed us to update the study performed by Butrón et al. (2011) with the port's data from 1997 to 2006. Thanks to the data gathered by the Port Authority of Bilbao for each vessel such as its Dead Weight Tonnage, quantity of cargo, type of cargo and cargo operations (*i.e.* cargo loading, unloading or both), we estimated the volume of ballast water discharged for each vessel by following the ballast water discharge assessment model developed by David et al. (2012). Over the 10 years of the data, 19,723,216 t of ballast water were discharged into the port of Bilbao coming from more than 900 international ports, but 40 % of the total volume discharged came from 10 European ports (Figure 24). It is worth mentioning that the last port of call was considered as the source port in the ballast water discharged model which is generally true for tankers but is not certain for containers and general cargo that can contain ballast water taken in several ports (David et al., 2012). For these "high risk" routes (Figure 24), it could be useful to integrate regular monitoring

of the ballast water coming from these ports to early detect new introductions and monitor the efficiency of ballast water managements required by the BWM Convention.

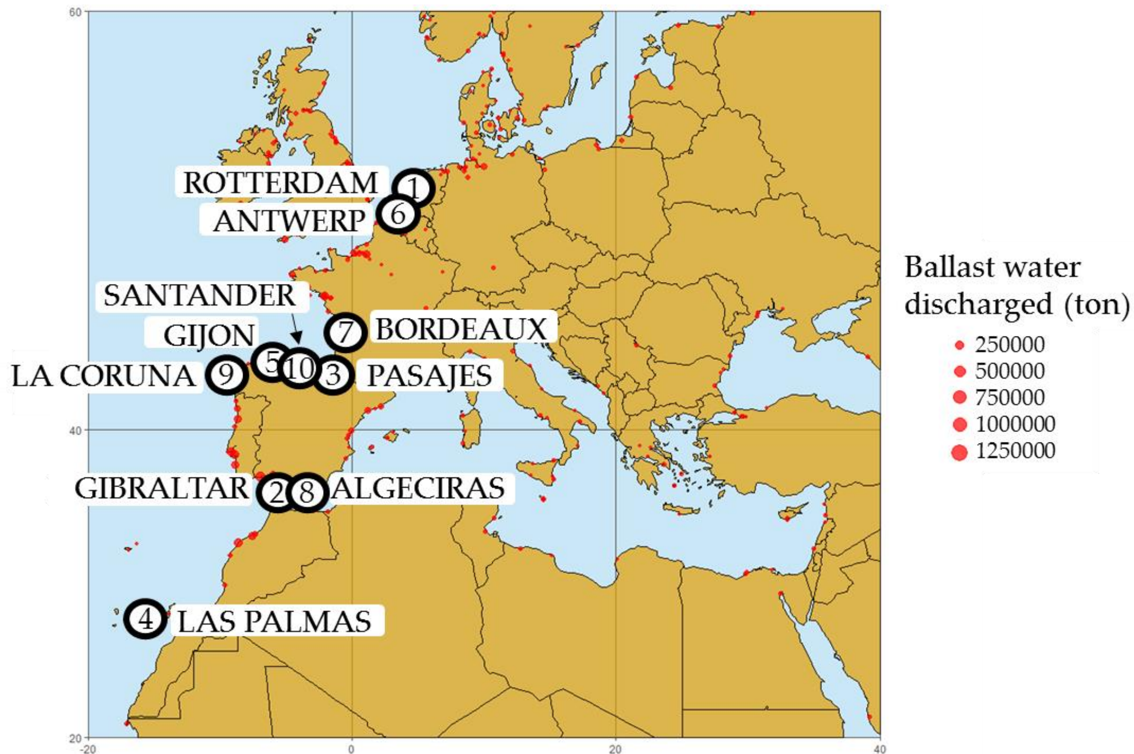


Figure 24. Top10 ports identified as source of ballast water discharged into the port of Bilbao during 2007-2017

In this thesis, we focused on the monitoring of ballast water as the main vector of introduction, but we acknowledge that biofouling and non-shipping related vectors should be monitored as well (Lacoursière-Roussel et al., 2016; Miller et al., 2018; Ojaveer et al., 2018). Special attention should be paid to the biofouling of recreational vessels, which often plays an important role in the secondary and coastwise spread of NICS (Ojaveer et al., 2018; Zabin et al., 2014). For instance, sequences from the PBBS dataset of the port of Bilbao in Chapter III matched with the non-indigenous amphipods *Grandidierella japonica* and *Melitida nitida,* and the sea-spider *Ammothea hilgendorfi*. While as mentioned in section 2.3., confidence in these taxonomic assignments must be further assessed, these species could have been introduced with different vectors of shipping.

Indeed, the dispersal of these species into new environments has been associated to ballast water, hull fouling on both commercial and recreational vessels and as well as to aquaculture dispersal vector (Droual et al., 2017; Faasse, 2013; Gouillieux et al., 2016; Marchini et al., 2016). Interestingly, for the sea-spider *Ammothea hilgendorfi*, its currently known distribution spans the north of Europe between Antwerp and Rotterdam in the North Sea and in the UK near Poole Harbor (Faasse, 2013). As we showed in Figure 24, Antwerp and Rotterdam present strong connection with the port of Bilbao, as does Poole Harbor that has a ferry-connection with Bilbao serving twice a week. This suggests that these maritime routes could potentially be involved with the transfer of this species either via ballast water or hull fouling. As this species lives on sediment or in fouling communities and does not have a larval phase, ballast water seems less likely than hull fouling (Eno et al., 1997; Faasse, 2013; Krapp and Sconfietti, 1983).

Overall, this thesis has highlighted the potential of metabarcoding for ship-borne biodiversity monitoring. The cost-efficiency of the technique allows to process many samples simultaneously (Aylagas et al., 2018; Ji et al., 2013). Thanks to this advantage and the use of maritime traffic data to understand shipping patterns, it is much recommended to continue monitoring port waters and include the surveillance of vectors of introduction to:

(1) inform the port authorities and environmental governmental agencies about the distribution of NICS within a port and potential "high risk" ports for the introduction of new NICS,

(2) provide biological baseline data against future changes in the environment and,

(3) provide biological data for the BWM Convention to support the exemption process and evaluate the efficiency of ballast water management.

# CONCLUSIONS

**(English version)**

Taking into account the objectives of this thesis, we conclude that:

(1) Genetic tools supported different monitoring actions of the BWM Convention, such as Port Biological Baseline Survey and testing the efficiency of ballast water management. Particularly, DNA metabarcoding for biodiversity assessment and detection of potential NIS provided relevant biological data for the granting exemption procedure of the BWM Convention.

(2) Guidelines for the future implementation of DNA metabarcoding for Port Biological Baseline Survey had been developed. We recommend sampling during spring and late summer to maximize the biodiversity recovery and always include sites with a wide range of salinities to increase the detection of NICS. We also suggest combining different conventional sampling methods (plankton nets, sediment grab, settlement plates) as not a single method will recover all taxa present in the diverse habitats of a port.

(3) The eDNA alone was not enough to recover the metazoan diversity present in the different habitats of the port when comparing with the combination of conventional sampling methods. More studies will be needed to increase the reliability of eDNA metabarcoding by assessing the efficiency of relying of group-specific primers (*e.g.* for crustacean) instead of universal primers as used in this thesis.

(4) The use of eDNA was reliable to perform ballast water monitoring on board of vessel as being a fast sampling method able to characterize a diverse range of taxa of ballast water community and to distinguish between ballast water exchanged and treated communities. The sampling point and method used impacted the communities which requires further work to assess which sampling point may be the most representative of the discharged communities detected with eDNA metabarcoding.

# CONCLUSIONES

## (Versión española)

Teniendo en cuenta los objetivos de esta tesis, concluimos que:

(1) Las herramientas genéticas pueden dar apoyo a diferentes acciones de monitoreo requeridas por el Convenio BWM, tales como el estudio de referencia biológica de puertos y la demostración de la eficacia de la gestión de las aguas de lastre. En particular, el ADN *metabarcoding* utilizado para la evaluación de la biodiversidad y la detección de posibles especies alóctonas proporcionan datos biológicos relevantes para el procedimiento de exención de concesión del Convenio BWM.

(2) Se desarrollaron directrices para la futura implementación del ADN *metabarcoding* para el estudio de referencia biológica de los puertos. Con el fin de aumentar la detección de las especies alóctonas y obtener la máxima biodiversidad posible, se recomienda tomar muestras durante la primavera y finales del verano e incluir siempre sitios con una amplia gama de salinidad. También se sugiere combinar diferentes métodos de muestreo convencionales (redes de plancton, toma de sedimentos, placas de asentamiento) ya que ningún método de forma individual detecta todos los taxones presentes en los diversos hábitats de un puerto.

(3) El ADN ambiental no fue suficiente por sí sólo de detectar la biodiversidad de metazoo presente en los diferentes hábitats del puerto en comparación con la combinación de métodos de muestreo convencionales. Se necesitarán más estudios para aumentar la fiabilidad del ADN ambiental *metabarcoding*. Esto podrá llevarse a cabo evaluando la eficacia de *primers* específicos de grupo (por ejemplo, para crustáceos) en lugar de *primers* universales como se usa en esta tesis.

(4) Se ha demostrado la fiabilidad del ADN ambiental como un método de muestreo rápido capaz de caracterizar una amplia gama de taxones de la comunidad presente en las aguas de lastre a bordo de los buques, permitiendo, en función de las comunidades, diferenciar entre la gestión aplicada a las aguas (intercambio de agua de lastre o tratamiento). El punto de muestreo y el método utilizado afecta a la detección de la presencia de comunidades. Esto requiere un trabajo adicional para evaluar qué punto de muestreo puede ser el más representativo para detectar con el ADN ambiental *metabarcoding* las comunidades presentes en las aguas de lastre descargadas.

## REFERENCES


# A

Adarraga, I., Martínez, J., 2012. First record of the invasive brackish water mytilid *Limnoperna securis* (Lamarck, 1819) in the Bay of Biscay. Aquat. Invasions 7.

Adarraga, I., Martínez, J., 2011. First record of invasive *Theora lubrica* (Mollusca: Bivalvia: Semelidae) in the Atlantic Ocean. Mar. Biodivers. Rec. 4. https://doi.org/10.1017/S1755267211001011

Agencia Vasca del Agua, 2014. Protocolo de muestreo, análisis y evaluación de fauna bentónica de macroinvertebrados de sustrato blando en masas de agua de transición y costeras.

Agnarsson, I., Kuntner, M., 2007. Taxonomy in a changing world: seeking solutions for a science in crisis. Syst. Biol. 56, 531–539.

Agrawal, N., Hassan, Y.A., Ugaz, V.M., 2007. A Pocket-Sized Convective PCR Thermocycler. Angew. Chem. Int. Ed. 46, 4316–4319.

Aguirre-Macedo, M.L., Vidal-Martinez, V.M., Herrera-Silveira, J.A., Valdés-Lozano, D.S., Herrera-Rodríguez, M., Olvera-Novoa, M.A., 2008. Ballast water as a vector of coral pathogens in the Gulf of Mexico: the case of the Cayo Arcas coral reef. Mar. Pollut. Bull. 56, 1570–1577. https://doi.org/10.1016/j.marpolbul.2008.05.022

Ahyong, S.T., Costello, M.J., Galil, B.S., Gollasch, S., Hutchings, P.A., Katsanevakis Stylianos, M., Lejeusne, C., Marchini, A., Occhipinti-Ambrogi, A., Pagad, S., Poore, G.C.B., Ruis, M., Robinson, T.B., Sterrer, W., Turon, X., Willan, R.C., Zhan, A., 2019. World Register of Introduced Marine Species (WRiMS). Accessed at http://www.marinespecies.org/introduced on 2019-01-25.

Albaina, A., Uriarte, I., Aguirre, M., Abad, D., Iriarte, A., Villate, F., Estonba, A., 2016. Insights on the origin of invasive copepods colonizing Basque estuaries; a DNA barcoding approach. Mar. Biodivers. Rec. 9, 51. https://doi.org/10.1186/s41200-016-0045-2

Albert, R.J., Lishman, J.M., Saxena, J.R., 2013. Ballast water regulations and the move toward concentration-based numeric discharge limits. Ecol. Appl. 23, 289–300.

Amaral-Zettler, L.A., McCliment, E.A., Ducklow, H.W., Huse, S.M., 2009. A Method for Studying Protistan Diversity Using Massively Parallel Sequencing of V9 Hypervariable Regions of Small-Subunit Ribosomal RNA Genes. PLOS ONE 4, e6372. https://doi.org/10.1371/journal.pone.0006372

Ammon, U. von, Wood, S.A., Laroche, O., Zaiko, A., Tait, L., Lavery, S., Inglis, G.J., Pochon, X., 2018. Combining morpho-taxonomy and metabarcoding enhances the detection of non-indigenous marine pests in biofouling communities. Sci. Rep. 8, 16290. https://doi.org/10.1038/s41598-018-34541-1

Andrews, S., 2010. FastQC: a quality control tool for high throughput sequence data.

Antonella, P., Luca, G., 2013. The quantitative real-time PCR applications in the monitoring of marine harmful algal bloom (HAB) species. Environ. Sci. Pollut. Res. Int. 20, 6851–6862. https://doi.org/10.1007/s11356-012-1377-z

AquaNIS. Editorial board., 2015. Information system on Aquatic Non-Indigenous and Cryptogenic Species. World Wide Web electronic publication. www.corpi.ku.lt/databases/aquanis. Version 2.36+.

Ardura, A., Zaiko, A., Martinez, J.L., Samuiloviene, A., Borrell, Y., Garcia-Vazquez, E., 2015a. Environmental DNA evidence of transfer of North Sea molluscs across tropical waters through ballast water. J. Molluscan Stud. 81, 495–501. https://doi.org/10.1093/mollus/eyv022

Ardura, A., Zaiko, A., Martinez, J.L., Samulioviene, A., Semenova, A., Garcia-Vazquez, E., 2015b. eDNA and specific primers for early detection of invasive species–A case study on the bivalve *Rangia cuneata*, currently spreading in Europe. Mar. Environ. Res. 112, 48–55.

Asai, S., Ianora, A., Lauritano, C., Lindeque, P.K., Carotenuto, Y., 2015. High-quality RNA extraction from copepods for Next Generation Sequencing: A comparative study. Mar. Genomics, SI:StructureRegulationEvolution 24, Part 1, 115–118. https://doi.org/10.1016/j.margen.2014.12.004

Ashton, G.V., Boos, K., Shucksmith, R., Cook, E.J., 2006. Rapid assessment of the distribution of marine non-native species in marinas in Scotland. Aquat. Invasions 1, 209–213.

Awad, A., Haag, F., Anil, A.C., Abdulla, A., 2014. Guidance on Port Biological Baseline Surveys (PBBS). GEF-UNDP-IMO GloBallast Partnerships, IOI, NIO, IUCN.

Aylagas, E., 2017. DNA metabarcoding derived biotic indices for marine monitoring and assessment.

Aylagas, E., Borja, Á., Irigoien, X., Rodríguez-Ezpeleta, N., 2016a. Benchmarking DNA Metabarcoding for Biodiversity-Based Monitoring and Assessment. Front. Mar. Sci. 3. https://doi.org/10.3389/fmars.2016.00096

Aylagas, E., Borja, Á., Muxika, I., Rodríguez-Ezpeleta, N., 2018. Adapting metabarcoding-based benthic biomonitoring into routine marine ecological status assessment networks. Ecol. Indic. 95, 194–202. https://doi.org/10.1016/j.ecolind.2018.07.044

Aylagas, E., Borja, Á., Rodríguez-Ezpeleta, N., 2014. Environmental status assessment using DNA metabarcoding: towards a genetics based marine biotic index (gAMBI). PloS One 9, e90529.

Aylagas, E., Mendibil, I., Borja, Á., Rodríguez-Ezpeleta, N., 2016b. Marine Sediment Sample Pre-processing for Macroinvertebrates Metabarcoding: Mechanical Enrichment and Homogenization. Front. Mar. Sci. 3. https://doi.org/10.3389/fmars.2016.00203

Aylagas, E., Rodríguez-Ezpeleta, N., 2016. Analysis of Illumina MiSeq Metabarcoding Data: Application to Benthic Indices for Environmental Monitoring. Methods Mol. Biol. Clifton NJ 1452, 237–249. https://doi.org/10.1007/978-1-4939-3774-5_16

Ayres, D.R., Garcia-Rossi, D., Davis, H.G., Strong, D.R., 1999. Extent and degree of hybridization between exotic (*Spartina alterniflora*) and native (*S. foliosa*) cordgrass (Poaceae) in California, USA determined by random amplified polymorphic DNA (RAPDs). Mol. Ecol. 8, 1179–1186. https://doi.org/10.1046/j.1365-294x.1999.00679.x

## B

Bailey, S.A., Rajakaruna, H., 2017. Optimizing methods to estimate zooplankton concentration based on generalized patterns of patchiness inside ballast tanks and ballast water discharges. Ecol. Evol. 7, 9689–9698. https://doi.org/10.1002/ece3.3498

Barnes, M.A., Turner, C.R., 2016. The ecology of environmental DNA and implications for conservation genetics. Conserv. Genet. 17, 1–17.

Barnes, M.A., Turner, C.R., Jerde, C.L., Renshaw, M.A., Chadderton, W.L., Lodge, D.M., 2014. Environmental conditions influence eDNA persistence in aquatic systems. Environ. Sci. Technol. 48, 1819–1827.

Basurko, O.C., Mesbahi, E., 2011. Statistical representativeness of ballast water sampling. Proc. Inst. Mech. Eng. Part M J. Eng. Marit. Environ. 225, 183–190. https://doi.org/10.1177/1475090211412682

Bax, N., Williamson, A., Aguero, M., Gonzalez, E., Geeves, W., 2003. Marine invasive alien species: a threat to global biodiversity. Mar. Policy, Emerging Issues in Oceans, Coasts and Islands 27, 313–323. https://doi.org/10.1016/S0308-597X(03)00041-1

Berry, T.E., Saunders, B.J., Coghlan, M.L., Stat, M., Jarman, S., Richardson, A.J., Davies, C.H., Berry, O., Harvey, E.S., Bunce, M., 2019. Marine environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events. PLOS Genet. 15, e1007943. https://doi.org/10.1371/journal.pgen.1007943

Bijleveld, A.I., Compton, T.J., Klunder, L., Holthuijsen, S., Horn, J. ten, Koolhaas, A., Dekinga, A., Meer, J. van der, Veer, H.W. van der, 2018. Presence-absence of marine macrozoobenthos does not generally predict abundance and biomass. Sci. Rep. 8, 3039. https://doi.org/10.1038/s41598-018-21285-1

Bishop, M.J., Hutchings, P.A., 2011. How useful are port surveys focused on target pest identification for exotic species management? Mar. Pollut. Bull. 62, 36–42. https://doi.org/10.1016/j.marpolbul.2010.09.014

Bista, I., Carvalho, G.R., Walsh, K., Seymour, M., Hajibabaei, M., Lallias, D., Christmas, M., Creer, S., 2017. Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. Nat. Commun. 8, 14087. https://doi.org/10.1038/ncomms14087

Blatchley, E.R., Cullen, J.J., Petri, B., Bircher, K., Welschmeyer, N., 2018. The Biological Basis for Ballast Water Performance Standards: “Viable/Non-Viable” or “Live/Dead”? Environ. Sci. Technol. 52, 8075–8086. https://doi.org/10.1021/acs.est.8b00341

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Borcard, D., Legendre, P., Drapeau, P., 1992. Partialling out the Spatial Component of Ecological Variation. Ecology 73, 1045–1055. https://doi.org/10.2307/1940179

Borrell, Y.J., Miralles, L., Huu, H.D., Mohammed-Geba, K., Garcia-Vazquez, E., 2017. DNA in a bottle—Rapid metabarcoding survey for early alerts of invasive species in ports. PLOS ONE 12, e0183347. https://doi.org/10.1371/journal.pone.0183347

Bott, N.J., 2015. Detection of Invasive Species, in: Prof, S.-K.K. (Ed.), Springer Handbook of Marine Biotechnology. Springer Berlin Heidelberg, pp. 441–453. https://doi.org/10.1007/978-3-642-53971-8_16

Branstrator, D.K., Westphal, K.L., King, B.K., 2015. Analysis of invertebrate resting eggs and other biota in ballast tank sediment of domestic Great Lakes cargo ships. J. Gt. Lakes Res. 41, 200–207. https://doi.org/10.1016/j.jglr.2014.11.017

Brawley, S.H., Coyer, J.A., Blakeslee, A.M.H., Hoarau, G., Johnson, L.E., Byers, J.E., Stam, W.T., Olsen, J.L., 2009. Historical invasions of the intertidal zone of Atlantic North America associated with distinctive patterns of trade and emigration. Proc. Natl. Acad. Sci. 106, 8239–8244. https://doi.org/10.1073/pnas.0812300106

Brinkmeyer, R., 2016. Diversity of bacteria in ships ballast water as revealed by next generation DNA sequencing. Mar. Pollut. Bull. 107, 277–285. https://doi.org/10.1016/j.marpolbul.2016.03.058

Briski, E., Bailey, S.A., Casas-Monroy, O., DiBacco, C., Kaczmarska, I., Lawrence, J.E., Leichsenring, J., Levings, C., MacGillivary, M.L., McKindsey, C.W., Nasmith, L.E., Parenteau, M., Piercey, G.E., Rivkin, R.B., Rochon, A., Roy, S., Simard, N., Sun, B., Way, C., Weise, A.M., MacIsaac, H.J., 2013. Taxon- and vector-specific variation in species richness and abundance during the transport stage of biological invasions. Limnol. Oceanogr. 58, 1361–1372. https://doi.org/10.4319/lo.2013.58.4.1361

Briski, E., Bailey, S.A., Cristescu, M.E., Macisaac, H.J., 2010. Efficacy of "saltwater flushing" in protecting the Great Lakes from biological invasions by invertebrate eggs in ships' ballast sediment. Freshw. Biol. 55, 2414–2424. https://doi.org/10.1111/j.1365-2427.2010.02449.x

Briski, E., Cristescu, M.E., Bailey, S.A., MacIsaac, H.J., 2011. Use of DNA barcoding to detect invertebrate invasive species from diapausing eggs. Biol. Invasions 13, 1325–1340. https://doi.org/10.1007/s10530-010-9892-7

Briski, E., Ghabooli, S., Bailey, S.A., MacIsaac, H.J., 2016. Are genetic databases sufficiently populated to detect non-indigenous species? Biol. Invasions 18, 1911–1922. https://doi.org/10.1007/s10530-016-1134-1

Briski, E., Ghabooli, S., Bailey, S.A., MacIsaac, H.J., 2012. Invasion risk posed by macroinvertebrates transported in ships' ballast tanks. Biol. Invasions 14, 1843–1850. https://doi.org/10.1007/s10530-012-0194-0

Briski, E., Gollasch, S., David, M., Linley, R.D., Casas-Monroy, O., Rajakaruna, H., Bailey, S.A., 2015. Combining Ballast Water Exchange and Treatment To Maximize Prevention of Species Introductions to Freshwater Ecosystems. Environ. Sci. Technol. 49, 9566–9573. https://doi.org/10.1021/acs.est.5b01795

Briski, E., Linley, R.D., Adams, J., Bailey, S.A., 2014. Evaluating efficacy of a ballast water filtration system for reducing spread of aquatic species in freshwater ecosystems. Manag. Biol. Invasions 5, 245–253.

Brown, E.A., Chain, F.J.J., Crease, T.J., MacIsaac, H.J., Cristescu, M.E., 2015. Divergence thresholds and divergent biodiversity estimates: can metabarcoding reliably describe zooplankton communities? Ecol. Evol. 5, 2234–2251. https://doi.org/10.1002/ece3.1485

Brown, E.A., Chain, F.J.J., Zhan, A., MacIsaac, H.J., Cristescu, M.E., 2016. Early detection of aquatic invaders using metabarcoding reveals a high number of non-indigenous species in Canadian ports. Divers. Distrib. 22, 1045–1059. https://doi.org/10.1111/ddi.12465

Bucklin, A., Lindeque, P.K., Rodriguez-Ezpeleta, N., Albaina, A., Lehtiniemi, M., 2016. Metabarcoding of marine zooplankton: prospects, progress and pitfalls. J. Plankton Res. 38, 393–400. https://doi.org/10.1093/plankt/fbw023

Burkholder, J.M., Hallegraeff, G.M., Melia, G., Cohen, A., Bowers, H.A., Oldach, D.W., Parrow, M.W., Sullivan, M.J., Zimba, P.V., Allen, E.H., others, 2007. Phytoplankton and bacterial assemblages in ballast water of US military ships as a function of port of origin, voyage time, and ocean exchange practices. Harmful Algae 6, 486–518.

Butrón, A., Orive, E., Madariaga, I., 2011. Potential risk of harmful algae transport by ballast waters: The case of Bilbao Harbour. Mar. Pollut. Bull. 62, 747–757.

# C

CABI, 2019a. *Mytilus edulis*. In: Invasive Species Compendium. Wallingford, UK: CAB International. www.cabi.org/isc.

CABI, 2019b. *Hemigrapsus takanoi*. In: Invasive Species Compendium. Wallingford, UK: CAB International. www.cabi.org/isc.

Cabrini, M., Cerino, F., de Olazabal, A., Di Poi, E., Fabbro, C., Fornasaro, D., Goruppi, A., Flander-Putrle, V., Francé, J., Gollasch, S., Hure, M., Lipej, L., Lučić, D., Magaletti, E., Mozetič, P., Tinta, T., Tornambè, A., Turk, V., Uhan, J., David, M., 2018. Potential transfer of aquatic organisms via ballast water with a particular focus on harmful and non-indigenous species: A survey from Adriatic ports. Mar. Pollut. Bull. https://doi.org/10.1016/j.marpolbul.2018.02.004

Campbell, M.L., Gould, B., Hewitt, C.L., 2007. Survey evaluations to assess marine bioinvasions. Mar. Pollut. Bull. 55, 360–378.

Cardeccia, A., Marchini, A., Occhipinti-Ambrogi, A., Galil, B., Gollasch, S., Minchin, D., Narščius, A., Olenin, S., Ojaveer, H., 2018. Assessing biological invasions in European Seas: Biological traits of the most widespread non-indigenous species. Estuar. Coast. Shelf Sci., Vectors of change in the marine environment 201, 17–28. https://doi.org/10.1016/j.ecss.2016.02.014

Carlton, J.T., 1999. The scale and ecological consequences of biological invasions in the World's oceans. Presented at the Population and Community Biology Series Volume 24, Kluwer Academic Publishers, pp. 195–212.

Carlton, J.T., 1996a. Pattern, process, and prediction in marine invasion ecology. Biol. Conserv. 78, 97–106.

Carlton, J.T., 1996b. Biological Invasions and Cryptogenic Species. Ecology 77, 1653–1655. https://doi.org/10.2307/2265767

Carlton, J.T., Hodder, J., 1995. Biogeography and dispersal of coastal marine organisms: experimental studies on a replica of a 16th-century sailing vessel. Mar. Biol. 121, 721–730. https://doi.org/10.1007/BF00349308

Carney, K.J., Basurko, O.C., Pazouki, K., Marsham, S., Delany, J.E., Desai, D.V., Anil, A.C., Mesbahi, E., 2013. Difficulties in obtaining representative samples for compliance with the Ballast Water Management Convention. Mar. Pollut. Bull. 68, 99–105.

Carney, K.J., Minton, M.S., Holzer, K.K., Miller, A.W., McCann, L.D., Ruiz, G.M., 2017. Evaluating the combined effects of ballast water management and trade dynamics on transfers of marine organisms by ships. PLOS ONE 12, e0172468. https://doi.org/10.1371/journal.pone.0172468

Cenciarini-Borde, C., Courtois, S., La Scola, B., 2009. Nucleic acids as viability markers for bacteria detection using molecular tools. Future Microbiol. 4, 45–64. https://doi.org/10.2217/17460913.4.1.45

Chain, F.J.J., Brown, E.A., MacIsaac, H.J., Cristescu, M.E., 2016. Metabarcoding reveals strong spatial structure and temporal turnover of zooplankton communities among marine and freshwater ports. Divers. Distrib. 22, 493–504. https://doi.org/10.1111/ddi.12427

Chan, F.T., Bradie, J., Briski, E., Bailey, S.A., Simard, N., MacIsaac, H.J., 2015. Assessing introduction risk using species' rank-abundance distributions. Proc. R. Soc. B Biol. Sci. 282. https://doi.org/10.1098/rspb.2014.1517

Choi, Y.-J., Beakes, G., Glockling, S., Kruse, J., Nam, B., Nigrelli, L., Ploch, S., Shin, H.-D., Shivas, R.G., Telle, S., Voglmayr, H., Thines, M., 2015. Towards a universal barcode of

oomycetes – a comparison of the cox1 and cox2 loci. Mol. Ecol. Resour. 15, 1275–1288. https://doi.org/10.1111/1755-0998.12398

Chu, K.H., Tam, P.F., Fung, C.H., Chen, Q.C., 1997. A biological survey of ballast water in container ships entering Hong Kong, in: Wong, Y.-S., Tam, N.F.-Y. (Eds.), Asia-Pacific Conference on Science and Management of Coastal Environment. Springer Netherlands, Dordrecht, pp. 201–206. https://doi.org/10.1007/978-94-011-5234-1_20

Clarke, L.J., Beard, J.M., Swadling, K.M., Deagle, B.E., 2017. Effect of marker choice and thermal cycling protocol on zooplankton DNA metabarcoding studies. Ecol. Evol. 7, 873–883. https://doi.org/10.1002/ece3.2667

Clavero, M., García-Berthou, E., 2006. Homogenization dynamics and introduction routes of invasive freshwater fish in the Iberian Peninsula. Ecol. Appl. 16, 2313–2324.

Collins, R.A., Wangensteen, O.S., O'Gorman, E.J., Mariani, S., Sims, D.W., Genner, M.J., 2018. Persistence of environmental DNA in marine systems. Commun. Biol. 1, 185. https://doi.org/10.1038/s42003-018-0192-6

Comtet, T., Sandionigi, A., Viard, F., Casiraghi, M., 2015. DNA (meta) barcoding of biological invasions: a powerful tool to elucidate invasion processes and help managing aliens. Biol. Invasions 17, 905–922.

Corell, J., Rodríguez-Ezpeleta, N., 2014. Tuning of protocols and marker selection to evaluate the diversity of zooplankton using metabarcoding. Rev Invest Mar 21, 19–39.

Cornils, A., Wend-Heckmann, B., 2015. First report of the planktonic copepod *Oithona davisae* in the northern Wadden Sea (North Sea): Evidence for recent invasion? Helgol. Mar. Res. 69, 243–248. https://doi.org/10.1007/s10152-015-0426-7

Cowart, D.A., Pinheiro, M., Mouchel, O., Maguer, M., Grall, J., Miné, J., Arnaud-Haond, S., 2015. Metabarcoding is powerful yet still blind: a comparative analysis of morphological and molecular surveys of seagrass communities. PloS One 10, e0117562. https://doi.org/10.1371/journal.pone.0117562

Creer, S., Deiner, K., Frey, S., Porazinska, D., Taberlet, P., Thomas, W.K., Potter, C., Bik, H.M., 2016. The ecologist's field guide to sequence-based identification of biodiversity. Methods Ecol. Evol. 7, 1008–1018. https://doi.org/10.1111/2041-210X.12574

Cristescu, M.E., 2014. From barcoding single individuals to metabarcoding biological communities: towards an integrative approach to the study of global biodiversity. Trends Ecol. Evol. 29, 566–571.

Cristescu, M.E., Hebert, P.D.N., 2018. Uses and Misuses of Environmental DNA in Biodiversity Science and Conservation. Annu. Rev. Ecol. Evol. Syst. 49, 209–230. https://doi.org/10.1146/annurev-ecolsys-110617-062306

Crooks, J.A., Suarez, A.V., 2006. Hyperconnectivity, invasive species, and the breakdown of barriers to dispersal, in: Crooks, K.R., Sanjayan, M. (Eds.), Connectivity Conservation. Cambridge University Press, Cambridge, pp. 451–478. https://doi.org/10.1017/CBO9780511754821.021

## D

Darling, J.A., 2015. Genetic studies of aquatic biological invasions: closing the gap between research and management. Biol. Invasions 17, 951–971.

Darling, J.A., Blum, M.J., 2007. DNA-based methods for monitoring invasive species: a review and prospectus. Biol. Invasions 9, 751–765.

Darling, J.A., Carlton, J.T., 2018. A Framework for Understanding Marine Cosmopolitanism in the Anthropocene. Front. Mar. Sci. 5. https://doi.org/10.3389/fmars.2018.00293

Darling, J.A., Frederick, R.M., 2017. Nucleic acids-based tools for ballast water surveillance, monitoring, and research. J. Sea Res. https://doi.org/10.1016/j.seares.2017.02.005

Darling, J.A., Galil, B.S., Carvalho, G.R., Rius, M., Viard, F., Piraino, S., 2017. Recommendations for developing and applying genetic tools to assess and manage biological invasions in marine ecosystems. Mar. Policy 85, 54–64. https://doi.org/10.1016/j.marpol.2017.08.014

Darling, J.A., Mahon, A.R., 2011. From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. Environ. Res. 111, 978–988.

Darling, J.A., Martinson, J., Gong, Y., Okum, S., Pilgrim, E., Lohan, K.M.P., Carney, K.J., Ruiz, G.M., 2018. Ballast Water Exchange and Invasion Risk Posed by Intracoastal Vessel Traffic: An Evaluation Using High Throughput Sequencing. Environ. Sci. Technol. 52, 9926–9936. https://doi.org/10.1021/acs.est.8b02108

Darling, J.A., Tepolt, C.K., others, 2008. Highly sensitive detection of invasive shore crab (*Carcinus maenas* and *Carcinus aestuarii*) larvae in mixed plankton samples using polymerase chain reaction and restriction fragment length polymorphisms (PCR-RFLP). Aquat Invasions 3, 141–152.

David, M., Gollasch, S. (Eds.), 2015. Global Maritime Transport and Ballast Water Management. Springer Netherlands, Dordrecht.

David, M., Gollasch, S., Pavliha, M., 2013. Global ballast water management and the "same location" concept: a clear term or a clear issue? Ecol. Appl. 23, 331–338. https://doi.org/10.1890/12-0992.1

David, M., Gollasch, S., Penko, L., 2018. Identification of ballast water discharge profiles of a port to enable effective ballast water management and environmental studies. J. Sea Res., Recent Advances in Ballast Water Research 133, 60–72. https://doi.org/10.1016/j.seares.2017.03.001

David, M., Perkovič, M., Suban, V., Gollasch, S., 2012. A generic ballast water discharge assessment model as a decision supporting tool in ballast water management. Decis. Support Syst. 53, 175–185. https://doi.org/10.1016/j.dss.2012.01.002

Davidson, A.D., Fusaro, A.J., Kashian, D.R., 2015. Using a Novel Spatial Tool to Inform Invasive Species Early Detection and Rapid Response Efforts. Environ. Manage. 56, 54–65. https://doi.org/10.1007/s00267-015-0484-2

Davidson, I.C., Scianni, C., Minton, M.S., Ruiz, G.M., 2018. A history of ship specialization and consequences for marine invasions, management and policy. J. Appl. Ecol. 55, 1799–1811. https://doi.org/10.1111/1365-2664.13114

De Cáceres, M.D., Legendre, P., 2009. Associations between species and groups of sites: indices and statistical inference. Ecology 90, 3566–3574.

Deagle, B.E., Bax, N., Hewitt, C.L., Patil, J.G., 2003. Development and evaluation of a PCR-based test for detection of Asterias (Echinodermata: Asteroidea) larvae in Australian plankton samples from ballast water. Mar. Freshw. Res. 54, 709–719.

Deagle, B.E., Jarman, S.N., Coissac, E., Pompanon, F., Taberlet, P., 2014. DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. Biol. Lett. 10, 20140562.

Deiner, K., Bik, H.M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D.M., Vere, N. de, Pfrender, M.E., Bernatchez, L., 2017. Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. Mol. Ecol. 26, 5872–5895. https://doi.org/10.1111/mec.14350

Deiner, K., Fronhofer, E.A., Mächler, E., Walser, J.-C., Altermatt, F., 2016. Environmental DNA reveals that rivers are conveyer belts of biodiversity information. Nat. Commun. 7, 12544. https://doi.org/10.1038/ncomms12544

Desai, D.V., Narale, D., Khandeparker, L., Anil, A.C., 2018. Potential ballast water transfer of organisms from the west to the east coast of India: Insights through on board sampling. J. Sea Res., Recent Advances in Ballast Water Research 133, 88–99. https://doi.org/10.1016/j.seares.2017.03.010

Dias, P.J., Fotedar, S., Munoz, J., Hewitt, M.J., Lukehurst, S., Hourston, M., Wellington, C., Duggan, R., Bridgwood, S., Massam, M., 2017. Establishment of a taxonomic and molecular reference collection to support the identification of species regulated by the Western Australian Prevention List for Introduced Marine Pests. Manag. Biol. Invasions 8, 215–225.

Díaz-Ferguson, E.E., Moyer, G.R., 2014. History, applications, methodological issues and perspectives for the use environmental DNA (eDNA) in marine and freshwater environments. Rev. Biol. Trop. 62, 1273–1284.

DiBacco, C., Humphrey, D.B., Nasmith, L.E., Levings, C.D., 2012. Ballast water transport of non-indigenous zooplankton to Canadian ports. ICES J. Mar. Sci. 69, 483–491. https://doi.org/10.1093/icesjms/fsr133

Djurhuus, A., Pitz, K., Sawaya, N.A., Rojas-Márquez, J., Michaud, B., Montes, E., Muller-Karger, F., Breitbart, M., 2018. Evaluation of marine zooplankton community structure through environmental DNA metabarcoding: Metabarcoding zooplankton from eDNA. Limnol. Oceanogr. Methods 16, 209–221. https://doi.org/10.1002/lom3.10237

Djurhuus, A., Port, J., Closek, C.J., Yamahara, K.M., Romero-Maraccini, O., Walz, K.R., Goldsmith, D.B., Michisaki, R., Breitbart, M., Boehm, A.B., Chavez, F.P., 2017. Evaluation of Filtration and DNA Extraction Methods for Environmental DNA Biodiversity Assessments across Multiple Trophic Levels. Front. Mar. Sci. 4, 314. https://doi.org/10.3389/fmars.2017.00314

Doblin, M.A., Coyne, K.J., Rinta-Kanto, J.M., Wilhelm, S.W., Dobbs, F.C., 2007. Dynamics and short-term survival of toxic cyanobacteria species in ballast water from NOBOB vessels transiting the Great Lakes—implications for HAB invasions. Harmful Algae 6, 519–530.

Dowle, E.J., Pochon, X., C Banks, J., Shearer, K., Wood, S.A., 2016. Targeted gene enrichment and high-throughput sequencing for environmental biomonitoring: a case study using freshwater macroinvertebrates. Mol. Ecol. Resour. 16, 1240–1254. https://doi.org/10.1111/1755-0998.12488

Doyle, T.K., De Haas, H., Cotton, D., Dorschel, B., Cummins, V., Houghton, J.D., Davenport, J., Hays, G.C., 2008. Widespread occurrence of the jellyfish *Pelagia noctiluca* in Irish coastal and shelf waters. J. Plankton Res. 30, 963–968.

Drake, J.M., Lodge, D.M., 2004. Global hot spots of biological invasions: Evaluating options for ballast–water management. Proc. R. Soc. Lond. B Biol. Sci. 271, 575–580.

Drake, L.A., Doblin, M.A., Dobbs, F.C., 2007. Potential microbial bioinvasions via ships' ballast water, sediment, and biofilm. Mar. Pollut. Bull. 55, 333–341.

Dray, S., Bauman, D., Blanchet, G., Borcard, D., Clappe, S., Guenard, G., Jombart, T., Larocque, G., Legendre, P., Madi, N., Wagner, H.H., 2018. adespatial: Multivariate Multiscale Spatial Analysis.

Droual, G., Le Garrec, V., Cabelguen, J., Gélinaud, G., Grall, J., 2017. The spread goes on: the non-indigenous species *Grandidierella japonica* Stephensen, 1938 (Amphipoda: Aoridae) has reached Brittany (Gulf of Morbihan). Aod- Cah. Nat. L'Observatoire Mar. 1, 21–29.

Drummond, A.J., Newcomb, R.D., Buckley, T.R., Xie, D., Dopheide, A., Potter, B.C., Heled, J., Ross, H.A., Tooman, L., Grosser, S., Park, D., Demetras, N.J., Stevens, M.I., Russell, J.C., Anderson, S.H., Carter, A., Nelson, N., 2015. Evaluating a multigene environmental DNA approach for biodiversity assessment. GigaScience 4. https://doi.org/10.1186/s13742-015-0086-1

Dufrene, M., Legendre, P., 1997. Species Assemblages and Indicator Species: The Need for a Flexible Asymmetrical Approach. Ecol. Monogr. 67, 345. https://doi.org/10.2307/2963459

Duggan, I.C., Bailey, S.A., Overdijk, C.D.A. van, MacIsaac, H.J., 2006. Invasion risk of active and diapausing invertebrates from residual ballast in ships entering Chesapeake Bay. Mar. Ecol. Prog. Ser. 324, 57–66. https://doi.org/10.3354/meps324057

## E

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200. https://doi.org/10.1093/bioinformatics/btr381

Edwards, A., Debbonaire, A.R., Sattler, B., Mur, L.A., Hodson, A.J., 2016. Extreme metagenomics using nanopore DNA sequencing: a field report from Svalbard, 78 N. bioRxiv 073965. https://doi.org/10.1101/073965

Elbrecht, V., 2017. Development of DNA metabarcoding methods for stream ecosystem assessment.

Elbrecht, V., Leese, F., 2015. Can DNA-Based Ecosystem Assessments Quantify Species Abundance? Testing Primer Bias and Biomass—Sequence Relationships with an Innovative Metabarcoding Protocol. PLoS ONE 10, e0130324. https://doi.org/10.1371/journal.pone.0130324

Elbrecht, V., Peinert, B., Leese, F., 2017a. Sorting things out: Assessing effects of unequal specimen biomass on DNA metabarcoding. Ecol. Evol. 7, 6918–6926. https://doi.org/10.1002/ece3.3192

Elbrecht, V., Vamos, E.E., Meissner, K., Aroviita, J., Leese, F., 2017b. Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. Methods Ecol. Evol. 8, 1265–1275. https://doi.org/10.1111/2041-210X.12789

Emami, K., Askari, V., Ullrich, M., Mohinudeen, K., Anil, A.C., Khandeparker, L., Burgess, J.G., Mesbahi, E., 2012. Characterization of bacteria in ballast water using MALDI-TOF mass spectrometry. PloS One 7, e38515.

Endresen, Ø., Lee Behrens, H., Brynestad, S., Bjørn Andersen, A., Skjong, R., 2004. Challenges in global ballast water management. Mar. Pollut. Bull. 48, 615–623. https://doi.org/10.1016/j.marpolbul.2004.01.016

Eno, N.C., Sanderson, W.G., Conservation, J.N., 1997. Non-native marine species in British waters: a review and directory.

Esquete, P., Moreira, J., Troncoso, J.S., 2011. Peracarid assemblages of Zostera meadows in an estuarine ecosystem (O Grove inlet, NW Iberian Peninsula): spatial distribution and seasonal variation. Helgol. Mar. Res. 65, 445.

# F

Faasse, M., 2013. Further dispersal of the sea-spider *Ammothea hilgendorfi* (Böhm, 1879) in the North Sea to The Netherlands. BioInvasions Rec. 2, 287–289.

Ferrari, F.D., Orsi, J., 1984. *Oithona davisae*, New Species, and *Limnoithona sinensis* (Burckhardt, 1912) (Copepoda: Oithonidae) from the Sacramento-San Joaquin Estuary, California. J. Crustac. Biol. 4, 106–126. https://doi.org/10.2307/1547900

Ferrario, J., Caronni, S., Occhipinti-Ambrogi, A., Marchini, A., 2017. Role of commercial harbours and recreational marinas in the spread of non-indigenous fouling species. Biofouling 33, 651–660. https://doi.org/10.1080/08927014.2017.1351958

Findenig, B.M., Chatzinotas, A., Boenigk, J., 2010. Taxonomic and ecological characterization of stomatocysts of Spumella-like flagellates (Chrysophyceae). J. Phycol. 46, 868–881. https://doi.org/10.1111/j.1529-8817.2010.00892.x

First, M.R., Robbins-Wamsley, S.H., Riley, S.C., Moser, C.S., Smith, G.E., Tamburri, M.N., Drake, L.A., 2013. Stratification of living organisms in ballast tanks: how do organism concentrations vary as ballast water is discharged? Environ. Sci. Technol. 47, 4442–4448.

Flynn, J.M., Brown, E.A., Chain, F.J.J., MacIsaac, H.J., Cristescu, M.E., 2015. Toward accurate molecular identification of species in complex environmental samples: testing the performance of sequence filtering and clustering methods. Ecol. Evol. 5, 2252–2266. https://doi.org/10.1002/ece3.1497

Fofonoff, P.W., Ruiz, G.M., Simkanin, S., Carlton, J.T., 2019. National Exotic Marine and Estuarine Species Information System. http://invasions.si.edu/nemesis/. Access Date: 8-Jan -2019.

Fonseca, V.G., Carvalho, G.R., Sung, W., Johnson, H.F., Power, D.M., Neill, S.P., Packer, M., Blaxter, M.L., Lambshead, P.J.D., Thomas, W.K., Creer, S., 2010. Second-generation environmental sequencing unmasks marine metazoan biodiversity. Nat. Commun. 1, 98. https://doi.org/10.1038/ncomms1095

Forster, D., Dunthorn, M., Mahé, F., Dolan, J.R., Audic, S., Bass, D., Bittner, L., Boutte, C., Christen, R., Claverie, J.-M., Decelle, J., Edvardsen, B., Egge, E., Eikrem, W., Gobet, A., Kooistra, W.H.C.F., Logares, R., Massana, R., Montresor, M., Not, F., Ogata, H., Pawlowski, J., Pernice, M.C., Romac, S., Shalchian-Tabrizi, K., Simon, N., Richards, T.A., Santini, S., Sarno, D., Siano, R., Vaulot, D., Wincker, P., Zingone, A., de Vargas, C., Stoeck, T., 2016. Benthic protists: the under-charted majority. FEMS Microbiol. Ecol. 92, fiw120. https://doi.org/10.1093/femsec/fiw120

Frazier, M., Miller, A.W., Lee, H., Reusser, D.A., 2013. Counting at low concentrations: the statistical challenges of verifying ballast water discharge standards. Ecol. Appl. 23, 339–351. https://doi.org/10.1890/11-1639.1

Fujimoto, M., Moyerbrailean, G.A., Noman, S., Gizicki, J.P., Ram, M.L., Green, P.A., Ram, J.L., 2014. Application of ion torrent sequencing to the assessment of the effect of

alkali ballast water treatment on microbial community diversity. PloS One 9, e107534. https://doi.org/10.1371/journal.pone.0107534

Fykse, E.M., Nilsen, T., Nielsen, A.D., Tryland, I., Delacroix, S., Blatny, J.M., 2012. Real-time PCR and NASBA for rapid and sensitive detection of *Vibrio cholerae* in ballast water. Mar. Pollut. Bull. 64, 200–206.

# G

Galili, T., 2015. dendextend: an R package for visualizing, adjusting and comparing trees of hierarchical clustering. Bioinformatics 31, 3718–3720.

Gao, Y., Wei, J., Yuan, J., Zhang, X., Li, F., Xiang, J., 2017. Transcriptome analysis on the exoskeleton formation in early developmetal stages and reconstruction scenario in growth-moulting in Litopenaeus vannamei. Sci. Rep. 7, 1098. https://doi.org/10.1038/s41598-017-01220-6

García-Garay, J., Franco-Herrera, A., Machuca-Martinez, F., 2018. Zooplankton sensitivity and phytoplankton regrowth for ballast water treatment with advanced oxidation processes. Environ. Sci. Pollut. Res. Int. 25, 35008–35014. https://doi.org/10.1007/s11356-018-2308-4

Gerhard, W.A., Gunsch, C.K., 2019. Metabarcoding and machine learning analysis of environmental DNA in ballast water arriving to hub ports. Environ. Int. 124, 312–319. https://doi.org/10.1016/j.envint.2018.12.038

Ghabooli, S., Zhan, A., Paolucci, E., Hernandez, M.R., Briski, E., Cristescu, M.E., MacIsaac, H.J., 2016. Population attenuation in zooplankton communities during transoceanic transfer in ballast water. Ecol. Evol. 6, 6170–6177. https://doi.org/10.1002/ece3.2349

Gillum, J.E., Jimenez, L., White, D.J., Goldstien, S.J., Gemmell, N.J., 2014. Development and application of a quantitative real-time PCR assay for the globally invasive tunicate *Styela clava*. Management 5, 133–142.

Gittenberger, A., Rensing, M., Niemantsverdriet, P., Schrieken, N., Stegenga, H., 2014. Port of Rotterdam survey and monitoring non-native species conform HELCOM/OSPAR protocol.

Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., Mckee, A., Oyler-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S., Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J., Taberlet, P., 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species 7, 1299–1307. https://doi.org/doi.org/10.1111/2041-210X.12595

Gollasch, S., Lenz, J., Dammer, M., Andres, H.-G., 2000. Survival of tropical ballast water organisms during a cruise from the Indian Ocean to the North Sea. J. Plankton Res. 22, 923–937. https://doi.org/10.1093/plankt/22.5.923

Gollasch, S., MacDonald, E., Belson, S., Botnen, H., Christensen, J.T., Hamer, J.P., Houvenaghel, G., Jelmert, A., Lucas, I.A.N., Masson, D., others, 2002. Life in ballast tanks, in: Invasive Aquatic Species of Europe. Distribution, Impacts and Management. Springer, pp. 217–231.

Gollasch, S., Rosenthal, H., Botnen, H., Crncevic, M., Gilbert, M., Hamer, J., Hülsmann, N., Mauro, C., McCann, L., Minchin, D., Öztürk, B., Robertson, M., Sutton, C., Villac,

M.C., 2003. Species Richness and Invasion Vectors: Sampling Techniques and Biases. Biol. Invasions 5, 365–377. https://doi.org/10.1023/B:BINV.0000005569.81791.25

Gómez-Rodríguez, C., Crampton-Platt, A., Timmermans, M.J., Baselga, A., Vogler, A.P., 2015. Validating the power of mitochondrial metagenomics for community ecology and phylogenetics of complex assemblages. Methods Ecol. Evol. 6, 883–894.

Gouillieux, B., Lavesque, N., Blanchet, H., Bachelet, G., 2016. First record of the non-indigenous *Melita nitida Smith*, 1873 (Crustacea: Amphipoda: Melitidae) in the Bay of Biscay (NE Atlantic). BioInvasions Rec. 5, 85–92.

Goulletquer, P., 2016. Guide des organismes exotiques marins. Belin.

Grey, E.K., Bernatchez, L., Cassey, P., Deiner, K., Deveney, M., Howland, K.L., Lacoursière-Roussel, A., Leong, S.C.Y., Li, Y., Olds, B., Pfrender, M.E., Prowse, T.A.A., Renshaw, M.A., Lodge, D.M., 2018. Effects of sampling effort on biodiversity patterns estimated from environmental DNA metabarcoding surveys. Sci. Rep. 8, 8843. https://doi.org/10.1038/s41598-018-27048-2

Grigorszky, I., Tihamér Kiss, K., Pór, G., Dévai, G., Nagy, A.S., Somlyai, I., Berta, C., Duleba, M., Trábert, Z., Ács, É., 2017. Temperature and growth strategies as the essential factors influencing the occurrence of *Stephanodiscus minutulus* (Kützing) Cleve & Möller and *Palatinus apiculatus* (Ehrenberg) Craveiro, Calado, Daugbjerg & Moestrup. Fundam. Appl. Limnol. Für Hydrobiol. 189, 167–175.

Grob, C., Pollet, B.G., 2016. Regrowth in ship's ballast water tanks: Think again! Mar. Pollut. Bull. 109, 46–48. https://doi.org/10.1016/j.marpolbul.2016.04.061

Grosholz, E.D., Ruiz, G.M., Dean, C.A., Shirley, K.A., Maron, J.L., Connors, P.G., 2000. The impacts of a nonindigenous marine predator in a california bay. Ecology 81, 1206–1224. https://doi.org/10.1890/0012-9658(2000)081[1206:TIOANM]2.0.CO;2

Guiry, M.D., Guiry, G.M., 2019. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; searched on 08 January 2019.

Günther, B., Knebelsberger, T., Neumann, H., Laakmann, S., Arbizu, P.M., 2018. Metabarcoding of marine environmental DNA based on mitochondrial and nuclear genes. Sci. Rep. 8, 14822. https://doi.org/10.1038/s41598-018-32917-x

# H

Hallegraeff, G.M., 2015. Transport of harmful marine microalgae via ship's ballast water: Management and mitigation with special reference to the Arabian Gulf region. Aquat. Ecosyst. Health Manag. 18, 290–298. https://doi.org/10.1080/14634988.2015.1027138

Hallegraeff, G.M., 2007. Foreword. Harmful Algae 6, 461–464. https://doi.org/10.1016/j.hal.2007.02.001

Hallegraeff, G.M., Bolch, C.J., 1991. Transport of toxic dinoflagellate cysts via ships' ballast water. Mar. Pollut. Bull. 22, 27–30.

Harvey, J.B.J., Hoy, M.S., Rodriguez, R.J., 2009. Molecular detection of native and invasive marine invertebrate larvae present in ballast and open water environmental samples collected in Puget Sound. J. Exp. Mar. Biol. Ecol. 369, 93–99. https://doi.org/10.1016/j.jembe.2008.10.030

Hawlitschek, O., Fernández-González, A., Puente, A.B. la, Castresana, J., 2018. A pipeline for metabarcoding and diet analysis from fecal samples developed for a small semi-aquatic mammal. PLOS ONE 13, e0201763. https://doi.org/10.1371/journal.pone.0201763

Hebert, P.D., Cywinska, A., Ball, S.L., others, 2003a. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B Biol. Sci. 270, 313–321.

Hebert, P.D., Ratnasingham, S., de Waard, J.R., 2003b. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. Lond. B Biol. Sci. 270, S96–S99.

HELCOM/OSPAR, 2013. Joint HELCOM/OSPAR Guidelines for the Contracting Parties of OSPAR and HELCOM on the granting of exemptions under International Convention for the Control and Management of Ships' Ballast Water and Sediments, Regulation A-4 (OSPAR Agreement 2013-09), 45 pp.

Heller, P., Casaletto, J., Ruiz, G., Geller, J., 2018. A database of metazoan cytochrome c oxidase subunit I gene sequences derived from GenBank with CO-ARBitrator. Sci. Data 5, 180156. https://doi.org/10.1038/sdata.2018.156

Hernandez, M.R., Johansson, M.L., Xiao, Y., Lewis, M.A., MacIsaac, H.J., 2017. Modeling sampling strategies for determination of zooplankton abundance in ballast water. Mar. Pollut. Bull. 115, 80–85. https://doi.org/10.1016/j.marpolbul.2016.11.050

Hess-Erga, O.-K., Blomv\a agnes-Bakke, B., Vadstein, O., 2010. Recolonization by heterotrophic bacteria after UV irradiation or ozonation of seawater; a simulation of ballast water treatment. Water Res. 44, 5439–5449.

Hewitt, C.L., Gollasch, S., Minchin, D., 2009. The Vessel as a Vector – Biofouling, Ballast Water and Sediments, in: Rilov, G., Crooks, J.A. (Eds.), Biological Invasions in Marine Ecosystems. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 117–131. https://doi.org/10.1007/978-3-540-79236-9_6

Hewitt, C.L., Martin, R.B., 2001. Revised protocols for baseline port surveys for introduced marine species–design considerations, sampling protocols and taxonomic sufficiency. Cent. Res. Introd. Mar. Pests Tech. Rep.

Hewitt, C.L., Thresher, R.E., Martin, R.B., Boyd, S., Cohen, B.F., Currie, D.R., Gomon, M.F., Keough, M.J., Lewis, J.A., Lockett, M.M., Mays, N., McArthur, M.A., O'Hara, T.D., Poore, G.C.B., Ross, D.J., Storey, M.J., Watson, J.E., Wilson, R.S., Campbell, M.L., 2004. Introduced and cryptogenic species in Port Phillip Bay, Victoria, Australia. Mar. Biol. 144, 183–202. https://doi.org/10.1007/s00227-003-1173-x

Hinlo, R., Gleeson, D., Lintermans, M., Furlan, E., 2017. Methods to maximise recovery of environmental DNA from water samples. PLOS ONE 12, e0179251. https://doi.org/10.1371/journal.pone.0179251

Hirst and Bott, 2016. Marine pests in the Gippsland Lakes: Existing threats and future monitoring.

Hulme, P.E., 2009. Trade, transport and trouble: managing invasive species pathways in an era of globalization. J. Appl. Ecol. 46, 10–18.

Hutchings, P., 1992. Ballast water introductions of exotic marine organisms into Australia: Current status and management options. Mar. Pollut. Bull. 25, 196–199. https://doi.org/10.1016/0025-326X(92)90225-U

# I

IMO, 2017. IMO Report of the Marine Environmental Protection Committee on its Seventy-First Session; International Maritime Organization: London, UK, 2017.

IMO, 2016. Global treaty to halt invasive aquatic species to enter into force in 2017. http://www.imo.org/en/MediaCentre/PressBriefings/Pages/22-BWM-.aspx (Accessed: 8/9/2016).

IMO, 2015. Guidance on ballast water sampling and analysis for trial use in accordance with the BWM Convention and Guidelines (G2), BWM.2/Circ.42.Rev.1 International Maritime Organization, 13 Feb 2004, London.

IMO, 2008. Guidelines for ballast water sampling (G2), IMO Resolution MEPC.173(58). International Maritime Organization, 13 Feb 2004, London.

IMO, 2007. Guidelines for risk assessment under regulation A-4 of the BWM Convention (G7). Marine Environment Protection Committee, Resolution MEPC.162(56), 13 July 2007. International Maritime Organization, 13 Feb 2004, London.

IMO, 2004. International Convention for the Control and Management of Ships' Ballast Water and Sediments, 2004. International Maritime Organization, 13 Feb 2004, London.

# J

Jeraldo, P., Kalari, K., Chen, X., Bhavsar, J., Mangalam, A., White, B., Nelson, H., Kocher, J.-P., Chia, N., 2014. IM-TORNADO: a tool for comparison of 16S reads from paired-end libraries. PloS One 9, e114804. https://doi.org/10.1371/journal.pone.0114804

Jerde, C.L., Mahon, A.R., Chadderton, W.L., Lodge, D.M., 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. Conserv. Lett. 4, 150–157.

Jeunen, G.-J., Knapp, M., Spencer, H.G., Lamare, M.D., Taylor, H.R., Stat, M., Bunce, M., Gemmell, N.J., 2018. Environmental DNA (eDNA) metabarcoding reveals strong discrimination among diverse marine habitats connected by water movement. Mol. Ecol. Resour. https://doi.org/10.1111/1755-0998.12982

Jeunen, G.-J., Knapp, M., Spencer, H.G., Taylor, H.R., Lamare, M.D., Stat, M., Bunce, M., Gemmell, N.J., 2019. Species-level biodiversity assessment using marine environmental DNA metabarcoding requires protocol optimization and standardization. Ecol. Evol. https://doi.org/10.1002/ece3.4843

Ji, Y., Ashton, L., Pedley, S.M., Edwards, D.P., Tang, Y., Nakamura, A., Kitching, R., Dolman, P.M., Woodcock, P., Edwards, F.A., others, 2013. Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. Ecol. Lett. 16, 1245–1257.

Johansson, M.L., Chaganti, S.R., Simard, N., Howland, K., Winkler, G., Rochon, A., Laget, F., Tremblay, P., Heath, D.D., MacIsaac, H.J., 2017. Attenuation and modification of the ballast water microbial community during voyages into the Canadian Arctic. Divers. Distrib. 23, 567–576. https://doi.org/10.1111/ddi.12552

## K

Kakkonen, J.E., Worsfold, T.W., Ashelby, C.W., Taylor, A., Beaton, K., 2019. The value of regular monitoring and diverse sampling techniques to assess aquatic non-native species: a case study from Orkney. Manag. Biol. Invasions 10.

Katoh, K., Standley, D.M., 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Mol. Biol. Evol. 30, 772. https://doi.org/10.1093/molbev/mst010

Katsanevakis Stylianos, M., Zenetos, A., Cardoso, A., Sumares Da Cruz Nunes Ana, L., 2014. Gateways to alien invasions in the European Seas. Aquat. Invasions 9, 133–144. https://doi.org/10.3391/ai.2014.9.2.02

Keller, R.P., Drake, J.M., Drew, M.B., Lodge, D.M., 2011. Linking environmental conditions and ship movements to estimate invasive species transport across the global shipping network. Divers. Distrib. 17, 93–102. https://doi.org/10.1111/j.1472-4642.2010.00696.x

Kelly, R.P., Closek, C.J., O'Donnell, J.L., Kralj, J.E., Shelton, A.O., Samhouri, J.F., 2017. Genetic and Manual Survey Methods Yield Different and Complementary Views of an Ecosystem. Front. Mar. Sci. 3. https://doi.org/10.3389/fmars.2016.00283

Kelly, R.P., Port, J.A., Yamahara, K.M., Martone, R.G., Lowell, N., Thomsen, P.F., Mach, M.E., Bennett, M., Prahler, E., Caldwell, M.R., others, 2014. Harnessing DNA to improve environmental management. Science 344, 1455–1456.

Kim, Y., Aw, T.G., Teal, T.K., Rose, J.B., 2015. Metagenomic investigation of viral communities in ballast water. Environ. Sci. Technol. 49, 8396–8407.

King, D.M., Tamburri, M.N., 2010. Verifying Compliance with Ballast Water Discharge Regulations. Ocean Dev. Int. Law 41, 152–165. https://doi.org/10.1080/00908321003733139

Klymus, K.E., Marshall, N.T., Stepien, C.A., 2017. Environmental DNA (eDNA) metabarcoding assays to detect invasive invertebrate species in the Great Lakes. PLOS ONE 12, e0177643. https://doi.org/10.1371/journal.pone.0177643

Knebelsberger, T., Landi, M., Neumann, H., Kloppmann, M., Sell, A.F., Campbell, P.D., Laakmann, S., Raupach, M.J., Carvalho, G.R., Costa, F.O., 2014. A reliable DNA barcode reference library for the identification of the North European shelf fish fauna. Mol. Ecol. Resour. 14, 1060–1071. https://doi.org/10.1111/1755-0998.12238

Koziol, A., Stat, M., Simpson, T., Jarman, S., DiBattista, J.D., Harvey, E.S., Marnane, M., McDonald, J., Bunce, M., 2018. Environmental DNA metabarcoding studies are critically affected by substrate selection. Mol. Ecol. Resour. https://doi.org/10.1111/1755-0998.12971

Krapp, F., Sconfietti, R., 1983. *Ammothea hilgendorfi* (Böhm, 1879), an Adventitious Pycnogonid new for the Mediterranean Sea. Mar. Ecol. 4, 123–132. https://doi.org/10.1111/j.1439-0485.1983.tb00291.x

Kraus, R., Ninčević-Gladan, Ž., Auriemma, R., Bastianini, M., Bolognini, L., Cabrini, M., Cara, M., Čalić, M., Campanelli, A., Cvitković, I., Despalatović, M., Drakulović, D., Flander-Putrle, V., Grati, F., Grego, M., Grilli, F., Jaklin, A., Janeković, I., Kolitari, J., Lipej, L., Magaletti, E., Marini, M., Mavrič, B., Mikus, J., Mozetič, P., Orlando-Bonaca, M., Petović, S., Precali, R., Supić, N., Trabucco, B., Travizi, A., Žuljević, A., 2018. Strategy of port baseline surveys (PBS) in the Adriatic Sea. Mar. Pollut. Bull. https://doi.org/10.1016/j.marpolbul.2018.08.067

Kvist, S., 2013. Barcoding in the dark?: A critical view of the sufficiency of zoological DNA barcoding databases and a plea for broader integration of taxonomic knowledge. Mol. Phylogenet. Evol. 69, 39–45. https://doi.org/10.1016/j.ympev.2013.05.012

Kvist, S., ErsÉus, C., 2018. Two new European species of the marine genus Tubificoides (Annelida: Clitellata: Naididae) with notes on the morphology of *T. pseudogaster* (Dahl, 1960). Zootaxa 4433, 561–573. https://doi.org/10.11646/zootaxa.4433.3.10

## L

Lacoursière-Roussel, A., Bock, D.G., Cristescu, M.E., Guichard, F., McKindsey, C.W., 2016. Effect of shipping traffic on biofouling invasion success at population and community levels. Biol. Invasions 18, 3681–3695. https://doi.org/10.1007/s10530-016-1258-3

Lacoursière-Roussel, A., Howland, K., Normandeau, E., Grey, E.K., Archambault, P., Deiner, K., Lodge, D.M., Hernandez, C., Leduc, N., Bernatchez, L., 2018. eDNA metabarcoding as a new surveillance approach for coastal Arctic biodiversity. Ecol. Evol. 8, 7763–7777. https://doi.org/10.1002/ece3.4213

Lance, R.F., Carr, M.R., 2012. Detecting eDNA of Invasive Dreissenid Mussels, Report on Capital Investment Project. ANSRP Tech. Notes Collect. ERDCTN ANSRP-12-2 Vicksbg. MS US Army Eng. Res. Dev. Cent. Available Httpel Erdc Usace Army Milpublications Cfm.

Lanzén, A., Lekang, K., Jonassen, I., Thompson, E.M., Troedsson, C., 2017. DNA extraction replicates improve diversity and compositional dissimilarity in metabarcoding of eukaryotes in marine sediments. PLOS ONE 12, e0179443. https://doi.org/10.1371/journal.pone.0179443

Lee, P.L.M., 2017. DNA amplification in the field: move over PCR, here comes LAMP. Mol. Ecol. Resour. 17, 138–141. https://doi.org/10.1111/1755-0998.12548

Legendre, P., Gallagher, E.D., 2001. Ecologically meaningful transformations for ordination of species data. Oecologia 129, 271–280. https://doi.org/10.1007/s004420100716

Lehtiniemi, M., Ojaveer, H., David, M., Galil, B., Gollasch, S., McKenzie, C., Minchin, D., Occhipinti-Ambrogi, A., Olenin, S., Pederson, J., 2015. Dose of truth—Monitoring marine non-indigenous species to serve legislative requirements. Mar. Policy 54, 26–35. https://doi.org/10.1016/j.marpol.2014.12.015

Lekang, K., Thompson, E., Troedsson, C., 2015. A comparison of DNA extraction methods for biodiversity studies of eukaryotes in marine sediments. Aquat. Microb. Ecol. 75, 15–25. https://doi.org/10.3354/ame01741

Leray, M., Ho, S.-L., Lin, I.-J., Machida, R.J., 2018. MIDORI server: a webserver for taxonomic assignment of unknown metazoan mitochondrial-encoded sequences using a curated database. Bioinforma. Oxf. Engl. 34, 3753–3754. https://doi.org/10.1093/bioinformatics/bty454

Leray, M., Yang, J.Y., Meyer, C.P., Mills, S.C., Agudelo, N., Ranwez, V., Boehm, J.T., Machida, R.J., 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Front. Zool. 10, 34. https://doi.org/10.1186/1742-9994-10-34

Leuven, R.S.E.W., van der Velde, G., Baijens, I., Snijders, J., van der Zwart, C., Lenders, H.J.R., bij de Vaate, A., 2009. The river Rhine: a global highway for dispersal of aquatic invasive species. Biol. Invasions 11, 1989. https://doi.org/10.1007/s10530-009-9491-7

Levin, 2006. Invasive cordgrass modifies wetland trophic function. Ecology 87, 419–432. https://doi.org/10.1890/04-1752

Levings, C.D., Cordell, J.R., Ong, S., Piercey, G.E., 2004. The origin and identity of invertebrate organisms being transported to Canada's Pacific coast by ballast water. Can. J. Fish. Aquat. Sci. 61, 1–11. https://doi.org/10.1139/f03-135

Li, R., Tun, H.M., Jahan, M., Zhang, Z., Kumar, A., Dilantha Fernando, W.G., Farenhorst, A., Khafipour, E., 2017. Comparison of DNA-, PMA-, and RNA-based 16S rRNA Illumina sequencing for detection of live bacteria in water. Sci. Rep. 7. https://doi.org/10.1038/s41598-017-02516-3

Liebich, V., Stehouwer, P.P., Veldhuis, M., 2012. Re-growth of potential invasive phytoplankton following UV-based ballast water treatment. Aquat. Invasions.

Lodge, D.M., Turner, C.R., Jerde, C.L., Barnes, M.A., Chadderton, L., Egan, S.P., Feder, J.L., Mahon, A.R., Pfrender, M.E., 2012. Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. Mol. Ecol. 21, 2555–2558. https://doi.org/10.1111/j.1365-294X.2012.05600.x

Lodge, D.M., Williams, S., MacIsaac, H.J., Hayes, K.R., Leung, B., Reichard, S., Mack, R.N., Moyle, P.B., Smith, M., Andow, D.A., others, 2006. Biological invasions: recommendations for US policy and management. Ecol. Appl. 16, 2035–2054.

Lohan, K.P., Fleischer, R.C., Carney, K.J., Holzer, K.K., Ruiz, G.M., 2015. Amplicon-Based Pyrosequencing Reveals High Diversity of Protistan Parasites in Ships' Ballast Water: Implications for Biogeography and Infectious Diseases. Microb. Ecol. 1–13.

López-Legentil, S., Legentil, M.L., Erwin, P.M., Turon, X., 2015. Harbor networks as introduction gateways: contrasting distribution patterns of native and introduced ascidians. Biol. Invasions 17, 1623–1638. https://doi.org/10.1007/s10530-014-0821-z

Lymperopoulou, D.S., Dobbs, F.C., 2017. Bacterial Diversity in Ships' Ballast Water, Ballast-Water Exchange, and Implications for Ship-Mediated Dispersal of Microorganisms. Environ. Sci. Technol. 51, 1962–1972. https://doi.org/10.1021/acs.est.6b03108

## M

Ma, Y., Xiong, H., Tang, S., Yang, Q., Li, M., 2009. Comparison of the community structure of planktonic bacteria in ballast water from entry ships and local sea water in Xiamen Port. Prog. Nat. Sci. 19, 947–953.

Macher, J.-N., Vivancos, A., Piggott, J.J., Centeno, F.C., Matthaei, C.D., Leese, F., 2018. Comparison of environmental DNA and bulk-sample metabarcoding using highly degenerate cytochrome c oxidase I primers. Mol. Ecol. Resour. https://doi.org/10.1111/1755-0998.12940

Mack, R.N., Simberloff, D., Mark Lonsdale, W., Evans, H., Clout, M., Bazzaz, F.A., 2000. Biotic invasions: causes, epidemiology, global consequences, and control. Ecol. Appl. 10, 689–710. https://doi.org/10.1890/1051-0761(2000)010[0689:BICEGC]2.0.CO;2

Mahé, F., Rognes, T., Quince, C., de Vargas, C., Dunthorn, M., 2014. Swarm: robust and fast clustering method for amplicon-based studies. PeerJ 2, e593. https://doi.org/10.7717/peerj.593
Mandelik, Y., Roll, U., Fleischer, A., 2010. Cost-efficiency of biodiversity indicators for Mediterranean ecosystems and the effects of socio-economic factors. J. Appl. Ecol. 47, 1179–1188. https://doi.org/10.1111/j.1365-2664.2010.01864.x
Marcelino, V.R., Verbruggen, H., 2016. Multi-marker metabarcoding of coral skeletons reveals a rich microbiome and diverse evolutionary origins of endolithic algae. Sci. Rep. 6. https://doi.org/10.1038/srep31508
Marchini, A., Ferrario, J., Nasi, E., 2016. Arrival of the invasive amphipod *Grandidierella japonica* to the Mediterranean Sea. Mar. Biodivers. Rec. 9. https://doi.org/10.1186/s41200-016-0049-y
Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17, 10–12. https://doi.org/10.14806/ej.17.1.200
Martínez, J., Adarraga, I., 2006. Programa de vigilancia y control de la introducción de especies invasoras en los ecosistemas litorales de la costa vasca. 1. Costa Gipuzkoa 2.
Mazzocchi, M.G., Paffenhöfer, G.-A., 1998. First observations on the biology of *Clausocalanus furcatus* (Copepoda, Calanoida). J. Plankton Res. 20, 331–342.
McCarthy, H.P., Crowder, L.B., 2000. An Overlooked Scale of Global Transport: Phytoplankton Species Richness in Ships' Ballast Water. Biol. Invasions 2, 321–322. https://doi.org/10.1023/A:1011418432256
McCarthy, S.A., Khambaty, F.M., 1994. International dissemination of epidemic Vibrio cholerae by cargo ship ballast and other nonpotable waters. Appl. Environ. Microbiol. 60, 2597–2601.
McCollin, T., Quilez-Badia, G., Josefsen, K.D., Gill, M.E., Mesbahi, E., Frid, C.L.J., 2007. Ship board testing of a deoxygenation ballast water treatment. Mar. Pollut. Bull. 54, 1170–1178. https://doi.org/10.1016/j.marpolbul.2007.04.013
McDonald, J.H., Koehn, R.K., 1988. The mussels *Mytilus galloprovincialis* and *M. trossulus* on the Pacific coast of North America. Mar. Biol. 99, 111–118. https://doi.org/10.1007/BF00644984
McMurdie, P.J., Holmes, S., 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLOS ONE 8, e61217. https://doi.org/10.1371/journal.pone.0061217
Meyer, C.P., Paulay, G., 2005. DNA Barcoding: Error Rates Based on Comprehensive Sampling. PLoS Biol 3, e422. https://doi.org/10.1371/journal.pbio.0030422
Miller, A.W., Davidson, I.C., Minton, M.S., Steves, B., Moser, C.S., Drake, L.A., Ruiz, G.M., 2018. Evaluation of wetted surface area of commercial ships as biofouling habitat flux to the United States. Biol. Invasions 20, 1977–1990.
Minchin, D., 2007. Rapid coastal survey for targeted alien species associated with floating pontoons in Ireland. Aquat. Invasions 2, 63–70. https://doi.org/10.3391/ai.2007.2.1.8
Minchin, D., Gollasch, S., Cohen, A.N., Hewitt, C.L., Olenin, S., 2009. Characterizing Vectors of Marine Invasion, in: Rilov, G., Crooks, J.A. (Eds.), Biological Invasions in Marine Ecosystems. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 109–116. https://doi.org/10.1007/978-3-540-79236-9_5
Miralles, L., Ardura, A., Arias, A., Borrell, Y.J., Clusa, L., Dopico, E., de Rojas, A.H., Lopez, B., Muñoz-Colmenero, M., Roca, A., Valiente, A.G., Zaiko, A., Garcia-Vazquez, E., 2016. Barcodes of marine invertebrates from north Iberian ports: Native diversity and

resistance to biological invasions. Mar. Pollut. Bull. 112, 183–188. https://doi.org/10.1016/j.marpolbul.2016.08.022

Mitchell, A.A., Webber, M.K., Buddo, D., Webber, D., 2014. Development of a protocol for sampling and analysis of ballast water in Jamaica. Rev. Biol. Trop. 62, 249–257.

Mitsuhashi, S., Kryukov, K., Nakagawa, S., Takeuchi, J.S., Shiraishi, Y., Asano, K., Imanishi, T., 2017. A portable system for metagenomic analyses using nanopore-based sequencer and laptop computers can realize rapid on-site determination of bacterial compositions. bioRxiv 101865.

Molnar, J.L., Gamboa, R.L., Revenga, C., Spalding, M.D., 2008. Assessing the global threat of invasive species to marine biodiversity. Front. Ecol. Environ. 6, 485–492.

Mooney, H.A., 2005. Invasive Alien Species: A New Synthesis. Island Press.

Moreira, J., Troncoso, J.S., 2007. Inventario de la macrofauna bentónica de sedimentos submareales de la Ensenada de Baiona (Galicia, NO Península Ibérica).

Moser, C.S., Wier, T.P., Grant, J.F., First, M.R., Tamburri, M.N., Ruiz, G.M., Miller, A.W., Drake, L.A., 2016. Quantifying the total wetted surface area of the world fleet: a first step in determining the potential extent of ships' biofouling. Biol. Invasions 18, 265–277. https://doi.org/10.1007/s10530-015-1007-z

Mountfort, D., Smith, K.F., Kirs, M., Kuhajek, J., Adamson, J.E., Wood, S.A., 2012. Development of single and multispecies detection methods for the surveillance and monitoring of marine pests in New Zealand. Aquat. Invasions 7, 125–128.

Murphy, K.R., Ritz, D., Hewitt, C.L., 2002. Heterogeneous zooplankton distribution in a ship's ballast tanks. J. Plankton Res. 24, 729–734. https://doi.org/10.1093/plankt/24.7.729

Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

## N

Nathan, L.M., Simmons, M., Wegleitner, B.J., Jerde, C.L., Mahon, A.R., 2014. Quantifying environmental DNA signals for aquatic invasive species across multiple detection platforms. Environ. Sci. Technol. 48, 12800–12806.

Ng, C., Le, T.H., Goh, S.G., Liang, L., Kim, Y., Rose, J.B., Yew-Hoong, K.G., 2015. A Comparison of Microbial Water Quality and Diversity for Ballast and Tropical Harbor Waters. PloS One 10, e0143123–e0143123. https://doi.org/10.1371/journal.pone.0143123

Nguyen, N.H., Smith, D., Peay, K., Kennedy, P., 2015. Parsing ecological signal from noise in next generation amplicon sequencing. New Phytol. 205, 1389–1393. https://doi.org/10.1111/nph.12923

Niessen, L., Luo, J., Denschlag, C., Vogel, R.F., 2013. The application of loop-mediated isothermal amplification (LAMP) in food testing for bacterial pathogens and fungal contaminants. Food Microbiol. 36, 191–206. https://doi.org/10.1016/j.fm.2013.04.017

# O

OBIS, 2019. Ocean Biogeographic Information System. Intergovernmental Oceanographic Commission of UNESCO. www.iobis.org.

O'Donnell, J.L., Kelly, R.P., Shelton, A.O., Samhouri, J.F., Lowell, N.C., Williams, G.D., 2017. Spatial distribution of environmental DNA in a nearshore marine habitat. PeerJ 5, e3044. https://doi.org/10.7717/peerj.3044

Ojaveer, H., Galil, B.S., Carlton, J.T., Alleway, H., Goulletquer, P., Lehtiniemi, M., Marchini, A., Miller, W., Occhipinti-Ambrogi, A., Peharda, M., Ruiz, G.M., Williams, S.L., Zaiko, A., 2018. Historical baselines in marine bioinvasions: Implications for policy and management. PLOS ONE 13, e0202383. https://doi.org/10.1371/journal.pone.0202383

Ojaveer, H., Galil, B.S., Minchin, D., Olenin, S., Amorim, A., Canning-Clode, J., Chainho, P., Copp, G.H., Gollasch, S., Jelmert, A., Lehtiniemi, M., McKenzie, C., Mikuš, J., Miossec, L., Occhipinti-Ambrogi, A., Pećarević, M., Pederson, J., Quilez-Badia, G., Wijsman, J.W.M., Zenetos, A., 2014. Ten recommendations for advancing the assessment and management of non-indigenous species in marine ecosystems. Mar. Policy 44, 160–165. https://doi.org/10.1016/j.marpol.2013.08.019

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2013. Package "vegan." Community Ecol. Package Version 2.

Olden, J.D., 2006. Biotic homogenization: a new research agenda for conservation biogeography. J. Biogeogr. 33, 2027–2039. https://doi.org/10.1111/j.1365-2699.2006.01572.x

Olenin, S., Alemany, F., Cardoso, A.C., Gollasch, S., Goulletquer, P., Lehtiniemi, M., McCollin, T., Minchin, D., Miossec, L., Ambrogi, A.O., 2010. Marine strategy framework directive—Task Group 2 Report. Non-indigenous species. Office for Official Publications of the European Communities. Luxembourg: EU/ICES. Task Group 2, 47 p.

Olenin, S., Ojaveer, H., Minchin, D., Boelens, R., 2016. Assessing exemptions under the ballast water management convention: preclude the Trojan horse. Mar. Pollut. Bull. 103, 84–92. https://doi.org/10.1016/j.marpolbul.2015.12.043

Ostenfeld, C.J., 1908. On the immigration of Biddluphia sinensis Grev. and its occurrence in the North Sea during 1903-1907. Meddelelser Fra Komm. Dan. Fisk.-Og Havundersogelser Ser. Plankton 1, 44.

# P

Pagenkopp Lohan, K.M., Fleischer, R.C., Carney, K.J., Holzer, K.K., Ruiz, G.M., 2017. Molecular characterisation of protistan species and communities in ships' ballast water across three U.S. coasts. Divers. Distrib. 680–691. https://doi.org/10.1111/ddi.12550

Pagenkopp Lohan, K.M., Fleischer, R.C., Carney, K.J., Holzer, K.K., Ruiz, G.M., 2016. Amplicon-Based Pyrosequencing Reveals High Diversity of Protistan Parasites in Ships' Ballast Water: Implications for Biogeography and Infectious Diseases. Microb. Ecol. 71, 530–542. https://doi.org/10.1007/s00248-015-0684-6

Palomares, M.L.D, Pauly, D., 2018. SeaLifeBase. World Wide Web electronic publication. www.sealifebase.org, version (10/2018).

Paolucci, E.M., Ron, L., MacIsaac, H.J., 2017. Combining ballast water treatment and ballast water exchange: Reducing colonization pressure and propagule pressure of phytoplankton organisms. Aquat. Ecosyst. Health Manag. 20, 369–377. https://doi.org/10.1080/14634988.2017.1404419

Patil, J.G., Gunasekera, R.M., Deagle, B.E., Bax, N.J., Blackburn, S.I., 2005. Development and evaluation of a PCR based assay for detection of the toxic dinoflagellate, *Gymnodinium catenatum* (Graham) in ballast water and environmental samples. Biol. Invasions 7, 983–994.

Pawlowski, J., Esling, P., Lejzerowicz, F., Cedhagen, T., Wilding, T.A., 2014. Environmental monitoring through protist next-generation sequencing metabarcoding: assessing the impact of fish farming on benthic foraminifera communities. Mol. Ecol. Resour. 14, 1129–1140. https://doi.org/10.1111/1755-0998.12261

Pazouki, Basurko, Mesbahi, Kemp, 2009. The control of spread of non-indigenous species through ballast water. Part B: Ballast Water Sampling: Methods, Analysis, Representativeness and Legal issues, MCA: 89.

Pejovic, I., Ardura, A., Miralles, L., Arias, A., Borrell, Y.J., Garcia-Vazquez, E., 2016. DNA barcoding for assessment of exotic molluscs associated with maritime ports in northern Iberia. Mar. Biol. Res. 12, 168–176. https://doi.org/10.1080/17451000.2015.1112016

Peng, P., Yang, Y., Cheng, S., Lu, F., Yuan, Z., 2019. Hub-and-spoke structure: Characterizing the global crude oil transport network with mass vessel trajectories. Energy 168, 966–974.

Pereira, F., Carneiro, J., Amorim, A., 2008. Identification of species with DNA-based technology: current progress and challenges. Recent Pat. DNA Gene Seq. 2, 187–199.

Pérez-Portela, R., Arranz, V., Rius, M., Turon, X., 2013. Cryptic speciation or global spread? The case of a cosmopolitan marine invertebrate with limited dispersal capabilities. Sci. Rep. 3, 3197. https://doi.org/10.1038/srep03197

Pfrender, M.E., Hawkins, C.P., Bagley, M., Courtney, G.W., Creutzburg, B.R., Epler, J.H., Fend, S., Ferrington, L.C., Hartzell, P.L., Jackson, S., Larsen, D.P., Lévesque, C.A., Morse, J.C., Petersen, M.J., Ruiter, D., Schindel, D., Whiting, M., 2010. Assessing Macroinvertebrate Biodiversity in Freshwater Ecosystems: Advances and Challenges in DNA-based Approaches. Q. Rev. Biol. 85, 319–340. https://doi.org/10.1086/655118

Pimentel, D., Zuniga, R., Morrison, D., 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol. Econ. 52, 273–288.

Pochon, X., Bott, N.J., Smith, K.F., Wood, S.A., 2013. Evaluating detection limits of next-generation sequencing for the surveillance and monitoring of international marine pests. PLoS One 8, e73935.

Pochon, X., Zaiko, A., Fletcher, L.M., Laroche, O., Wood, S.A., 2017. Wanted dead or alive? Using metabarcoding of environmental DNA and RNA to distinguish living assemblages for biosecurity applications. PLOS ONE 12, e0187636. https://doi.org/10.1371/journal.pone.0187636

Pochon, X., Zaiko, A., Hopkins, G.A., Banks, J.C., Wood, S.A., 2015. Early detection of eukaryotic communities from marine biofilm using high-throughput sequencing: an

assessment of different sampling devices. Biofouling 31, 241–251. https://doi.org/10.1080/08927014.2015.1028923

Podani, J., Schmera, D., 2011. A new conceptual and methodological framework for exploring and explaining pattern in presence - absence data. Oikos 120, 1625–1638. https://doi.org/10.1111/j.1600-0706.2011.19451.x

Port, J.A., O'Donnell, J.L., Romero-Maraccini, O.C., Leary, P.R., Litvin, S.Y., Nickols, K.J., Yamahara, K.M., Kelly, R.P., 2016. Assessing vertebrate biodiversity in a kelp forest ecosystem using environmental DNA. Mol. Ecol. 25, 527–541. https://doi.org/10.1111/mec.13481

Porter, J.S., Nunn, J.D., Ryland, J.S., Minchin, D., Jones, M.E.S., 2017. The status of non-native bryozoans on the north coast of Ireland. BIOINVASIONS Rec. 6, 321–330.

Pyšek, P., Hulme, P.E., Meyerson, L.A., Smith, G.F., Boatwright, J.S., Crouch, N.R., Figueiredo, E., Foxcroft, L.C., Jarošík, V., Richardson, D.M., Suda, J., Wilson, J.R.U., 2013. Hitting the right target: taxonomic challenges for, and of, plant invasions. AoB PLANTS 5. https://doi.org/10.1093/aobpla/plt042

Pyšek, P., Richardson, D.M., 2010. Invasive Species, Environmental Change and Management, and Health. Annu. Rev. Environ. Resour. 35, 25–55. https://doi.org/10.1146/annurev-environ-033009-095548

## R

Rajakaruna, H., VandenByllaardt, J., Kydd, J., Bailey, S., 2018. Modeling the distribution of colonial species to improve estimation of plankton concentration in ballast water. J. Sea Res., Recent Advances in Ballast Water Research 133, 166–176. https://doi.org/10.1016/j.seares.2017.08.005

Ramgren, A.C., Newhall, H.S., James, K.E., 2015. DNA barcoding and metabarcoding with the Oxford Nanopore MinION, in: GENOME. CANADIAN SCIENCE PUBLISHING, NRC RESEARCH PRESS 65 AURIGA DR, SUITE 203, OTTAWA, ON K2E 7W6, CANADA, pp. 268–268.

Rassati, D., Faccoli, M., Toffolo, E.P., Battisti, A., Marini, L., 2015. Improving the early detection of alien wood-boring beetles in ports and surrounding forests. J. Appl. Ecol. 52, 50–58. https://doi.org/10.1111/1365-2664.12347

Razouls, C., de Bovée, F., Kouwenberg, J., Desreumaux, N., 2005. Diversity and Geographic Distribution of Marine Planktonic Copepods. Sorbonne University, CNRS. Available at http://copepodes.obs-banyuls.fr/en [Accessed January 24, 2019].

Read, G.B., 2010. Comparison and history of *Polydora websteri* and *P. haswelli* (Polychaeta: Spionidae) as mud-blister worms in New Zealand shellfish. N. Z. J. Mar. Freshw. Res. 44, 83–100. https://doi.org/10.1080/00288330.2010.482969

Rees, H.C., Maddison, B.C., Middleditch, D.J., Patmore, J.R.M., Gough, K.C., 2014. REVIEW: The detection of aquatic animal species using environmental DNA – a review of eDNA as a survey tool in ecology. J. Appl. Ecol. 51, 1450–1459. https://doi.org/10.1111/1365-2664.12306

Rey, A., Basurko, O.C., Rodríguez-Ezpeleta, N., 2018. The challenges and promises of genetic approaches for ballast water management. J. Sea Res. 133, 134–145. https://doi.org/10.1016/j.seares.2017.06.001

Ricciardi, A., 2016. Tracking marine alien species by ship movements. Proc. Natl. Acad. Sci. 113, 5470–5471. https://doi.org/10.1073/pnas.1605152113

Rius, M., Bourne, S., Hornsby, H.G., Chapman, M.A., 2015. Applications of next-generation sequencing to the study of biological invasions. Curr. Zool. 61, 488–504.

Rohde, S., Schupp, P.J., Markert, A., Wehrmann, A., 2017. Only half of the truth: Managing invasive alien species by rapid assessment. Ocean Coast. Manag. 146, 26–35. https://doi.org/10.1016/j.ocecoaman.2017.05.013

Rublee, P.A., Remington, D.L., Schaefer, E.F., Marshall, M.M., 2005. Detection of the Dinozoans *Pfiesteria piscicida* and *P. shumwayae*: A Review of Detection Methods and Geographic Distribution 1. J. Eukaryot. Microbiol. 52, 83–89.

Ruiz, G.M., Fofonoff, P.W., Steves, B.P., Carlton, J.T., 2015. Invasion history and vector dynamics in coastal marine ecosystems: A North American perspective. Aquat. Ecosyst. Health Manag. 18, 299–311. https://doi.org/10.1080/14634988.2015.1027534

Ruiz, G.M., Rawlings, T.K., Dobbs, F.C., Drake, L.A., Mullady, T., Huq, A., Colwell, R.R., 2000. Global spread of microorganisms by ships. Nature 408, 49–50.

## S

Saitoh, S., Aoyama, H., Fujii, S., Sunagawa, H., Nagahama, H., Akutsu, M., Shinzato, N., Kaneko, N., Nakamori, T., 2016. A quantitative protocol for DNA metabarcoding of springtails (Collembola). Genome 59, 705–723. https://doi.org/10.1139/gen-2015-0228

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Horn, D.J.V., Weber, C.F., 2009. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Appl. Environ. Microbiol. 75, 7537–7541. https://doi.org/10.1128/AEM.01541-09

Seebens, H., Gastner, M.T., Blasius, B., 2013. The risk of marine bioinvasion caused by global shipping. Ecol. Lett. 16, 782–790. https://doi.org/10.1111/ele.12111

Seebens, H., Schwartz, N., Schupp, P.J., Blasius, B., 2016. Predicting the spread of marine species introduced by global shipping. Proc. Natl. Acad. Sci. 113, 5646–5651. https://doi.org/10.1073/pnas.1524427113

Shaw, J.L.A., Weyrich, L., Cooper, A., 2017. Using environmental (e)DNA sequencing for aquatic biodiversity surveys: a beginner’s guide. Mar. Freshw. Res. 68, 20–33. https://doi.org/10.1071/MF15361

Shikata, T., Nagasoe, S., Matsubara, T., Yoshikawa, S., Yamasaki, Y., Shimasaki, Y., Oshima, Y., Jenkinson, I.R., Honjo, T., 2008. Factors influencing the initiation of blooms of the raphidophyte *Heterosigma akashiwo* and the diatom *Skeletonema costatum* in a port in Japan. Limnol. Oceanogr. 53, 2503–2518.

Shokralla, S., Spall, J.L., Gibson, J.F., Hajibabaei, M., 2012. Next-generation sequencing technologies for environmental DNA research. Mol. Ecol. 21, 1794–1805.

Simard, N., Plourde, S., Gilbert, M., Gollasch, S., 2011. Net efficacy of open ocean ballast water exchange on plankton communities. J. Plankton Res. 33, 1378–1395. https://doi.org/10.1093/plankt/fbr038

Simmons, M., Tucker, A., Chadderton, W.L., Jerde, C.L., Mahon, A.R., Taylor, E., 2015. Active and passive environmental DNA surveillance of aquatic invasive species. Can. J. Fish. Aquat. Sci. 73, 76–83.

Slijkerman, D.M.E., Glorius, S.T., Gittenberger, A., van der Weide, B.E., Bos, O.G., Rensing, M., de Groot, G.A., 2017. Monitoring Groningen Sea Ports, Non-indigenous species and risks from ballast water in Eemshaven and Delfzijl. Wagening. Mar. Res. Univ. Res. Cent. Wagening. Mar. Res. Rep. C04517 A 81. https://doi.org/https://doi.org/10.18174/417717

Spens, J., Evans, A.R., Halfmaerten, D., Knudsen, S.W., Sengupta, M.E., Mak, S.S.T., Sigsgaard, E.E., Hellström, M., 2017. Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods Ecol. Evol. 8, 635–645. https://doi.org/10.1111/2041-210X.12683

Srivathsan, A., Sha, J.C.M., Vogler, A.P., Meier, R., 2015. Comparing the effectiveness of metagenomics and metabarcoding for diet analysis of a leaf-feeding monkey (*Pygathrix nemaeus*). Mol. Ecol. Resour. 15, 250–261. https://doi.org/10.1111/1755-0998.12302

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313.

Stat, M., Huggett, M.J., Bernasconi, R., DiBattista, J.D., Berry, T.E., Newman, S.J., Harvey, E.S., Bunce, M., 2017. Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. Sci. Rep. 7, 12240. https://doi.org/10.1038/s41598-017-12501-5

Stefanni, S., Stanković, D., Borme, D., de Olazabal, A., Juretić, T., Pallavicini, A., Tirelli, V., 2018. Multi-marker metabarcoding approach to study mesozooplankton at basin scale. Sci. Rep. 8, 12085.

Stehouwer, P.P., Buma, A., Peperzak, L., 2015. A comparison of six different ballast water treatment systems based on UV radiation, electrochlorination and chlorine dioxide. Environ. Technol. 36, 2094–2104. https://doi.org/10.1080/09593330.2015.1021858

Stehouwer, P.P., Liebich, V., Peperzak, L., 2013a. Flow cytometry, microscopy, and DNA analysis as complementary phytoplankton screening methods in ballast water treatment studies. J. Appl. Phycol. 25, 1047–1053.

Stehouwer, P.P., van Slooten, C., Peperzak, L., 2013b. Microbial dynamics in acetate-enriched ballast water at different temperatures. Ecotoxicol. Environ. Saf. 96, 93–98.

Steichen, J.L., Quigg, A., 2015. Assessing the viability of microorganisms in the ballast water of vessels transiting the North Atlantic Ocean. Mar. Pollut. Bull. 101, 258–266.

Steichen, J.L., Schulze, A., Brinkmeyer, R., Quigg, A., 2014. All aboard! A biological survey of ballast water onboard vessels spanning the North Atlantic Ocean. Mar. Pollut. Bull. 87, 201–210.

Stevens, W., 1993. Ships are culprits as foreign species invade ecosystems. New York Times. July 6, 1993;C4 https://www.nytimes.com/1993/07/06/news/ships-are-culprits-as-foreign-species-invade-ecosystems.html.

Stiger-Pouvreau, V., Thouzeau, G., 2015. Marine Species Introduced on the French Channel-Atlantic Coasts: A Review of Main Biological Invasions and Impacts. Open J. Ecol. 05, 227–257. https://doi.org/10.4236/oje.2015.55019

Strayer, D.L., 2008. Twenty years of zebra mussels: lessons from the mollusk that made headlines. Front. Ecol. Environ. 7, 135–141.

Sutherland, T.F., Levings, C.D., Elliott, C.C., Hesse, W.W., 2001. Effect of a ballast water treatment system on survivorship of natural populations of marine plankton. Mar. Ecol. Prog. Ser. 210, 139–148. https://doi.org/10.3354/meps210139

Sutton, C.A., Murphy, K.R., Martin, R.B., Hewitt, C.L., 1998. A review and evaluation of ballast water sampling protocols.

## T

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E., 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Mol. Ecol. 21, 2045–2050.

Tajadura, J., Bustamante, M., Salinas, J.I.S., 2016. Temporal and spatial variability in the distribution of non-indigenous zoobenthic species in the intertidal hard substrates in the "Abra of Bilbao" Poster ISOBAY conference 2016 https://www.ehu.eus/documents/5019180/0/Book-of-abstract-ISOBAY15.pdf. ISOBAY Conf.

Takahashi, C.K., Lourenço, N., Lopes, T.F., Rall, V.L.M., Lopes, C. a. M., 2008. Ballast water: a review of the impact on the world public health. J. Venom. Anim. Toxins Trop. Dis. 14, 393–408. https://doi.org/10.1590/S1678-91992008000300002

Tan, B., Ng, C., Nshimyimana, J.P., Loh, L.L., Gin, K.Y.-H., Thompson, J.R., 2015. Next-generation sequencing (NGS) for assessment of microbial water quality: current progress, challenges, and future opportunities. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.01027

Tang, M., Hardman, C.J., Ji, Y., Meng, G., Liu, S., Tan, M., Yang, S., Moss, E.D., Wang, J., Yang, C., Bruce, C., Nevard, T., Potts, S.G., Zhou, X., Yu, D.W., 2015. High-throughput monitoring of wild bee diversity and abundance via mitogenomics. Methods Ecol. Evol. 6, 1034–1043. https://doi.org/10.1111/2041-210X.12416

Tang, M., Tan, M., Meng, G., Yang, S., Su, X., Liu, S., Song, W., Li, Y., Wu, Q., Zhang, A., others, 2014. Multiplex sequencing of pooled mitochondrial genomes—a crucial step toward biodiversity analysis using mito-metagenomics. Nucleic Acids Res. 42, e166–e166.

Taylor, A.H., Rigby, G., 2001. Suggested designs to facilitate improved management and treatment of ballast water on new and existing ships. Department of Agriculture, Fisheries & Forestry, Australia.

Team, Rs., 2015. RStudio: integrated development for R. RStudio Inc Boston MA URL Httpwww Rstudio Com 42.

Thines, M., Kamoun, S., 2010. Oomycete-plant coevolution: recent advances and future prospects. Curr. Opin. Plant Biol. 13, 427–433. https://doi.org/10.1016/j.pbi.2010.04.001

Thomsen, P., Kielgast, J.O.S., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L., Willerslev, E., 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Mol. Ecol. 21, 2565–2573.

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M.A., Roussel, J.-M., 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. J. Appl. Ecol. 51, 871–879. https://doi.org/10.1111/1365-2664.12262

Tsiamis, K., Zenetos, A., Deriu, I., Gervasini, E., Cardoso, A.C., 2018. The native distribution range of the European marine non-indigenous species. Aquat. Invasions 13, 187–198.
Tsolaki, E., Diamadopoulos, E., 2010. Technologies for ballast water treatment: a review. J. Chem. Technol. Biotechnol. 85, 19–32. https://doi.org/10.1002/jctb.2276
Turner, C.R., Uy, K.L., Everhart, R.C., 2015. Fish environmental DNA is more concentrated in aquatic sediments than surface water. Biol. Conserv., Special Issue: Environmental DNA: A powerful new tool for biological conservation 183, 93–102. https://doi.org/10.1016/j.biocon.2014.11.017

# U

Uriarte, I., Villate, F., Iriarte, A., 2016. Zooplankton recolonization of the inner estuary of Bilbao: influence of pollution abatement, climate and non-indigenous species. J. Plankton Res. 38, 718–731. https://doi.org/10.1093/plankt/fbv060
Urrutia, J., Casamitjana, I., 1981. Estudio de la Productividad Primaria y Distribución Estacional del Fitoplláncton en el Abra de Bilbao (Noviembre, 1977-Noviembre, 1978). Munibe 33, 31–50.

# V

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P.F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G.H., Geniez, P., Pont, D., Argillier, C., Baudoin, J.-M., Peroux, T., Crivelli, A.J., Olivier, A., Acqueberge, M., Le Brun, M., Møller, P.R., Willerslev, E., Dejean, T., 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Mol. Ecol. 25, 929–942. https://doi.org/10.1111/mec.13428
Van Steenkiste, N., Locke, S.A., Castelin, M., Marcogliese, D.J., Abbott, C.L., 2015. New primers for DNA barcoding of digeneans and cestodes (Platyhelminthes). Mol. Ecol. Resour. 15, 945–952. https://doi.org/10.1111/1755-0998.12358
Vander Zanden, M.J., Hansen, G.J., Higgins, S.N., Kornis, M.S., 2010. A pound of prevention, plus a pound of cure: early detection and eradication of invasive species in the Laurentian Great Lakes. J. Gt. Lakes Res. 36, 199–205.
Verna, D.E., Harris, B.P., 2016. Review of ballast water management policy and associated implications for Alaska. Mar. Policy 70, 13–21. https://doi.org/10.1016/j.marpol.2016.04.024
Verna, D.E., Harris, B.P., Holzer, K.K., Minton, M.S., 2016. Ballast-borne marine invasive species: exploring the risk to coastal Alaska, USA. Manag. Biol. Invasions 7, 199–211.
Viard, F., David, P., Darling, J., 2016. Marine invasions enter the genomic era: three lessons from the past, and the way forward. Curr. Zool. zow053. https://doi.org/10.1093/cz/zow053
Vilgalys, R., 2003. Taxonomic misidentification in public DNA databases. New Phytol. 160, 4–5. https://doi.org/10.1046/j.1469-8137.2003.00894.x
Visco, J.A., Apothéloz-Perret-Gentil, L., Cordonier, A., Esling, P., Pillet, L., Pawlowski, J., 2015. Environmental Monitoring: Inferring the Diatom Index from Next-Generation Sequencing Data. Environ. Sci. Technol. 49, 7597–7605. https://doi.org/10.1021/es506158m

## W

Walsh, J.R., Spear, M.J., Shannon, T.P., Krysan, P.J., Zanden, M.J.V., 2018. Using eDNA, sediment subfossils, and zooplankton nets to detect invasive spiny water flea (*Bythotrephes longimanus*). Biol. Invasions 1–13. https://doi.org/10.1007/s10530-018-1862-5

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267. https://doi.org/10.1128/AEM.00062-07

Wang, Shengling, Wang, C., Wang, Shenling, Ma, L., 2018. Big data analysis for evaluating bioinvasion risk. BMC Bioinformatics 19, 2272. https://doi.org/10.1186/s12859-018-2272-5

Wangensteen, O.S., Cebrian, E., Palacín, C., Turon, X., 2018a. Under the canopy: Community-wide effects of invasive algae in Marine Protected Areas revealed by metabarcoding. Mar. Pollut. Bull. 127, 54–66. https://doi.org/10.1016/j.marpolbul.2017.11.033

Wangensteen, O.S., Palacín, C., Guardiola, M., Turon, X., 2018b. DNA metabarcoding of littoral hard-bottom communities: high diversity and database gaps revealed by two molecular markers. PeerJ 6, e4705. https://doi.org/10.7717/peerj.4705

Wangensteen, O.S., Turon, X., 2017. Metabarcoding Techniques for Assessing Biodiversity of Marine Animal Forests, in: Rossi, S., Bramanti, L., Gori, A., Orejas, C. (Eds.), Marine Animal Forests: The Ecology of Benthic Biodiversity Hotspots. Springer International Publishing, Cham, pp. 445–473. https://doi.org/10.1007/978-3-319-21012-4_53

Ward, R.D., Andrew, J., 1995. Population genetics of the northern Pacific seastar *Asterias amurensis* (Echinodermata: Asteriidae): allozyme differentiation among Japanese, Russian, and recently introduced Tasmanian populations. Mar. Biol. 124, 99–109. https://doi.org/10.1007/BF00349151

Ware, C., Berge, J., Jelmert, A., Olsen, S.M., Pellissier, L., Wisz, M., Kriticos, D., Semenov, G., Kwaśniewski, S., Alsos, I.G., 2016. Biological introduction risks from shipping in a warming Arctic. J. Appl. Ecol. 53, 340–349. https://doi.org/10.1111/1365-2664.12566

Ware, C., Berge, J., Sundet, J.H., Kirkpatrick, J.B., Coutts, A.D., Jelmert, A., Olsen, S.M., Floerl, O., Wisz, M.S., Alsos, I.G., 2014. Climate change, non-indigenous species and shipping: assessing the risk of species introduction to a high-Arctic archipelago. Divers. Distrib. 20, 10–19.

Williams, S.L., Grosholz, E.D., 2008. The Invasive Species Challenge in Estuarine and Coastal Environments: Marrying Management and Science. Estuaries Coasts 31, 3–20. https://doi.org/10.1007/s12237-007-9031-6

Wonham, M.J., Walton, W.C., Ruiz, G.M., Frese, A.M., Galil, B.S., 2001. Going to the source: role of the invasion pathway in determining potential invaders. Mar. Ecol. Prog. Ser. 215, 1–12. https://doi.org/10.3354/meps215001

Wood, S.A., Smith, K.F., Banks, J.C., Tremblay, L.A., Rhodes, L., Mountfort, D., Cary, S.C., Pochon, X., 2013. Molecular genetic tools for environmental monitoring of New Zealand's aquatic habitats, past, present and the future. N. Z. J. Mar. Freshw. Res. 47, 90–119.

WoRMS Editorial Board, 2019. World Register of Marine Species. Available from http://www.marinespecies.org at VLIZ. Accessed 2019-01-08. https://doi.org/doi:10.14284/170

Wright, D.A., Welschmeyer, N.A., 2015. Establishing benchmarks in compliance assessment for the ballast water management convention by port state control. J. Mar. Eng. Technol. 14, 9–18.

Wu, H., Chen, C., Wang, Q., Lin, J., Xue, J., 2017. The biological content of ballast water in China: A review. Aquac. Fish. 2, 241–246. https://doi.org/10.1016/j.aaf.2017.03.002

# X

Xiong, W., Li, H., Zhan, A., 2016. Early detection of invasive species in marine ecosystems using high-throughput sequencing: technical challenges and possible solutions. Mar. Biol. 163, 1–12.

Xu, D., Li, R., Hu, C., Sun, P., Jiao, N., Warren, A., 2017. Microbial Eukaryote Diversity and Activity in the Water Column of the South China Sea Based on DNA and RNA High Throughput Sequencing. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.01121

Xu, X.-W., Huo, Y.-Y., Bai, X.-D., Wang, C.-S., Oren, A., Li, S.-Y., Wu, M., 2011. *Kordiimonas lacus* sp. nov., isolated from a ballast water tank, and emended description of the genus Kordiimonas. Int. J. Syst. Evol. Microbiol. 61, 422–426.

# Y

Yamamoto, S., Masuda, R., Sato, Y., Sado, T., Araki, H., Kondoh, M., Minamoto, T., Miya, M., 2017. Environmental DNA metabarcoding reveals local fish communities in a species-rich coastal sea. Sci. Rep. 7, 40368.

# Z

Zabin, C.J., Ashton, G.V., Brown, C.W., Davidson, I.C., Sytsma, M.D., Ruiz, G.M., 2014. Small boats provide connectivity for nonindigenous marine species between a highly invaded international port and nearby coastal harbors. Manag. Biol. Invasions 5, 97–112.

Zaiko, A., Martinez, J.L., Ardura, A., Clusa, L., Borrell, Y.J., Samuiloviene, A., Roca, A., Garcia-Vazquez, E., 2015a. Detecting nuisance species using NGST: methodology shortcomings and possible application in ballast water monitoring. Mar. Environ. Res. 112, 64–72.

Zaiko, A., Martinez, J.L., Schmidt-Petersen, J., Ribicic, D., Samuiloviene, A., Garcia-Vazquez, E., 2015b. Metabarcoding approach for the ballast water surveillance–An advantageous solution or an awkward challenge? Mar. Pollut. Bull. 92, 25–34.

Zaiko, A., Pochon, X., Garcia-Vazquez, E., Olenin, S., Wood, S.A., 2018. Advantages and Limitations of Environmental DNA/RNA Tools for Marine Biosecurity: Management and Surveillance of Non-indigenous Species. Front. Mar. Sci. 5. https://doi.org/10.3389/fmars.2018.00322

Zaiko, A., Schimanski, K., Pochon, X., Hopkins, G.A., Goldstien, S., Floerl, O., Wood, S.A., 2016. Metabarcoding improves detection of eukaryotes from early biofouling communities: implications for pest monitoring and pathway management. Biofouling 32, 671–684. https://doi.org/10.1080/08927014.2016.1186165

Zenetos, A., Cinar, M.E., Pancucci-Papadopoulou, M.A., Harmelin, J.G., Furnari, G., Andaloro, F., Bellou, N., Streftaris, N., Zibrowius, H., 2005. Annotated list of marine alien species in the Mediterranean with records of the worst invasive species. Mediterr. Mar. Sci. 6, 63–118. https://doi.org/10.12681/mms.186

Zetsche, E.-M., Meysman, F.J.R., 2012. Dead or alive? Viability assessment of micro- and mesoplankton. J. Plankton Res. 34, 493–509. https://doi.org/10.1093/plankt/fbs018

Zhan, A., He, S., Brown, E.A., Chain, F.J., Therriault, T.W., Abbott, C.L., Heath, D.D., Cristescu, M.E., MacIsaac, H.J., 2014. Reproducibility of pyrosequencing data for biodiversity assessment in complex communities. Methods Ecol. Evol. 5, 881–890.

Zhan, A., Hulák, M., Sylvester, F., Huang, X., Adebayo, A.A., Abbott, C.L., Adamowicz, S.J., Heath, D.D., Cristescu, M.E., MacIsaac, H.J., 2013. High sensitivity of 454 pyrosequencing for detection of rare species in aquatic communities. Methods Ecol. Evol. 4, 558–565. https://doi.org/10.1111/2041-210X.12037

Zhang, H., Finiguerra, M., Dam, H.G., Huang, Y., Xu, D., Liu, G., Lin, S., 2013. An improved method for achieving high-quality RNA for copepod transcriptomic studies. J. Exp. Mar. Biol. Ecol. 446, 57–66. https://doi.org/10.1016/j.jembe.2013.04.021

Zhang, H., Litaker, W., Vandersea, M.W., Tester, P., Lin, S., 2008. Geographic distribution of *Karlodinium veneficum* in the US east coast as detected by ITS–ferredoxin real-time PCR assay. J. Plankton Res. 30, 905–922. https://doi.org/10.1093/plankt/fbn047

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30, 614–620. https://doi.org/10.1093/bioinformatics/btt593

Zorita, I., Solaun, O., Borja, A., Franco, J., Muxika, I., Pascual, M., 2013. Spatial distribution and temporal trends of soft-bottom marine benthic alien species collected during the period 1989–2008 in the Nervión estuary (southeastern Bay of Biscay). J. Sea Res. 83, 104–110.

Zuccarello, G.C., West, J., Rueness, J., 2002. Phylogeography of the cosmopolitan red alga *Caulacanthus ustulatus* (Caulacanthaceae, Gigartinales). Phycol. Res. 50, 163–172. https://doi.org/10.1046/j.1440-1835.2002.00270.x