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DNA metabarcoding monitoring of zooplankton for the detection of non-indigenous species (NIS): a seasonal study in a recreational marina of the northwest of Portugal

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and
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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Monitorização do zooplâncton usando *DNA metabarcoding* para deteção de espécies não indígenas (NIS): estudo sazonal numa marina recreacional no noroeste de Portugal

Resumo

O *DNA metabarcoding* é uma ferramenta poderosa para avaliar a biodiversidade. Tem o potencial de ser mais eficaz na monitorização das comunidades zooplanctónicas e na deteção de potenciais NIS, mas extremamente dependente das várias metodologias adotadas ao longo da sua cadeia analítica. Como tal, os objetivos desta tese foram: i) fazer um estado da arte das metodologias que têm sido adotadas para monitorizar as comunidades de zooplâncton usando *DNA metabarcoding* (desde a amostragem à sequenciação), com base na literatura; ii) avaliar a capacidade de dois marcadores genéticos, o gene que codifica para subunidade I da enzima citocromo C oxidase mitocondrial (COI) e a região variável V4 do gene que codifica para pequena subunidade do gene ribossomal nuclear (18S), na monitorização da dinâmica sazonal das comunidades de zooplâncton, incluindo NIS, através de *DNA metabarcoding*, na marina recreacional de Viana do Castelo, localizada no estuário do rio Lima (NO Portugal) e iii) comparar posteriormente a diversidade obtida com uma lista compilada de espécies de zooplâncton com ocorrência confirmada no estuário do rio Lima, e que foram previamente identificadas com base na morfologia. Para tal, as comunidades de zooplâncton foram amostradas ao longo de 3 estações consecutivas (primavera, outono e inverno 2020/2021), em 3 pontos amostragem na marina recreacional. A área de estudo foi dominada pelo meroplâncton, uma vez que o *DNA metabarcoding* detetou 155 espécies diferentes, das quais os Annelida, Arthropoda e Mollusca contabilizaram cerca de 68%. Aproximadamente 12% das espécies detetadas foram previamente reportadas no estuário em comunidades de zooplâncton e macrozoobentos, incluindo uma NIS. Cinco novas potenciais NIS foram também detetadas. A maioria das espécies foram recuperadas através do marcador 18S. Esta ferramenta providenciou ainda informação mais pormenorizada acerca dos padrões sazonais da diversidade zooplanctónica no estuário do rio Lima, algo que ainda se encontrava por revelar. A maioria das espécies, incluindo as NIS, foram detetadas na primavera-outono. Assim sendo, este estudo vem destacar ainda mais a eficácia desta ferramenta em avaliar as dinâmicas do zooplâncton, e ainda o seu uso na monitorização de comunidades naturais de zooplâncton, particularmente no caso em que as NIS são o alvo principal. A padronização dos protocolos do *DNA metabarcoding* é um passo chave, rumo ao desenvolvimento de programas de monitorização de invertebrados não indígenas utilizando o zooplâncton, o que envolve algumas melhorias técnicas, como por exemplo, o uso de múltiplos pares de *primers* para a mesma região genética, o desenvolvimento de bibliotecas de sequências de referência locais e uma base de dados regional ou local de NIS, e a aplicação de uma amostragem mais extensiva (ao nível espacial e temporal). Finalmente, a comparação das espécies recuperadas por *DNA metabarcoding* com listas compiladas de espécies ocorrentes no local de estudo, é essencial de forma a avaliar a eficácia desta ferramenta, embora algumas discrepâncias possam ser encontradas.

Palavras-chave: ecossistemas costeiros; zooplâncton; espécies não indígenas; *DNA metabarcoding*; abordagem com múltiplos marcadores

DNA metabarcoding monitoring of zooplankton for the detection of non-indigenous species (NIS): a seasonal study in a recreational marina of the northwest of Portugal

Abstract

DNA metabarcoding is a powerful technique for assessing biodiversity that has the potential to be a more effective and reliable tool for monitoring zooplankton communities and detecting new putative NIS. However, the efficacy of this tool is highly dependent on the methodologies adopted along its analytical chain. As a result, the current thesis aims were i) make a state-of-the-art of the methodologies that have been used in several studies found in the literature, using DNA metabarcoding to monitor zooplankton communities (from sampling to sequencing); ii) to examine the capacity of two genetic markers, the mitochondrial cytochrome C oxidase subunit I gene (COI) and the variable region V4 of the nuclear ribosomal small subunit gene (18S), to assess the seasonal dynamics of zooplankton communities, including NIS, through DNA metabarcoding, in the recreational marina of Viana do Castelo, located in the Lima River estuary (NW Portugal), and iii) to further compare the recovered diversity with a compiled list of zooplanktonic species with reported occurrence in the Lima River estuary, using morphology-based assessments. To this end, zooplankton communities, spanning three consecutive seasons (spring, autumn, and winter 2020/2021), were sampled in three sampling points in the recreational marina. The studied area was dominated by meroplankton, since metabarcoding, in general, recovered 155 distinct species, with Annelida, Arthropoda and Mollusca accounting for around 68%. Approximately 12% of the zooplanktonic and macrozoobenthos species recovered, were reported previously to occur in the estuary, including one NIS. Five additional potential NIS were detected. Majority of species were detected with 18S. Metabarcoding provided further insights into seasonal patterns within Lima River estuary zooplankton biodiversity, not yet uncovered. Most of the species (including NIS) were found during spring-autumn period. This study further highlights the effectiveness of this tool in assessing the dynamics of zooplankton, and in using it for monitoring naturally occurring zooplankton, particularly if newly introduced NIS are the main target. Standardization of DNA metabarcoding protocols is a critical step towards the development of invertebrate NIS monitoring programs targeting zooplankton communities, therefore, further improvements are critical, such as, employment of multiple primers pairs targeting the same genetic regions, the generation of local reference sequences libraries and a regional or local NIS database, allied with an extensive spatial and temporal sampling. Finally, the comparison of DNA metabarcoding recovered diversity with compiled lists of previously reported occurring species is crucial to evaluate the efficiency of this tool, although some discrepancies are expected to occur.

Keywords: coastal ecosystems; zooplankton; NIS; DNA metabarcoding; multi-marker approach

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List of Abbreviations and Acronyms

ºC	Degree Celsius
µL	Microliter
µm	Micrometer
ANOVA	Analysis of variance
APA	Portuguese Agency of the Environment
BIN	Barcode Index Number
BOLD	Barcode of Life Database
bp	Base pairs
CMarZ	Census of Marine Zooplankton
COI	Cytochrome *c* Oxidase subunit 1
DAISIE	Delivering Alien Species Inventories for Europe
DNA	Deoxyribonucleic Acid
EASIN	European Alien Species Information Network
HTS	High-Throughput Sequencing
IPMA	
JNCC	Joint Nature Conservation Committee
MOTU	Molecular Operational Taxonomic Unit
NA	Not Available
NIS	Non-Indigenous Species
NISA	National Invasive Species Act
nMDS	Non-metric Multidimensional Scaling
OTU	Operational Taxonomic
PCR	Polymerase Chain Reaction

PERMANOVA Permutational analysis of variance

PGM Personal Genome Machine

rRNA Ribosomal Ribonucleic Acid

WoRMS World Register Marine Species

1. Introduction

1.1. Coastal ecosystems

Coastal systems are found along continental margins as they are a result of intensive and extensive interaction between the land and the sea that extends 1,636,701 km long (Burke et al., 2001). As a result of uninterrupted continental and oceanic pressure, coastlines are in constant change in their geomorphologic features and weather regime through time and space, which culminates in a high variety of biomes. The coastal region encompasses around 8% of the Earth's surface area and the ecosystems in it are considered to be the most productive and diverse in the world, hence it harbors roughly 50% of the human population and a disproportionate number of species (Ray, 1988; Suchanek, 1994). Although there is some disagreement on how coastal ecosystems can be defined due to multiple disciplinary points of view, ecologically, they can be described as the transitional area between terrestrial and marine systems, generally including coastal plains, continental platforms, and associated water columns, bays, and transitional systems, such as estuaries, deltas, lagoons and rias (Inman & Nordstrom, 1971; Inman & Brush, 1973; Burke et al., 2001). Further, these usually encompass a wide variety of ecosystems like dunes, salt marshes, tidal lagoons, mangroves, peat swamps, coral reefs, barrier islands, seagrass forests, and others that are widely different from each other - in physical and chemical conditions, services provided, and pressures that undergo (Burke et al., 2001; Schwartz, 2006). Coastal ecosystems are regions with great biological productivity, which can be associated with their higher biodiversity compared to open sea (Griffiths, 2010; Miloslavich et al., 2010), and are of great accessibility, which for millennia made them hotspots of human activity. Therefore, coastal systems are remarkably dynamic and are constantly loaded with nutrients from the continent that have a great impact on biodiversity and ecosystem integrity.

For thousands of years, human history has been strongly integrated into coastal ecosystems, as the first settlements were established near or on them, particularly on estuaries. At the time of the first settlements, these were established in zones of the estuaries where it was still possible to cross the water body by foot. Still, until today, with the fast growth of the human population and industry, such settlements expanded more and more near the entry point of the sea. Nowadays such expansions are reflected in the locations of today's biggest cities. Initially, newly established settlements did not affect the sustainability of these ecosystems, but the technological developments allowed rapid growth in the human population, which per se incremented resource demand (Ngoile & Horrill, 1993). Consequently, coastal ecosystems

play a critical role in today's economic activities such as commercial and recreational fisheries of which about 90% are from the coast, sports activities, oil exploration/extraction, mining, sand dredging, tourism, aquacultures, genetic stocks with potential application on biotechnology and medicine and many others (Alongi, 2016; Gruber et al., 2003; Martínez et al., 2006). In response to the continuously increasing demand for resources, various coastal ecosystems have been under pressure and degraded to extreme levels, in the last two centuries, due to negligence and bad-to-none administration (Suchanek, 1994). For instance, besides the current economic climate and environmental conditions, fishing and aquaculture industries are still the main sources of nourishment to many countries, and merely 10 countries represent 69.16% output of the total global fisheries production, excluding aquaculture. Regarding the most populated country, with 1.41 billion people (Jizhe, 2021), China's aquatic products alone accounted for 35% of global aquatic production, in 2016, but 40 years earlier it represented just 5% (Zhao & Shen, 2016). Further, roughly 60% of global aquaculture production was estimated to have originated from China. The rapid growth of fisheries and aquaculture exploration in China has brought interest in the resulting ecological impacts. Though pond aquaculture has been the main technique of aquaculture in China, according to the authors, most of the ponds are old and built with poor construction standards which do not meet the requirements of culturing sanitation. Regarding mariculture in China, such has been characterized by increasing intensification and modernization and further problems of discoordination, misbalance, and unsustainability (Zhao & Shen, 2016).

With increasing resource demand, transportation technology would create a bottleneck in commercial supply rates. Thus, the revolutionary containerization of cargo ships was promoted to minimize the turn-round time of the ship by speeding up the loading and unloading time and also decreasing the labor required in doing it (Wilson, 1988). This modernization of transportation, in response to the increasing demand for resources, also allowed the increment of the amount of cargo per ship and decreased the number of ships docking at ports, therefore reducing traffic congestion (Hayut, 1981). Responding to the resource demand with high supply rates, promoted even more the expansion of already established societies and industry seawards. However, the modernization of overall ships introduced the engineering of storing water in special tanks - ballast water - to reduce the load on the hull, improve lateral stability, propulsion, and maneuverability, and further compensate for weight changes at different load levels. Even though ballast waters are nowadays critical to stabilizing ships at sea, it typically contains a variety of biological materials, e.g., propagules of locally never-settled species, that can be hazardous to recipient estuarine ecosystems. Other than that, boats and ships consist of additional substrates for

organisms to adhere to and colonize and be transported to areas beyond their natural distribution. Overall national and international, either recreational or commercial, shipping traffic represents a major threat to coastal ecosystems' health as it can transport new species to the recipient ecosystems beyond their natural range.

1.1.1. Non-indigenous species and introduction in coastal ecosystems

Although the dispersion of organisms beyond a species' natural region is known to be a natural process, such occurrence via anthropogenic means is prone to a wider range of dispersion to other places around the world, otherwise naturally impossible to reach (Burke et al., 2001). Besides pollution, overexploitation of resources, climate changes, and land reclamation, the introduction of non-indigenous species (NIS) represents a major threat to coastal ecosystems, including estuaries (Dukes & Mooney, 1999; Rogers & McCarty, 2000; Xu et al., 2006).

In a review of 151 papers, Gallardo et al. (2016) observed a strong negative influence of invasive species on the abundance of communities of macrophytes, zooplankton, and fish. Further, the ubiquity of such species showed an increase in water turbidity, nitrogen, and organic matter concentration, and subsequently eutrophication. Additionally, NIS often constitute new functional components in the recipient community, in some cases being able to decrease trophic interactions, modulate the structure and composition of native communities, and even influence physiological conditions of native species with whom they compete (Angeler et al., 2002; Clavero et al., 2009; Ordóñez et al., 2010; Gallardo et al., 2016; Ferreira-Rodríguez et al., 2018). There is also evidence that NIS are capable of altering ecosystems' overall productivity, decomposition rates, efficiency in water use, and even the frequency and intensity of fires (Shrader-Frechette, 2001; Allen et al., 2011; Charles & Dukes, 2008; Tait et al., 2015; McLeod et al., 2016; South et al., 2016). Consequently, in Europe NIS activities and impacts were estimated to have caused a loss of at least €12.5 billion, probably reaching over €20 billion (Kettunen et al., 2009). Also, in China, 283 NIS were reported from 2001 until 2003, which caused economic losses to agriculture, forestry, stockbreeding, fishery, road and water transportation, storage, water conservancy, environment and public facilities, and human health estimated at US$ 14.45 billion, where indirect economic losses alone accounted for 83.41% of total economic losses (Xu et al., 2006). Although in some areas some NIS could benefit the regional economy, Charles & Dukes (2008) show that a single NIS can account for hundreds of billions in economic losses. Hence, NIS introductions are a critical source of possible ecological and economic impacts and, therefore, have been receiving more and more attention worldwide.

The biology of invasions is merely the process of species overcoming a series of stages to dispersion and geographical expansion, where each stage presents stress factors as barriers to the next stage (Figure 1). Thus, the definition of indigenous and non-indigenous species is one factor that limits governmental and non-governmental entities and institutions in establishing priorities for monitoring, either for prevention of their introduction or mitigation of the impacts, when these species are already established (Copp et al., 2005; Richardson et al., 2000). There is no general consensus on how to define non-indigenous and indigenous species. Therefore, there are a plethora of arbitrary and stipulative ways to define NIS (Shrader-Frechette, 2001), many of which are specific to groups of taxa, or either do not recognize human intervention (intentionally or accidentally) or do not include in the definition the subsequent impacts on the recipient regions (e.g., NISA, 1996; Richardson et al., 2000; European Commission, 2008; JNCC, 2021). Albeit, not yet firmly defined, NIS are herein considered as species, sub-species, race, or variety - including gametes, propagules, or part of an organism able to survive and subsequently reproduce - which were transported beyond their natural geographical area via human actions (intentionally or not), regardless of their eventual impact in recipient ecosystems – adapted from Copp et al. (2005) and Lockwood et al. (2007) definitions.

Further, the biology of invasions is merely the process of species overcoming a series of stages to dispersion and geographical expansion, where each stage presents stress factors as barriers to the next stage, as represented in the model in Figure 1. The first stage of the process of invasion is the

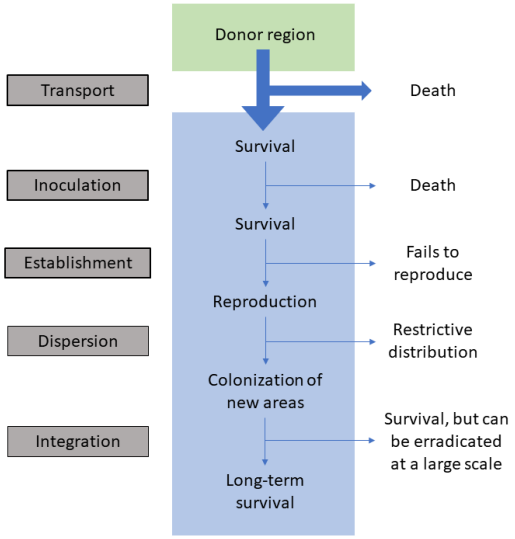

Figure 1. Invasion process model with every stage that a propagule must go through to a successful integration in the recipient ecosystem. The donor region is represented by the green square from where the propagules originate. The recipient region is represented by the blue square to where propagules are transported and face various barriers in their process of establishment and invasion. The model was based on the Lockwood et al (2007), Sakai et al (2001) and Moyle & Marchetti (2006) model.

transport from the donor region to the recipient region, where its success can be impaired by the survival of the organisms when exposed to various altering environmental, physical, and chemical conditions. Those organisms that tolerate and survive transport conditions, face the overall environmental conditions of the recipient ecosystem. Many introductions fail because propagules are released into habitats that are totally or partially unsuitable (Sax & Brown, 2000). Only when the habitat conditions are suitable, a new population can be established in the recipient ecosystem. After this step, the reproductive capability of a NIS consists of their growth in abundance and in the expansion of its geographical range. Finally, NIS populations become integrated into the recipient ecosystem when they respond to local environmental conditions and to biotic processes of other members of the community, in ways that are apparently indistinguishable from native species (Moyle & Marchetti, 2006). Usually, when a non-indigenous taxon is capable of establishing and presenting a strong population growth in the recipient ecosystems, ecological and economic damages are manifested, which from now on are described as invasive. NIS are then classified as invasive in the region where their activity has impacted the local communities and overall ecology and the region's economy (Lockwood et al., 2007).

1.2. Biomonitoring of coastal ecosystems

Globalization led to the integration of widely dispersed human communities into a worldwide economy, which has facilitated the spread of NIS across the planet - a problem which has increasingly shown to have more attention to how they impact local and regional ecology, populations, genetic pool and economy, and how to prevent it (Krueger & May, 1991; Ojaveer et al., 2004; Xu et al., 2006; Charles & Dukes, 2008; Kettunen et al., 2009). While improvement actions partaken in case of pollution for recovering to the previous ecological state, the damages of NIS introductions are most often irreversible (Streftaris et al., 2005); accordingly, the introduction of NIS is a global threat to biodiversity, particularly in estuaries, where it has been shown that ten to hundreds of NIS can be accumulated per estuary (Ruiz et al., 1997). Particularly, the Convention on Biological Diversity considered the occurrence of NIS as the second most significant threat to global biodiversity (EEA, 2012). In such matters, monitoring their presence and impacts should be considered a prerequisite for marine environmental management and sustainable development (Lehtiniemi et al., 2015).

In developing programs aiming the preservation and conservation of ecosystems, it is crucial the adequate planning, which demands comprehensive methodological approaches to assess the current state of these ecosystems and to monitor the rates of how human disturbances have changed them

(Johnston, 1981; Facca, 2020). Biomonitoring or biological monitoring is a method of observing the impact of external factors on ecosystems and their development over a long period, or of ascertaining differences between one location and another using biological factors (Markert et al., 1999), which in short is the use of biological response to assess changes in the environment, in the majority, due to anthropogenic means (Oertel & Salánki, 2003). In fact, chlorophyll a was the first suggested as an index of productivity and trophic conditions, in transitional and coastal waters, in the second half of the 20th century (Steele, 1962; Boyer et al., 2009). Following, bioindicators encompass a wide scale of biological systems and functions, in which reactions are observed representatively to evaluate a situation, giving clues for the condition of the whole ecosystem (Gerhardt, 2002). In terms of the parameter measured as an index to productivity and trophic conditions, primary producers are the bioindicators targeted. In aquatic systems, it is mainly phytoplankton. So, the involvement in the monitoring of biological variables in management programs is usually cheaper, requires less sophisticated instruments and, of most concern, it reflects the integrated expressions of pollution intake. Nonetheless, physical and chemical monitoring should not be ignored under all circumstances as they can explain the cause of the effect.

Generally, biomonitoring involves the use of numerous levels of biological organization, ranging from subcellular to communities, although in means of ecological assessment the higher levels - species, populations, and communities - are the main focus. Over the last century, a range of studies has suggested numerous groups of organisms for biomonitoring programs in aquatic systems. A review of the literature up to 1970, ranked the groups of organisms recommended for biological monitoring of aquatic ecosystems (although most were centered in freshwater ecosystems) as benthic macroinvertebrates, algae, and protozoa the most suggested (around 69% of the studies), followed by bacteria and fish - 10% and 6% respectively - with rare recommendations of fungi, macrophytes, yeasts, and viruses (Hellawell, 1986). However, the perception of bioindicator groups of organisms has changed, where benthic macroinvertebrates, fish, and phytoplankton have received the most attention in biomonitoring programs. Macroinvertebrates are usually considered to be better bioindicators than fish according to ecological and logistical reasons. The smaller number of species and lower densities of the latter makes them less useful for statistical analyses, as well as their greater mobility allows them to swim to locations less impacted (Morse et al., 2007). Furthermore, macroinvertebrates constitute the link between organic matter and nutrient resources to higher trophic levels, hence are critical to the aquatic food webs (Li et al., 2010). Additionally, according to Ruaro et al. (2016), biomonitoring planning for macroinvertebrates is a better choice in situations in which time and funds are a concern. Otherwise, fish monitoring has been applied

for a long time to monitor the health of the aquatic ecosystem, particularly in long-term assessments, as they include a range of species that represent a variety of trophic levels and are at the top of the aquatic food webs. The interest in their monitoring is also favored as they constitute resources for human nourishment (Plafkin et al., 1989). Phytoplankton growth, on the other hand, is one of the primary symptoms of surplus nutrient loading as they present rapid responses to nutrient availability, and phytoplankton itself is a relevant vector in the metal biogeochemical cycling, as they are efficient scavengers of trace elements and control their concentration in the water (González-Dávila, 1995; Bricker et al., 2003; Cabrita et al., 2020). Notwithstanding, in more recent years, zooplankton has been highlighted as a promising indicator of water quality, since zooplanktonic communities have been shown to react to subtle variations in water quality (Gannon & Stemberger, 1978; Gajbhiye, 2002; Kour et al., 2022) and further represent the step between primary production and higher trophic levels, as well as, they include numerous larvae, eggs and other propagules of economic-relevant species, as well as of species used for biomonitoring purposes.

1.2.1. Zooplankton communities

The word zooplankton originates from the Greek word *zoon* referring to animal and the adjective *planktos* which in turn means errant, wanderer, or drifter. Thereby, zooplankton organisms are described as animals that habit the water column and do not present any kind of significant volunteer locomotion, hence are transported by the currents. Although their movement in the horizontal plane is due to moving water, on the vertical plane some of their movements are significant, usually daily and vertical diel migrations, which are seen all over the world are considered to be the biggest animal migration in terms of biomass (Hempel & Weikert, 1972; Hays, 2003). These migrations involve movements from shallow depths at night to greater depths during the day. Microscopic organisms compose the majority of zooplankton, however, some species at the macro scale are also included in the definition of zooplankton, such as jellyfish.

Zooplankton encompasses almost every taxon in the animal kingdom - with emphasis on invertebrates - and organisms that spend their entire life cycle drifting in the water column (holoplankton) or merely some stages, e.g., eggs and larvae (meroplankton). Marine zooplankton is mainly composed of Protozoa and Copepoda, with the latter being the most common in terms of abundance and species richness. Other major groups include Cladocera, Ostracoda, Isopoda, Amphipoda, Mysidacea, Euphausiacea, Decapoda, Hydrozoa, Siphnophora, Cubozoa, Scyphozoa, Ctenophora, Rotifera,

Platyhelminthes, Nematomorpha, Nemertea, Polychaeta, Mollusca, Chaetognatha, and Chordata (CMarZ, 2011; Srichandan et al., 2018; Bucklin et al., 2021). These play an important role in the study of the biodiversity of aquatic ecosystems. The energy flow between primary producing phytoplankton and benthic and nektonic species in higher trophic levels is mediated by zooplankton communities (Suthers & Rissik, 2009; Steinberg, 2017). For instance, Landry & Hassett (1982) estimated that zooplankton predation over phytoplankton consumed 6 to 24% of its biomass and impacted 17 to 52% of daily primary production. Their grazing activities lower phytoplankton populations, then excrete nutrient substances in the water, which nourishes back phytoplankton by accelerating their growth (Ikeda, 1974). Bacteria also have some effect on phytoplankton biomass, which in return also supplies zooplankton as a food source. This way, zooplankton play a crucial role in the complex trophic networks, as well as in biochemical processes occurring in aquatic ecosystems (Suthers & Rissik, 2009; Le Quéré et al., 2016; Steinberg, 2017). Zooplankton occurrence and distribution subsequently affect pelagic fisheries' location and potential yield either by contributing to species newer generations - via meroplanktonic species, especially in nursery zones (e.g., estuaries) - or as food supply to fisheries (Goswami, 2004).

In terms of monitoring, zooplanktonic organisms are usually larger than phytoplankton, which enhances the process of identification and processing of samples (Gannon & Stemberger, 1978). Further due to being easier to identify than phytoplankton, the authors infer researchers can be trained in zooplankton taxonomy faster. Regarding new introductions of NIS monitoring, zooplankton is a key element since it is possible to detect species in early development stages. Also, sampling pelagic stages involve less effort than benthic stages or organisms of high mobility. Still, zooplankton morphological identification consists of a low-throughput process that requires a high sample size, and obtaining results can be a slow process. Other than that, identification of early stages of development (eggs or larvae) to species level might be impossible due to ambiguous morphology between taxa. Moreover, there is evidence that traditional methods of identification are less likely to detect species unless population density is high (C. T. Harvey et al., 2009; Xiong et al., 2016). Rare species are inherently hard to find (Jerde et al., 2011; Zhan et al., 2013); therefore, their detection via morphological traits is usually compelled to the high amounts of samples. For instance, in the Great Lakes, it was estimated that more than 750 samples would be needed to detect 95% of the zooplankton species present, and for a high-probability of early NIS detection (Hoffman et al., 2011). Recently introduced NIS would otherwise be undetected unless in the case of already established, considerable sized and/or spread populations (Crooks & Soulé, 1999). In sum, morphology-based methods of identification and monitoring of newly

introduced NIS are in general sluggish due to the critical need for high sampling size and high experience requirement in the taxonomy of zooplankton, which in long-term projects/programs, is less efficient and appears to relate to high expenses - about 30% of the bioassessments (Stein et al., 2014). However, the use of molecular tools simultaneous to the traditional processing of zooplankton samples is becoming more vulgar in response to aforesaid problems (Leray & Knowlton, 2015).

1.3. Use of molecular tools in zooplankton biomonitoring and NIS early detection in coastal ecosystems

Molecular tools development based on DNA has shown great potential in the identification of organisms, and high efficiency in the assessment of zooplankton communities (Carroll et al., 2019; Djurhuus et al., 2018; Lavrador et al., 2021), overcoming various limiting factors inherent to low-throughput methods. DNA fragment sequencing, as a form of organisms' identification, presents a methodology from which obtaining results can be greatly faster, from a fraction of the sampling size needed when taken for the identification via morphology. Such differs in many ways from conventional taxonomic identification approaches, over which it offers several advantages as it allows the identification of species fragments, at any stage of its life-history, and further allows the standardization of the overall process of identification in a way that reduces ambiguity (Costa & Carvalho, 2007). The efficiency of such methods in detecting biodiversity has been emphasized by a wide range of areas that applied it, such as, taxonomy, ecology, forensic sciences, and the food industry (Galimberti et al., 2013; Bell et al., 2016; Harris et al., 2016; Abad et al., 2017; Lobo et al., 2017; Carroll et al., 2019; Speranskaya et al., 2018; Teixeira et al., 2020).

DNA barcoding *sensu lato* corresponds to the identification of any taxonomic level using any DNA fragment, however, regarding Consortium for the Barcode of Life (CBOL) point of view, DNA barcoding *sensu stricto* corresponds to the identification at the species level using a single standardized DNA fragment, which is better fitted in terms for the accomplishment of CBOL aim (Hebert et al., 2003b). The concept of DNA barcoding has already been in use for two decades (Floyd et al., 2002; Hebert et al., 2003a,b); yet the term "DNA barcode" was firstly inferred almost three decades ago (Arnot et al., 1993). In general, the DNA barcoding system should account for some criteria that allow the viable practice and respond to the limitations of conventionally used taxonomic approaches (Valentini et al., 2009). Following this, the sequencing of the genetic region should be standardized and nearly identical among individuals of the same species, but different between species, where it should still contain enough phylogenetic

information to easily identify and assign to higher taxonomic groups to unknown species and/or that are not yet barcoded. Still, it should present highly conserved priming sites and highly reliable DNA amplification and sequencing (Valentini et al., 2009). Then, sequences are grouped in Molecular Operational Taxonomic Units (MOTU) based on sequence identity, which itself does not necessarily correspond to the identity of Operational Taxonomic Units (OTU) - based on biological and morphological parameters. This approach allows the assignment of putative species to clusters that emerge from the molecular divergence date and then enables further testing on species groupings under various scenarios (Costa et al., 2009).

Nonetheless, DNA barcoding is not an ideal tool for detecting and identifying a bulk of organisms that are encompassed in natural communities since this methodology is focused on the amplification and sequencing of genetic markers of single organisms. The concept of DNA metabarcoding came to revolutionize the employment of molecular tools in ecological studies, and eventually potential monitoring. Such tool development was possible due to continuous improvement of the sequencing technology that allowed greater sequencing rates and depth. This approach comprehends the application of the concept of DNA barcoding, of detecting species via amplification and sequencing of standardized genetic markers, to the identification of multiple species, in a single experiment, from a single sample of complex communities of organisms - either bulk or environmental samples (Hajibabaei et al., 2011; Taberlet et al., 2012). The DNA from environmental samples (such as soil, water, and feces), often referenced as eDNA, usually consists of highly degraded DNA disregarding the presence of biological material, and the amplification of long fragments of several base pairs (bp) is not reliable. Bulk samples, on the other hand, encompass the isolation of biological material from the environmental matrix, and therefore its genetic contents are of greater quality - this process is usually designed as DNA metabarcoding (Taberlet et al., 2012).

Unlike DNA barcoding which is usually based on Sanger DNA sequencing of individual specimens and species, DNA metabarcoding follows a high-throughput sequencing (HTS) of DNA fragments that allows a greater amount of DNA sequence data derived and, consequently, allows taxonomy of sampled communities to be rapidly assigned to various existent individuals. The rapid progress of HTS technologies led to the development of various sequencing systems - e.g., PCR-based technologies, such as 454 Roche, Illumina MiSeq, or IonTorrent PGM, and single-molecule sequencing - but, in terms of ecological analysis and studies, PCR-based platforms appear to be better suited (Shokralla et al., 2012).

The diversity detected from the metabarcoding analysis is dependent on the specificity of the primers employed and the reference database that link genetic sequence to taxonomic morphology (Seymour, 2021). There is a plethora of universal primers that aim to amplify the most taxa possible, but no primer is perfect, with many designed to detect specific taxonomic groups. In many cases, depending on the aim of the study, a complementary multi-primer approach is used.

DNA barcodes are then standardized small fragments of DNA, that are generally shared to some degree by all individuals of the same taxa, which allows differentiation of organisms from different species. In animals, the standardized barcode region is a 658 bp segment of the gene encoding the mitochondrial cytochrome c oxidase subunit 1, commonly designed as COI (Hebert et al., 2003b). COI choice was due to: i) the generally uniparental nature of inheritance, ii) the considerably high mutation rate, iii) the existence of a large number of copies in cells, iv) the lack of recombination and introns, v) the relatively small genome, and vi) the rare occurrence of indels (Saccone et al., 1999; Mueller, 2006; Salas et al., 2007; Andújar et al., 2018), that subsequently allowed high sequences representativity in databases and significant capability to discriminate organisms to species level (Hebert et al., 2003b; Costa et al., 2009; Baek et al., 2016). However, applying efficiently COI in sequencing DNA fragments still has some limitations, namely inefficiency due to the occurrence of pseudogenes (Song et al., 2008), or due to low primer affinity to certain taxonomic groups (Jorge Lobo et al., 2013), further incapability of discriminating recently discovered species, hybrids and highly genetic conservative species (McFadden et al., 2011), and in some cases biparental mitochondrial DNA inheritance (Hoeh et al., 1991; Śmietanka et al., 2014).

Various other nuclear and organellar genetic markers can be targeted for sequencing in identifying biodiversity (Taberlet et al., 2012). Nuclear genetic markers include the nuclear gene ITS (internal transcribed spacer) and the nuclear/mitochondrial ribosomal RNA genes (12S, 16S, and 18S), while organellar genetic markers include the chloroplast genes matK (maturase K) and rbcl (ribulose-bisphosphate carboxylase) (Stoeckle, 2003). Although the mitochondrial COI marker is considered to be the universal barcode for metazoans, 18S has been the standardized marker adopted for the analysis of microbial eukaryotic marine diversity (Gouy & Li, 1989; Amaral-Zettler et al., 2009). Sometimes applying 18S sequencing underestimates the number of species since its mutation rate is not enough to distinguish organisms at the species level (Tang et al., 2012). Then, COI and 18S are sometimes amplified and sequenced complementarily for better species detection (Stefanni et al., 2018; Brandão et al., 2021). In regard to zooplankton studies, species have sequenced records of one or the other molecular marker, therefore their complementary use benefits biodiversity detection.

In summary, DNA metabarcoding is a potential tool to implement in monitoring coastal ecosystems, particularly in the early detection of NIS. DNA metabarcoding is a molecular-based tool that: i) is morphology-independent, therefore is capable to detect new introductions of NIS, independently of the available development stage; ii) appears to be more efficient in cases of morphological ambiguity, particularly when NIS can be difficult to differentiate from similar indigenous species; iii) requires less sampling effort as species detection is more efficient, particularly to less abundant species (rare species and recently introduced NIS); iv) requires less to none morphological taxonomic experience; v) is less time-consuming in samples processing; vi) appears to be more cost-effective. Regarding the latter, according to Stein et al. (2014), HTS is comparable to or slightly less expensive than when applying traditional methods of identification of organisms. This emphasizes even further that DNA metabarcoding is a cost-effective tool, providing an overall greatly taxonomic resolution on biodiversity.

1.3.1. Workflows in use for DNA-based monitoring of zooplankton communities

In order to make a state of the art on use of DNA metabarcoding, and the methodologies that have been employed through its analytical chain, in zooplankton communities monitoring in marine and coastal ecosystems, a literature review was made and the results are included as part of the next sub-sections of this chapter. For that, a search was conducted in December of 2021 on the Web of Science by querying the following: ''((“zooplankton”) AND (“estuar*” OR “transition*” OR “marine” OR “lagoon*” OR “sea*” OR “coast*”) AND (“metabarcoding” OR “high throughput sequencing” OR “high-throughput sequencing” OR “HTS” OR “next generation sequencing” OR “next-generation sequencing” OR “NGS” OR “eDNA” OR “environmental DNA”))'', which include the term ''zooplankton'' with the combination of all terms designing coastal and marine ecosystems, as well as metabarcoding. The combinations of terms were searched by topic, which included the words in the title, abstract, and keywords. The search yielded a total of 123 publications, of which 36 were retained for further analysis since they met the defined criteria. To these, 6 more publications were added from personal collections and that were not displayed in the initial search. The information retrieved from each selected publication, when available, included: sampling location and method, including details such as type of net used and its mesh size; sample preservation method; sample pre-processing prior to DNA extraction where method, filter material and mesh size were taken into account; DNA extraction kit or protocol used; markers and primers opted for amplification and sequencing of zooplankton samples; as well as sequencing platforms chosen.

The increasing implementation of DNA-based species identification is extensively encouraged by worldwide efforts in building curated taxonomic reference libraries of standardized genetic regions. Particularly, the implementation of DNA metabarcoding for assessing marine and brackish natural zooplankton communities is gaining significantly increased interest, particularly in the last 4 years (Figure 2A). Curiously, the majority of studies were developed in the northern hemisphere, where the USA was the focus of 6 publications, followed by Canada, Italy, Spain, and South Korea, with 3 papers found for each. For the southern hemisphere, only 8 studies were conducted in South Africa and Oceania, but the latter has been the focus of more studies than the former.

Consequently, standardization of such an approach is an important step towards an efficient and comparable method to be employed in monitoring programs. However, the overall process of sample gathering and processing, DNA extraction, DNA fragments amplification, and sequencing are also required to be standardized to obtain comparable timely and spatially dispersed results in general monitoring programs, particularly when it takes to early detection of NIS in coastal ecosystems.

1.3.1.1. Zooplankton sampling strategies

This analysis showed that only 2 studies did not specify which technique was employed in the sampling of zooplankton communities (Mohrbeck et al., 2015; Walters et al., 2019). In short, 4 different techniques were applied throughout the papers herein analyzed: continuous plankton recorder (CPR), conductivity-temperature-depth instrument (CTD), Niskin bottles, and planktonic nets (Figure 2B). The majority of the studies used planktonic nets for sample gathering. This can be related to the fact that the aim of the studies do not include fine details in spatial patterns (e.g., vertical variation) in zooplankton communities, as zooplankton hauls generally imply the mixing and integration across the space (Alcaraz & Calbet, 2003), although plankton nets are an economical sampler for large, rare or more active animals (Karjalainen et al., 1996). However, there were some cases that either employed planktonic nets in conjunction with Niskin bottles (Abad et al., 2016, 2017) or different techniques other than planktonic nets (Deagle et al., 2018; Sun et al., 2021). Nisking bottles are usually advantageous for very small organisms, which seemed to be the case for those that referenced its use, however, the limited water volume sampled compared to plankton nets (Gajbhiye, 2002) might explain why plankton nets dominate Niskin bottles in studies of DNA metabarcoding targeting zooplankton communities. Further, CPR, an instrument that has been implemented in zooplankton surveys for almost a century, can be towed at high speed from whatever ship (not necessarily a research ship), overextended transects, but that lacks

calibration against other more widely used sampling techniques (John et al., 2001). On the other hand, CTD is a package of electronic sensors that detect conductivity and temperature according to depth and is usually used to sample zooplankton communities from specific depths with the simultaneous measurement of environmental parameters. In regard to inferred plankton nets used, up to 20 articles did not specify which kind of net applied, while, on the other hand, one study did design a new Cruising Speed Net (CSN) that, according to the authors, demonstrated better results in planktonic biodiversity recovered than traditional plankton nets at higher tow speed (not specified which one used; von Ammon et al., 2020). In such a manner, the Bongo and WP2 nets were the most adopted choice, each with 6 and 7, respectively, reports of use (Lindeque et al., 2013; Casas et al., 2017; Clarke et al., 2017; Harvey et al., 2018; Stefanni et al., 2018; Bucklin et al., 2019; Couton et al., 2019; Kim et al., 2019; Brandão et al., 2021; Cicala et al., 2021; Coguiec et al., 2021; Ershova et al., 2021), followed by Apstein nets with 2 citations (Schroeder et al., 2020, 2021). The MANTA, the NORPAC, the Pairovet and the Fao nets were only mentioned once (Casas et al., 2017; Rey et al., 2020; Hirai et al., 2021; Govender et al., 2022b) (Figure 2B). Only 2 studies employed multiple plankton nets, the newly designed CSN with a non-specified net - merely mentioned as traditional plankton nets (von Ammon et al., 2020) - and the combination with the Bongo net along with the MANTA and WP2 nets (Casas et al., 2017).

Skjoldal et al. (2013) demonstrated in a multi-plankton net study that mesh size is the major factor influencing the biomass and species composition of zooplankton communities, even though towing speed, patchiness and avoidance also play a role in sample quality (Karjalainen et al., 1996; Gajbhiye, 2002; von Ammon et al., 2020). The authors even recommended the employment of 150 μm mesh for zooplankton communities, particularly when targeting neritic zooplankton. Hence, mesh size varied with the study itself. Most used nets with a mesh size between 20 μm and 200 μm (35 cases), followed by meshes beyond 200 μm (11 cases) and plankton nets with a mesh size lower than 20 μm (3 cases). Although, 200 μm sized meshes were the most applied, with 14 citations, followed by 150 μm, in 4 studies. However, the overuse of 200 μm mesh size nets has shown to often underestimate small copepods of mesozooplankton communities. Nonetheless, using smaller-sized meshes is usually correlated with clogging due to the accumulation of debris, hence >100 μm-mesh nets are eventually more efficient in the event of planktonic debris being less abundant in the water column (Riccardi, 2010; Mack et al., 2012). All things considered, the net design choice is basically determined according to the study aims, the conditions of the study area, the overall resources available, the target biological groups, and the sampling design (either day or night-time tows, vertical or oblique tows, among others) (Keen,

2013; e.g., Casas et al., 2017). Even though the lack of information regarding sampling instruments has not been a critical problem, the majority of the plankton nets mentioned did not specify which kind was used. This can pose a limiting factor to replication by other projects and/or comparability of results, which play a critical role in monitoring programs to extrapolate reliable analysis of timely and spatial patterns and trends, where the replicability of the protocols employed is crucial.

1.3.1.2. Zooplankton samples preservation and pre-processing

For bulk zooplankton samples, preservation with formalin, that has been formally adopted for identification using traditional methods, is not recommended for DNA analysis as it is known to alter and degrade DNA (Williams et al., 1999). Nonetheless, ethanol has been the preferred choice for sampling storage and preservation of the genetic material - about 80% (see also van der Loos & Nijland, 2021). Merely 5 studies opted to freeze-dried all samples, whereas the use of RNALater (-80 °C) seems a rare choice (Figure 2C). For freeze-dried zooplankton samples, subsequent storage at very low temperatures has been frequently adopted (-80 °C). Although the majority of ethanol-based preservations opted for further storage at room temperature, some cases also complemented with the use of low temperatures, with -20°C being the most opted (ca. 26%). Freezing samples and/or adding ethanol is a very common and standardized technique in preserving zooplankton samples for DNA metabarcoding, however, RNALater is an uncommon choice (van der Loos & Nijland, 2021) – mostly used for dietary analysis (Rey et al., 2020b). Additionally high % ethanol solutions are also the most preferred choice (95%-100%; Rey et al., 2020b; van der Loos & Nijland, 2020), as it dehydrates the cells and protects the DNA by coagulating proteins including those that could degrade genetic content. However lower concentrated solutions have also been employed, as low as 60%, but there is no information regarding the effect of ethanol concentration on the recovered biodiversity, especially if it can affect NIS detection. Nonetheless, ethanol is acidic which is not suitable for long-term sample preservation since there is evidence of high DNA degradation already 24 hours after sampling (Oosting et al., 2020). RNALater has been a rare option (Figure 2C), even though it is highly recognized as a good DNA preserving solution, since it denatures proteases and RNases preventing RNA and protein degradation, however, lower quantities of DNA have been found further downstream, that other preserving solutions.

Henceforth, ethanol usage for zooplankton sample preservation has been the most chosen option, which might be due to its price and accessibility. Ethanol is very accessible, particularly where molecular studies are performed, which allied to the fact of its cost effectiveness, it poses as a favorable

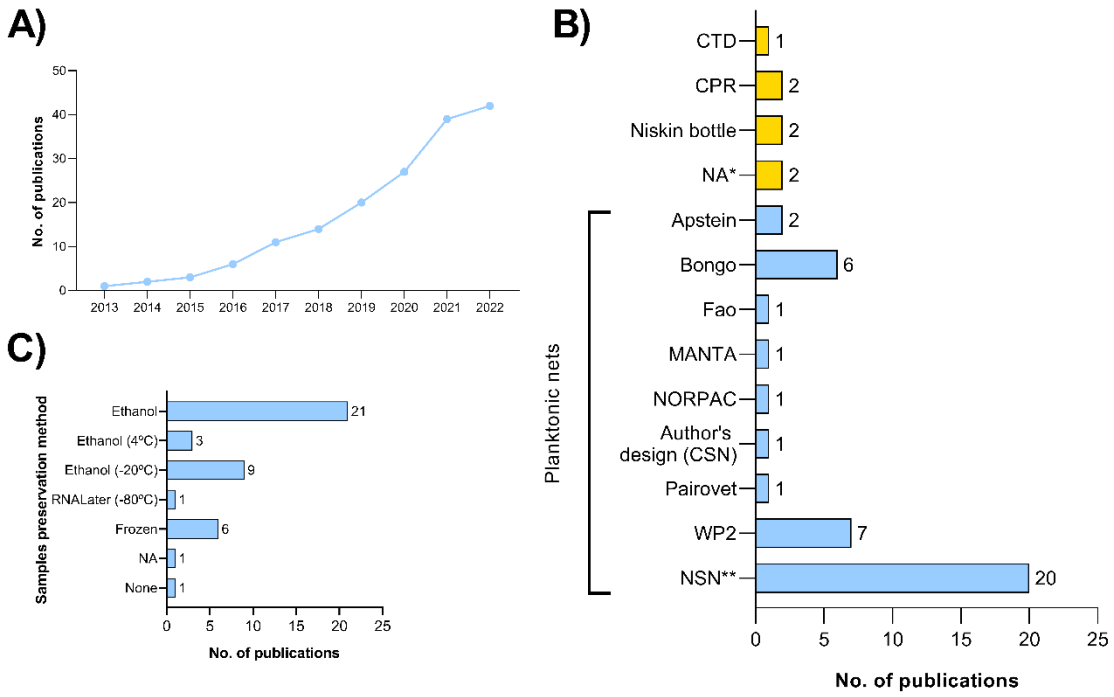

Figure 2. A) Cumulative number of papers, published over the last 8 years, and that met the criteria of the search conducted in the current thesis on the use of DNA metabarcoding to assess natural occurring zooplankton communities; B) Methods applied for sampling zooplankton communities. Yellow bars represent techniques that were used (other than plankton nets) and publications that did not describe the technique used for sampling (NA), while blue bars represent all types of plankton nets that were used; C) Summary of the sampling preservation methods practiced in the publications.

*NA – includes studies that did not have information of which sampling method was used.
** NSN (not specified net) – includes studies that did not specify which kind of net was used and studies that referred to it as traditional plankton net.

choice. DESS (salt-saturated DMSO buffers containing EDTA; Yoder et al., 2006) has been widely used for the preservation of other taxa from hard substrates and sediment samples (van der Loos & Nijland, 2021), and is also known for better long-term DNA preservation and even providing higher DNA quantity and quality. This preservative prevents DNA degradation by deactivating metal-dependent enzymes using EDTA. DESS has also been shown to increase amplification success (Gaither et al., 2011; Ransome et al., 2017). Yet, our and van der Loos & Nijland (2020) findings did not report any use of DESS as a preservative of zooplankton samples before DNA extraction. The only exception was its use to preserve gelatinous zooplankton (Silke Laakmann & Holst, 2014), while for corals, nematodes, and hard substrate communities, has been commonly adopted. van der Loos & Nijland (2020) even suggested a possible switch from ethanol-based preservation methods to DESS but did not refer specifically to zooplankton. In addition to DNA extracts of high quality and higher amplification success, the latter poses an extra

advantage, since it can be easily removed from the samples through centrifugation, while ethanol needs to be completely dried off, before DNA extraction.

There is a current lack of information on the effect of sample pre-processing methods on zooplankton samples to be used in DNA metabarcoding, particularly in NIS detection. However, the methodology choice during the processing steps may influence OTUs recovering, particularly rare and low abundant, such as newly introduced NIS (Pagenkopp Lohan et al., 2019). Analysis of pre-processing steps taken before DNA extraction for NIS detection via eDNA found that such may strongly influence biodiversity reports (Duarte et al., 2021b). The current analysis showed that filtration has been the preferred technique in sample concentration, before DNA extraction (ca. 48%). Technically, this step usually includes a second filtration stage, where the zooplankton organisms are concentrated and retained on filters, whereas removing ethanol or water residuals, before DNA extraction. Centrifugation of bulk samples, before DNA extraction, has also been widely employed in zooplankton samples processing (ca. 41%), although grinding samples prior to DNA extraction has been rarely used (Rey et al., 2020a; Westfall et al., 2020). Centrifugation can be employed together with the homogenization of zooplankton samples, where homogenization seems to enhance the diversity recovered, thereby improving the chances of NIS detection (Pagenkopp Lohan et al., 2019). Centrifugation has been mainly used for ethanol removal, and the pellet is then used for DNA extraction (Rey et al., 2020b). Filtering the samples allows the complete separation of the biological material from the liquid matrix and the residual adhered genetic material, which was not filtered while sampling. With this, it is possible using the samples as a whole, but in cases of very concentrated samples and/or small pores, the filter can easily clog and extend the filtration process even more. Whereas centrifugation also allows ethanol or water removal from samples, but to a fraction of sample content. However, the removal of the former involves additional steps to remove residual ethanol through evaporation. Nonetheless, one study has been found to not have adopted preservation and processing zooplankton samples prior to DNA extraction (Brandão et al., 2021). Such has reported to have performed the extraction of DNA content right after sampling collection. Up until today, no comparisons have been performed to assess the effect of the use of different fluid removal techniques, from zooplankton samples on further downstream results. However, centrifugation of the entire samples is highly time-consuming, which might contribute to the higher adoption of filtration.

In general, only 3 filter materials were reported to be used in the filtration step description: nylon-based filters (Bucklin et al., 2019; Hirai et al., 2021; Questel et al., 2021), cellulose-based filters (Casas et al., 2017; von Ammon et al., 2020; Zhao et al., 2021), and polyethersulfone-based filters (Walters et

al., 2019). Only 3 papers mentioned the use of sieves during filtration, but did not specify the material used (Figure 3A). On the other hand, still regarding the filters, the majority opted to use a mesh of the same size or smaller than the one used for sampling, where pores between 20-200 µm have been the mostly adopted (from these, 200 µm have been used 14 times), followed by a pore size higher than 200 µm (Figure 3B). Although there is no evidence of the influence of the filter pore size and material composition in the recovered diversity, when employing DNA metabarcoding to analyze zooplankton communities, cellulose-based filters were shown to perform better than glass-fiber filters on NIS detection through metabarcoding of water eDNA (Jeunen et al., 2019; Deiner et al., 2018). In terms of the overall efficiency of the sample processing, larger pore sizes in theory perform better, as they allow faster filtrations and fewer chances of clogging.

1.3.1.3. DNA extraction

Nowadays there is a very competitive market for DNA extraction kits resulting in a great variety of commercial kits. Non-commercial protocols such as phenol-chloroform or salt based are also greatly adopted. The current analysis showed that commercial extraction kits are the preferred option among studies characterizing zooplankton communities through DNA metabarcoding (ca. 65% of the studies), in particular DNeasy Blood & Tissue Kit (from Qiagen), DNeasy PowerSoil DNA Isolation Kit (from Qiagen, former MoBio) and E.Z.N.A. Mollusc DNA Kit (from Omega Bio-Tek) (Figure 3C; see also Jeunen et al., 2019; Liu et al., 2019). Similar results have already been observed in previous methodological reviews (van der Loos & Nijland, 2020; Duarte et al., 2021a,b). The use of only one DNA extraction kit is commonly adopted, though some opted to apply multiple kits due either to different zooplankton size fractions or sampling dates (Abad et al., 2016, 2017; Coguiec et al., 2021). Steep inclination towards the use of commercial DNA extraction kits could be associated with consistently providing DNA suitable (in quantity and purity) for amplification and sequencing, while being generally more reproducible and less time-consuming than non-commercialized protocols. Additionally, Liu et al. (2019) demonstrated that the DNA extraction kits' choice can have a greater impact on rare taxa detection compared with abundant taxa (see also Deiner et al., 2018). Nonetheless, commercial extraction kits have high prices associated, and, thus, can be less cost-effective (Dell'Anno et al., 2015; Duarte et al., 2021b).

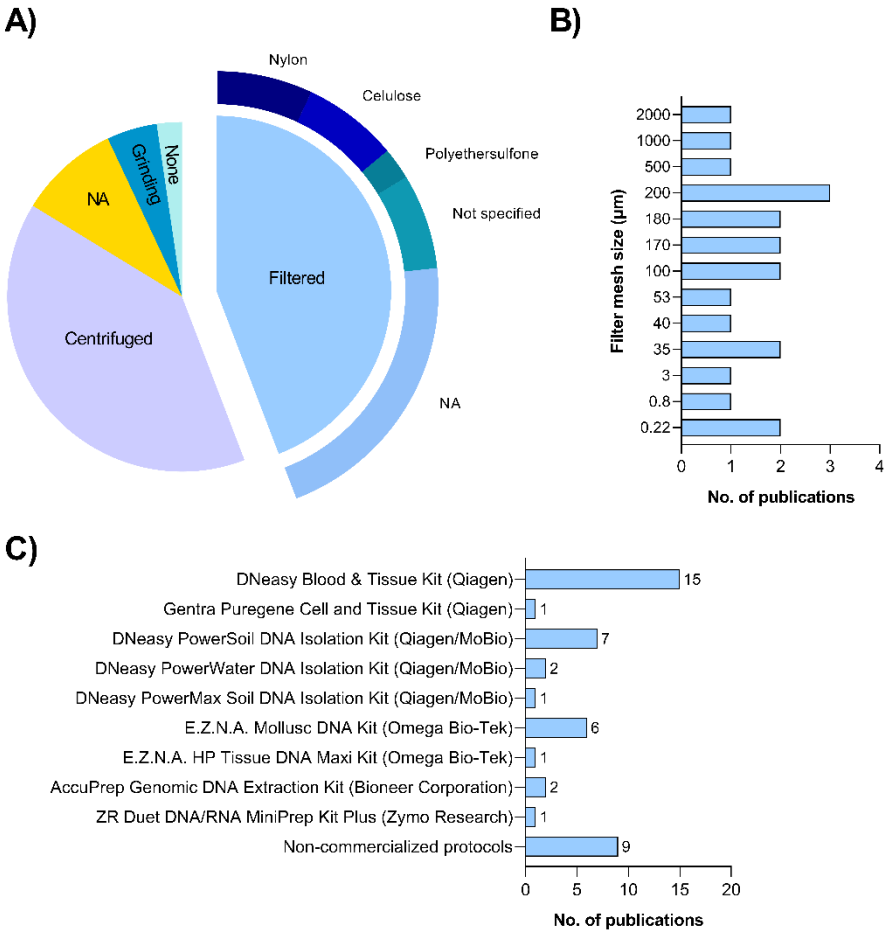

Figure 3. A) Pre-processing methodology applied to capture or concentrate zooplankton communities, before DNA extraction. The inner circle demonstrates the proportions of the three techniques found, whereas the outer semi-circle represents the proportions of the material of the filters used in the filtration process; B) Proportions of the mesh sizes of the filters used upon the process of filtration prior to DNA extraction; C) DNA extraction methodologies used for assessing zooplankton diversity through DNA metabarcoding in marine and brackish ecosystems.

1.3.1.4. Genetic marker and primers choice

In any metabarcoding study, the targeted genetic marker and subsequent primers used to amplify it, are one of the most crucial considerations to take. The marker of choice should be variable enough for interspecific differentiation and simultaneously conserved for the development of primers capable of amplifying a wide range of taxonomic groups (Rey et al., 2020b). The resulting data from DNA metabarcoding is dependent on marker taxonomic coverage and resolution of the target taxa, with the former being preferable, in the case of zooplankton, but with the cost of fewer taxa resolution (Clarke et al., 2017).

To date, the use of the 18S rRNA gene has dominated the studies on DNA metabarcoding of zooplankton communities, followed by COI (Figure 4) (improved results from Bucklin et al., 2016). These markers are a standard barcode (COI) and a historically amplified region for aquatic microbial eukaryotes

(18S), that demonstrate moderate to high specificity to zooplankton species, species coverage, and species identification (Clarke et al., 2017; Xu et al., 2020; Meredith et al., 2021). For that reason, the 18S rRNA gene has an extensive reference database, but is too conservative to discriminate organisms to lower than genus level (Tang et al., 2012; Questel et al., 2021), whereas COI allows discrimination at species level due to faster mutation rates (Mueller, 2006). Complete sequencing of COI and 18S rRNA genes is rare when characterizing zooplankton communities (Questel et al., 2021). Several hypervariable regions of the 18S rRNA gene have been used to characterize zooplankton communities, including V1-V2 (Lindeque et al., 2013; Mohrbeck et al., 2015; Sommer et al., 2017; Couton et al., 2019; Pitz et al., 2020; Brandão et al., 2021), V4 (Zhan et al., 2014; Brown et al., 2016; Chain et al., 2016; Clarke et al., 2017; Walters et al., 2019; Rodas et al., 2020; von Ammon et al., 2020; Questel et al., 2021; Suter et al., 2021; Zhao et al., 2021; Lin et al., 2022), V4-V5 (Walters et al., 2019) and V9 (Abad et al., 2016, 2017; Casas et al., 2017; Stefanni et al., 2018; Bucklin et al., 2019; Carroll et al., 2019; Blanco-Bercial, 2020; Kim et al., 2020; Rey, Basurko, et al., 2020; Cicala et al., 2021; Questel et al., 2021; Sun et al., 2021). The latter has been the most employed, probably due to the amount of available information on reference databases, however, it is not variable enough for reliable species screening in zooplankton communities.

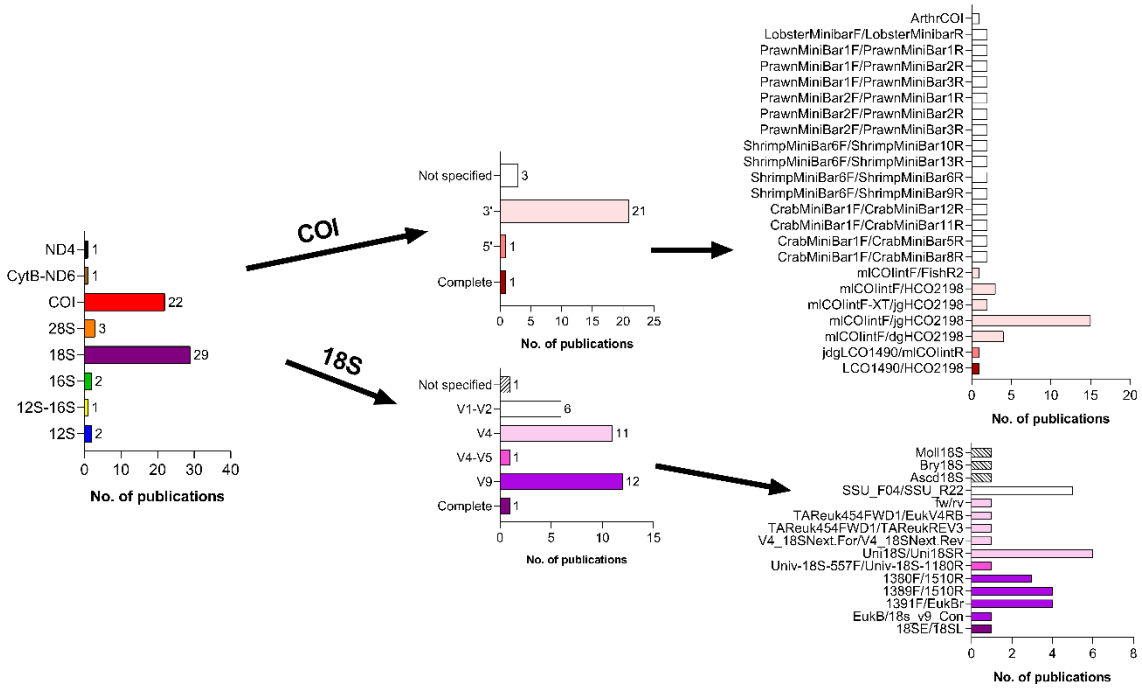

Figure 4. Genetic markers and sets of primers, particularly for COI and 18S, used for assessing zooplankton diversity via DNA metabarcoding.

This molecular marker has been generally targeted to a similar degree by using three different primer pairs: 1380F/1510R, 1389F/1510R, and 1391/FEukBr. The hypervariable regions V4 and V1-V2 have been the second and third most used, respectively. Unlike the V9 region, their amplification has been dominated by applying a set of primers, particularly the region V1-V2 has been mainly amplified with the primer set SSU_F04/SSU_R22 (Lindeque et al., 2013; Mohrbeck et al., 2015; Sommer et al., 2017; Couton et al., 2019; Pitz et al., 2020; Brandão et al., 2021). Although the hypervariable region V9 prevalence in these studies (Figure 4), V1, V2, and V4 regions appear to equally display highly nucleotide divergence, hence might be as well as good options for species-level identifications via 18S (Ki, 2012; see also Questel et al., 2021). In what respects COI, the region 3' strongly dominated among the most targeted fragments, which in turn prevailed by the use of the forward primer mlCOIintF (Leray et al., 2013) in combination with the reverse primer jgHCO2198 (Geller et al., 2013), and to a lesser extent with dgHCO2198 (Meyer, 2003), that amplify a shorter fragment than the full barcode region, allowing full-length sequencing with HTS (Leray et al., 2013; Rey et al., 2020b). Additionally, the prevalent employment of the internal forward primer mlCOIintF is due to higher efficiency than its reverse complement used with LCO1490 (Leray et al., 2013) or the degenerated versions of this primer (jg/dg; Figure 4). The tendency towards 3' region might be due to findings of Leray et al. (2013), where sets of primers that amplified the 3' end performed better across metazoan phylogenetic diversity. Further, Schroeder et al. (2021), found that this region showed a higher number of exclusive genus/species when compared with 5' region of the COI barcoding region. The high variability of COI provides a suitable molecular marker for species-level identification; however, it is hard to identify regions that are conservative enough for designing universal primers suitable for DNA metabarcoding (Capra et al., 2016). Zooplankton metabarcoding studies have also used regions of the 28S rRNA gene, but due to its considerable conservative nature, it can greatly underestimate species richness in a community (Tang et al., 2012).

At least 13 studies applied more than one marker for the characterization of zooplankton communities, from which 10 studies comprised the employment of at least 18S rRNA and COI genes (Clarke et al., 2017; Carroll et al., 2019; Couton et al., 2019; Pitz et al., 2020; Rey et al., 2020; Stefanni et al., 2018; Westfall et al., 2020; Brandão et al., 2021; Cicala et al., 2021; Questel et al., 2021; Suter et al., 2021; Zhao et al., 2021). Most studies emphasized the complementary advantages of using both markers in overall zooplankton diversity screening, and few aimed at the early detection of NIS (Couton et al., 2019; Rey al., 2020a); 18S rRNA gene for a broader range of taxa and COI for a better taxonomic

resolution. Additionally, COI primer-template mismatch in some taxonomic groups, such as cnidarians, promoted the use of alternative mitochondrial genes, such as 16S rRNA, which is considered by some a more reliable marker for the aforesaid taxa (Zheng et al., 2014). Nonetheless, the 16S rRNA gene report of use was merely for demonstrative purposes of overall efficiency (Clarke et al., 2017), and further complementary use of this molecular marker for NIS introduction assessments should be a balance of taxonomic resolution with amplification efficiency (Westfall et al., 2020).

1.3.1.5. Sequencing platform

In total 3 sequencing platforms were reported to be used in zooplankton DNA metabarcoding studies (Figure 5). Illumina MiSeq has been the most adopted, however, that has not always been the case; after all, sequencing platforms have changed throughout the years. The sudden rise in the number of reports of Illumina MiSeq and Ion Torrent PGM (Personal Genome Machine), between 2016 and 2018, was related to the discontinuation of the platform Roche 454 pyrosequencing by mid-2016. However, compared to other platforms, these two have performed better read accuracy in microbiological communities and, to a lesser degree, are capable of higher read lengths (Vincent et al., 2017; see also Zaiko et al., 2015).

The disproportionate use of sequencing platforms comprises an additional factor to have in mind in developing routine monitoring programs/projects in detecting NIS in the early stages of their introduction in zooplankton communities, using DNA metabarcoding as an identification tool. Illumina uses a sequencing-by-synthesis approach, utilizing fluorescently labeled reversible-terminator nucleotides on clonally amplified DNA templates immobilized on the surface of a flow cell, while Ion Torrent and 454

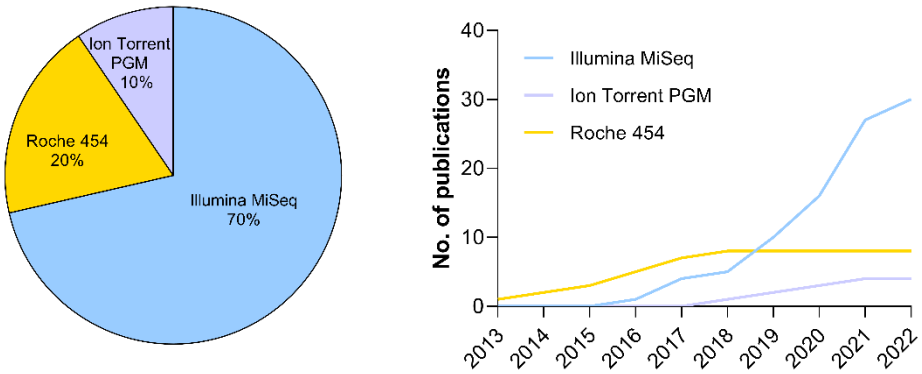

Figure 5. Sequencing platforms used for assessing zooplankton diversity via DNA metabarcoding. The pie chart on the left represents the overall proportion of the studies employing each sequencing platform. On the right is represented the trending use of Illumina MiSeq (light blue), Ion Torrent (yellow) and Roche 454 (grey) sequencing platforms for assessing zooplankton diversity over the last 9 years.

Roche also use DNA fragments sequenced-by-synthesis, but after clonally amplified by emulsion PCR on the surface of microbeads (Zaiko et al., 2015). Actually, no study was found that compared the performance and efficiency of the still currently used Illumina MiSeq and Ion Torrent platforms, or alternative sequencing platforms, on sequencing DNA fragments of zooplankton and in detecting NIS. Still, Illumina MiSeq is known for its higher potential throughput capability, but not for its sequencing speed - which otherwise is one of Ion Torrent's advantages (Vincent et al., 2017; Reuter et al., 2015). Nonetheless, other biological groups have been analyzed: on bacterial mock communities using the 16S rRNA gene, both sequencing platforms were in general in good agreement (Salipante et al., 2014; Allali et al., 2017; Braukmann et al., 2019); similar conclusions were reached with arthropods mock communities, where sequencing platforms did not influence species recovery significantly, but Illumina MiSeq provided better quality sequences (Braukmann et al., 2019).

In general, there is a disproportionate distribution of information available on how each step from sampling gathering to sample sequencing influences the resulting data of zooplankton communities metabarcoding, especially if NIS are the main target. Many studies try to understand which molecular marker/primer choice is more efficient in detecting most zooplankton taxa, and fewer had more focus on NIS (Brown et al., 2016; Couton et al., 2019; Rey et al., 2020a). Sampling methodology, sample preservation, DNA extraction method, and further downstream sequencing platforms, demonstrate trending choices, but still lack particular understanding of how it affects resulting zooplankton data. Further, molecular marker and/or primer choice are the most critical decisions in the development of a NIS monitoring program through zooplankton DNA metabarcoding. These decisions greatly influence taxonomic richness and reliability, particularly for cryptic, rare, and less abundant species, such as newly introduced NIS. Therefore, there is a need for research to be developed prior to setting efficient monitoring programs, since reproducibility, standardization and sensibility are key parameters in defining more reliable data and information in the early detection of NIS in coastal ecosystems (Duarte et al., 2021b).

1.4. Aim of thesis

The aim of the present thesis is to demonstrate the applicability of DNA metabarcoding as a viable tool for monitoring zooplankton communities in coastal ecosystems and in the early detection of marine invertebrate non-indigenous species (NIS), in coastal ecosystems, using as a case study the recreational marina of Viana do Castelo. To fulfill this, the work was conducted in several stages. In the first one, in order to understand what is the state of the art in the use of this high-throughput method to

target naturally occurring marine and brackish zooplankton communities, a literature review was performed and presented as part of the general introduction. For that, the data from papers published on the subject was compiled, which included the geographic region, the year of the study, and all the methodologies employed in the analytical chain of the DNA metabarcoding approach, namely: devices used to collect the samples, samples preservation and DNA extraction, genetic markers and primers employed and sequencing platforms. Further objectives of the thesis included:

i) The compilation of a list of zooplankton species occurring in the Lima River estuary, by analyzing all possible existing literature. Since several marine macrozoobenthos species are known to display larval pelagic stages during their development cycles, these were also included in the list, since they can make part of the temporal zooplanktonic communities;

ii) A gap-analysis in order to verify the presence of sequences on genetic databases, namely BOLD and GenBank, for all species in the lists compiled in i);

iii) The investigation of the seasonal dynamics of zooplankton communities in the recreational marina of Viana do Castelo, which is located in the Lima River estuary, using DNA metabarcoding, as well as, to test the efficiency of two different marker genes - COI and 18S - in the zooplankton diversity monitoring, as well as in assessing their complementarity;

iv) The investigation of the DNA metabarcoding efficiency in the early detection of marine invertebrate NIS, located in a potential entry-point in the Lima River estuary (recreational marina), and their temporal dynamics;

v) To verify similarities between the zooplankton communities detected through metabarcoding in the current thesis and the species reported in the literature (i), which includes both the zooplankton taxa and the macrozoobenthos.

This thesis was developed in the scope of the project NIS-DNA: Early detection and monitoring of non-indigenous species (NIS) in coastal ecosystems based on high-throughput sequencing tools.

2. Materials and Methods

2.1. Characterization of the study area

The temperate Lima River estuary is located in the vicinity of the city of Viana do Castelo, NW Portugal. This is a narrow body of water with the formation of submerged bars (Alves, 2003), with water flow-oriented ENE-WSW. In terms of the estuary's mouth amplitude and hydrologic characterization, it can be classified as intermediate (Sousa et al., 2006b). Due to human alteration of the shores of the Lima River estuary, it has a quite consistent width of around 400 m, however, the upstream part is shallower and wider, reaching a maximum of 1 km, although, with high tides, it can attain greater width (Sousa et al., 2006b).

Based on the morphology, bathymetry, salinity, and the presence/absence of saltmarshes, the estuary can be divided into three different zones (Ramos, 2007): 1) the lowermost part, located in the initial 3 km from the mouth of the river, is a narrow, deep navigational channel, with walled banks, where salinity is generally higher than 30 PSU. Ship navigation is possible by maintenance of the depth through regular dredging activities. The river mouth is an artificial and deep channel with two jetties protecting the river mouth, one of which reaches 2 km southward, directing the water flow to the south. While the middle and upper areas of the estuary have retained most of their natural banks, the downstream part has been subject to extensive modifications within the last century with the additional development of a large shipyard, a commercial seaport, a fishing harbor, and a marina. 2) the middle estuary, located beyond the Eiffel bridge (3-7 km; Figure X), encompasses a broad shallow salt marsh zone and longitudinal bars, mainly colonized by the common rush (*Juncus* spp.). The occurring mixture of freshwater and saltwater contributes to salinity ranging from 18 to 30 PSU. 3) the upper part of the estuary which extends from 7 km of the mouth of the river to 20 km upstream, is a narrow and shallow channel where depth decreases upstream, as well as salinity (5-18 PSU).

The study encompassed particularly the downstream dock of the recreational marina located on the north bank of the Lima estuary, in the city of Viana do Castelo (41° 40.5' N 8° 50.3' W). Both docks together consist of the recreational marina with most mooring posts from north of Portugal, with 307 slots for vessels with 20 meters of length and 3 meters drafts at maximum (Costa, 2012), from which most are encompassed in the study area (163 slots). The upstream dock is mostly for smaller vessels, where it can dock only those with a draft of less than 1.5 m. Both docks cover around 2,100 meters in length and 150 m in width. Most downstream dock has an area of 25,000 m^2 of wet surface and a depth

of 3 meters, usually maintained by dredging (Porto de Viana do Castelo, 2017). Several hundreds of boats attract at the recreational marina annually, the majority from France, the United Kingdom, the Netherlands, and Germany (Porto de Viana do Castelo, 2017). Although international and national dockings data were not found for this marina, according to PwC (2017) analysis of recreational dockings in Portugal, 13% of the total vessel dockings were in the northern region, in 2017, from which most were international visitors (87%).

Overall, the recreational marina of Viana do Castelo presents lower current flow and ship traffic since it is located further downstream where commercial and fishing ports are located. According to Ramos (2007), estuarine salinity increases from spring to summer and autumn and then decreases strongly in the winter, a pattern that has been also observed for water temperature.

2.2. Compilation of a list of zooplankton and macrozoobenthos species with occurrence in the Lima River estuary

For evaluating the DNA metabarcoding efficiency in zooplanktonic species detection, the compilation of a list of species that can occur in zooplankton (both definitive or temporary), already documented in the Lima River estuary, comprehends a crucial step. It was taken into account the zooplanktonic species that have been documented through morphology, as well as macrozoobenthic species. The inclusion of macrozoobenthos in the compiled list is justified by the fact that several species included in this group of organisms might present a planktonic stage of life.

According to Sousa (2003), the majority of biological studies made in the estuary were conducted during the decade 1980 (e.g., Fontoura, 1984; Fontoura & Moura, 1984; Guimarães & Galhano, 1987, 1988, 1989), up until the early 2000s, and even though more studies have been developed in the estuary, are still scarce until today (reports on chapter 3).

The manual literature research was conducted on the Web of Science and Google Scholar, which included all the terms displaying “zooplankton” and “macrozoobenthos” with the combination of all terms specifying the sample location (Lima River estuary): [("invertebrate*" OR "macroinvertebrate*" OR "benth*" OR "*plankton") AND ("Lima") AND (estuar*) AND (Portugal)]. A total of 16 publications were retained for further analysis since they met the defined criteria. To these publications, additional data on documented macrozoobenthos species from APA (Portuguese Agency of Environment) report from 2004, was included from personal collections and that was not displayed in the initial search. These studies were then analyzed in terms of species-level identifications and sampling area to further exclude species

found outside the estuarine environment. The resulting lists of zooplankton species and invertebrate macrozoobenthos were then taxonomically curated comparing it with the WoRMS database (World Register of Marine Species - www.marinespecies.org) (October, 2021), for further data analysis.

2.3. Gap-analysis for zooplankton and macrozoobenthos species occurring in the Lima River estuary for COI and 18S genetic markers

2.3.1. Availability of DNA sequences on the Barcode of Life Datasystem (BOLD) for the COI marker

A gap analysis of molecular information consists of evaluating available molecular records for a defined list of species, which in this case correspond to the lists of zooplankton and macrozoobenthos documented in the Lima River estuary and compiled in 2.2. This analysis will serve to assess which species will not be detected a priori through DNA metabarcoding, due to the absence of sequences on genetic databases, but also to assess DNA barcode representation or completeness status for the target organisms of the current study.

To assess the availability of COI information, regarding the compiled lists of zooplankton and macrozoobenthos, a combination of the use of the tool "Barcode, Audit & Grade System – BAGS" (Fontes et al., 2021), of a R script and the BOLD Systems database (www.boldsystems.org), was employed. BAGS is an R script with a user-friendly interface that allows to pull the information available on sequence data from the BOLD Systems database and further analyze available and public records for an uploaded list of species. The script provides all existing Barcode Index Numbers (BIN; Ratnasingham & Hebert, 2013) of grouped molecular records, that most of the times, and ideally, on BIN corresponds to one species. The two resulting lists of BINs were then pooled into BOLD Systems v4, from where all records were downloaded. The search of records was done by COI-5P records with a minimum of 300 bp, resulting in three datasets of singleton, concordant and discordant records for each group.

The resulting data was further analyzed, particularly for discordant records, as these include any BIN with different nomenclatures, either being synonymous or not. In order to do that, discordant BINs were classified into 3 different groups: ambiguous, synonymous, and misidentified BINs. The former included any BIN for which was not possible to conclude to which taxa it corresponded, due to multiple different taxa being included in the same BIN, with a similar number of records. In contrast, the remaining groups (synonymous and misidentified) included BINs for which was possible to attribute a correspondent

taxon. This was possible by either taking into account the synonymous nomenclatures (according to WoRMS) or due to, in the case of different taxa being grouped in the same BIN, existing a disproportionate distribution of records. For the latter, the correct nomenclature is attributed to the species harboring more sequence records (the majority rule), and those with less records (usually 1), being considered as misidentified. Only concordant and conclusive synonymous and misidentified discordant BINs were considered for gap assessment, in molecular data available, for the lists of species compiled in 2.2.

2.3.2. Availability of DNA sequences on GenBank for the 18S marker

In terms of the gap analysis for 18S, particularly the hypervariable region V4, the gap analysis was conducted in a different genetic database - the GenBank (www.ncbi.nlm.nih.gov/genbank/). This was conducted by using an ingroup-developed custom R script, that provided a semi-automated system to pull the wanted information from GenBank. The use of this script comprises two phases: the manual phase comprised the development of a query to the specific molecular marker under study in GenBank, followed by the automated download of the metadata associated, obtained with the R script where the developed query is applied. The query included the lists of species that were previously compiled, each followed by [ORGANISM] and with the command "OR" between each species, then followed by "AND" and subsequently a set of keywords specific for the marker under study (18S V4), such as: Species1[ORGANISM] OR Species2[ORGANISM] OR Species3[ORGANISM] OR (...)) AND (18S ribosomal RNA[GENE] OR 18S small subunit ribosomal RNA[GENE] OR 18S rRNA[GENE] OR 18S ribosomal RNA[Title] OR 18S small subunit ribosomal RNA[Title] OR 18S rRNA[Title] OR complete sequence[Title] OR variable region V4[Title]).

2.4. Zooplankton communities sampling in the recreational marina of Viana do Castelo

2.4.1. Samples collection and processing

In total 3 different points were considered in this work design, the two inner docks, and the dock right beside the entrance of the recreational marina (Figure 6). Sampling gathering was conducted among three different seasons, namely late spring (15/06/2020), autumn (12/10/2020), and late stage of winter (01/03/2021). At each sampling point, 3 separate oblique tows were performed for 1.5 min, using a plankton net with a 40 cm opening diameter, 100 cm length, and a mesh size of 55 μm. After each tow deployment, the end-cup content was poured into a storage bottle, previously washed with bleach

(10%), and rinsed with ultra-pure water. Any considerable residual content inside the end-cup was washed into the bottle with water from the sampling site. This process was repeated a total of 9 times for each season (3 times per point). Surface water samples were also collected for measurement of salinity, conductivity, and pH parameters, with a WTW Multiline F/set 3 no. 400327 (WTW, Weilheim, Germany). Temperature data were obtained in a daily basis through the Portuguese Institute of the Sea and Atmosphere (IPMA) (www.ipma.pt/pt/maritima/costeira/), for each sampled season.

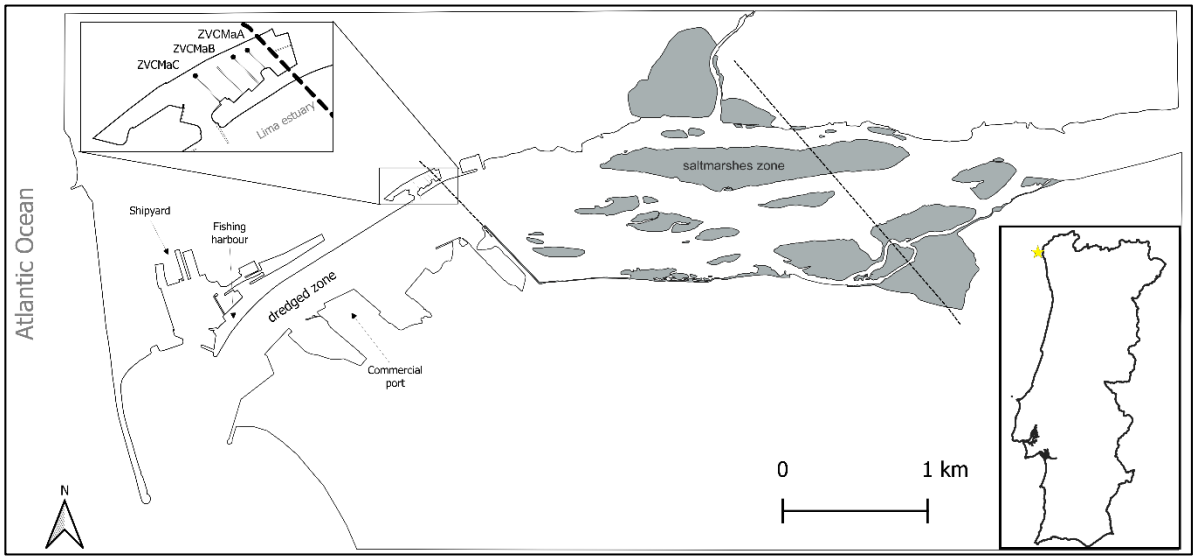

Figure 6. Location of the study area and sampling points in the Lima estuary.

Zooplankton samples were preserved in a cool box filled with thermoregulators, during sampling, and at 4 °C overnight, after reaching the lab, prior to sample processing. Zooplankton samples were filtered using an EZ-Fit™ Manifold filtration ramp, with 3-place for Microfil® funnels and membranes (Merck-Millipore) attached to an EZ-Stream Vacuum Filtration Pump (Merck-Millipore). Before each filtration, all removable parts of the filtration ramp were submerged in bleach (10%) for 30 minutes and rinsed with ultra-pure water 3 times, and all surfaces were also cleaned with bleach 10% and ethanol 96%. Between each sample, the porous stones, where the membranes are placed, were burned with ethanol 96% to avoid any cross-contamination between samples. For sample filtration, sterilized membranes of mixed cellulose esters with 47 mm diameter and 45 µm pore-mesh size (Merck-Millipore), were used. Filtration took part to extract the water matrix, and concentrate the organisms, and was only considered filtered when no humidity was observed. The filter membranes were then preserved in Petri dishes (sealed with parafilm), at -20°C, until DNA extraction. All filtrations took place on the next day of sampling collection.

2.4.2. DNA extraction, amplification and HTS

DNA contents were extracted from zooplankton filtered samples using the DNeasy PowerSoil Kit (Qiagen), following the manufacturer's protocol with minor changes. During extraction, 2 technical replicates were considered, consisting of ¼ of scraped-off zooplankton from the filters. As a final step, 30 µL of each technical replicate, from the same sample, was mixed into the same 2 mL collection tube. Extract products were quantified using a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific) and then stored at -20 °C, until further downstream steps. Details of the concentrations and DNA quality are provided in the Table S1 (Supplementary Material).

Samples were prepared for Illumina MiSeq sequencing by 18S rRNA and COI genes amplification of the eukaryotic communities, at Genoinseq (Biocant, Cantanhede, Portugal). Two different sets of primers pairs were used: the forward primer mlCOIintF 5'-GGWACWGGWTGAACWGTWTAYCCYCC-3' (Leray et al., 2013) with the reverse primer LoboR1 5'-TAAACYTCWGGRTGWCCRAARAAYCA-3' (Jorge Lobo et al., 2013), which target the 3' region of COI (~313 bp); and the forward primer TAReuk454FWD1 5′-CCAGCASCYGCGGTAATTCC-3′ with the reverse primer TAReukREV3 5′-CTTTCGTTCTTGATYRA-3′ (Stoeck et al., 2010), which targets the hypervariable region V4 (~380 bp) of the eukaryotic 18S rRNA gene. 18S rRNA gene was selected because it has been widely used in metabarcoding studies targeting zooplankton communities, while COI is the standardized DNA barcode marker used in metazoan identification, as it provides more reliable species-level identifications (see sub-section 1.3). The DNA was amplified for the hypervariable regions with specific primers (Table 1) and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes.

Table 1. Specific PCR conditions used for each primer set employed in the current study, at Genoinseq (Biocant, Cantanhede, Portugal).

Primer pair	PCR conditions
mlCOIintF/LoboR1	95 °C 3' (initial denaturation) 98 °C 20'' (denaturation) | 60 °C 30'' (annealing) | 72 °C 30'' (extension) 35x 72 °C 5' (final extension)
TAReuk454FWD1/ TAReukREV3	95 °C 3' (initial denaturation) 98 °C 20'' (denaturation) | 57 °C 30'' (annealing) | 72 °C 30'' (extension) 10X 98 °C 20'' (denaturation) | 47 °C 30'' (annealing) | 72 °C 30'' (extension) 25X 72 °C 5' (final extension)

Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to the manufacturer's recommendations (Illumina, 2013). PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017), pooled and paired-end sequenced in the Illumina MiSeq® sequencer with the MiSeq reagent Kit v3 (600 cycles), according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

2.4.3. Bioinformatic processing and taxonomic assignment

Quality filtration was performed on Illumina reads (fastq files) using PRINSEQ v0.20.4 (Schmieder & Edwards, 2011) to remove sequencing adapters, trim bases with an average quality lower than Q25 in a window of 5 bases and eliminate reads with less than 100 bases for 18S and 150 bases for COI. Forward and reverse reads were merged by overlapping paired-end reads with AdapterRemoval v2.1.5 (Schubert et al., 2016), using default parameters. This initial processing was performed at Genoinseq (Biocant, Cantanhede, Portugal).

Prior to taxonomic assignment, primer sequences were trimmed from quality-filtered reads using a Mothur script (Schloss et al., 2009). The resulting fasta format file (COI sequencing data) was uploaded to the Multiplex: Barcode Research and Visualization Environment platform (mBrave; www.mbrave.net), for comparison of COI sequenced reads with those taxonomically identified reference records from BOLD. Settings and libraries used to analyze the results from HTS sequencing can be found in the Table S2 (Supplementary Material). Species-level assignments were accepted at a similarity threshold higher than 97% for COI. The resulting fastq format file (18S sequencing data) was uploaded to SILVAngs (www.ngs.arb-silva.de) to compare 18S sequenced reads with reference records taxonomically assigned from their own database (Quast et al., 2013). Settings used to analyze the results from HTS sequencing can be found in the Table S3 (Supplementary Material). Species-level assignments for 18S were accepted at a higher threshold of similarity (>99%). The taxonomic nomenclature assigned to the resulting reads was confirmed according to the WoRMS database (February, 2022). Throughout further analysis and discussion, only marine and brackish metazoans with more than 8 reads were considered (Fais et al., 2020; Leite et al., 2021).

2.4.4. Data processing and analysis

Venn diagrams were used to i) compare the proportion of species and classes overlapping or exclusive on both compiled lists (zooplankton versus macrozoobenthos); ii) compare the proportion of species with overlapping or exclusive detections, previously found in the Lima River estuary by morphology-based identification (compiled lists), and metabarcoding-based identification in the current study; and iii) analyze the proportion of species with overlapping or exclusive detections by each genetic marker (COI versus 18S) and season (spring versus autumn versus winter).

Two-way analyses of variance (ANOVA), followed by Tukey's honestly significant difference tests (Tukey's HSD) were performed to determine whether season and marker choice affected richness detection via DNA metabarcoding (Zar, 2010). The number of species detected per sample dataset was previously transformed (log10), as Anderson-Darling, D'Agostino-Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests accused a non-normal distribution of the data (Zar, 2010).

Nonmetric multidimensional scaling (nMDS) (Taguchi & Oono, 2005)was performed using a Jaccard similarity index to visualize similarities of samples obtained in different seasons and with different genetic markers. A one-way - for season and marker separately - and a two-way permutation analysis (PERMANOVA) were performed to address the significance of these factors on the taxonomic composition differences at the species level. A more comprehensive analysis of the season and marker choice effects on taxonomic composition was performed with comparative vertical slice charts using the phyla detected. In more detail, to evaluate zooplankton composition in the samples from spring, autumn, and winter, a heat map was constructed of the top 30 most relatively abundant families. This evaluation was performed using the number of reads per species per sample dataset and without any transformation.

Statistical analysis on species richness was performed using GraphPad Prism 8 software (www.graphpad.com), while on the overall taxonomic composition the we PAST software v4.09 (Hammer et al., 2001), was used. For all statistical analyses, differences were considered significant when $p < 0.05$. Plots were mainly made with Graphpad Prism 8, while all Venn diagram analyses were done using InteractiVenn online platform (Heberle et al., 2015).

2.4.4.1. Detailed analysis of the zooplankton composition detected with COI and 18S

The life cycle of each species was analyzed to further evaluate the composition of the zooplankton community. This analysis divided COI and 18S datasets of species between 7 categories: i) non-planktonic

species, that encompassed every species without any known planktonic developmental stage, or at least no information was found regarding their presence in plankton communities; ii) holoplankton which includes species with all of their developmental stages in the plankton; and iii) meroplankton, which included species with developmental or adult non-plankton stages, although some are planktonic (temporary plankton). The latter was further divided between species almost fully planktonic, but that display benthonic stages at some point in their life cycle; species that at least display a planktonic larval stage, although no information regarding to the eggs were found; species that only the larvae were planktonic; and species that both eggs and larvae are planktonic. An additional category of pseudo-plankton was considered, which included free-living or benthic species and other non-planktonic taxa that are carried to the water column due to environmental disturbance e.g., water flow currents – their presence in the plankton are not related to any life stage (tychoplankton; Simberloff & Rejmánek, 2011). Species without any information related to their life cycle were either categorized based on information regarding higher taxonomic groups which they were included or were categorized as no available information.

3. Results

3.1. Lists compilation of zooplankton and macrozoobenthos species occurring in the Lima River estuary

3.1.1. Taxonomic composition

The lists of species reported in zooplankton and macrozoobenthos communities, with occurrence in the Lima River estuary, were retrieved from a total of 16 papers, 2 master's degree theses, and a report from APA, published between 1988 and 2015. Most of the studies and report from APA were used to compile the list of macrozoobenthos species (Guimarães & Galhano, 1988; Cortes et al., 2002; Fidalgo & Gerhardt, 2002; Sousa, 2003; Sousa et al., 2006a,b, 2007; Conde et al., 2011; Sampaio, 2012; Azevedo et al., 2013; Sampaio & Rodil, 2014; Rubal et al., 2021), while only 4 papers were used to compile the list of zooplanktonic species (Ramos et al., 2006, 2010, 2015; Vieira et al., 2015).

Overall, 216 species were reported for both groups in the Lima River estuary, from which 113 were reported as zooplanktonic species, whereas 115 as macrozoobenthos (Table S4, Supplementary Material). Only 12 species (ca. 5.6%), were shared between both groups (Figure 7). Almost all of the shared species belonged to Arthropoda, namely, the green crab *Carcinus maenas* (Linnaeus, 1758), the common shrimp *Crangon crangon* (Linnaeus, 1758), the *Grastrosaccus spinifer* (Goës, 1864), the *Idotea chelipes* (Pallas, 1766), the *Neomysis integer* (Leach, 1814), the rockpool shrimp *Palaemon elegans* (Rathke, 1836), the small hermit crab *Diogenes pugilator* (Roux, 1829), the common prawn *Palaemon*

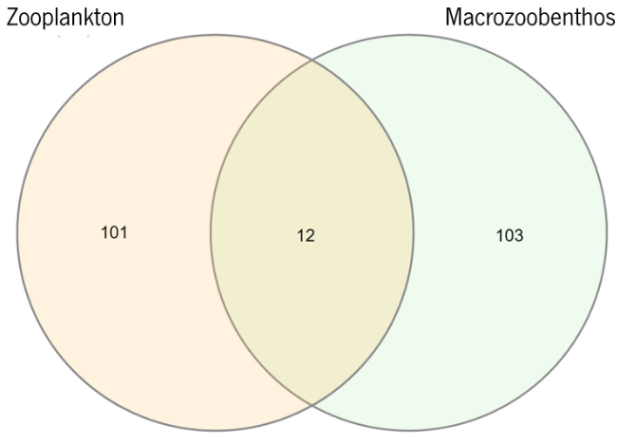

Figure 7. Venn diagram representing the partitioning of species found in zooplankton (orange) and macrozoobenthos (green) communities, from the compiled lists of species occurring in the Lima River estuary. The area between each circle represents the number of shared species.

serratus (Pennant, 1777) and *Paragnathia formica* (Hesse, 1864), and only a few representative species belonged to Annelida (the sandmason worm *Lanice conchilega* (Pallas, 1766)), Echinodermata (the common heart urchin *Echinocardium cordatum* (Pennant, 1777)) and Mollusca (the liver spire shell *Peringia ulvae* (Pennant, 1777)).

Regarding the reported zooplankton, Arthropoda and Chordata were the major contributors to the compiled list, representing ca. 82% of the total number of species. The species found to belong to the phylum Arthropoda were taxonomically fairly distributed - Branchiopoda (11 species) contributed with 2 times fewer species than Hexanauplia (20 species) and 1.5 times less than Malacostraca (16 species). On the other hand, all Chordata were mostly Actinopterygii, with only 2 Appendicularia species. In the end, with roughly 39% of the species, Actinopterygii showed to be the major contributor to the diversity of zooplankton communities (Figure 8). Cnidaria was only represented by 12 hydrozoan species (10.6%). The remaining groups were represented by 2 species each from the same class, excluding Echinodermata that belong to two distinct classes - Echinoidea and Ophiuroidea.

Annelida, Arthropoda, and Mollusca were the major contributors to the diversity of macrozoobenthos reported in the Lima River estuary, contributing ca. 93.9% to the total number of species. Mollusca by itself accounted for 38.3% of the species from the compiled list, from which the majority were Bivalvia. Unlike what was found in the zooplankton species list, Arthropoda was strongly dominated by Malacostraca, with 27 species, as opposed to 2 Thecostraca species. The remaining 6.1% of the species were represented by 3 phyla, namely Echinodermata (5 species), Cnidaria (1 species), and Platyhelminthes (1 species) (Figure 8).

In general, the Lima River estuary communities' compositions here analyzed were very distinct from each other. Firstly, even though the general number of species did not differ considerably, however the representative phyla, otherwise, did (Figures 7 and 8). Zooplankton species were distributed by 7 different phyla, from which Chordata and Chaetognatha were found exclusively, while macrozoobenthos among 6 different phyla, from which Platyhelminthes were reported exclusively. In addition, the class composition within each phylum also differed between lists. In particular, Hexanauplia and Branchiopoda were solely reported in zooplankton communities, whereas Thecostraca were reported uniquely in the macrozoobenthos list. In terms of the Mollusca, from the 2 classes reported, only Gastropoda was represented in both groups. In both groups, Cnidaria was poorly represented, but belonged to two different

classes, namely Hydrozoa and Anthozoa. In terms of Echinodermata diversity, an additional class (Asteroidea) was reported in macrozoobenthos communities (Figure 8).

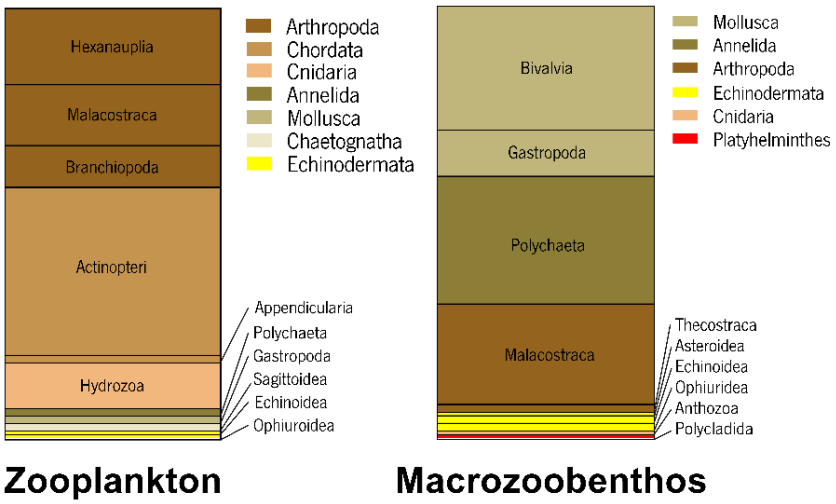

Figure 8. Proportion of taxa that composed zooplankton (left) and macrozoobenthos (right) species lists reported to occur in the Lima River estuary. Colors are specific to each reported phylum. For the species, which according to WoRMS do not have any designed class, the immediate lower taxonomic group was instead used (order).

3.1.2. Gap analysis

In general, only 3 species in the compiled list of zooplankton – *Caligus coryphaenae* (Steenstrup & Lütken, 1861), *Ceriodaphnia reticulata* (Jurine, 1820), and *Clytia islandica* (Kramp, 1919) – are not yet barcoded, while 85.2% of the macrozoobenthos in the compiled list were covered with sequence records in the genetic databases (Table S4, Supplementary Material). COI records were available for 89.4% of the zooplankton and for 75.6% of the macrozoobenthos species, totaling 5476 and 5561 sequence records, respectively. On the other hand, for 18S, 1364 and 1597 records were found to be available for zooplankton and macrozoobenthos, respectively, covering ca. 73.5% and 71.3% of species on each of the respective lists (Figure 9). Generally, the variation in the representation of zooplankton species did not vary much, between genetic markers, but Chordata presented a considerable informational gap for the 18S marker since its representation fell from 97.8% (COI) to 58.7% (18S). Additionally, Chaetognatha were better represented with 18S sequences than COI in genetic databases. For macrozoobenthos, the representation for both markers in the genetic databases did also not vary much. Cnidaria and Platyhelminthes species did not have any molecular information available in either BOLD or GenBank, for COI and 18S, respectively.

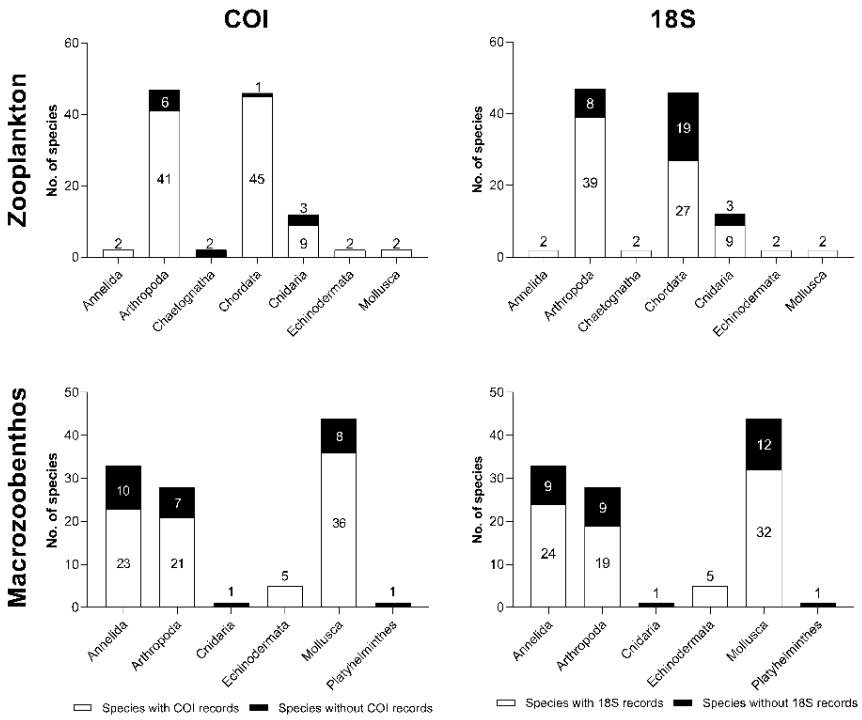

Figure 9. Proportion of zooplankton (upper) and macrozoobenthos (lower) taxa that occur in the Lima River estuary with available COI (left) and 18S (right) reference sequence records in BOLD and GenBank, respectively.

3.2. Assessing zooplankton communities through DNA metabarcoding in the recreational marina of Viana do Castelo

3.2.1. Environmental characterization

Surface water profile data showed that in the spring the recreational marina was characterized by the highest pH values and intermediate conductivity and salinity values. During autumn, pH decreased slightly whereas an increase was observed for conductivity and salinity. During winter, surface water was more acidic and presented an oligohaline profile (Table 2).

Further, throughout the duration of the three seasons, the average temperature only varied 0.1 °C from spring to autumn, even though maximum and minimum temperatures were found to be more variable between these two seasons. In the winter, average temperatures were lower, in fact 2 °C less. Minimal temperature was similar to spring, whereas the maximum temperature did not differ significantly from average temperature.

Table 2. Physical and chemical characterization of the surface water, during the field survey, from the recreational marina of Viana do Castelo, NW Portugal.

Parameter	Spring	Autumn	Winter
pH	8.1	7.7	6.6
Conductivity (mS/cm)	37.3	44.1	4.6
Salinity	23.1	27.5	2.3
TMean (°C)	15.3	15.2	13.3
TMax (°C)	17.6	18.6	14.1
TMin (°C)	12.8	13.7	12.5

3.2.2. Initial metabarcoding datasets

A total of 331,585 and 328,322 sequenced raw reads were generated for COI and 18S genes, respectively, for a total of 9 samples, by Illumina MiSeq. Of these, 239,922 and 182,919 reads passed quality checks (further details in Table 3). COI quality-filtered sequenced data were clustered at a 97% similarity level, while 18S was clustered at a 99% similarity level, with 25,490 and 75,211 of the reads being taxonomically assigned to metazoans, respectively. Further data filtration ended up with 7.1% and 21.6% of marine and brackish metazoans, of the initial datasets, and which accounted for more than 8 reads on each dataset. Overall, 155 species were detected in all the experiments (Figure 10), with 97 species (62.6%) being exclusively detected with 18S while 51 species (32.9%) were exclusively detected with COI. Only 7 species were simultaneously detected with both markers.

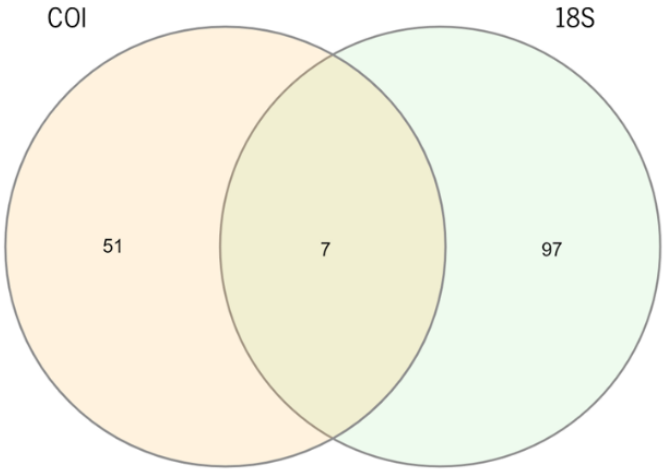

Figure 10. Partitioning of the total number of species detected with both markers (COI and 18S), from samples collected in the recreational marina of Viana do Castelo, NW Portugal.

Table 3. Summary of the numbers of reads, before and after all filtration steps, until obtaining a dataset with taxonomic species-level assignments to marine and brackish metazoans, and with more than 9 sequences.

Samples	Raw reads		Filtered		Mothur		Usable reads*	
	COI	18S	COI	18S	COI	18S	COI	18S
Spring	58447	75799	57693	75441	47708	56735	47651	31957
Autumn	201473	195269	193917	192273	149059	162335	147270	109392
Winter	71665	57254	71017	56799	45214	51841	45001	41570
TOTAL	**100.00%**	**100.00%**	**97.30%**	**98.84%**	**72.98%**	**82.51%**	**72.36%**	**55.71%**
Samples	**Taxonomic assignment**		**Species level ID (metazoan)**		**> 9 sequences**		**Only marine & brackish**	
	COI	**18S**	**COI**	**18S**	**COI**	**18S**	**COI**	**18S**
Spring	13634	13256	16941	12257	5153	11904	5153	11377
Autumn	42743	56190	16941	54199	16777	53991	16777	53982
Winter	3878	11627	3227	8755	3058	8289	1458	5421
TOTAL	**18.17%**	**24.69%**	**11.19%**	**22.91%**	**7.54%**	**22.59%**	**7.05%**	**21.56%**

* Usable reads encompass the resulting reads after Mothur processing (primers sequences cut and forward and reverse reads assembling) and that were used in mBrave and SILVAngs for taxonomic assignment.

3.2.3. Effect of the genetic marker and season on the taxonomic composition and diversity

In general, the most diverse groups were Annelida and Arthropoda, with 37 species detected within each phylum (47.7% in total), followed by Mollusca (31 species), which contributed to 20% of the overall species reported here. Henceforth, annelids, mollusks, and arthropods were the main contributors to the number of species recovered, though the latter were more abundant in the 18S dataset (30 species), in comparison with COI (7 species). Hexanauplia and Thecostraca composed almost half of all the arthropods detected, which were particularly species-rich, with a total of 18 and 8 species, respectively. All Hexanauplia species were identified with 18S, same as all the species of Thecostraca, with exception of 3 species that were detected with COI. Arachnids and ostracods were exclusively detected with 18S, while springtails (Collembola) and insects were exclusively detected with COI. For the remaining arthropod groups, COI and 18S detected the same number of species (Tables S5 and S6, Supplementary Material). Annelids were almost fully composed by polychaetes, excluding 2 species, which belonged to Clitellata and Myzostomida. Two of the shared detections by both markers were annelids, namely *Malacoceros fuliginosus* (Claparède, 1868) and *Spiophanes bombyx* (Clarapède, 1870).

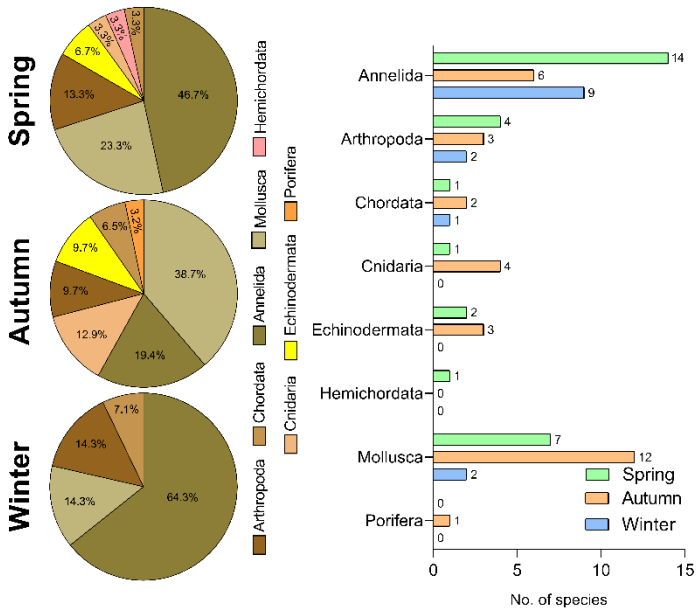

Figure 11. Proportion of detected phyla with COI, on all three sampled seasons (left) and species richness variation, within each phylum, among seasons (right). Only species that accounted for more than 1% of the reads were included in the analysis.

Mollusks here reported included 19 gastropods and 12 bivalves, from which *Ancula gibbosa* (Risso, 1818), *P. ulvae*, *Tergipes tergipes* (Forsskål, 1775), and *Mytilus edulis* (Linnaeus, 1758) were detected with both markers. Disregarding the latter, all bivalves were solely identified by 18S, whereas more than half of gastropods were only detected in the COI dataset (ca. 58%).

The remaining reported phyla corresponded altogether to 32.3% of the total species detected. COI detected more bryozoans (Table S5, Supplementary Material) and chordates, and the only Hemichordata species reported. On the other hand, 18S recovered a higher number of phyla, with 9 additional phyla reported (Table S6, Supplementary Material). Nematoda and Platyhelminthes were exclusively detected with 18S and included more than 2 species. Both markers detected the same number of echinoderms, but different species were recovered. The remaining shared species was a cnidarian – *Obelia geniculata* (Linnaeus, 1758) – and 18S had more exclusive species detected. Species belonging to Chaetognatha, Entoprocta, Nematoda, Nemertea, Phoronida, Rotifera, Sipuncula, and Xenacoelomorpha, accounted for less than 1% of the total reads each on each datasets (Tables S5 and S6, Supplementary Material).

The proportion of the number of annelid species in zooplankton decreased from spring to autumn and then increased towards winter, a pattern that was observed for both COI and 18S (Figure 11 and 12), but the number of significant species (more than 1% of the reads) decreased for 18S throughout all season (Figure 12). Also, with both markers, Porifera species only recovered a considerable proportion

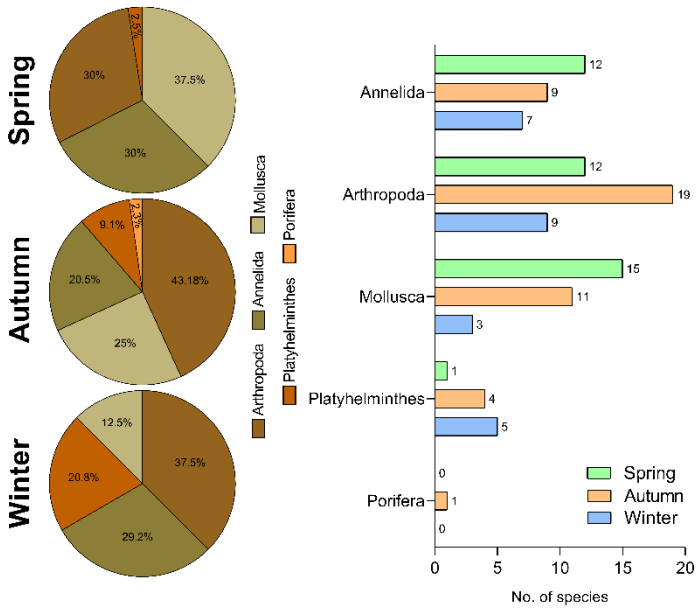

Figure 12. Proportion of detected phyla with 18S, on all three sampled seasons (left) and species richness variation, within each phylum, among seasons (right). Only species that accounted for more than 1% of the reads were included in the analysis.

during autumn (Figure 11 and 12). Contrariwise, patterns for mollusks were more variable between markers; while for COI the number of species increased from spring to autumn, and then decreased from autumn to winter, for 18S the number of species decreased through all seasons. The proportion of the arthropods, for COI, slightly decreased between spring and autumn followed by a slight increase in winter, however arthropods richness from autumn to winter was characterized by a decrease (Figure 11). For 18S, arthropods proportion displayed the opposite distribution. Although arthropods richness, detected with 18S, was higher in the spring than in the winter, their proportion was considerably higher in the winter than in the spring (Figure 12).

An analysis of variance (Two-way ANOVA) of the effects of the marker gene and season on the number of species detected indicated a significant effect of boths factors ($F_{1,12} = 53.74$, $p < 0.01$, for the marker, and $F_{2,12} = 41.52$, $p < 0.01$, for the season). In general, a higher number of species were recovered via 18S sequencing. A Tukey's HSD analysis showed that COI species richness was lower than 18S during spring ($p = 0.03$) and a stronger effect was observed during winter ($p < 0.01$). Autumn samples were the only for which COI and 18S sequencing recovered a comparable number of marine and brackish metazoan species ($p = 0.11$) (Figure 13). Overall, the number of species did not vary between spring and autumn ($p > 0.05$), but in winter the species richness retrieved with both markers was much lower than in spring ($p < 0.01$, for COI, and $p = 0.02$, for 18S) and autumn ($p < 0.01$, for both markers). So, both

markers' datasets presented similar seasonal patterns; the number of species did not vary significantly from spring to autumn, but strongly decreased in winter (Figure 13).

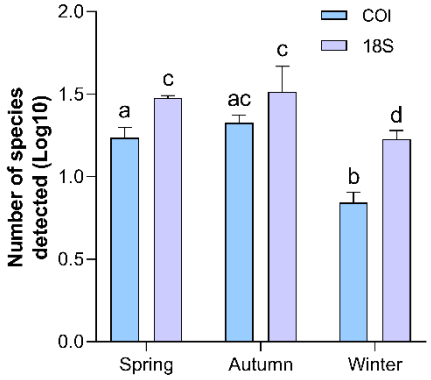

Figure 13. Seasonal and markers choice effect on the number of zooplankton species detected via metabarcoding. Blue bars correspond to COI and purple bars correspond to 18S identifications. Similar letters above each bar indicate absence of significant differences ($p > 0.05$).

3.2.4. Effect of the genetic marker and season on zooplankton community structure

COI and 18S sequencing detected seasonal differences for several groups of species. In fact, both COI and 18S datasets suggested similar seasonal patterns to several shared taxa, although with some exceptions. This analysis further supported what was already mentioned in the sub-section 3.2.3; that winter was a very distinctive season for zooplankton communities, when comparing it with spring and autumn. Particularly, annelids displayed similar taxa from spring to autumn and in the winter occurred a switch in dominant taxa. For COI, Spionidae displayed a higher relative abundance during spring and autumn, but during the winter, two additional groups dominated the annelids found in the zooplankton – Sabeliidae and Sabellariidae (found in all seasons, but prevailed in the overall diversity found in the winter). Additionally major contributors to the diversity of annelids found in the spring and autumn ceased to appear in the winter (Figure 14). For 18S the distribution of Spionidae was similar, but to a lesser extent since it was less relevant to the overall sequenced samples. Capitellidae prevailed in the first two seasons analyzed in the dataset of 18S, and later its dominance was switched to Sabellariidae (similar seasonal distribution found with COI) (Figure 15). However less considerable abundant groups ceased to be detected comparing to what has been observed with COI.

The most relative abundant arthropods detected with COI demonstrated strict exclusive seasonal distribution, where Balanidae were the only that were detected in more than one sampled season, but did not appear in the zooplankton sampled in the winter (Figure 14). Still, 18S showed a stronger diversity

of arthropods in the zooplankton, especially during the autumn. Majority of taxa found in spring and autumn were different from those that prevailed during the winter (Figure 16). According to the 18S dataset, arthropods were the most relative abundant organisms in zooplankton (Figure 12) (Table S6, Supplementary Material), overcoming the annelids that were more abundantly detected with this marker in the other seasons. Conflicting findings between heat plots of each marker for the arthropods might be related to the different species sequenced – 18S identified 30 different arthropods and 7 additional species were identified with COI. Still, these findings supported the fact that arthropods were major contributors to zooplankton organisms, even though fewer species were identified with COI.

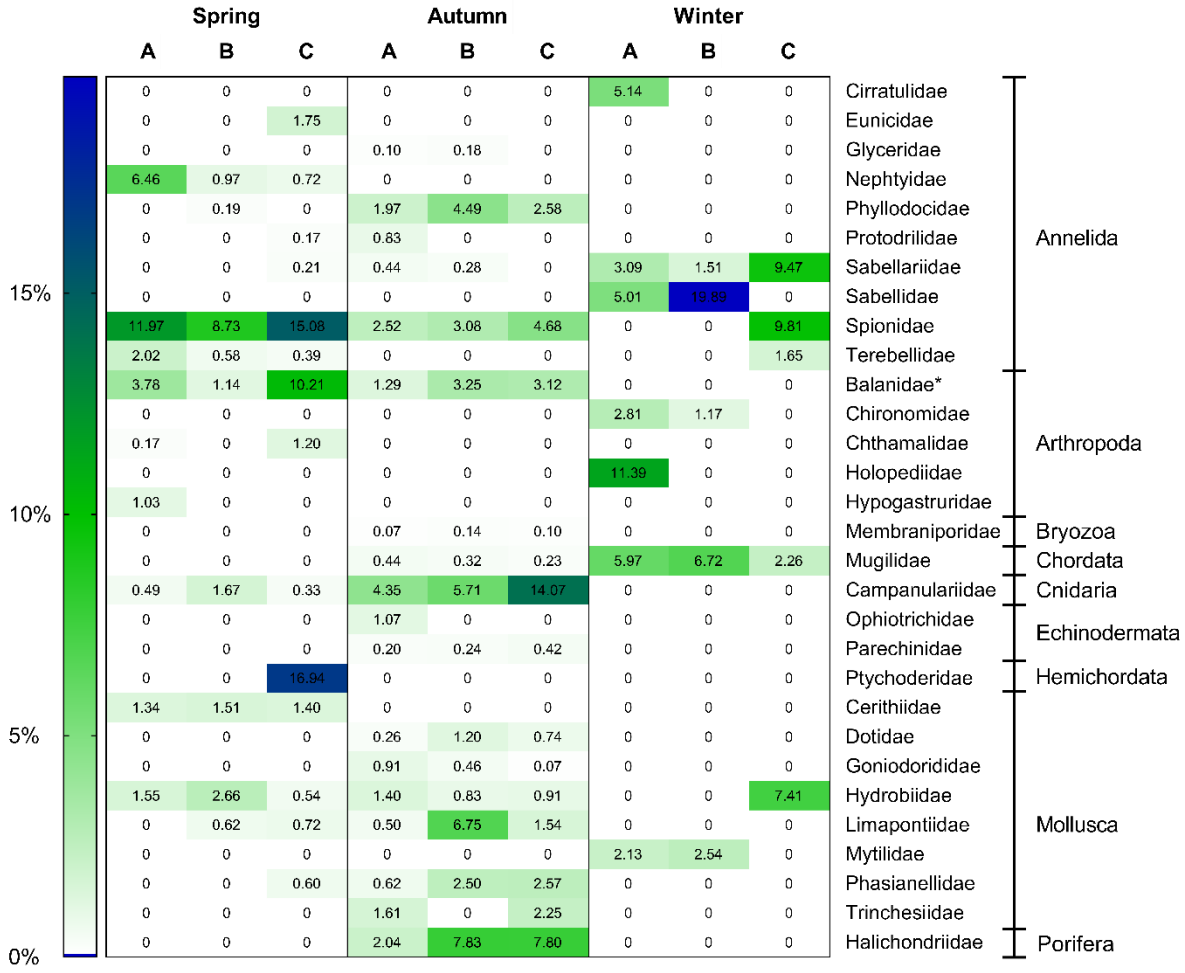

Figure 14. Seasonal distribution of the most relatively abundant families recovered with the COI marker. Letters on the top of the heap plot indicate the replicate code of each sample collected from the recreational marina of Viana do Castelo: A and B correspond to the two most inner docks, respectively, and C corresponds to the most outer dock. The families highlighted with (*) indicate those that harbor the non-indigenous taxa detected. The different colors express the proportional weight of represented taxa on the overall sampled season, where white represents absence and from light to darker green and from light to darker blue, represents an increasing degree of relative contribution (in %) to the overall dataset that are represented in the y-axis color graded bar in the left side of the plot.

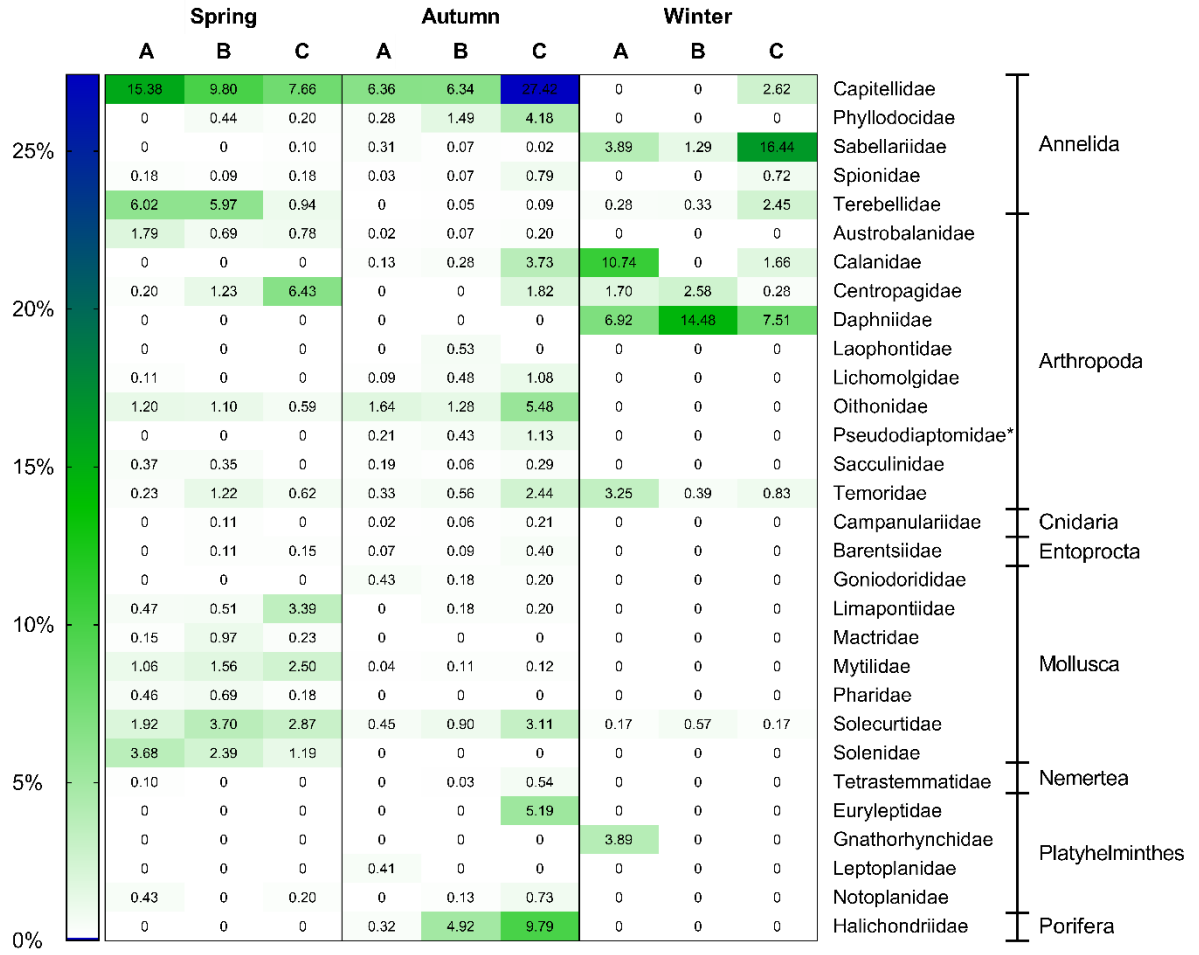

Figure 15. Seasonal distribution of the most relatively abundant families recovered with the 18S marker. Letters on the top of the heap plot indicate the replicate code of each sample collected from the recreational marina of Viana do Castelo: A and B correspond to the two most inner docks, respectively, and C corresponds to the most outer dock. The families highlighted with (*) indicate those that harbor the non-indigenous taxa detected. The different colors express the proportional weight of represented taxa on the overall sampled season, where white represents absence and from light to darker green and from light to darker blue, represents an increasing degree of relative contribution (in %) to the overall dataset that are represented in the y-axis color graded bar in the left side of the plot.

Mollusks were a group of organisms that seemed to be the most affected by the winter. From several taxa identified with COI and 18S, only few were still found in the winter. In fact, merely Solecurtidae (Figure 15), Hydrobiidae and Mytilidae (Figure 14) were reported in winter samples. The first two families were represented in all three seasons and in the winter the number of reads identified as such, was lower than what was obtained in the other seasonal samples. However, Hydrobiidae species were considerably more relevant in zooplankton diversity in the winter, than in spring and autumn. *Mytilus edulis* was the only representative taxon of Mytilidae and that displayed the most conflictive seasonal distribution observed between markers. Overall, this taxon was reported throughout spring to winter, however the markers applied reported its occurrence in different seasons. With sequencing of COI this species was

recovered only in winter, whereas with 18S, both in spring and autumn, even though the same species was identified. Limapontidae species displayed similar patterns, although with opposite findings. Species from this family were reported during spring and autumn with COI (Figure 14), while 18S detected them only in the winter (Figure 15). However, this taxon grouped several representative species, which might explain discordant seasonal distribution since these markers detected mostly different species.

In COI and 18S datasets, sponges were found to contribute very little to zooplankton richness (Figures 11 and 12). Only two species were identified - one with each marker - and both were from the genus *Hymeniacidon*. Although different species, these were strictly found only in the samples collected in the autumn (Figures 14 and 15). A similar case was observed within the annelids. A total of 3 species were identified as grouped in the family Phyllodocidae with both markers. *Notophyllum foliosum* (Sars, 1835) was detected with 18S and was represented with a total of 23 reads; the remaining two species were identified within the genus *Eumida* which dominated the Phyllodocidae detections (Tables S5 and S6, Supplementary Material). Although identified as two taxonomically separate species, these displayed similar seasonal distributions (Figures 14 and 15); increasing relative abundance from spring to autumn and then in the winter no fragment reads were identified as such (*N. foliosum* was only found in the autumn, but was less relevant to Phyllodocidae in the autumn). On the other hand, Campanulariidae was found to occur in the zooplankton during spring and autumn, with both markers, but with greater representation in the latter. This family was represented by 3 species in total, where only *Obelia geniculata* was recovered with both markers, and was the only species detected by 18S. Even though it was detected by COI, *Obelia dichotoma* (Linnaeus, 1758) was the major representative, with 4152 reads compared to 25 reads total between the remaining two cnidarians (Tables S5 and S6, Supplementary Material).

Exclusively to COI dataset, samples from the winter lacked any echinoderms, hemichordats and bryozoans. Disregarding the increasing number of annelids from autumn to winter observed with COI (Figure 11), flatworms were the only organisms that displayed an increasing species richness towards the winter (Figure 12). However, their representativity peaked in autumn (Figure 15). In fact, from the 5 species found in winter samples, only one species was included in the list of the most relative abundant taxa analysis that was reported to be found in the winter (Figure 12). During autumn all 4 species presented a considerable number of reads, particularly *Prostheceraeus vittatus* (Montagu, 1815) (Eurylepidae) that accounted for ca. 5.2 % of the reads sequenced in overall autumn (Figure 15).

Non-Metric-Multidimensional Scaling analysis showed that for each marker, zooplanktonic community structure clearly differed between seasons (Figure 16). When both marker datasets were used in the nMDS analysis, a clear effect of the genetic marker on community composition was also observed, which is in accordance with the taxonomic differences found between markers and already detailed in previous sub-sections. These differences in the zooplankton communities linked to the two markers and the different seasons were further supported by a Two-way PERMANOVA analysis ($F_{1,12}$ = 9.19, $p < 0.01$ and $F_{2,12}$ = 4.35, $p < 0.01$, for marker and season, respectively). The effect of the marker choice seemed to be stronger than seasonal variation on structuring zooplankton communities (Figure 16).

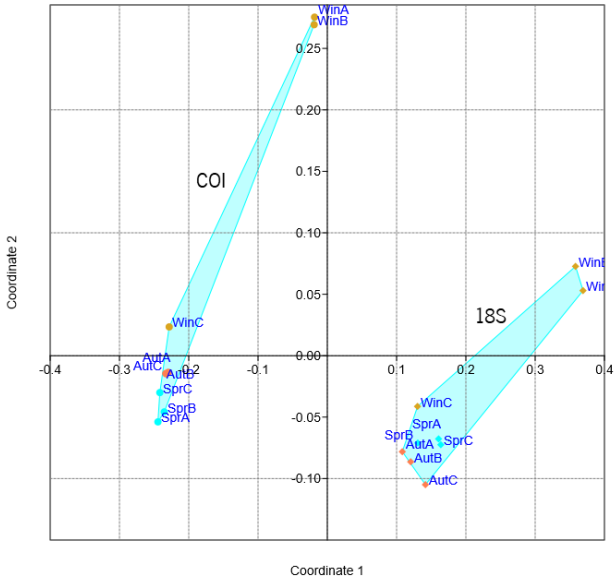

Figure 16. nMDS ordination diagram of the zooplankton communities detected in the recreational marina of Viana do Castelo, accordingly to the molecular marker and season, based on Jaccard's similarity index. This plot is color-coded where symbols in blue, orange and yellow correspond to samples collected in spring, autumn and winter respectively.

A separate analysis conducted for each marker dataset further highlighted that the zooplankton community structure found in the spring was more related to autumn than winter, since these two were distributed in the negative side of the x-axis; and sampled zooplankton from the winter were distributed in the positive side of the same axis (Figure 17). Still, spring and autumn samples were different from each other due to opposite patterns observed throughout the y-axis. A One-way PERMANOVA analysis further supported the differences detected in both datasets (F_2 = 4.48, p = 0.03 and F_2 = 3.98, p = 0.03, for COI and 18S, respectively).

In both datasets, samples from winter varied more between each other than from samples collected in other seasons, in fact zooplankton sampled from the most inner docks were less related to the rest of the samples. This was also displayed in the nMDS plot (Figure 16), where the aforesaid

samples were emphasized by being distributed towards higher values in x-axis, compared to the overall samples sequenced with the same marker. However, a more detailed analysis showed that this variation was more predominant in the COI dataset (Figure 17A). Still, no significant statistical variation was observed between sampling sites. Although in the first two seasons the zooplankton structure was different, 18S dataset further showed more differentiation in the composition of species from spring to autumn, with the x-axis more strongly highlighting the divergences found between spring and autumn (Figure 17B).

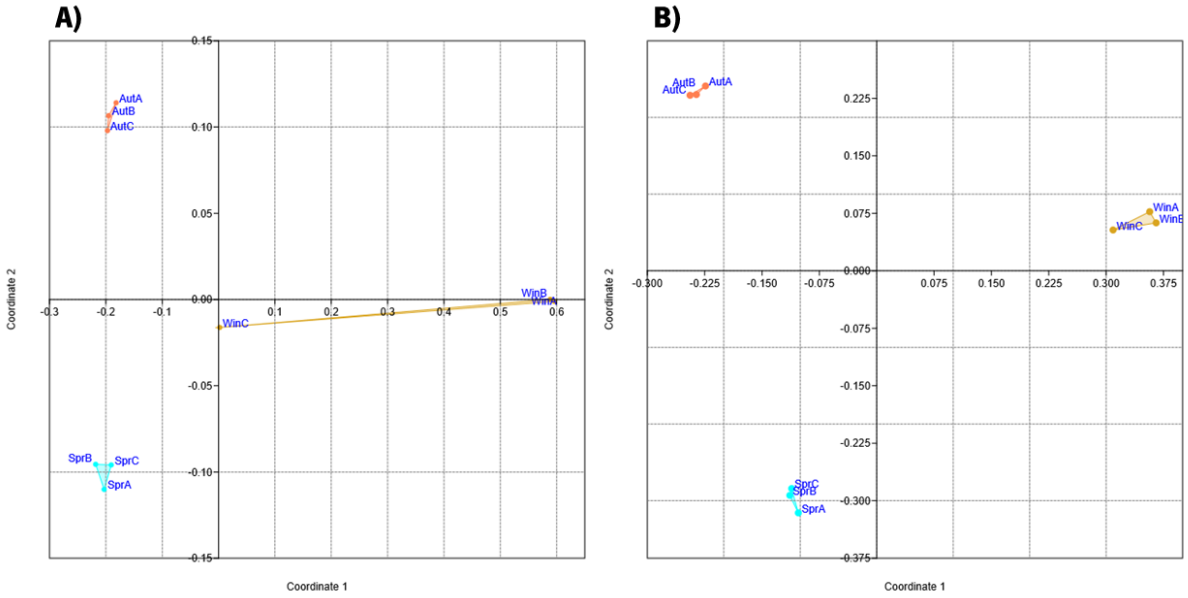

Figure 17. nMDS ordination diagram of the zooplankton communities detected in the recreational marina of Viana do Castelo, accordingly to the season for the COI (A) and 18S (B) datasets, based on Jaccard's similarity index. These plots are color-coded where symbols in blue, orange and yellow correspond to samples collected in spring, autumn and winter respectively.

The highlighted difference of winter samples from the other two seasons supports the observed community shift. Fewer species were found in the latter season due to ceasing reports and, in addition, various groups of already documented phyla were exclusive or more predominant during the winter. Both datasets are in accordance in the occurrence of annelids and arthropods species composition shift, and further in the disappearance of representative cnidarians, mollusks and sponges from zooplankton communities. Null identifications of several relevant taxa were also reported with either marker, that further highlights the contrast between spring plus autumn and winter.

3.2.5. Detailed analysis of the zooplankton composition

A more detailed analysis of the life cycle of each species showed that the majority here reported with DNA metabarcoding were temporary in the plankton. As a matter of fact, COI identified 58 metazoan

species known to be from marine and/or brackish environments (Figure 10), from which 54 were classified as occurring temporarily in the plankton (ca. 93.1%). To the best of our knowledge, from these, every species was documented to have at least a planktonic larval stage (ca. 20.7%), only the larval stage (ca. 36.2%) or both eggs and larvae to be planktonic (ca. 32.8%) (Figure 18). Two cnidarians were the only exceptions – *Clytia hemisphaerica* (Linnaeus, 1758) and *Rhizostoma luteum* (Quoy & Gaimard,1827) – since these species are mostly planktonic, but at some stage in the life cycle are benthonic. Two arthropods, namely *Holopedium gibberum* (Zaddach, 1855) and *Hypogastrura viatica* (Tullberg, 1872), were found to be the only permanent plankton species that were identified with COI, in the recreational marina.

A similar trend was observed in the 18S dataset, where majority of the species detected were meroplankton (ca. 63.5%), but with different proportions. As this molecular marker detected more species than COI, 18S detected six times more holoplanktonic species which accounted for ca. 11.5%; and even though meroplankton richness was higher, it was proportionally less relevant to the dataset. In fact, species that display planktonic eggs and larvae or only larval stages in the plankton accounted both for ca. 45.2%, whereas species for which information was found regarding the presence of planktonic eggs or to be non-planktonic covered 17 species (ca. 16.3%) (Figure 18). Additionally, two cnidarians were also encompassed as meroplankton – *Lizzia blondina* (Forbes, 1848) and *Rhopilema verrilli* (Fewkes, 1887) – but included in a separate group, since most of their life cycle is planktonic and are temporarily benthonic.

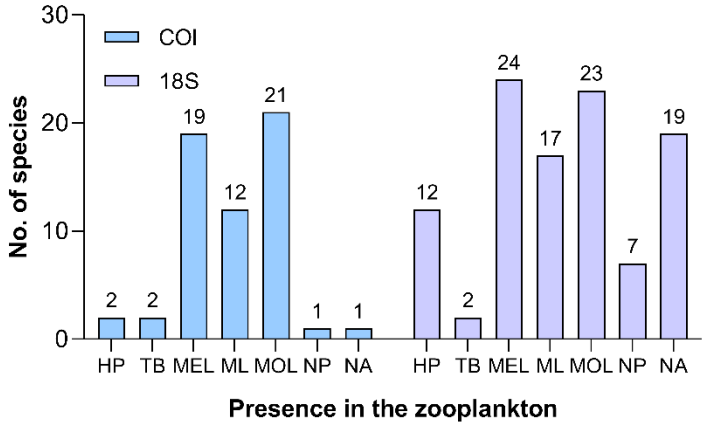

Figure 18. Influence of the marker choice over the proportion of the different categories of organisms in terms of the time of occurrence in the plankton. Numerical values over the bars represent the number of species categorized. Blue bars correspond to the species identified with COI and purple bars correspond to 18S. X-axis present all different categories analyzed: holoplankton (HP), temporary benthos (TB), meroplankton eggs and larvae (MEL), meroplankton at least for the larvae (ML), meroplankton only for the larvae (MOL), non-planktonic (NP). NA, correspond to species for which no information concerning its occurrence in plankton was not found.

Overall, 8 non-planktonic species were detected with DNA metabarcoding as occurring in the zooplankton communities (Figure 18). *Melita palmata* (Montagu, 1804) was a tychoplanktonic species identified with COI and the remaining were all identified with 18S, which encompassed *Thelepus cincinnatus* (Fabricius, 1780) and all Platyhelminthes found. Additionally, no information was found in terms of the life cycle for 14 species, from which 2 were identified with COI and the remaining with 18S.

As already analyzed on previous sections, the number of species detected and the structure of the zooplankton communities from the recreational marina were influenced by the molecular markers opted and the seasons sampled. Similarly, a Two-way PERMANOVA analysis found that COI and 18S detected divergent zooplankton composition (species richness and relative abundance), in terms of temporal presence of species in the zooplankton ($F_{1,12}$ = 11.08, $p < 0.01$, for both), and that there was a variation throughout sampled seasons ($F_{2,12}$ = 3.42, $p = 0.02$, for both). However, regarding seasonal variation, a separate analysis conducted for each marker indicated absence of differences in the species richness (One-way PERMANOVA, COI: $F = 2.39$, $p = 0.17$; and 18S: $F = 3.03$, $p = 0.07$) and on the relative abundance of the different groups (One-way PERMANOVA, COI: $F = 2.39$, $p = 0.17$; and 18S: $F = 3.03$, $p = 0.07$).

3.2.6. Comparison of DNA metabarcoding with morphology-based identified zooplankton species in the Lima River estuary

The comparison of the metabarcoding data obtained in the current study with the data of the compiled lists of species previously reported to occur in the Lima River estuary, implied that only 26 species of these lists were detected via COI and 18S sequencing (18.1% of the species recovered through metabarcoding) (Figure 19). From these lists, both markers appear to be complementary. While COI detected exclusively 5 zooplanktonic species – *C. hemisphaerica*, *L. conchilega*, *Ophiothrix fragilis* (Abildgaard, 1789), *Pomatoschistus microps* (Krøyer, 1838), and *Trisopterus luscus* (Linnaeus, 1758) – and 7 species of previously reported macrozoobenthos - *Amphiura filiformis* (Müller, 1776), *Bittium reticulatum* (da Costa, 1778), *L. conchilega*, *M. palmata*, *Nephtys homberguii* (Savigny, 1818), *Psamathe fusca* (Johnston, 1836), and *Pseudopotamilla reniformis* (Bruguière, 1789) – 18S detected exclusively 5 zooplanktonic species – *Centropages typicus* (Krøyer, 1849), *Daphnia pulex* (Leydig, 1860), *Lizza blondina* (Forbes, 1848), *Parasagitta friderici* (Ritter-Záhony, 1911), and *Temora longicornis* (Müller, 1785) – and 8 macrozoobenthos species - *Cerastoderma edule* (Linnaeus, 1758), *Ensis ensis* (Linnaeus, 1758), *Hediste diversicolor* (Müller, 1776), *Heteromastus filiformis* (Clarapède, 1864), *Hiatella arctica*

(Linnaeus, 167), *Mya arenaria* (Linnaeus, 1758), *Pharus legumen* (Linnaeus, 1758), and *Pollicipes pollicipes* (Gmelin, 1791). Additionally, *M. fuliginosus* – a species never documented in zooplankton communities in the Lima River estuary (here reported only as a species included in the compiled list of macrozoobenthos) – was detected with both markers. *Peringia ulvae* was the only species that was compiled in both lists and simultaneously reported in COI and 18S datasets.

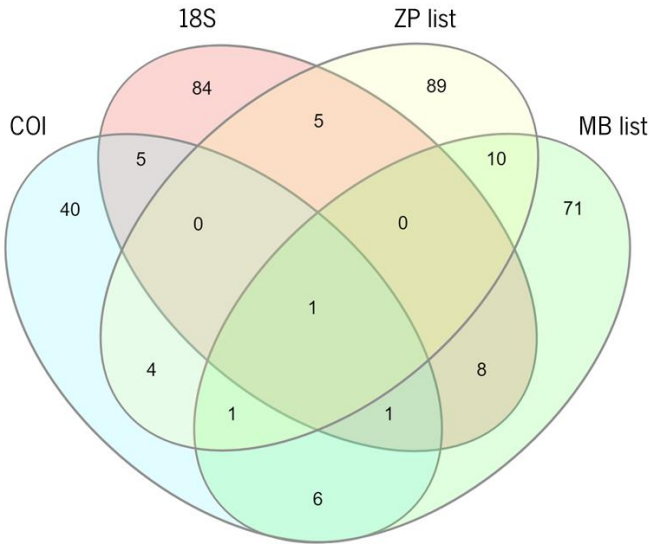

Figure 19. Partitioning of zooplankton species, between morphologically-based identifications, from studies previously conducted in the Lima River estuary, and based DNA-identifications, from the current study. ZP and MB correspond to zooplankton and macrozoobenthos species with sequenced records in online databases (BOLD and GenBank), respectively.

3.2.7. Detection of non-indigenous species (NIS)

A compiled list of 105 acknowledged marine and brackish waters NIS, found on Portugal's mainland, was used to determine whether any of the detected species in zooplankton were already reported as NIS in the Portuguese territory. A total of 5 NIS not reported in the compiled lists, were detected in the recreational marina, while 3 NIS that were already documented in the estuary – *Austrominius modestus* (Darwin, 1854), *Corbicula fluminea* (Müller, 1774), and *Acartia (Acanthacartia) tonsa* (Dana, 1849) – were not found in the current study. The 5 NIS detected included *Amphibalanus eburneus* (Gould, 1841) and *Balanus trigonus* (Darwin, 1854), both detected with COI, and *Cordylophora caspia* (Pallas, 1771), *Eriocheir sinensis* (Edwards, 1853), and *Pseudodiaptomus marinus* (Sato, 1913), that were detected with 18S (Table 3). *Mya arenaria*, a known NIS to occur in Portugal, has been already reported in the Lima River estuary and was also recovered in the current study, in the 18S dataset. NIS here detected accounted for 3.9% of the species recovered. Almost all species were detected with a reasonable number of reads, but *M. arenaria* was an exception that displayed a minimal number of reads

(9 reads) (Table 3). The majority of NIS were arthropods, with a single report of a cnidarian and a mollusk. These presented similar trends to the overall diversity detected. The first two sampled seasons demonstrated a greater and similar number of NIS, whereas in the winter only one species was detected.

Both NIS detected with COI belonged to the Balanidae, which encompassed one of the 30 most relative abundant groups in the COI dataset (Figure 14). In the 18S dataset, another family was also included, Pseudodiaptomidae, represented by *P. marinus* (Figure 15). The remaining NIS were detected in fewer samples and fewer reads were recovered. Balanidae species was the only group to be detected during both spring and autumn, however *B. trigonus* had a weaker representation in spring, since it was detected only in the innermost dock of the marina. *Pseudodiaptomus marinus* was the only NIS recovered only in autumn, whereas *E. sinensis* and *M. arenaria* were detected only in spring. *Cordylophora caspia* was the only NIS detected in the winter, but was also found in autumn. Therefore, the highest number of NIS were detected in spring and autumn, with 4 species each, however, species identity differed.

Table 4. Number of reads recovered and taxonomic assigned to non-indigenous species. Samples docks are represented by the letters, with A and B corresponding to the two most inner docks and C to the dock closest to the entrance of the marina.

NIS	Spring			Autumn			Winter		
	A	B	C	A	B	C	A	B	C
Amphibalanus eburneus	125	59	526	-	23	158	-	-	-
Balanus trigonus	70	-	-	217	522	366	-	-	-
Cordylophora caspia	-	-	-	-	37	-	-	72	-
Eriocheir sinensis	-	142	-	-	-	-	-	-	-
Mya arenaria	10	18	9	-	-	-	-	-	-
Pseudodiaptomus marinus	-	-	-	116	234	611	-	-	-

4. Discussion

4.1. Gap analysis

The completeness and reliability of the reference sequences libraries is crucial to the accurate identification of species using molecular-based approaches. In fact, the absence of sequences in reference databases is still recognized as one of the major limitations that affects the use of metabarcoding for zooplankton biodiversity monitoring, as well as the early detection of newly introduced NIS and identifications at species level (Bucklin et al., 2016; Duarte et al., 2021b). In this study, COI and 18S reference libraries displayed an acceptable coverage (ca. 98% and 86%, respectively) over the species found in the literature with known occurrence in the Lima River estuary. These results are much better than what has been observed on a larger scale. For instance, Weigand et al. (2019) reported that only 48% of marine macrobenthos and fish species, from Europe, have at least a COI reference sequence available on genetic databases, while Leite et al. (2020) found that 63% had at least a reference COI DNA barcode, for invertebrate macrozoobenthos of the Atlantic coast of the Iberia Peninsula. On the other hand, few studies were found that focused in analyzing the gaps in the reference databases for planktonic metazoan in Europe, but Singh et al. (2021) found that for marine zooplankton from South Africa, only 45% of the species were covered by reference COI sequences (see also Duarte et al., 2020; Jazdzewska et al., 2021; Vieira et al., 2021). The more local focused analysis partaken in this study, might explain higher values of representation of species in molecular reference libraries, than what has been reported at larger scale – COI coverage at European extent was considerably lower (Weigand et al., 2019) than at a more regional range (Iberian Peninsula coast) (Leite et al., 2020), and subsequently in the scope of the Lima River estuary, it was expected that species representation was more complete in reference libraries. Usually at a larger scale, the number of species analyzed is higher than those that were reported in the Lima River estuary, which *per se* might increase the number of rare, endemic and vaguely studied species to be covered in the analysis, and consequently increasing the number of species that are not yet barcoded and included in reference databases. Nonetheless, coverage of molecular markers can vary strongly among geographic regions (Weigand et al., 2019).

Since COI is the standard barcode region for metazoan species, the number of species already included the reference libraries was considerably higher than for 18S. This trend seems to be consistent in various marine metazoan groups. For instance, Vieira et al. 2021 reported that COI covered more marine macroinvertebrates than 18S, from both native and non-indigenous species in Macaronesia, and

Duarte et al. 2020 also found a similar pattern for NIS with reported occurrence in Europe. Still, few studies were found that performed a reference sequence gap analysis (regional or local) for 18S records of macrozoobenthos and zooplankton species (Vieira et al., 2021), which is curious since hypervariable regions of 18S have been the major genetic targets used for taxonomically characterization of zooplankton communities, using DNA metabarcoding. Furthermore, molecular markers representation of species also varies considerably among taxonomic groups (Weigand et al., 2019; Duarte et al., 2020), and in the present study, gap analysis of BOLD (COI) and GenBank (18S) displayed that taxonomic groups reported as occurring in the Lima River estuary exhibited different levels of completion in the surveyed reference databases (Figure 9).

In the present gap analysis, ca. 8.9% of the compiled species occurring in the Lima River estuary are not be yet represented with sequences in BOLD and GenBank genetic databases. However, it is crucial to take into account that GenBank records do not fully reflect SILVAngs reference records, therefore the gap analysis can be biased, as the aforesaid platform includes more curated molecular records. But, in the current study, it was not possible to perform a gap analysis with data from such database. Additionally, the analysis only included the current accepted nomenclature. Hence, the records with synonymous taxonomy and that were absent from the species lists, might have reference sequences on genetic databases, but under another designation, which would even decrease more the gaps found.

Though the informational gap for species morphologically reported to occur in the Lima River estuary is acceptable, and more species are represented in reference libraries than what has been observed in literature, the completeness of reference databases with comprehensive taxonomic identification and geographical representation for each species (and molecular marker) is still a critical step for more reliable identifications of organisms by using DNA-based approaches. Studies at a larger scale showed that the gap in reference libraries is still persistent (Weigand et al., 2019; Duarte et al., 2020; Leite et al., 2020; Jazdzewska et al., 2021; Singh et al., 2021; Vieira et al., 2021), which remains an impediment to the general use of DNA metabarcoding for species-level characterization of complex communities such as zooplankton (Bucklin et al., 2016). As long as reference databases are far from completion, the taxonomic assignments when using DNA-based approaches for species identification may be improved by generating local reference sequences libraries (Abad et al., 2016; Yang et al., 2017). Abad et al. (2016) was able to increase the taxonomic assignment success from 23.7 to 50.5% by generating DNA barcodes for local plankton species in the estuary of Bilbao (Spain).

4.2. Zooplankton communities in the recreational marina of Viana do Castelo

4.2.1. Effect of the genetic marker on taxonomic composition and diversity

As expected, recovered diversity was highly dependent on the marker choice. 18S rRNA gene is considerably more conservative than COI; and has been mostly used for studies targeting higher taxonomic levels and for a broader approach of taxa detection (e.g., Stefanni et al., 2018; Zhang et al., 2018; Xu et al., 2020; Questel et al., 2021). Even so, 18S recovered 2 times greater diversity from zooplankton samples in the current study, than COI (Figure 13), which was also reflected in NIS detection (Table 4), in the current study. Similarly, but to a lesser extent, Zhang et al. (2018) also reported a higher species richness recovered with 18S comparing to COI, for zooplankton mock communities and when employing different primers for both markers.

On one hand, the obtained results could be related with the different efficiency of both sets of universal PCR primers in recovering different organisms (Zhao et al., 2021). Amplification efficiency is in fact dependent on the molecular maker choice, particularly under the use of different universal primers, which may preferentially target different taxonomic groups. In this study, several PCR-related bias may have been introduced due to the universality of the primers and in the amplification steps (Engelbrektson et al., 2010; Govender et al., 2022a). In fact, both primers pairs used in the current study were chosen based on previous studies that showed their high efficiency in recovering macroinvertebrates diversity using DNA metabarcoding (Fais et al., 2020; Leite et al., 2021). Furthermore, it is also feasible that during primer annealing, due to mismatches that may occur between primer region sequences and the target sequences, there is a competition between the sequences belonging to different taxa, and those with a greater affinity will be preferentially amplified in detriment of others displaying lower affinity (Deagle et al., 2014; Piñol et al., 2014; Leite et al., 2021).

On the other hand, the use of different reference libraries for taxonomic assignment of the generated sequences may also lead to distinctive taxonomic identifications (Zhao et al., 2021). For reliable molecular-based identification of species it is crucial the existence of taxonomically complete and geographically comprehensive reference databases of DNA sequences (Bucklin et al., 2016). In the current study two different reference databases were used for taxonomic assignment of the recovered sequences from complex zooplankton samples (see section 2.3.3). SILVAngs has been shown to be suitable for identification of sequences from nuclear genes, such as 18S rRNA gene (Lindeque et al., 2013), whereas BOLD database has been primarily used for the taxonomic assignment of COI sequence

records. Furthermore, taxonomic assignment may also have a bias related with the scarcity of reliable sequence records belonging to specific taxa, such was the case of *Notoplana* spp., in the current study (further details in the section 4.5).

Nevertheless, if COI was the only marker employed to analyze the zooplankton communities sampled in the recreational marina of Viana do Castelo, it would not be able to detect several taxonomic groups that were recovered exclusively in the 18S dataset. Several previous studies have suggested the complementary use of COI and 18S for DNA metabarcoding of zooplankton (e.g., Clarke et al., 2017; Stefanni et al., 2018; Zhang et al., 2018; Carroll et al., 2019; Couton et al., 2019; Pitz et al., 2020; Rey et al., 2020a; Cicala et al., 2021; Questel et al., 2021; Suter et al., 2021; Zhao et al., 2021) and the current study further supports it, particularly when the target is newly introduced NIS. For instance, more than half of the recovered species from DNA metabarcoding of zooplankton, from the recreational marina of Viana do Castelo, were detected exclusively with 18S (Figure 10 and 19), which included exclusive phyla such as Nemertea, Nematoda, Platyhelminthes, Rotifera, Chaetognatha, Entoprocta, Phoronida, Sipuncula, Xenacoelomorpha (Table S6, Supplementary Material). Regarding species richness, platyhelminths and nematodes were the most relevant groups. According to León-Reògagnon (2010) the platyhelminths COI barcoding region is usually shorter than the standard barcodes; while according to Deagle et al. (2014), COI is unsuitable for species-level identification of nematodes due to sequence diversity in the primer annealing regions. Furthermore, 18S seems to be a more suitable alternative for the identification of these taxa (Haenel et al., 2017; Carroll et al., 2019; Fais et al., 2020; Castro et al., 2021; Duarte et al., 2021a,b; Leite et al., 2021). For instance, a multi-marker DNA metabarcoding analysis of zooplankton communities in New Zealand, also reported exclusive-to-greater representation of Nematoda, Nemertea, Platyhelminthes and Rotifera (Carroll et al., 2019). However, the exclusive detection of Rotifera, Chaetognatha and Sipuncula, in the current study, may have been a result possibly related to amplification or geographical location bias (e.g., Wangensteen et al., 2018). On the other hand, Entoprocta, Phoronida, Xenacoelomorpha are poorly represented in the BOLD database, from which the majority are specimens from the western Atlantic Ocean and the Pacific Ocean.

4.2.2. Effect of the season on taxonomic composition and diversity

Though there have been some discrepancies between the recovered diversity using COI and 18S, both displayed a decrease in the overall species richness and for the majority of the phyla from spring-autumn to winter. Sampled seasons were characterized by temperature (maximum, mean and minimum),

pH, salinity and conductivity (Table 2), which may have contributed to shape the structure of zooplankton communities (Figure 17). Indeed, high water temperature, salinity and subsequent conductivity were among the abiotic factors reported to be associated with higher zooplankton biomass in the Lima River estuary (Vieira et al., 2015). Although inferred as the hardness of the water (calcium carbonate), pH levels seem to have a negative relation with zooplankton biomass – high levels of calcium carbonate in the water increase pH, which is associated with higher zooplankton biomass (Suthers & Rissik, 2009).

The winter was the most different season, in terms of community diversity and structure, highlighted by the decrease of salinity and subsequent conductivity levels, pH and mean temperatures – maximum and minimum decreased slightly – resulting in the lowest recovered species richness, as well as NIS, and with a greater representation of Platyhelminthes. The Lima River estuary is also characterized by low primary production in the winter, reflected by low levels of chlorophyll *a*, which per se can be associated to acidification of the water column (Ladhar et al., 2015). Spring-autumn salinity variations observed in the current study seemed to be more coherent with the variation found in Ramos et al. (2006), than in Vieira et al. (2015), where it fluctuated very slightly from spring-summer-autumn, to then display a sharper decrease in the winter. Therefore, DNA metabarcoding seemed to be able to uncover the seasonal variation within the zooplankton communities' structure in the recreational marina of Viana do Castelo (Casas et al., 2017;Blanco-Bercial, 2020; Schroeder et al., 2020; Rey et al., 2020a; Coguiec et al., 2021; Zhao et al., 2021) (sections 3.2.3).

In the Lima River estuary, zooplankton community dynamics has been poorly studied and described. For instance, more studies regarding ichthyoplankton were found (Ramos et al., 2006, 2010, 2015) and only Vieira et al. (2015) targeted the general zooplankton composition, however with more focus in copepods, which have been reported to be the main contributors to the composition of zooplankton communities (see also Guimarães & Galhano, 1987, 1988, 1989). Although Guimarães & Galhano (1987, 1988, 1989) also studied the zooplankton in the estuary, they have only considered the seasonal and annual biomass fluctuation of holo- and meroplankton. Therefore, for several taxonomic groups the seasonal dynamics has not yet been thoroughly reported or analyzed, and the current study was the first attempt to address this gap.

Indeed, only ichthyoplankton and copepods variation have been more thoroughly analyzed throughout several sampling times (Ramos et al., 2006, 2010, 2015; Vieira et al., 2015). Oithonidae family has been analyzed in more detail (Vieira et al., 2015) and was also recovered, in the current study, with DNA metabarcoding. Although different species were recovered with DNA metabarcoding (further

depicted in the section 4.3.1), in general Oithonidae (Figure 15) variation was similar to the dynamics of *Oithona nana* (Giesbrecht, 1893) and *Oithona* spp. dynamics, since *Oithona plumifera* (Baird, 1943) was more seasonally and spatially restricted. Vieira et al. (2015) reported their absence during winter, but occurrence in the remaining seasons. However, the highest relative abundance of Oithonidae was recovered in autumn samples through DNA metabarcoding in the present study, some months after the previously reported peak of *O. nana*, which occurred during the summer (August). The differences found may be related to annual abiotic changes. For instance, there was a greater discrepancy between Vieira et al. (2015) reported salinity and conductivity in sampling locations near the recreational marina (lower than 4 and 4 to 8 mS/cm, respectively) and the study area of the current study (27.5 and 44.1 mS/cm).

In the current study, DNA metabarcoding was able to detect a switch in the structure of zooplankton community within the recreational marina (Figure 14 and 15), though this was significantly dependent on the molecular markers herein employed. Yet, shared taxonomic families – Phyllodocidae, Sabellariidae, Spionidae and Terebellidae (Annelida); Campanulariidae (Cnidaria); Gniodoridiidae, Limapontiidae and Mytilidae (Mollusca), Halichondriidae (Porifera) – displayed consistent seasonal patterns disregarding the marker used. Mytilidae was the only exception, with opposite seasonal patterns being found with both markers (18S in spring-autumn and COI in winter). No clear evidence was found for such discrepancy. For instance, *Mytilus* spp. larvae are usually associated to spring-autumn periods, but the occurrence during the winter may be associated to slower growth rates due temperature and salinity changes. Nevertheless, different seasonal patterns, found for COI and 18S, may be a result of amplification-related bias. In fact, the primer pair employed for COI had less affinity to *Mytilus* spp. sequences, it is expected that this would be more evident in samples from spring and autumn where due to a higher number of taxa found, the competition of the different sequences for the same primers would be higher. On the other hand, the lower zooplankton biomass and species richness associated with winter samples, might have increased the chances of detection, however this does not explain the absence of *Mytilus* spp. in winter samples in the 18S dataset.

The majority of NIS recovered were assigned to sequenced reads from spring and autumn samples. Balanidae taxa were associated with the first two sampled seasons, however recovered reads seem to report a putative dominance shift between *A. eburneus* larvae in the late spring towards *B. trigonus* larvae in autumn, which can be associated with temperature/salinity variations (Scheltema & Williams, 1982; Thiyagarajan et al., 2003). Both *M. arenaria* and *E. sinensis* were exclusively detected in the spring with a few recovered reads, probably suggesting a low biomass for this species. Indeed, adult

specimens of *M. arenaria* were previously reported in the estuary, however in low abundances (Sousa, 2003; Sousa et al., 2007; Conde et al., 2011), which may be due to the occurrence of a low pool of reproductive individuals in the estuary (Conde et al., 2011).

Pseudodiaptomus marinus was the only holoplanktonic NIS recovered, thus its detection does not necessarily mean that it is displaying reproductive activity, therefore such phenomenon does not explain its exclusive detection in autumn samples. *Pseudodiaptomus marinus* was recently first recorded in Portugal in 2011– Mondego River estuary (Uttieri et al., 2020), but not information regarding its reproduction was found, however, *P. marinus* was recovered exclusively during the autumn in the present study, which coincides with the secondary population peak in a population studied in Japan (Liang & Uye, 1997). Henceforward it is feasible that this NIS occurs more frequently in the estuary. However, its occurrence in the recreational marina may be predominantly mediated by water movements than by environmental conditions, due to similarities between spring and autumn. Furthermore, a *P. marinus* population in the North Sea seemed to be more mediated by temperature and primary producers, but not by salinity changes (Deschutter et al., 2018). However, salinity varies more drastically within an estuarine ecosystem than in open sea. Indeed, the recreational marina became oligohaline in the winter, which *P. marinus* does not seem to tolerate (Svetlichny et al., 2019), therefore probably explaining its absence.

According to the environmental characterization performed in the current study, the recreational marina of Viana do Castelo seem to display acceptable abiotic conditions for establishment of *C. caspia*. Indeed, this species has high salinity and temperature tolerance. Furthermore, in the presence of particular conditions, such as those found in the winter, *C. caspia* is one of the species able to tolerate, in particular lower values of pH, as the ones that were found in the current study (Hellawell, 1986; Gutierre, 2012; Mora et al., 2021). Nevertheless, recovered data through DNA metabarcoding may not allow to precisely analyze *C. caspia* planktonic larvae seasonal patterns in the recreational marina. In fact, Tyler-Walters & Pizzolla (2007) inferred the dormancy of this species during winter, therefore a lack of reproductive specimens (Muskó et al., 2008). The occurrence of *C. caspia* planktonic larvae during the autumn may explain the recovered reads in the present study, however the occurrence of dormant and resting stages (menonts) as part of the tychoplankton, could also explain overwinter recovered reads.

4.2.3. Detailed analysis of the zooplankton composition

A more detailed analysis of the life cycle of each species detected, further suggested that the most common abundant group of zooplankton – holoplankton (Duggan et al., 2008) – was underrepresented (Guimarães & Galhano, 1987, 1988, 1989). In the current study, zooplankton diversity found in the Lima estuary was mostly comprised by meroplankton throughout the three sampled seasons, despite some variation between both markers. The sampling of zooplankton in the opposite margin from the recreational marina, in the Lima River estuary, also reported meroplankton to be the major component of zooplankton communities (Guimarães & Galhano, 1988). In the current study, detected meroplankton diversity was mostly composed of eggs and/or larvae of macrozoobenthos, such as polychaetas, mollusks and crustaceans (Guimarães & Galhano, 1988) – with the latter being more relevant in the 18S dataset. However, no dataset suggested any seasonal influence over meroplankton species richness, unlike what has been previously found by Guimarães & Galhano (1988) and in other estuaries that used traditional methods of identification based on morphology (Vieira et al., 2003).

Species richness of holoplankton was lower in both datasets (COI and 18S) and were potentially underestimated. For instance, holoplankton encompass the majority of biomass and abundance of zooplankton, of which the major contributors are copepods (Guimarães & Galhano, 1987, 1988, 1989; Pardal & Azeiteiro, 2001; Marques et al., 2007; Duggan et al., 2008; Vieira et al., 2015). Previously, in the Lima River estuary, Vieira et al. (2015) concluded that Copepoda encompassed 43 to 76% of the organisms sampled. Therefore, it is possible that underestimation of holoplankton (contributor taxa described in the section 1.2) may be a result of the universal primers choice. Govender et al. (2022b) pointed out the lack of taxon-specific primers employed as the most feasible reason for the underestimation of several taxa, including Copepoda (see also Bucklin et al., 2016). For instance, several studies have displayed and increased performance of taxon-specific primers for specific taxonomic groups, since the lack of effective universal primers constitutes a limitation for DNA metabarcoding underestimation of group-specific diversity (Kelly et al., 2017; Zhang et al., 2018; Kim et al., 2021; Govender et al., 2022a).

Nevertheless, DNA metabarcoding further uncovered not yet documented meroplanktonic diversity in the Lima River estuary, particularly in the recreational marina (Figure 19). Resulting diversity also highlighted the ecological role of the Lima River estuary as a nursery spot for various macrozoobenthos, which in return provide nourishment for other harbored organisms (Ramos et al.,

2006, 2010). Hydrodynamics of the Lima estuary might be a key factor for a stronger representation of meroplankton than reported by Vieira et al. (2015), however plankton nets mesh size may also influence previously reported meroplankton (Vieira et al., 2003; Intxausti et al., 2012). Despite residence time, the Lima River estuary has a semidiurnal and mesotidal regime (Sousa, 2003; Ramos, 2007); and it is dominated by a stationary wave (Ramos, 2007), which might allow enough time for development of meroplankton species inside the estuary (Largier et al., 1997). Some species are known to actively migrate on a vertical axis in terms of tidal regime to avoid being swept out of an estuary (Ré, 1984). Such kind of behavior has been observed for *Eurytemora affinis* (Poppe, 1880) in the Seine River estuary (France). Densities of *E. affinis* were higher at the bottom than in the surface during ebb tides, which suggests such behavior of avoiding of being swept to the ocean (for futher details see Devreker et al., 2008; Menéndez et al., 2012). However, such behavior is not observable within non-motile macrozoobenthos eggs and larvae, where studies on the distributions of holo- and meroplankton, in a semidiurnal estuary, depicted higher densities of non-motile meroplankton in the deeper locations in the upper parts of the estuary (Hsieh et al., 2010; Menéndez et al., 2012), where slow water flows are possible. Though the recreational marina of Viana do Castelo is not very deep (section 2.1), the characteristic semi-enclosed system is prone to slower flowing waters, hence displaying good environmental conditions for harboring a greater meroplankton diversity (however no comparison has yet been reported).

Furthermore, DNA metabarcoding also uncovered a more relevant contribution of tychoplankton in the Lima River estuary, from which the major contributors were Platyhelminthes (Table S7, Supplementary Material). Tychoplankton correspond to benthic organisms transported to the water column by means of physical disturbances. In the chapter 2 (section 2.1) the Lima River estuary is briefly characterized into 3 parts, based on the Ramos (2007) description, where the lowermost part of the estuary comprises deep navigational waters. Though wind-based water flow is not a feasible reason for introduction of tychoplankton in the water column, ship traffic may create water movements and subsequently disturb bottom substrates. However, no relevant tychoplankton diversity has been previously reported in the Lima River estuary; in fact, only a Platyhelminthes species has been found (Table S4, Supplementary Material). However, in the recreational marina, depth is more or less maintained at 3 meters, where the constant movement of recreational vessels might mostly control water disturbances, which may explain the highest relevance of tychoplankton to the composition of zooplankton communities found in the current study, despite the wind influence.

4.2.4. Comparison of DNA metabarcoding with previous morphology-based assessments

The majority of the species already reported to occur in the Lima River estuary, in the compiled lists, were not detected via sequencing of COI and 18S of zooplankton samples collected in the recreational marina of Viana do Castelo (Figure 19). However, several DNA sequences matched with congeneric species previously reported such as, *Magelona* spp. recovered in the COI dataset and *Oithona* spp., in the 18S dataset. Additional several cases of multiple congeneric species were reported in the estuary, based on their morphology, but only one was recovered through DNA metabarcoding, in the current study.

Within the compiled lists of planktonic metazoans, the majority of singular or different congeneric species detected with DNA metabarcoding were recovered through 18S sequencing. Such conflictive reports, between previously reported species and resulting taxonomic-matched sequences from DNA metabarcoding, can be mainly related to the lower resolution power of the molecular marker used for taxa identification (in particular for 18S), which can represent a critical limitation of molecular-based identification methods, such as DNA metabarcoding (Bucklin et al., 2016). Conflicting species identifications between previously morphology-based and current DNA metabarcoding were found within 5 genera: *Calanus*, *Caligus*, *Oithona*, *Ophiothrix* and *Magelona*. According to several studies, most of the divergences between morphology-based reports and 18S sequencing of *Calanus* spp., *Oithona* spp., may be related to the lack of resolution at species level for the aforesaid marker, as above-mentioned (Øines & Schram, 2008; Freeman et al., 2013; Stefanni et al., 2018; Bernard et al., 2019; Di Capua et al., 2021; Questel et al., 2021; Lindemood, 2022). However, more recently Parshukov et al. (2021) inferred that species-level identification of calagids (Caligidae) is possible with 18S.

For instance, *Magelona mirabilis* (Johnston, 1865) was found in the compiled list (Vieira et al., 2015), while COI recovered reads belonging to *Magelona johnstoni* (total of 36 reads). This species has been recently described as a cryptic species, and has been mistakenly identified as *M. mirabilis* (Fiege et al., 2000). An analysis of all COI records, belonging to the Magelonidae family in BOLD, depicted a clear clustering of the comprehended taxa; even though that both species (*M. mirabilis* and *M. johnstoni*) grouped in multiple BINs, but with a clear separation of the records belonging to both species. In fact, *M. mirabilis* and *M. johnstoni* can coexist in European waters, and thus, while we cannot completely discard

a misidentification of *M. mirabilis*, in fact both species may indeed occur in the Lima River estuary (Fiege et al., 2000).

Additionally, several multiple species belonging to the genera *Centropages*, *Clytia*, *Daphnia*, *Parasagitta* and *Temora* have been previously reported in the plankton sampled in the Lima River estuary, but in the current study only one species per genus was reported. Such findings could probably be related to sampling location, since for most of them there is a clear discrimination of the species within each genus (e.g., *Clytia* sp. for COI, and *Temora* sp., for 18S). Within the *Centropages* genus, COI is not able to discriminate *Centropages chierchiae* (Giesbrecht, 1889) (compiled list) from *Centropages typicus* (compiled list and recovered through 18S in the current study), but these were not detected with COI (similar to Blanco-Bercial et al., 2014), and no information was found that compared the ability of 18S to distinguish both species. On the other side, Laakmann et al. (2013) found that both COI and 18S were capable of discriminate *Centropages hamatus* (Liljeborg, 1853) (compiled list) from *C. typicus*, but only the latter was taxonomically assigned to 18S reads. Brown et al. (2015) further listed a group of taxa problematic in terms of taxonomic assignment, which included *Daphnia* spp., and thus, may have affected the detection of *Daphnia longispina* (Müller, 1776) (compiled list) through DNA metabarcoding in the current study. 18S also seems to have low species-level resolution (at 99% threshold) within Sagittidae, hence the present DNA metabarcoding analysis could not resolve *Parasagitta* species in zooplankton samples.

Since several macrozoobenthos species can display planktonic stages, we also compared the data obtained in the current study with the data from a compiled list of macrozoobenthos, previously reported in the Lima River estuary. Similar to the list of zooplankton, differences were found between the taxa previously reported and the taxa recovered in the current study, and which were more evident for the 18S dataset.

Such cases included: *Capitella capitata* (Fabricius, 1780)*, Glycera tridactyla* (Schmarda, 1861), *Mytilus galloprovincialis* (Lamarck, 1819), *N. homberguii*, *Nephtys cirrosa* (Ehlers, 1868), *N. atomata*, *Solen capensis* (Fischer, 1881), *Spisula solida* (Linnaeus, 1758), *Spisula subtruncata* (da Costa, 1778) and *Venerupis corrugata* (Gmelin, 1791), that have been previously reported to occur in the Lima River estuary using morphology-based identification (Table S4, Supplementary Material). The 18S rRNA gene has been reported to not be able to distinguish species from genera *Capitella, Ensis*, *Mytilus*, *Nephtys*, *Spisula* and *Venerupis* (Rice et al., 1993; Westheide & Schmidt, 2003; Larsen et al., 2005; Vierna et al., 2014; Devriese et al., 2016; Guo et al., 2021). A blast of the 18S reads assigned to *Capitella teleta* (Blake

et al., 2009), *Ensis siliqua* (Linnaeus, 1758), *M. edulis*, *Nephtys incisa* (Malmgren, 1865), *Spisula solidissima* (Dillwyn, 1817) and *V. corrugata*, against GenBank revealed that even when employing a similarity threshold of 99%, this was not enough to distinguish the species detected in the current study through DNA metabarcoding from the species from the same genera and that were reported previously in the Lima River estuary, through morphology. Although COI seems unsuitable in discriminating *Glycera tridactyla* from *G. alba* (Müller, 1776), only two records of the former were found in BOLD (Teixeira, 2013). Similarly, assignment of recovered reads of *N. atomata* and *S. capensis* was probably not possible due to the lack of available reference records (in GenBank). Regarding *Mytilus* genus, COI is not able to distinguish species (Harris et al., 2016; Giusti et al., 2020), due to the unusual inheritance mechanism (Hoeh et al., 1991) and introgression that is found among this genus (Śmietanka et al., 2014). Such characteristics then lead to the occurrence of misidentifications in reference libraries, limiting species-level identifications with COI. Therefore, in the current study it is impossible to confirm neither the occurrence of *M. galloprovincialis* nor *M. edulis* in the Lima River estuary, with DNA metabarcoding, but instead the occurrence of *Mytilus* spp. However, the reads attributed to *Mytilus* spp. belong most probably to *M. galloprovincialis*, since it has been previously reported in the Lima River estuary, and it is the only *Mytilus* species occurring in the Portuguese coast (Śmietanka et al., 2014).

Polydora was also a genus from which the species recovered through DNA metabarcoding in the current study – *Polydora onagawaensis* (21 reads in the 18S dataset) – differed from the one identified previously in the estuary, which was *Polydora ciliata* (Johnston, 1838) (Table S4, Supplementary Material). The former was recently redescribed from a *P. ciliata/websteri* complex from specimens collected in Japan (Teramoto et al., 2013). Furthermore, a blast of the recovered 18S reads attributed to *P. onagawaensis* against GenBank displayed a clear interspecific dissimilarity. Nonetheless, *P. ciliata* occurrence in the Lima River estuary was reported before Teramoto et al. (2013) first description of *P. onagawaensis*, and thus, even if this species was present at the time, it could not be identified. In fact, since its first description in the Onagawa Bay, in Japan (Teramoto et al., 2013), it has been reported in several other regions of Japan (Abe et al., 2014, 2019), China (W. Sato-Okoshi et al., 2013), USA (Silverbrand et al., 2021; Wright, 2022), France/England (Sato-Okoshi et al., 2022), and this study might in fact be the first report of *P. onagawaensis* in Portugal. According to COI analysis, Sato-Okoshi et al. (2022) also concluded that *P. onagawaensis* specimens from France were genetically closer to specimens from USA, than specimens from Japan.

Henceforth, although 18S rRNA gene has been widely used for characterization of zooplankton diversity (see section 1.3), the lack of variability between taxa grouped within the same family or genus might underestimate species diversity in a community (Bucklin et al., 2016). The lack of taxonomic resolution was also observed within the COI dataset, but to a lesser extent than for 18S, as expected. COI ensures a better species-level characterization of zooplankton diversity; however, its amplification success is inconsistent in some taxonomic groups, and it is also limited by highly genetic conservative species, unusual mtDNA inheritance, hybridization (e.g., *Mytilus* spp.), and pseudogenes (Hoeh et al., 1991; Song et al., 2008; McFadden et al., 2011; Lobo et al., 2013; Śmietanka et al., 2014). DNA Metabarcoding can also be affected by the incompleteness of reference libraries (e.g., some of *Ophiothrix*, *Glycera*, *Notoplana* and *Solen* species lack sequence records; Table S4, Supplementary Material), though acknowledging the lack of species-level resolution for some taxa was also above-mentioned.

4.2.5. Detection of NIS

On one hand, a total of 4 NIS has been previously reported in the Lima River estuary – *M. arenaria*, *A. modestus*, *C. fluminea* and *A. tonsa* (Table S4, Supplementary Material) – of which only the former was recovered in the current study through DNA metabarcoding of zooplankton samples. Different reasons may have contributed to this outcome. For instance, the lack of detection of *A. modestus* may have been affected by the lack of 18S reference records (see also Fernández et al., 2018). Further, Fernández et al. (2019) also reported a possible primer-associated bias when detecting *A. modestus* using COI primers, since it generated a low number of reads even though the same DNA amount as of other species was used. Additionally, Rubal et al. (2021) only reported *A. modestus* specimens from the south margin, and sampling location might also have affected its detection with DNA metabarcoding, since the recreational marina is located in the north margin. Similarly, *C. fluminea* was only reported upstream in a more freshwater-dominated environment (Sousa, 2003; Sousa et al., 2006a,b, 2007; Sampaio, 2012; Sampaio & Rodil, 2014). In fact, such population seems to be restricted from further population growth and expansion (Sousa et al., 2006a). Due to its reported distribution throughout the estuary, it is possible that planktonic larvae of *C. fluminea* do not reach the sampling location due to poor physiological resistance to environmental conditions (McMahon, 2002; Sousa et al., 2006a; Sousa et al., 2008), and thus, it was not recovered by any of the molecular markers employed. However, bias related to PCR performance and primer selection are also a feasible explanation for the absence of *A. tonsa* from both molecular datasets. For instance, Haenel et al. (2017) used two sets of primers,

mlCOIintF/dgHCO2198 (Leray et al., 2013) and mlCOIintF/LoboR1 (also used in the present study), of which *A. tonsa* was recovered only with the former primer set (see also Rey et al., 2020a). On the other hand, Wu et al. (2015) discussed that 18S rRNA gene is capable of species-level identification within *Acartia* spp., however no species belonging to this genus was recovered in the current study, which could also be related to the sampling location.

On the other hand, 5 new putative NIS and *M. arenaria* were recovered through DNA metabarcoding in sampled zooplankton communities, in the recreational marina of Viana do Castelo; of which all have been previously reported in Portuguese mainland (Chainho et al., 2015). For instance, *E. sinensis* has been documented particularly within Minho River and Tagus River estuaries (Cigoña & Ferreira, 1996; Cabral & Costa, 1999; Coelho, 2013), *C. caspia* throughout the Portuguese coast and in several estuaries (Cancela da Fonseca et al., 1989; Servia et al., 2006; Correia et al., 2012; Conde et al., 2013; Seyer et al., 2017; Encarnação et al., 2020), and the remaining species, *B. trigonus* and *P. marinus*, have been recently reported in the Sado River estuary (ICES, 2014) and Mondego River estuary (Uttieri et al., 2020), respectively. *Amphibalanus eburneus* has been the exception, only being documented in Azores (Southward, 1998; Torres et al., 2012), but a molecular-based analysis using eDNA has also recovered *A. eburneus* reads in Portuguese mainland marinas (Lavrador et al., 2021, unpublished data). According to GenBank, the absence of Balanidae species may be related to the lack of sequence records, where only one and two 18S records of *A. eburneus* and *B. trigonus*, respectively, were found. Curiously, a low number of sequenced 18S records were found both for *P. marinus* (7 records) and *C. caspia* (1 record) in GenBank. However, it is possible to distinguish *Pseudodiaptomus* species, but no evidence was found regarding 18S suitability for discriminating *Cordylophora* spp., according to GenBank. Further, 18S appears to not be suitable for discriminating at species level within *Eriocheir* and *Mya* genera, from which it is known of being difficult to distinguish *E. sinensis* from *E. japonica* and *E. hepuensis* (Chu et al., 2003; Costa & Carvalho, 2007), and *M. arenaria* from *M. truncata* (Brown et al., 2016). However, only *E. japonica* has already been reported in Europe, but not yet in Portugal, and no European record was found of *E. hepuensis*. Although *M. truncata* has been reported in Europe, no record has been documented in Portugal.

5. Final considerations

Overall, there is still room for improvement, in what respects the protocols employed through the analytical chain of DNA metabarcoding for assessing and monitoring zooplankton communities in coastal ecosystems. However, the high diversity allied, to considerable informational gaps, related with the methodologies employed to recover zooplankton diversity via molecular-based approaches, is limiting the implementation of DNA metabarcoding for monitoring zooplankton communities in coastal ecosystems in a more regular basis. Indeed, DNA metabarcoding offers a great potential tool for a more reliable species detection within zooplankton communities, which otherwise would not be detectable through visual identification of the organisms. However, and as suggested by the review conducted in the current thesis, it is still urgent to understand how different protocols may impact the effectiveness of zooplankton species detection with DNA metabarcoding, particularly if newly introduced NIS are the main target. Additionally, the choice of the bioinformatic pipelines employed may be also a crucial step to consider when analyzing sequence data produced from complex zooplankton communities with DNA metabarcoding. For instance, previous studies have shown some evidence of the influence of raw data processing over resulting diversity, when using different settings or bioinformatic pipelines (Pitz et al., 2020; Pappalardo et al., 2021).

The present study, although not using the most trending methodologies applied through the DNA metabarcoding analytical chain found in the literature, provides a protocol that has high potential in detecting high species diversity in naturally occurring estuarine zooplankton, and potential NIS. For instance, the methodologies employed displayed a small, but relevant overlap regarding the species previously reported in the Lima River estuary (through morphology) and the recovered in the current study (through metabarcoding), in the recreational marina of Viana do Castelo, which is also located in the lower part of the estuary. In fact, the sampled location of the current study is connected with the Lima River estuary through only one opening, which allied to the patchy nature of zooplankton, may be a justification more than acceptable for the differences found. However, employed primer pairs may require improvement for better diversity detection and resolution. For instance, a multi-marker approach, associated with the employment of multiple group-specific primers, may improve the aforesaid limitations (Zhang et al., 2018; Questel et al., 2021; Govender et al., 2019, 2022a; e.g., Schroeder et al., 2021; Govender et al., 2022b) by overcoming the primer-related bias, and increasing the changes of detecting taxonomic groups that may display less affinity with universal primers. Nonetheless, the present study

further supports DNA metabarcoding as a great tool for species identification, allowing further insight of the planktonic metazoan diversity, and imperative detection of newly introduced and not yet documented NIS in coastal ecosystems, that might be overseen by traditional methods of identification.

Nevertheless, DNA metabarcoding allowed the identification of a broader diversity within the recreational marina, compared to previous studies that used traditional methods of identification of zooplanktonic organisms in the Lima River estuary, since the latter appear to have been mostly limited to the identification of mero- and tychoplankton species. Additionally, recovered diversity also allowed to uncover several group-specific seasonal patterns which were not yet documented in the estuary. Therefore, implementing DNA metabarcoding in monitoring programs may provide further information of ecological dynamics throughout coastal ecosystems, promising a great potential of its use in conservation and monitoring programs. For instance, continuous molecular-based monitoring of zooplankton communities – e.g., either monthly or seasonally – may provide data on communities' dynamics and responses to direct or indirect anthropogenic activities. The results from the current study further supports the use of DNA metabarcoding for the early detection of NIS in coastal ecosystems, through zooplankton communities. Five new putative NIS were recovered in zooplankton samples, which are suspected to have been introduced either by incrustation or by ballast waters from recreational vessels or commercial ships. Additionally, DNA metabarcoding further confirmed *M. arenaria* occurrence in zooplankton samples, whose occurrence in Portugal, Conde et al. (2012) suspects to have been intentionally introduced. For instance, DNA metabarcoding detected *E. sinensis*, included in the 100 most invasive NIS in the world and Europe (Lowe et al., 2000; DAISIE, 2008), previously documented in two basins in Portugal. Additionally, two of the three remaining NIS included in the list of the worst invasive species in Europe, *C. caspia* and *M. arenaria* (DAISIE, 2008) were also detected in the current study. Only the latter has been previously reported in the Lima River estuary.

Standardization of DNA metabarcoding protocols is a critical step towards the development of invertebrate NIS monitoring programs targeting zooplankton communities. Prior to the deployment of such programs, based on DNA metabarcoding, a number of potential enhancements should be taken into consideration for the creation of a molecular-based protocol: i) Since some species seemed to be underrepresented in the current study, beyond the use of different marker genes, the use of more than one primer pair or primers cocktails targeting the same marker, may also improve species detection. ii) Although species representation by sequence records on BOLD and GenBank were herein acceptable for the compiled lists of species, the generation of local reference sequences libraries may further improve

the detection of species, has been shown in studies conducted in other geographic locations (Abad et al., 2016; Yang et al., 2017). iii) Similarly, the development of a regional or local NIS database, since these species may actually be underrepresented in available international NIS databases, such as DAISIE, AquaNIS and EASIN. For instance, Chainho et al. (2015) reported that Portugal is one of the European countries with the least NIS reports, which may be a result of a lack of available online NIS databases specific to Portugal. iv) Extensive spatial sampling allied to the generation of a high density of temporal data (monthly or bimonthly) may also enable a better spatial and seasonal resolution within the high environmental heterogeneity that may be experienced in coastal ecosystems, more particularly in estuarine environments. v) Finally, comparison of DNA metabarcoding data with species occurrence lists is essential for the evaluation of the efficiency of the DNA-based methodology, although some discordances are expected, as well as between molecular markers, which may require further in-depth morphological analysis or more targeted molecular approaches (e.g., qPCR, ddPCR).

Bibliography

Abad, D., Albaina, A., Aguirre, M., & Estonba, A. (2017). 18S V9 metabarcoding correctly depicts plankton estuarine community drivers. *Marine Ecology Progress Series*, *584*, 31–43. DOI: 10.3354/meps12373

Abad, D., Albaina, A., Aguirre, M., Laza-Martínez, A., Uriarte, I., Iriarte, A., Villate, F., & Estonba, A. (2016). Is metabarcoding suitable for estuarine plankton monitoring? A comparative study with microscopy. *Marine Biology*, *163*(7), 1–13. DOI: 10.1007/s00227-016-2920-0

Abe, H., Sato-Okoshi, W., Nishitani, G., & Endo, Y. (2014). Vertical distribution and migration of planktonic polychaete larvae in Onagawa. *Memoirs of Museum Victoria*, *71*, 1–9.

Abe, H., Takeuchi, T., Taru, M., Sato-Okoshi, W., & Kenji Okoshi. (2019). Habitat availability determines distribution patterns of spionid polychaetes (Annelida: Spionidae) around Tokyo Bay. *Marine Biodiversity Records*, *12*(1), 1–12. DOI: 10.1186/s41200-019-0167-4

Alcaraz, M., & Calbet, A. (2003). Zooplankton Ecology. In C. E. Duarte & A. L. Helgueras (Eds.), *Marine Ecology* (United Nat, pp. 295–318). EOLS. DOI: 10.1201/9781351021821

Allali, I., Arnold, J. W., Roach, J., Cadenas, M. B., Butz, N., Hassan, H. M., Koci, M., Ballou, A., Mendoza, M., Ali, R., & Azcarate-Peril, M. A. (2017). A comparison of sequencing platforms and bioinformatics pipelines for compositional analysis of the gut microbiome. *BMC Microbiology*, *17*(1), 1–16. DOI: 10.1186/s12866-017-1101-8

Allen, E. B., Steers, R. J., & Dickens, S. J. (2011). Impacts of fire and invasive species on desert soil ecology. *Rangeland Ecology and Management*, *64*(5), 450–462. doi: 10.2111/REM-D-09-00159.1

Alongi, D. M. (2016). Coastal Ecosystem Processes. In *The Wetland Book*. DOI: 10.1007/978-94-007-6172-8_68-1

Alves, A. M. C. (2003). O estuário do rio Lima: pressão antrópica e caracterização ambiental. *Ciências Da Tierra (UNL)*, *5*, H5–H9.

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*(7), 1–9. DOI: 10.1371/journal.pone.0006372

Andújar, C., Arribas, P., Yu, D. W., Vogler, A. P., & Emerson, B. C. (2018). Why the COI barcode should be the community DNA metabarcode for the metazoa. *Molecular Ecology*, *27*(20), 3968–3975. DOI: 10.1111/mec.14844

Angeler, D. G., Álvarez-Cobelas, M., Sánchez-Carrillo, S., & Rodrigo, M. A. (2002). Assessment of exotic fish impacts on water quality and zooplankton in a degraded semi-arid floodplain wetland. *Aquatic Sciences*, *64*(1), 76–86. DOI: 10.1007/s00027-002-8056-y

Arnot, D. E., Roper, C., & Bayoumi, R. A. L. (1993). Digital codes from hypervariable tandemly repeated DNA sequences in the Plasmodium falciparum circumsporozoite gene can genetically barcode isolates. *Molecular and Biochemical Parasitology*, *61*(1), 15–24. DOI: 10.1016/0166-6851(93)90154-P

Azevedo, I., Ramos, S., Mucha, A. P., & Bordalo, A. A. (2013). Applicability of ecological assessment tools for management decision-making: A case study from the Lima estuary (NW Portugal). *Ocean and Coastal Management*, *72*, 54–63. DOI: 10.1016/j.ocecoaman.2011.08.008

Baek, S. Y., Jang, K. H., Choi, E. H., Ryu, S. H., Kim, S. K., Lee, J. H., Lim, Y. J., Lee, J., Jun, J., Kwak, M., Lee, Y. S., Hwang, J. S., Maran, B. A. V., Chang, C. Y., Kim, I. H., & Hwang, U. W. (2016). DNA barcoding of metazoan zooplankton copepods from South Korea. *PLoS ONE*, *11*(7), 1–20. DOI: 10.1371/journal.pone.0157307

Bell, K. L., Burgess, K. S., Okamoto, K. C., Aranda, R., & Brosi, B. J. (2016). Review and future prospects for DNA barcoding methods in forensic palynology. *Forensic Science International: Genetics*, *21*, 110–116. DOI: 10.1016/j.fsigen.2015.12.010

Bernard, M. S., Tonk, L., de Groot, G. A., Glorius, S., & Jansen, H. M. (2019). Biodiversity monitoring in seaweed farms by DNA metabarcoding using settlement plates and water samples. DOI: 10.18174/496237

Blanco-Bercial, L. (2020). Metabarcoding Analyses and Seasonality of the Zooplankton Community at BATS. *Frontiers in Marine Science*, *7*(March), 1–16. DOI: 10.3389/fmars.2020.00173

Blanco-Bercial, L., Cornils, A., Copley, N. J., & Bucklin, A. (2014). DNA barcoding of marine copepods: assessment of analytical approaches to species identification. *PLoS Currents*, 6. DOI: 10.1371/currents.tol.cdf8b74881f87e3b01d56b43791626d2

Boyer, J. N., Kelble, C. R., Ortner, P. B., & Rudnick, D. T. (2009). Phytoplankton bloom status: Chlorophyll a biomass as an indicator of water quality condition in the southern estuaries of Florida, USA. *Ecological Indicators*, *9*(6 SUPPL.), 56–67. DOI: 10.1016/j.ecolind.2008.11.013

Brandão, M. C., Comtet, T., Pouline, P., Cailliau, C., Blanchet-Aurigny, A., Sourisseau, M., Siano, R., Memery, L., Viard, F., & Nunes, F. (2021). Oceanographic structure and seasonal variation contribute to high heterogeneity in mesozooplankton over small spatial scales. *ICES Journal of Marine Science*, *78*(9), 3288–3302. DOI: /10.1093/icesjms/fsab127

Braukmann, T. W. A., Ivanova, N. V., Prosser, S. W. J., Elbrecht, V., Steinke, D., Ratnasingham, S., de Waard, J. R., Sones, J. E., Zakharov, E. V., & Hebert, P. D. N. (2019). Metabarcoding a diverse arthropod mock community. *Molecular Ecology Resources*, *19*(3), 711–727. DOI: 10.1111/1755-0998.13008

Bricker, S. B., Ferreira, J. G., & Simas, T. (2003). An integrated methodology for assessment of estuarine trophic status. *Ecological Modelling*, *169*(1), 39–60. DOI: 10.1016/S0304-3800(03)00199-6

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? *Ecology and Evolution*, *5*(11), 2234–2251. DOI: 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. *Diversity and Distributions*, *22*(10), 1045–1059. DOI: 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. *Journal of Plankton Research*, *38*(3), 393–400. DOI: 10.1093/plankt/fbw023

Bucklin, A., Peijnenburg, K. T. C. A., Kosobokova, K. N., O'Brien, T. D., Blanco-Bercial, L., Cornils, A., Falkenhaug, T., Hopcroft, R. R., Hosia, A., Laakmann, S., Li, C., Martell, L., Questel, J. M., Wall-Palmer, D., Wang, M., Wiebe, P. H., & Weydmann-Zwolicka, A. (2021). Toward a global reference database of COI barcodes for marine zooplankton. *Marine Biology*, *168*(6), 1–26. DOI: 10.1007/s00227-021-03887-y

Bucklin, A., Yeh, H. D., Questel, J. M., Richardson, D. E., Reese, B., Copley, N. J., & Wiebe, P. H. (2019). Time-series metabarcoding analysis of zooplankton diversity of the NW Atlantic continental shelf. *ICES Journal of Marine Science*, *76*(4), 1162–1176. DOI: 10.1093/icesjms/fsz021

Burke, L., Kura, Y., Kassem, K., Revenga, C., Spalding, M., & McAllister, D. (2001). Coastal ecosystems. In *Encyclopedia of Earth Sciences Series*. Washington, DC: World Resources Institute. DOI: 10.1007/978-0-387-36699-9_19

Cabral, H. N., & Costa, M. J. (1999). On the occurrence of the Chinese mitten crab, *Eriocheir sinensis*, in Portugal (Decapoda, Brachyura). *Crustaceana*, *72*(1), 55–58. DOI: 10.1163/156854099502853

Cabrita, M. T., Brito, P., Caçador, I., & Duarte, B. (2020). Impacts of phytoplankton blooms on trace metal recycling and bioavailability during dredging events in the Sado estuary (Portugal). *Marine Environmental Research*, *153*(October 2019), 104837. DOI: 10.1016/j.marenvres.2019.104837

Cancela da Fonseca, L., Costa, A. M., & Bernardo, J. M. (1989). Seasonal variation of benthic and fish communities in a shallow land-locked coastal lagoon (St. André, SW Portugal). *Scientia Marina*, *53*(2–3), 663–669.

Capra, E., Giannico, R., Montagna, M., Turri, F., Cremonesi, P., Strozzi, F., Leone, P., Gandini, G., & Pizzi, F. (2016). A new primer set for DNA metabarcoding of soil Metazoa. *European Journal of Soil Biology*, *77*, 53–59. DOI: 10.1016/j.ejsobi.2016.10.005

Carroll, E. L., Gallego, R., Sewell, M. A., Zeldis, J., Ranjard, L., Ross, H. A., Tooman, L. K., O'Rorke, R., Newcomb, R. D., & Constantine, R. (2019). Multi-locus DNA metabarcoding of zooplankton communities and scat reveal trophic interactions of a generalist predator. *Scientific Reports*, *9*(1), 1–14. DOI: 10.1038/s41598-018-36478-x

Casas, L., Pearman, J. K., & Irigoien, X. (2017). Metabarcoding reveals seasonal and temperature-dependent succession of zooplankton communities in the red sea. *Frontiers in Marine Science*, *4*(AUG). DOI: 10.3389/fmars.2017.00241

Castro, L. R., Meyer, R. S., Shapiro, B., Shirazi, S., Cutler, S., Lagos, A. M., & Quiroga, S. Y. (2021). Metabarcoding meiofauna biodiversity assessment in four beaches of Northern Colombia: effects of sampling protocols and primer choice. *Hydrobiologia*, *848*(15), 3407–3426. DOI: 10.1007/s10750-021-04576-z

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. *Diversity and Distributions*, *22*(5), 493–504. DOI: 10.1111/ddi.12427

Chainho, P., Fernandes, A., Amorim, A., Ávila, S. P., Canning-Clode, J., Castro, J. J., Costa, A. C., Costa, J. L., Cruz, T., Gollasch, S., Grazziotin-Soares, C., Melo, R., Micael, J., Parente, M. I., Semedo, J., Silva, T., Sobral, D., Sousa, M., Torres, P., Veloso, V., & Costa, M. J. (2015). Non-indigenous species in Portuguese coastal areas, coastal lagoons, estuaries and islands. *Estuarine, Coastal and Shelf Science*, *167*, 199–211. DOI: 10.1016/j.ecss.2015.06.019

Charles, H., & Dukes, J. S. (2008). Impacts of Invasive Species on Ecosystem Services. *Springer*, *193*, 217–237. DOI: 10.1007/978-3-540-36920-2_13

Chu, K. H., Ho, H. Y., Li, C. P., & Chan, T.-Y. (2003). Molecular phylogenetics of the mitten crab species in *Eriocheir*, *sensu lato* (Brachyura: Grapsidae). *Journal of Crustacean Biology*, *23*(3), 738–746.

Cicala, F., Arteaga, M. C., Herzka, S. Z., Hereu, C. M., Jimenez-Rosenberg, S. P. A., Saavedra-Flores, A., Robles-Flores, J., Gomez, R., Batta-Lona, P. G., & Galindo-Sánchez, C. E. (2021). Environmental conditions drive zooplankton community structure in the epipelagic oceanic water of the southern Gulf of Mexico: A molecular approach. *Molecular Ecology*, *31*(2), 546–561. DOI: 10.1111/mec.16251

Cigoña, E. F., & Ferreira, S. (1996). Tres Crustáceos del Bajo Miño: el carangrejo chino *Eriocheir sinensis*; el carangrejo de río Ibérico *Austrapotamobius pallipes* y el carangrejo de río Americano *Procambarus clarkii*. *Actas Do I Simpósio Ibérico Sobre a Bacia Hidrográfica Do Rio Minho*.

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. *Ecology and Evolution*, *7*(3), 873–883. DOI: 10.1002/ece3.2667

Clavero, M., Brotons, L., Pons, P., & Sol, D. (2009). Prominent role of invasive species in avian biodiversity loss. *Biological Conservation*, *142*(10), 2043–2049. DOI: 10.1016/j.biocon.2009.03.034

CMarZ, Census of Marine Zooplankton (2011). Marine Zooplankton Groups. Weblink: www.cmarz.org/ (Accessed at 08/05/2022)

Coelho, A. F. S. (2013). Distribuição e abundância da espécie exótica *Eriocheir sinensis* no estuário do Tejo [Master's thesis, University of Évora]. ProQuest Dissertations and Theses Global.

Coguiec, E., Ershova, E. A., Daase, M., Vonnahme, T. R., Wangensteen, O. S., Gradinger, R., Præbel, K., & Berge, J. (2021). Seasonal Variability in the Zooplankton Community Structure in a Sub-Arctic Fjord as Revealed by Morphological and Molecular Approaches. *Frontiers in Marine Science*, *8*, 1–26. DOI: 10.3389/fmars.2021.705042

Comeau, A. M., Douglas, G. M., & Langille, M. G. I. (2017). Microbiome Helper : a custom and streamlined workflow for microbiome research. *MSystems*, *2*(1), 1–11. DOI: 10.1128/mSystems.00127-16

Conde, A., Aira, M., Novais, J. M., & Domínguez, J. (2011). On an early record of the alien clam *Mya arenaria* in the Iberian Peninsula and its likely confusion with *Scrobicularia plana* (Bivalvia). *Vie et Milieu*, *61*(3), 151–157.

Conde, A., Novais, J. M., & Domínguez, J. (2012). The presence of *Mya arenaria* in the Ria de Aveiro is

the third confirmed record of this invasive clam on the Portuguese coast. *ANZIAM Journal*, *5*(3), 1–6. DOI: 10.1017/S1755267212000784

Conde, Anxo, Domínguez, J., Novais, J. M., & Ramil, F. (2013). First record of *Cordylophora caspia* (Hydrozoa: Cnidaria) in the Tagus estuary, central Portugal. *Marine Biodiversity Records*, *6*, 1–6. DOI: 10.1017/S1755267213000833

Copp, G. H., Bianco, P. G., Bogutskaya, N. G., Erös, T., Falka, I., Ferreira, M. T., Fox, M. G., Freyhof, J., Gozlan, R. E., Grabowska, J., Kováč, V., Moreno-Amich, R., Naseka, A. M., Peňáz, M., Povž, M., Przybylski, M., Robillard, M., Russell, I. C., Stakenas, S., Šumer, S., Vila-Gispert, A., & Wiesner, C. (2005). To be, or not to be, a non-native freshwater fish? *Journal of Applied Ichthyology*, *21*(4), 242–262. DOI: 10.1111/j.1439-0426.2005.00690.x

Correia, M. J., Costa, J. L., Chainho, P., Félix, P. M., Chaves, M. L., Medeiros, J. P., Silva, G., Azeda, C., Tavares, P., Costa, A., Costa, A. M., Bernardo, J., Cabral, H. N., Costa, M. J., & Cancela da Fonseca, L. (2012). Inter-annual variations of macrobenthic communities over three decades in a land-locked coastal lagoon (Santo André, SW Portugal). *Estuarine, Coastal and Shelf Science*, *110*, 168–175. DOI: 10.1016/j.ecss.2012.04.028

Cortes, R. M. V., Ferreira, M. T., Oliveira, S. V., & Oliveira, D. (2002). Macroinvertebrate community structure in a regulated river segment with different flow conditions. *River Research and Applications*, *18*(4), 367–382. DOI: 10.1002/rra.679

Costa, F. O., & Carvalho, G. R. (2007). The Barcode of Life Initiative: Reply to Dupré, Hollingsworth and Holm. *Genomics, Society and Policy*, *3*(2), 29–40. DOI: 10.1186/1746-5354-3-2-52

Costa, F. O., Henzler, C. M., Lunt, D. H., Whiteley, N. M., & Rock, J. (2009). Probing marine Gammarus (Amphipoda) taxonomy with DNA barcodes. *Systematics and Biodiversity*, *7*(4), 365–379. DOI: 10.1017/S1477200009990120

Costa, P. S. (2012). Marinas , portos , docas e núcleos de recreio [Master's thesis, University of Porto]. University of Porto repositorium.

Couton, M., Comtet, T., Le Cam, S., Corre, E., & Viard, F. (2019). Metabarcoding on planktonic larval stages: An efficient approach for detecting and investigating life cycle dynamics of benthic aliens. *Management of Biological Invasions*, *10*(4), 657–689. DOI: 10.3391/mbi.2019.10.4.06

Crooks, J., & Soulé, M. E. (1999). Invasive Species and Biodiversity Management. *Invasive Species and Biodiversity Management*, *October*. DOI: 10.1007/978-94-011-4523-7

DAISIE. (2008). Handbook of Alien Species in Europe. In J. A. Drake (Ed.), *Springer* (Vol. 3). DOI: 10.1038/208008a0

Deagle, B. E., Clarke, L. J., Kitchener, J. A., Polanowski, A. M., & Davidson, A. T. (2018). Genetic monitoring of open ocean biodiversity: An evaluation of DNA metabarcoding for processing continuous plankton recorder samples. *Molecular Ecology Resources*, *18*(3), 391–406. DOI: 10.1111/1755-0998.12740

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. *Biology Letters*, *10*(9), 2–5. DOI: 10.1098/rsbl.2014.0562

Deiner, K., Lopez, J., Bourne, S., Holman, L. E., Seymour, M., Grey, E. K., Lacoursière-Roussel, A., Li, Y., Renshaw, M. A., Pfrender, M. E., Rius, M., Bernatchez, L., & Lodge, D. M. (2018). Optimising the detection of marine taxonomic richness using environmental DNA metabarcoding: The effects of filter material, pore size and extraction method. *Metabarcoding and Metagenomics*, *2*. DOI: 10.3897/mbmg.2.28963

Dell'Anno, A., Carugati, L., Corinaldesi, C., Riccioni, G., & Danovaro, R. (2015). Unveiling the biodiversity of deep-sea nematodes through metabarcoding: Are we ready to bypass the classical taxonomy? *PLoS ONE*, *10*(12), 1–18. DOI: 10.1371/journal.pone.0144928

Deschutter, Y., Vergara, G., Mortelmans, J., Deneudt, K., De Schamphelaere, K., & De Troch, M. (2018). Distribution of the invasive calanoid copepod *Pseudodiaptomus marinus* (Sato, 1913) in the Belgian part of the North Sea. *BioInvasions Records*, *7*(1), 33–41. DOI: 10.3391/bir.2018.7.1.05

Devreker, D., Souissi, S., Molinero, J. C., & Nkubito, F. (2008). Trade-offs of the copepod *Eurytemora affinis* in mega-tidal estuaries: Insights from high frequency sampling in the Seine estuary. *Journal of Plankton Research*, *30*(12), 1329–1342. DOI: 10.1093/plankt/fbn086

Devriese, L., Haegeman, A., Maes, S., Ruttink, T., De Backer, A., Van Hoey, G., Wittoeck, J., Hillewaert, H., De Tender, C., & Hostens, K. (2016). A DNA (meta)barcoding approach to tackle marine benthic biodiverisity. *North Sea Open Science Conference*.

Di Capua, I., Piredda, R., Mazzocchi, M. G., & Zingone, A. (2021). Metazoan diversity and seasonality through eDNA metabarcoding at a Mediterranean long-term ecological research site. *ICES Journal of Marine Science*, *78*(9), 3303–3316. DOI: 10.1093/icesjms/fsab059

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. *Limnology and Oceanography: Methods*, *16*(4), 209–221. DOI: 10.1002/lom3.10237

Duarte, S., Leite, B. R., Feio, M. J., Costa, F. O., & Filipe, A. F. (2021a). Integration of DNA-based approaches in aquatic ecological assessment using benthic macroinvertebrates. *Water (Switzerland)*, *13*(3), 1–25. DOI: 10.3390/w13030331

Duarte, S., Vieira, P. E., & Costa, F. O. (2020). Assessment of species gaps in DNA barcode libraries of nonindigenous species (NIS) occurring in European coastal regions. *Metabarcoding and Metagenomics*, *4*, 35–46. DOI: 10.3897/mbmg.4.55162

Duarte, S., Vieira, P. E., Lavrador, A. S., & Costa, F. O. (2021b). Status and prospects of marine NIS detection and monitoring through (e)DNA metabarcoding. *Science of the Total Environment*, *751*. DOI: 10.1016/j.scitotenv.2020.141729

Duggan, S., McKinnon, A. D., & Carleton, J. H. (2008). Zooplankton in an Australian tropical estuary. *Estuaries and Coasts*, *31*(2), 455–467. DOI: 10.1007/s12237-007-9011-x

Dukes, J. S., & Mooney, H. A. (1999). Does global change increase the success of biological invaders? *Trends in Ecology and Evolution*, *14*(4), 135–139.

Encarnação, J., Seyer, T., Teodósio, M. A., & Leitão, F. (2020). First Record of the Nudibranch *Tenellia*

adspersa. *Diversity*, *12*(24), 1–7.

Engelbrektson, A., Kunin, V., Wrighton, K. C., Zvenigorodsky, N., Chen, F., Ochman, H., & Hugenholtz, P. (2010). Experimental factors affecting PCR-based estimates of microbial species richness and evenness. *ISME Journal*, *4*(5), 642–647. DOI: 10.1038/ismej.2009.153

Ershova, E. A., Wangensteen, O. S., Descoteaux, R., Barth-Jensen, C., & Præbel, K. (2021). Metabarcoding as a quantitative tool for estimating biodiversity and relative biomass of marine zooplankton. *ICES Journal of Marine Science*, *78*(9), 3342–3355. DOI: 10.1093/icesjms/fsab171

European Commission. (2008). Directive 2008/56/EC of the European Parliament and of the Council Establishing a Framework for Community Action in the Field of Marine Environmental Policy (Marine Strategy Framework Directive). Weblink: environment.ec.europa.eu/

European Environmental Agency (EEA). (2012). Invasive alien species indicators in Europe. A review of streamlining European biodiversity (SEBI) Indicator 10. *EEA Technical Report No. 15/2012*.

Facca, C. (2020). Ecological status assessment of transitional waters. *Water (Switzerland)*, *12*(11), 1–5. DOI: 10.3390/w12113159

Fais, M., Duarte, S., Vieira, P. E., Sousa, R., Hajibabaei, M., Canchaya, C. A., & Costa, F. O. (2020). Small-scale spatial variation of meiofaunal communities in Lima estuary (NW Portugal) assessed through metabarcoding. *Estuarine, Coastal and Shelf Science*, *238*(March), 106683. DOI: 10.1016/j.ecss.2020.106683

Fernández, S., Rodríguez-Martínez, S., Martínez, J. L., Garcia-Vazquez, E., & Ardura, A. (2019). How can eDNA contribute in riverine macroinvertebrate assessment? A metabarcoding approach in the Nalón River (Asturias, Northern Spain). *Environmental DNA*, *1*(4), 385–401. DOI: 10.1002/edn3.40

Fernández, S., Rodríguez, S., Martínez, J. L., Borrell, Y. J., Ardura, A., & García-Vázquez, E. (2018). Evaluating freshwater macroinvertebrates from eDNA metabarcoding : A river Nalón case study. *PLoS ONE*, *3*(8), 1–17.

Ferreira-Rodríguez, N., Sousa, R., & Pardo, I. (2018). Negative effects of *Corbicula fluminea* over native freshwater mussels. *Hydrobiologia*, *810*(1), 85–95. DOI: 10.1007/s10750-016-3059-1

Fidalgo, M. L., & Gerhardt, A. (2002). Distribution of the freshwater shrimp, *Atyaephyra desmarestii* (Millet, 1831) in Portugal (Decapoda, Natantia). *Crustaceana*, *75*(11), 1375–1385. DOI: 10.1163/156854002321629808

Fiege, D., Licher, F., & Mackie, A. S. Y. (2000). A partial review of the European Magelonidae (Annelida: Polychaeta): *Magelona mirabilis* redefined and *M. johnstoni* sp. nov. distinguished. *Journal of Marine Biological Association of the United Kingdom*, *80*, 215–234.

Floyd, R., Abebe, E., Papert, A., & Blaxter, M. (2002). Molecular barcodes for soil nematode identification. *Molecular Ecology*, *11*(4), 839–850. DOI: 10.1046/j.1365-294X.2002.01485.x

Fontes, J. T., Vieira, P. E., Ekrem, T., Soares, P., & Costa, F. O. (2021). BAGS : An automated Barcode, Audit & Grade System for DNA barcode reference libraries. *Molecular Ecology*, *21*, 573–583. DOI: 10.1111/1755-0998.13262

Fontoura, A. P. (1984). Les communautés de macro-invertebrés du bassin hydrographique du fleuve Lima comme indicateurs de la qualité biologique de l'eua. Zoology Institute "Dr. Augusto Nobre" Sciences Faculty of Porto.

Fontoura, A. P., & Moura, A. M. G. (1984). Effects of some industrial effluents in the biological quality of the water of the river Lima. Zoology Institute "Dr. Augusto Nobre" Sciences Faculty of Porto.

Freeman, M. A., Anshary, H., & Ogawa, K. (2013). Multiple gene analyses of caligid copepods indicate that the reduction of a thoracic appendage in Pseudocaligus represents convergent evolution. *Parasites and Vectors*, *6*(1), 1–9. DOI: 10.1186/1756-3305-6-336

Gaither, M. R., Szabó, Z., Crepeau, M. W., Bird, C. E., & Toonen, R. J. (2011). Preservation of corals in salt-saturated DMSO buffer is superior to ethanol for PCR experiments. *Coral Reefs*, *30*(2), 329–333. DOI: 10.1007/s00338-010-0687-1

Gajbhiye, S. N. (2002). Zooplankton study methods, importance and significant observations. In *Proceedings of the National Seminar on Creeks, Estuaries and Mangroves - Pollution and Conservation* (pp. 21–27).

Galimberti, A., De Mattia, F., Losa, A., Bruni, I., Federici, S., Casiraghi, M., Martellos, S., & Labra, M. (2013). DNA barcoding as a new tool for food traceability. *Food Research International*, *50*(1), 55–63. DOI: 10.1016/j.foodres.2012.09.036

Gallardo, B., Clavero, M., Sánchez, M. I., & Vilà, M. (2016). Global ecological impacts of invasive species in aquatic ecosystems. *Global Change Biology*, *22*(1), 151–163. DOI: 10.1111/gcb.13004

Gannon, J. E., & Stemberger, R. S. (1978). Zooplankton (especially Crustaceans and Rotifers) as indicators of water quality. *Transactions of the American Microscopical Society*, *97*(1), 16–35.

Geller, J., Meyer, C., Parker, M., & Hawk, H. (2013). Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Resources*, *13*(5), 851–861. DOI: 10.1111/1755-0998.12138

Gerhardt, A. (2002). Bioindicator Species and Their Use in Biomonitoring. In H. I. Inyang & J. L. Daniels (Eds.), *Environmental Monitoring* (pp. 77–123). United Nations Educational, Scientific and Cultural Organization.

Giusti, A., Tosi, F., Tinacci, L., Guardone, L., Corti, I., Arcangeli, G., & Armani, A. (2020). Mussels (*Mytilus* spp.) products authentication: A case study on the Italian market confirms issues in species identification and arises concern on commercial names attribution. *Food Control*, *118*(May), 107379. DOI: 10.1016/j.foodcont.2020.107379

González-Dávila, M. (1995). The role of phytoplankton cells on the control of heavy metal concentration in seawater. *Marine Chemistry*, *48*(3–4), 215–236. DOI: 10.1016/0304-4203(94)00045-F

Goswami, S. C. (2004). Zooplankton Methodology , Collection & Identification - a field manual (V. K. Dhargalkar & X. N. Verlecar (eds.); First Ed..

Gouy, M., & Li, W. H. (1989). Molecular phylogeny of the kingdoms Animalia, Plantae, and Fungi. *Molecular Biology and Evolution*, *6*(2), 109–122. DOI: 10.1093/oxfordjournals.molbev.a040536

Govender, A., Groeneveld, J., Singh, S., & Willows-Munro, S. (2019). The design and testing of mini-barcode markers in marine lobsters. *PLoS ONE*, *14*(1), 1–11. DOI: 10.1371/journal.pone.0210492

Govender, A., Singh, S., Groeneveld, J., Pillay, S., & Willows-Munro, S. (2022a). Experimental validation of taxon-specific mini-barcode primers for metabarcoding of zooplankton. *Ecological Applications*, *32*(1), 1–15. DOI: 10.1002/eap.2469

Govender, A., Singh, S., Groeneveld, J., Pillay, S., & Willows-Munro, S. (2022b). Metabarcoding analysis of marine zooplankton confirms the ecological role of a sheltered bight along an exposed continental shelf. *Molecular Ecology*, *December 2021*, 1–13. DOI: 10.1111/mec.16567

Griffiths, H. J. (2010). Antarctic marine biodiversity - what do we know about the distribution of life in the southern ocean? *PLoS ONE*, *5*(8). DOI: 10.1371/journal.pone.0011683

Gruber, N. L. S., Barboza, E. G., & Nicolodi, J. L. (2003). Geografia dos Sistemas Costeiros e Oceanográficos: Subsídios para Gestão Integrada da Zona Costeira. *Gravel*, *1*, 81–89.

Guimarães, C., & Galhano, H. (1987). Ecological study of the estuary of River Lima (Portugal): I - The north bank saltmarshes. *Publ. Inst. Zool. Dr. Augusto Nobre*, *199*, 1–54.

Guimarães, C., & Galhano, H. (1988). Ecological study of the estuary of River Lima (Portugal): II - A mud-sandybeach. *Publicações Do Instituito de Zoologia "Dr. Augusto Nobre" Faculdade de Ciências Do Porto*, *205*, 1–73.

Guimarães, C., & Galhano, H. (1989). Ecological study of the estuary of River Lima (Portugal): III - Channels of Darque. *Publicações Do Instituito de Zoologia "Dr. Augusto Nobre" Faculdade de Ciências Do Porto*, *206*, 1–52.

Guo, Z., Wang, Z., & Hou, X. (2021). Comparative analysis of the nrDNA repeat unit of manila clam *Ruditapes philippinarum* and quahog *Mercenaria mercenaria*. *Fishes*, *6*(3). DOI: 10.3390/fishes6030042

Gutierre, S. M. M. (2012). pH tolerance of the biofouling invasive hydrozoan *Cordylophora caspia*. *Hydrobiologia*, *679*(1), 91–95. DOI: 10.1007/s10750-011-0855-5

Haenel, Q., Holovachov, O., Jondelius, U., Sundberg, P., & Bourlat, S. J. (2017). NGS-based biodiversity and community structure analysis of meiofaunal eukaryotes in shell sand from Hållö island, Smögen, and soft mud from Gullmarn Fjord, Sweden. *Biodiversity Data Journal*, *5*(1). DOI: 10.3897/BDJ.5.e12731

Hajibabaei, M., Shokralla, S., Zhou, X., Singer, G. A. C., & Baird, D. J. (2011). Environmental Barcoding: A Next-Generation Sequencing Approach for Biomonitoring Applications Using River Benthos. *PLoS ONE*, *6*(4), 17497. DOI: 10.1371/journal.pone.0017497

Hammer, Ø., Harper, D. A. . T., & Ryan, P. D. (2001). PAST : Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, *4*(1), 9.

Harris, D. J., Rosado, D., & Xavier, R. (2016). DNA Barcoding Reveals Extensive Mislabeling in Seafood Sold in Portuguese Supermarkets. *Journal of Aquatic Food Product Technology*, *25*(8), 1375–1380. DOI: 10.1080/10498850.2015.1067267

Harvey, C. T., Qureshi, S. A., & MacIsaac, H. J. (2009). Detection of a colonizing, aquatic, non-indigenous species. *Diversity and Distributions*, *15*(3), 429–437. DOI: 10.1111/j.1472-4642.2008.00550.x

Harvey, J. B. J., Fisher, J. L., Ryan, J. P., Johnson, S. B., Peterson, W. T., & Vrijenhoek, R. C. (2018). Changes in zooplankton assemblages in northern Monterey Bay, California, during a fall transition. *Marine Ecology Progress Series*, *604*, 99–120. DOI: 10.3354/meps12742

Hays, G. C. (2003). A review of the adaptive significance and ecosystem consequences of zooplankton diel vertical migrations. *Hydrobiologia*, *503*, 163–170. DOI: 10.1023/B:HYDR.0000008476.23617.b0

Hayut, Y. (1981). Containerization and the Load Center Concept. *Economic Geography*, *57*(2), 160–176. DOI: 10.1126/science.ns-15.362.18

Heberle, H., Meirelles, G. V., Silva, F. R., Telles, G. P., & Minghim, R. (2015). InteractiVenn : a web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics*, *16*(1), 7. DOI: 10.1186/s12859-015-0611-3

Hebert, P. D. N., Cywinska, A., Ball, S. L., & DeWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, *270*(1512), 313–321. DOI: 10.1098/rspb.2002.2218

Hebert, P. D. N., Ratnasingham, S., & DeWaard, J. R. (2003). Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society B: Biological Sciences*, *270*(SUPPL. 1), 96–99. DOI: 10.1098/rsbl.2003.0025

Hellawell, J. M. (1986). *Biological Indicatiors of Freshwaters Pollution and Environmental Managment* (K. Mellanby (ed.)). Elsevier Applied Science London and New York.

Hempel, G., & Weikert, H. (1972). The neuston of the subtropical and boreal North-eastern Atlantic Ocean. A review. *Marine Biology*, *13*(1), 70–88. DOI: 10.1007/BF00351141

Hirai, J., Yamazaki, K., Hidaka, K., Nagai, S., Shimizu, Y., & Ichikawa, T. (2021). Characterization of diversity and community structure of small planktonic copepods in the kuroshio region off Japan using a metabarcoding approach. *Marine Ecology Progress Series*, *657*(Turner 2004), 25–41. DOI: 10.3354/meps13539

Hoeh, W. R., Blakley, K. H., & Brown, W. M. (1991). Heteroplasmy suggests limited biparental inheritance of *Mytilus* mitochondrial DNA. *Science*, *251*(5000), 1488–1490. DOI: 10.1126/science.1672472

Hoffman, J. C., Kelly, J. R., Trebitz, A. S., Peterson, G. S., & West, C. W. (2011). Effort and potential efficiencies for aquatic nonnative species early detection. *Canadian Journal of Fisheries and Aquatic Sciences*, *68*(12), 2064–2079. DOI: 10.1139/F2011-117

Hsieh, H. L., Fan, L. F., Chen, C. P., Wu, J. T., & Liu, W. C. (2010). Effects of semidiurnal tidal circulation on the distribution of holo- and meroplankton in a subtropical estuary. *Journal of Plankton Research*, *32*(6), 829–841. DOI: 10.1093/plankt/fbq026

ICES. (2014). Report of the ICES Working Group on Introductions and Transfers of Marine Organisms *(WGITMO)*.

Ikeda, T. (1974). Nutritional ecology of marine zooplankton [Doctoral thesis, Hokkaido University].

Inman, D. L., & Brush, B. M. (1973). The Coastal Challenge. *Science*, *181*(4094), 20–32.

Inman, D. L., & Nordstrom, C. E. (1971). On the Tectonic and Morphologic Classification of Coasts. *The Journal of Geology*, *79*(1), 1–21.

Intxausti, L., Villate, F., Uriarte, I., Iriarte, A., & Ameztoy, I. (2012). Size-related response of zooplankton to hydroclimatic variability and water-quality in an organically polluted estuary of the Basque coast (Bay of Biscay). *Journal of Marine Systems*, *94*, 87–96. DOI: 10.1016/j.jmarsys.2011.10.015

Jazdzewska, A. M., Tandberg, A. H. S., Horton, T., & Brix, S. (2021). Global gap-analysis of amphipod barcode library. *PeerJ*, *9*, 1–28. DOI: 10.7717/peerj.12352

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, *4*(2), 150–157. DOI: 10.1111/j.1755-263X.2010.00158.x

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. *Ecology and Evolution*, *9*(3), 1323–1335. DOI: 10.1002/ece3.4843

Jizhe, N. (2021). Main Data of the Seventh National Population Census News Release. Main Data of the Seventh National Population Census; National Bureau of Statistics of China. Weblink: www.stats.gov.cn/

JNCC, Joint Nature Conservation Committee (2021). *UKBI - B6. Pressure from invasive species*.

John, E. H., Batten, S. D., Harris, R. P., & Hays, G. C. (2001). Comparison between zooplankton data collected by the Continuous Plankton Recorder survey in the English Channel and by WP-2 nets at station L4, Plymouth (UK). *Journal of Sea Research*, *46*(3–4), 223–232. DOI: 10.1016/S1385-1101(01)00085-5

Johnston, S. A. (1981). Estuarine dredge and fill activities: A review of impacts. *Environmental Management*, *5*(5), 427–440. DOI: 10.1007/BF01866820

Karjalainen, J., Rahkola, M., Viljanen, M., Andronikova, I. N., & Avinskii, V. A. (1996). Comparison of methods used in zooplankton sampling and counting in the joint Russian-Finnish evaluation of the trophic state of Lake Ladoga. *Hydrobiologia*, *322*(1–3), 249–253. DOI: 10.1007/BF00031836

Keen, E. (2013). A practical designer's guide to mesozooplankton nets.

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. *Frontiers in Marine Science*, *3*(JAN), 1–11. DOI: 10.3389/FMARS.2016.00283

Kettunen, M., Genovesi, P., Gollasch, S., Pagad, S., & Starfinger, U. (2009). Technical support to eu strategy on invasive alien species (IAS) Assessment of the impacts of IAS in Europe and the EU. Institute for European Environmental Policy (IEEP), Brussels, Belgium.

Ki, J. S. (2012). Hypervariable regions (V1-V9) of the dinoflagellate 18S rRNA using a large dataset for marker considerations. *Journal of Applied Phycology*, *24*(5), 1035–1043. DOI: 10.1007/s10811-

011-9730-z

Kim, A. R., Yoon, T. H., Lee, C. Il, Kang, C. K., & Kim, H. W. (2021). Metabarcoding Analysis of Ichthyoplankton in the East/Japan Sea Using the Novel Fish-Specific Universal Primer Set. *Frontiers in Marine Science*, *8*(March). DOI: 10.3389/fmars.2021.614394

Kim, E. B., Lee, S. R., Lee, C. Il, Park, H., & Kim, H. W. (2019). Development of the cephalopod-specific universal primer set and its application for the metabarcoding analysis of planktonic cephalopods in Korean waters. *PeerJ*, *2019*(6), 1–22. DOI: 10.7717/peerj.7140

Kim, H., Lee, C. R., Lee, S. kyu, Oh, S. Y., & Kim, W. (2020). Biodiversity and community structure of mesozooplankton in the marine and coastal national park areas of Korea. *Diversity*, *12*(6). DOI: 10.3390/D12060233

Kour, S., Slathia, D., Sharma, N., Kour, S., & Verma, R. (2022). Zooplankton as Bioindicators of Trophic Status of a Lentic Water Source, Jammu (J&K) with Remarks on First Reports. *Proceedings of the National Academy of Sciences India Section B - Biological Sciences*, *92*(2), 393–404. DOI: 10.1007/s40011-022-01349-z

Krueger, C. C., & May, B. (1991). Ecological and genetic effects of salmonid introductions in North America. *Canadian Journal of Fisheries and Aquatic Sciences*, *48*(Suppl.1), 66–77. DOI: 10.1139/f91-305

Laakmann, S., Gerdts, G., Erler, R., Knebelsberger, T., Martínez Arbizu, P., & Raupach, M. J. (2013). Comparison of molecular species identification for North Sea calanoid copepods (Crustacea) using proteome fingerprints and DNA sequences. *Molecular Ecology Resources*, *13*(5), 862–876. DOI: 10.1111/1755-0998.12139

Laakmann, Silke, & Holst, S. (2014). Emphasizing the diversity of North Sea hydromedusae by combined morphological and molecular methods. *Journal of Plankton Research*, *36*(1), 64–76. DOI: 10.1093/plankt/fbt078

Ladhar, C., Tastard, E., Casse, N., Denis, F., & Ayadi, H. (2015). Strong and stable environmental structuring of the zooplankton communities in interconnected salt ponds. *Hydrobiologia*, *743*(1), 1–13. DOI: 10.1007/s10750-014-1998-y

Landry, M. R., & Hassett, R. P. (1982). Estimating the grazing impact of marine micro-zooplankton. *Marine Biology*, *67*(3), 283–288. DOI: 10.1007/BF00397668

Largier, J., Delgadillo, F., & Grierson, P. (1997). Seasonally Hypersaline Estuaries in Mediterranean-climate Regions. *Estuarine, Coastal and Shelf Science*, *45*, 789–797.

Larsen, J. B., Frischer, M. E., Rasmussen, L. J., & Hansen, B. W. (2005). Single-step nested multiplex PCR to differentiate between various bivalve larvae. *Marine Biology*, *146*(6), 1119–1129. DOI: 10.1007/s00227-004-1524-2

Lavrador, A., Amaral, F., Vieira, P. E., Costa, F., & Duarte, S. (2021). Surveillance of non-indigenous invertebrate species through DNA metabarcoding in recreational marinas in the North and Center of Portugal. In *ARPHA Conference Abstracts* (Vol. 4). DOI: 10.3897/aca.4.e64900

Le Quéré, C., Buitenhuis, E. T., Moriarty, R., Alvain, S., Aumont, O., Bopp, L., Chollet, S., Enright, C., Franklin, D. J., Geider, R. J., Harrison, S. P., Hirst, A. G., Larsen, S., Legendre, L., Platt, T.,

Prentice, I. C., Rivkin, R. B., Sailley, S., Sathyendranath, S., Stephens, N., Vogt, M., & Vallina, S. M. (2016). Role of zooplankton dynamics for Southern Ocean phytoplankton biomass and global biogeochemical cycles. *Biogeosciences*, *13*(14), 4111–4133. DOI: 10.5194/bg-13-4111-2016

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. *Marine Policy*, *54*, 26–35. DOI: 10.1016/j.marpol.2014.12.015

Leite, B. R., Vieira, P. E., Teixeira, M. A. L., Lobo-Arteaga, J., Hollatz, C., Borges, L. M. S., Duarte, S., Troncoso, J. S., & Costa, F. O. (2020). Gap-analysis and annotated reference library for supporting macroinvertebrate metabarcoding in Atlantic Iberia. *Regional Studies in Marine Science*, *36*, 101307. DOI: 10.1016/j.rsma.2020.101307

Leite, Barbara R., Vieira, P. E., Troncoso, J. S., & Costa, F. O. (2021). Comparing species detection success between molecular markers in DNA metabarcoding of coastal macroinvertebrates. *Metabarcoding and Metagenomics*, *5*, 249–260. DOI: 10.3897/MBMG.5.70063

León-Reògagnon, V. (2010). Evidence of new species of *Haematoloechus* (Platyhelminthes: Digenea) using partial cox1 sequences. *Mitochondrial DNA*, *21*(SUPPL. 1), 12–17. DOI: 10.3109/19401736.2010.523700

Leray, M., & Knowlton, N. (2015). DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. *Proceedings of the National Academy of Sciences*, *112*(7), 2076–2081.

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. *Frontiers in Zoology*, *10*(1). DOI: 10.1186/1742-9994-10-34

Li, L., Zheng, B., & Liu, L. (2010). Biomonitoring and bioindicators used for river ecosystems: Definitions, approaches and trends. *Procedia Environmental Sciences*, *2*, 1510–1524. DOI: 10.1016/j.proenv.2010.10.164

Liang, D., & Uye, S. (1997). Population dynamics and production of the planktonic copepods in a eutrophic inlet of the Inland Sea of Japan. II. *Acartia omorii*. *Marine Biology*, *125*(1), 109–117. DOI: 10.1007/BF00350765

Lin, Y., Vidjak, O., Ezgeta-Balić, D., Bojanić Varezić, D., Šegvić-Bubić, T., Stagličić, N., Zhan, A., & Briski, E. (2022). Plankton diversity in Anthropocene: Shipping vs. aquaculture along the eastern Adriatic coast assessed through DNA metabarcoding. *Science of the Total Environment*, *807*(xxxx). DOI: 10.1016/j.scitotenv.2021.151043

Lindemood, A. R. (2022). DNA Metabarcoding of the San Diego Copepod Community [Master's thesis, University of California]. UC San Diego Electronic Theses and Dissertations. Weblink: escholarship.org

Lindeque, P. K., Parry, H. E., Harmer, R. A., Somerfield, P. J., & Atkinson, A. (2013). Next generation sequencing reveals the hidden diversity of zooplankton assemblages. *PLoS ONE*, *8*(11), 1–14. DOI: 10.1371/journal.pone.0081327

Liu, M., Xue, Y., & Yang, J. (2019). Rare plankton subcommunities are far more affected by DNA extraction kits than abundant plankton. *Frontiers in Microbiology*, *10*(MAR), 1–12. DOI: 10.3389/fmicb.2019.00454

Lobo, J., Ferreira, M. S., Antunes, I. C., Teixeira, M. A. L., Borges, L. M. S., Sousa, R., Gomes, P. A., Costa, M. H., Cunha, M. R., & Costa, F. O. (2017). Contrasting morphological and DNA barcode-suggested species bounderies among shallow-water amphiphod fauna from the southern European Atlantic coast. *Genome*, *60*(2), 147–157.

Lobo, J., Costa, P. M., Teixeira, M. A. L., Ferreira, M. S. G., Costa, M. H., & Costa, F. O. (2013). Enhanced primers for amplification of DNA barcodes from a broad range of marine metazoans. *BMC Ecology*, *13*, 1–8. DOI: 10.1186/1472-6785-13-34

Lockwood, J. L., Hoopes, M. F., & Marchetti, M. P. (2007). Invasion Ecology. In *John Wiley & Sons*. DOI: 10.1524/ncrs.2001.216.14.683

Lowe, S., Browne, M., Boudjelas, S., & De Poorter, M. (2000). 100 de las Especies Exóticas Invasoras más dañinas del mundo. Una selección del Global Invasive Species Database. In *Grupo Especialista de Especies Invasoras; Grupo Especialista Comisión de Supervivenciua Especies UICN.* Invasive Species Special Group (ISSG).

Mack, H. R., Conroy, J. D., Blocksom, K. A., Stein, R. A., & Ludsin, S. A. (2012). A comparative analysis of zooplankton field collection and sample enumeration methods. *Limnology and Oceanography: Methods*, *10*, 41–53. DOI: 10.4319/lom.2012.10.41

Markert, B., Wappelhorst, O., Weckert, V., Herpin, U., Siewers, U., Friese, K., & Breulmann, G. (1999). The use of bioindicators for monitoring the heavy-metal status of the environment. *Journal of Radioanalytical and Nuclear Chemistry*, *240*(2), 425–429. DOI: 10.1007/BF02349387

Marques, S. C., Pardal, M. A., Pereira, M. J., Gonçalves, F., Marques, J. C., & Azeiteiro, U. M. (2007). Zooplankton distribution and dynamics in a temperate shallow estuary. *Hydrobiologia*, *587*(1), 213–223. DOI: 10.1007/s10750-007-0682-x

Martínez, M. L., Intralawan, A., Vázquez, G., Pérez-Maqueo, O., Sutton, P., & Landgrave, R. (2006). The coasts of our world: Ecological, economic and social importance. *Ecological Economics2*, 254–272. DOI: 10.1016/j.ecolecon.2006.10.02

McFadden, C. S., Benayahu, Y., Pante, E., Thoma, J. N., Nevarez, P. A., & France, S. C. (2011). Limitations of mitochondrial gene barcoding in Octocorallia. *Molecular Ecology Resources*, *11*(1), 19–31. DOI: 10.1111/j.1755-0998.2010.02875.x

McLeod, M. L., Cleveland, C. C., Lekberg, Y., Maron, J. L., Philippot, L., Bru, D., & Callaway, R. M. (2016). Exotic invasive plants increase productivity, abundance of ammonia-oxidizing bacteria and nitrogen availability in intermountain grasslands. *Journal of Ecology*, *104*(4), 994–1002. DOI: 10.1111/1365-2745.12584

McMahon, R. F. (2002). Evolutionary and physiological adaptations of aquatic invasive animals: r selection versus resistance. *Canadian Journal of Fisheries and Aquatic Sciences*, *59*(7), 1235–1244. DOI: 10.1139/f02-105

Menéndez, M. C., Piccolo, M. C., & Hoffmeyer, M. S. (2012). Short-term variability on mesozooplankton

community in a shallow mixed estuary (Bahía Blanca, Argentina): Influence of tidal cycles and local winds. *Estuarine, Coastal and Shelf Science*, *112*, 11–22. DOI: 10.1016/j.ecss.2011.08.014

Meredith, C., Hoffman, J., Trebitz, A., Pilgrim, E., Okum, S., Martinson, J., & Cameron, E. S. (2021). Evaluating the performance of DNA metabarcoding for assessment of zooplankton communities in Western Lake Superior using multiple markers. *Metabarcoding and Metagenomics*, *5*, e64735. DOI: 10.3897/MBMG.5.64735

Meyer, C. P. (2003). Molecular systematics of cowries (Gastropoda: Cypraeidae) and diversification patterns in the tropics. *Biological Journal of the Linnean Society*, *79*(3), 401–459. DOI: 10.1046/j.1095-8312.2003.00197.x

Miloslavich, P., Díaz, J. M., Klein, E., Alvarado, J. J., Díaz, C., Gobin, J., Escobar-Briones, E., Cruz-Motta, J. J., Weil, E., Cortés, J., Bastidas, A. C., Robertson, R., Zapata, F., Martín, A., Castillo, J., Kazandjian, A., & Ortiz, M. (2010). Marine biodiversity in the caribbean: Regional estimates and distribution patterns. *PLoS ONE*, *5*(8). DOI: 10.1371/journal.pone.0011916

Mohrbeck, I., Raupach, M. J., Arbizu, P. M., Knebelsberger, T., & Laakmann, S. (2015). High-throughput sequencing-the key to rapid biodiversity assessment of marine metazoa? *PLoS ONE*, *10*(10), 1–24. DOI: 10.1371/journal.pone.0140342

Mora, D., Kleinteich, J., Zimmermann, J., Fischer, H., & Krenek, S. (2021). Influence of preservation methods for phytobenthos samples on the detection of microbial communities through eDNA metabarcoding - first results. *Ergebnisse Der Jahrestagung 2021*, 29–36.

Morse, J. C., Bae, Y. J., Munkhjargal, G., Sangpradub, N., Tanida, K., Vshivkova, T. S., Wang, B., Yang, L., & Yule, C. M. (2007). Freshwater biomonitoring with macroinvertebrates in East Asia. *Frontiers in Ecology and the Environment*, *5*(1), 33–42. DOI: 10.1890/1540-9295(2007)5[33:FBWMIE]2.0.CO;2

Moyle, P. B., & Marchetti, M. P. (2006). Predicting Invasion Success: Freshwaer Fishes in California as a Model. *BioScience*, *56*(6), 515–524.

Mueller, R. L. (2006). Evolutionary rates, divergence dates, and the performance of mitochondrial genes in Bayesian phylogenetic analysis. *Systematic Biology*, *55*(2), 289–300. DOI: 10.1080/10635150500541672

Muskó, I. B., Bence, M., & Balogh, C. (2008). Occurrence of a new Ponto-Caspian invasive species, Cordylophora caspia (Pallas, 1771) (Hydrozoa: Clavidae) in Lake Balaton (Hungary). *Acta Zoologica Acadamiae Scientiarum Hungaricae*, *54*(2), 169–179.

Ngoile, M. A. K., & Horrill, C. J. (1993). Coastal ecosystems, productivity and ecosystem protection: Coastal ecosystem management. *Ambio*, *22*(7), 461–467. DOI: 10.2307/4314127

NISA, National Invasive Species Act (1996). Public Law 104-332 (USA).

Oertel, N., & Salánki, J. (2003). Biomonitoring and Bioindicators in Aquatic Ecosystems. In R. S. Ambasht & N. Ambasht (Eds.), *Moderns Trends in Applied Aquatic Ecology*. Springer Science and Business Media New York. DOI: 10.1007/978-1-4615-0221-0

Øines, Ø., & Schram, T. (2008). Intra- or inter-specific difference in genotypes of *Caligus elongatus*

Nordmann 1832? *Acta Parasitologica*, *53*(1), 93–105. DOI: 10.2478/s11686-008-0002-2

Ojaveer, H., Simm, M., & Lankov, A. (2004). Population dynamics and ecological impact of the non-indigenous *Cercopagis pengoi* in the Gulf of Riga (Baltic Sea). *Hydrobiologia*, *522*(1–3), 261–269. DOI: 10.1023/B:HYDR.0000029927.91756.41

Oosting, T., Hilario, E., Wellenreuther, M., & Ritchie, P. A. (2020). DNA degradation in fish: Practical solutions and guidelines to improve DNA preservation for genomic research. *Ecology and Evolution*, *10*(16), 8643–8651. DOI: 10.1002/ece3.6558

Ordóñez, J., Armengol, J., Moreno-Ostos, E., Caputo, L., García, J. C., & Marcé, R. (2010). On non-Eltonian methods of hunting Cladocera, or impacts of the introduction of planktontivorous fish on zooplankton composition and clear-water phase occurrence in a Mediterranean reservoir. In L. Naselli-Flores & G. Rossetti (Eds.), *Fifty Years After the "Homage to Santa Rosalia": Old and New Paradigms on Biodiversity in Aquatic Ecosystems* (pp. 119–129). Springer.

Pagenkopp Lohan, K. M., Campbell, T. L., Guo, J., Wheelock, M., Dimaria, R. A., & Geller, J. B. (2019). Intact vs. Homogenized subsampling: Testing impacts of pre-extraction processing of multi-species samples on invasive species detection. *Management of Biological Invasions*, *10*(2), 324–341. DOI: 10.3391/mbi.2019.10.2.08

Pappalardo, P., Collins, A. G., Pagenkopp Lohan, K. M., Hanson, K. M., Truskey, S. B., Jaeckle, W., Ames, C. L., Goodheart, J. A., Bush, S. L., Biancani, L. M., Strong, E. E., Vecchione, M., Harasewych, M. G., Reed, K., Lin, C., Hartil, E. C., Whelpley, J., Blumberg, J., Matterson, K., Redmond, N. E., Becker, A., Boyle, M. J., & Osborn, K. J. (2021). The role of taxonomic expertise in interpretation of metabarcoding studies. *ICES Journal of Marine Science*, *78*(9), 3397–3410. DOI: 10.1093/icesjms/fsab082

Pardal, M. Â., & Azeiteiro, U. M. (2001). Zooplankton biomass, abundance and diversity in a shelf area of Portugal (the Berlenga Marine Natural Reserve). *Life and Marine Sciences*, *18-A*, 25–33. https://repositorio.uac.pt/bitstream/10400.3/151/1/pp25_33_Pardal_Azeiteiro_18A.pdf

Parshukov, A., Vlasenko, P., Simonov, E., Ieshko, E., Burdukovskaya, T., Anikieva, L., Kashinskaya, E., Andree, K. B., & Solovyev, M. (2021). Parasitic copepods *Caligus lacustris* (Copepoda: Caligidae) on the rainbow trout *Oncorhynchus mykiss* in cage aquaculture: morphology, population demography, and first insights into phylogenetic relationships. *Parasitology Research*, *120*(7), 2455–2467. DOI: 10.1007/s00436-021-07198-5

Piñol, J., Mir, G., Gomez-Polo, P., & Agustí, N. (2014). Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Molecular Ecology Resources*, *15*(4), 819–830. DOI: 10.1111/1755-0998.12355

Pitz, K. J., Guo, J., Johnson, S. B., Campbell, T. L., Zhang, H., Vrijenhoek, R. C., Chavez, F. P., & Geller, J. (2020). Zooplankton biogeographic boundaries in the California Current System as determined from metabarcoding. *PLoS ONE*, *15*(6), 1–20. DOI: 10.1371/journal.pone.0235159

Plafkin, J. L., Barbour, M. T., Porter, K. D., Gross, S. K., & Hughes, R. M. (1989). Rapid bioassessment protocols for use in streams and rivers: benthic macroinvertebrates and fish. In *United States Environmental Protection Agency (EPA)*.

Porto de Viana do Castelo. (2017). *Porto de Viana do Castelo HandBook 16/17* (Enigma Editores).

Calameo.

PwC. (2017). Recreational Boating in Portugal - A Prespective on Demand.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), 590–596. DOI: 10.1093/nar/gks1219

Questel, J. M., Hopcroft, R. R., DeHart, H. M., Smoot, C. A., Kosobokova, K. N., & Bucklin, A. (2021). Metabarcoding of zooplankton diversity within the Chukchi Borderland, Arctic Ocean: improved resolution from multi-gene markers and region-specific DNA databases. *Marine Biodiversity*, *51*(1). DOI: 10.1007/s12526-020-01136-x

Ramos, S. (2007). Ichthyoplankton of the Lima estuary (NW Portugal): Ecology of the early life stages of Pleuronectiformes [Doctoral thesis, University of Porto] . University of Porto open repositorium.

Ramos, S., Cabral, H., & Elliott, M. (2015). Do fish larvae have advantages over adults and other components for assessing estuarine ecological quality? *Ecological Indicators*, *55*, 74–85. DOI: 10.1016/j.ecolind.2015.03.005

Ramos, S., Cowen, R. K., Paris, C., Ré, P., & Bordalo, A. A. (2006a). Environmental forcing and larval fish assemblage dynamics in the Lima River estuary (northwest Portugal). *Journal of Plankton Research*, *28*(3), 275–286. DOI: 10.1093/plankt/fbi104

Ramos, Sandra, Cowen, R. K., Ré, P., & Bordalo, A. A. (2006b). Temporal and spatial distributions of larval fish assemblages in the Lima estuary (Portugal). *Estuarine, Coastal and Shelf Science*, *66*(1–2), 303–314. DOI: 10.1016/j.ecss.2005.09.012

Ramos, S., Ré, P., & Bordalo, A. A. (2010). Recruitment of flatfish species to an estuarine nursery habitat (Lima estuary, NW Iberian Peninsula). *Journal of Sea Research*, *64*(4), 473–486. DOI: 10.1016/j.seares.2010.01.010

Ransome, E., Geller, J. B., Timmers, M., Leray, M., Mahardini, A., Sembiring, A., Collins, A. G., & Meyer, C. P. (2017). The importance of standardization for biodiversity comparisons: A case study using autonomous reef monitoring structures (ARMS) and metabarcoding to measure cryptic diversity on Mo'orea coral reefs, French Polynesia. *PLoS ONE*, *12*(4), 1–19. DOI: 10.1371/journal.pone.0175066

Ratnasingham, S., & Hebert, P. D. N. (2013). A DNA-Based Registry for All Animal Species: The Barcode Index Number (BIN) System. *PLoS ONE*, *8*(7). DOI: 10.1371/journal.pone.0066213

Ray, G. C. (1988). Ecological Diversity in Coastal Zones and Oceans. In E. O. Wilson & F. M. Peter (Eds.), *Biodiversity* (pp. 36–50). National Academy Press.

Ré, P. (1984). Ictioplâncton da região central da costa portuguesa e do estuário do Tejo. Ecologia da postura e da fase planctónica de *Sardina pilchardus* (Walbaum, 1792) e de *Engraulis encrasicolus* (Linné, 1758) [Thesis, University of Lisbon].

Reuter, J. A., Spacek, D., & Snyder, M. P. (2015). High-throughput sequencing technologies. *Molecular Cell*, *58*(4), 586–597. DOI: 10.1016/j.molcel.2015.05.004.High-Throughput

Rey, A., Basurko, O. C., & Rodriguez-Ezpeleta, N. (2020). Considerations for metabarcoding-based port

biological baseline surveys aimed at marine nonindigenous species monitoring and risk assessments. *Ecology and Evolution*, *10*(5), 2452–2465. DOI: 10.1002/ece3.6071

Rey, A., Corell, J., & Rodriguez-Ezpeleta, N. (2020). Metabarcoding to Study Zooplankton Diversity. In *Zooplankton Ecology* (pp. 252–263). DOI: 10.1201/9781351021821-14

Riccardi, N. (2010). Selectivity of plankton nets over mesozooplankton taxa: Implications for abundance, biomass and diversity estimation. *Journal of Limnology*, *69*(2), 287–296. DOI: 10.3274/JL10-69-2-10

Rice, E. L., Roddick, D., & Singh, R. K. (1993). A comparison of molluscan (Bivalvia) phylogenies based on palaeontological and molecular data. *Molecular Marine Biology and Biotechnology*, *2*(3), 137–146.

Richardson, D. M., Pyšek, P., Rejmánek, M., Barbour, M. G., Panetta, F. D., & West, C. J. (2000). Naturalization and invasion of alien plants: Concepts and definitions. *Diversity and Distributions*, *6*(2), 93–107. DOI: 10.1046/j.1472-4642.2000.00083.x

Rodas, A. M., Wright, R. M., Buie, L. K., Aichelman, H. E., Castillo, K. D., & Davies, S. W. (2020). Eukaryotic plankton communities across reef environments in Bocas del Toro Archipelago, Panamá. *Coral Reefs*, *39*(5), 1453–1467. DOI: 10.1007/s00338-020-01979-7

Rogers, C. E., & McCarty, J. P. (2000). Climate change and ecosystems of the Mid-Atlantic Region. *Climate Research*, *14*(3 SPECIAL 7), 235–244. DOI: 10.3354/cr014235

Ruaro, R., Gubiani, É. A., Cunico, A. M., Moretto, Y., & Piana, P. A. (2016). Comparison of fish and macroinvertebrates as bioindicators of Neotropical streams. *Environmental Monitoring and Assessment*, *188*(1), 1–13. DOI: 10.1007/s10661-015-5046-9

Rubal, M., Maldre, K., Sousa-Pinto, I., & Veiga, P. (2021). Current distribution and abundance of *Austrominius modestus* (Darwin, 1854) and other non-indigenous barnacles along the northern coast of Portugal. *Regional Studies in Marine Science*, *41*, 101586. DOI: 10.1016/j.rsma.2020.101586

Ruiz, G. M., Carlton, J. T., Grosholz, E. D., & Hines, A. H. (1997). Global invasions of marine and estuarine habitats by non-indigenous species: mechanisms, extent, and consequences. *American Zoologist*, *37*(6), 621–632. DOI: 10.1093/icb/37.6.621

Saccone, C., De Giorgi, C., Gissi, C., Pesole, G., & Reyes, A. (1999). Evolutionary genomics in Metazoa: The mitochondrial DNA as a model system. *Gene*, *238*(1), 195–209. DOI: 10.1016/S0378-1119(99)00270-X

Salas, A., Bandelt, H. J., Macaulay, V., & Richards, M. B. (2007). Phylogeographic investigations: The role of trees in forensic genetics. *Forensic Science International*, *168*(1), 1–13. DOI: 10.1016/j.forsciint.2006.05.037

Salipante, S. J., Kawashima, T., Rosenthal, C., Hoogestraat, D. R., Cummings, L. A., Sengupta, D. J., Harkins, T. T., Cookson, B. T., & Hoffman, N. G. (2014). Performance comparison of Illumina and Ion Torrent next-generation sequencing platforms for 16S rRNA-based bacterial community profiling. *Applied and Environmental Microbiology*, *80*(24), 7583–7591. DOI: 10.1128/AEM.02206-14

Sampaio, Eduardo, & Rodil, Iván F. (2014). Effects of the invasive clam *Corbicula fluminea* (Müller, 1774) on a representative macrobenthic community from two estuaries at different stages of invasion. *Limnetica*, *33*(33), 249–262. DOI: 10.23818/limn.33.20

Sampaio, E. A. S. de. (2012). Comparação da diversidade e estrutura das comunidades de macroinvertebrados bentónicos associados ao bioinvasor Corbicula fluminea na área estuarina de água doce dos rios Minho e Lima [Master's thesis, University of Porto]. University of Porto open repusitorium

Sato-Okoshi, W., Okoshi, K., Abe, H., & Li, J. Y. (2013). Polydorid species (Polychaeta, Spionidae) associated with commercially important mollusk shells from eastern China. *Aquaculture*, *406–407*, 153–159. DOI: 10.1016/j.aquaculture.2013.05.017

Sato-Okoshi, Waka, Okoshi, K., Abe, H., & Dauvin, J. (2022). Polydorid species (Annelida: Spionidae) associated with commercially important oyster shells and their shell infestation along the coast of Normandy, in the English Channel, France. In *Aquaculture International*. Springer International Publishing. DOI: 10.1007/s10499-022-00971-y

Sax, D. F., & Brown, J. H. (2000). The paradox of invasion. *Global Ecology and Biogeography*, *9*(5), 363–371. DOI: 10.1046/j.1365-2699.2000.00217.x

Scheltema, R., & Williams, I. (1982). Significance of Temperature to Larval Survival and Length of Development in *Balanus ebumeus* (Crustacea: Cirripedia). *Marine Ecology Progress Series*, *9*(4950), 43–49. DOI: 10.3354/meps009043

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., Van Horn, D. J., & Weber, C. F. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*(23), 7537–7541. DOI: 10.1128/AEM.01541-09

Schmieder, R., & Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics*, *27*(6), 863–864. DOI: 10.1093/bioinformatics/btr026

Schroeder, A., Pallavicini, A., Edomi, P., Pansera, M., & Camatti, E. (2021). Suitability of a dual COI marker for marine zooplankton DNA metabarcoding. *Marine Environmental Research*, *170*(August), 105444. DOI: 10.1016/j.marenvres.2021.105444

Schroeder, A., Stanković, D., Pallavicini, A., Gionechetti, F., Pansera, M., & Camatti, E. (2020). DNA metabarcoding and morphological analysis - Assessment of zooplankton biodiversity in transitional waters. *Marine Environmental Research*, *160*. DOI: 10.1016/j.marenvres.2020.104946

Schubert, M., Lindgreen, S., & Orlando, L. (2016). AdapterRemoval v2 : rapid adapter trimming , identification , and read merging. *BMC Research Notes*, 1–7. DOI: 10.1186/s13104-016-1900-2

Schwartz, M. L. (2006). Encyclopedia of Coastal Science. In R. W. Fairbridge & M. Rampino (Eds.), *Encyclopedia of Earth Sciences Series*. Springer.

Servia, M. J., Vieira-Lanero, R., Cobo, F., González, M. A., Sánchez, J., & Barca, S. (2006). Notas sobre la presencia de *Cordilophora caspia* (Pallas, 1771) *Dugesia tigrina* (Girard, 1850) y *Elodea canadensis* (Michaux, 1803), en los rios gallegos. *Actas 28 Congreso Nacional Sobre Especies*

Exóticas Invasoras, GEIB, 19–22.

Seyer, T., Morais, P., Amorim, K., Leitão, F., Martins, F., & Teodósio, M. A. (2017). On the presence of the ponto-caspian hydrozoan *Cordylophora caspia* (Pallas, 1771) in an Iberian estuary: Highlights on the introduction vectors and invasion routes. *BioInvasions Records*, *6*(4), 331–337. DOI: 10.3391/bir.2017.6.4.05

Seymour, M. (2021). Environmental DNA Advancing Our Understanding and Conservation of Inland Waters. In *Encyclopedia of Inland Waters* (2nd ed, pp. 685–698). Elsevier. DOI: 10.1016/B978-0-12-819166-8.00070-0

Shokralla, S., Spall, J. L., Gibson, J. F., & Hajibabaei, M. (2012). Next-generation sequencing technologies for environmental DNA research. *Molecular Ecology*, *21*(8), 1794–1805. DOI: 10.1111/j.1365-294X.2012.05538.x

Shrader-Frechette, K. (2001). Non-indigenous species and ecological explanation. *Biology and Philosophy*, *16*(4), 507–519. DOI: 10.1023/A:1011953713083

Silverbrand, S. J., Lindsay, S. M., & Rawson, P. D. (2021). Detection of a novel species complex of shell-boring polychaetes in the northeastern United States. *Invertebrate Biology*, *140*(3). DOI: 10.1111/ivb.12343

Simberloff, D., & Rejmánek, M. (2011). Encyclopedia of Biological Invasions. In *Encyclopedia of the Natural World*. University of California Press.

Singh, S. P., Groeneveld, J. C., Huggett, J., Naidoo, D., Cedras, R., & Willows-Munro, S. (2021). Metabarcoding of marine zooplankton in South Africa. *African Journal of Marine Science*, *43*(2), 147–159. DOI: 10.2989/1814232X.2021.1919759

Skjoldal, H. R., Wiebe, P. H., Postel, L., Knutsen, T., Kaartvedt, S., & Sameoto, D. D. (2013). Intercomparison of zooplankton (net) sampling systems: Results from the ICES/GLOBEC sea-going workshop. *Progress in Oceanography*, *108*, 1–42. DOI: 10.1016/j.pocean.2012.10.006

Śmietanka, B., Burzyński, A., Hummel, H., & Wenne, R. (2014). Glacial history of the European marine mussels *Mytilus*, inferred from distribution of mitochondrial DNA lineages. *Heredity*, *113*(3), 250–258. DOI: 10.1038/hdy.2014.23

Sommer, S. A., Van Woudenberg, L., Lenz, P. H., Cepeda, G., & Goetze, E. (2017). Vertical gradients in species richness and community composition across the twilight zone in the North Pacific Subtropical Gyre. *Molecular Ecology*, *26*(21), 6136–6156. DOI: 10.1111/mec.14286

Song, H., Buhay, J. E., Whiting, M. F., & Crandall, K. A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(36), 13486–13491. DOI: 10.1073/pnas.0803076105

Sousa, R., Antunes, C., & Guilhermino, L. (2008). Ecology of the invasive Asian clam *Corbicula fluminea* (Müller, 1774) in aquatic ecosystems: An overview. *Annales de Limnologie*, *44*(2), 85–94. DOI: 10.1051/limn:2008017

Sousa, Ronaldo. (2003). Estrutura das comunidades de macroinvertebrados bentónicos presentes no estuário do rio Lima [Master's thesis, University of Porto]. University of Porto open repositorium.

Sousa, Ronaldo, Antunes, C., & Guilhermino, L. (2006). Factors influencing the occurrence and distribution of *Corbicula fluminea* (Müller, 1774) in the River Lima estuary. *Annales de Limnologie*, *42*(3), 165–171. DOI: 10.1051/limn/2006017

Sousa, Ronaldo, Dias, S., & Antunes, C. (2007). Subtidal macrobenthic structure in the lower Lima estuary, NW of Iberian Peninsula. *Annales Zoologici Fennici*, *44*(4), 303–313.

Sousa, Ronaldo, Dias, S., & Antunes, J. C. (2006). Spatial subtidal macrobenthic distribution in relation to abiotic conditions in the Lima estuary, NW of Portugal. *Hydrobiologia*, *559*(1), 135–148. DOI: DOI: 10.1007/s10750-005-1371-2

South, P. M., Lilley, S. A., Tait, L. W., Alestra, T., Hickford, M. J. H., Thomsen, M. S., & Schiel, D. R. (2016). Transient effects of an invasive kelp on the community structure and primary productivity of an intertidal assemblage. *Marine and Freshwater Research*, *67*(1), 103–112. DOI: 10.1071/MF14211

Southward, A. J. (1998). New Observations on Barnacles (Crustacea: Cirripedia of the Azores Region. *Arquipélago. Life and Marine Science*, *16*, 11–27.

Speranskaya, A. S., Khafizov, K., Ayginin, A. A., Krinitsina, A. A., Omelchenko, D. O., Nilova, M. V., Severova, E. E., Samokhina, E. N., Shipulin, G. A., & Logacheva, M. D. (2018). Comparative analysis of Illumina and Ion Torrent high-throughput sequencing platforms for identification of plant components in herbal teas. *Food Control*, *93*, 315–324. DOI: 10.1016/j.foodcont.2018.04.040

Srichandan, S., Baliarsingh, S. K., Prakash, S., Panigrahy, R. C., & Sahu, K. C. (2018). Zooplankton Research in Indian Seas: A Review. *Journal of Ocean University of China*, *17*(5), 1149–1158. DOI: 10.1007/s11802-018-3463-4

Steele, J. H. (1962). Environmental control of photosynthesis in the sea. *Limnology and Oceanography*, *7*(2), 137–150. DOI: 10.1016/B978-0-12-812487-1.00006-5

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. *Scientific Reports*, *8*(1), 1–13. DOI: 10.1038/s41598-018-30157-7

Stein, E. D., Martinez, M. C., Stiles, S., Miller, P. E., & Zakharov, E. V. (2014). Is DNA barcoding actually cheaper and faster than traditional morphological methods: Results from a survey of freshwater bioassessment efforts in the United States? *PLoS ONE*, *9*(4). DOI: 10.1371/journal.pone.0095525

Steinberg, D. (2017). Zooplankton biogeochemical cycles. In Castellani, C. & Edwards, M. (Eds.), *Marine Plankton: A practical guide to ecology, methodology, and taxonomy* (1st ed., pp. 52–66). Oxford University Press. DOI: 10.1093/acprof:oso/9780199233267.001.0001

Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M. D. M., Breiner, H. W., & Richards, T. A. (2010). Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. *Molecular Ecology*, *19*, 21–31. DOI: 10.1111/J.1365-294X.2009.04480.X

Stoeckle, M. (2003). Taxonomy, DNA, and the Bar Code of Life. *BioScience*, *53*(9), 796–797.

Streftaris, N., Zenetos, A., & Papathanassiou, E. (2005). Globalisation in marine ecosystems: The story of non-indigenous marine species across European seas. *Oceanography and Marine Biology*, *43*, 419–453. DOI: 10.1201/9781420037449-10

Suchanek, T. H. (1994). Temperate coastal marine communities: Biodiversity and threats. *American Zoologist*, *34*(1), 100–114. DOI: 10.1093/icb/34.1.100

Sun, Y., Liu, Y., Wu, C., Fu, X., Guo, C., Li, L., & Sun, J. (2021). Characteristics of eukaryotic plankton communities in the cold water masses and nearshore waters of the south yellow sea. *Diversity*, *13*(1), 1–19. DOI: 10.3390/d13010021

Suter, L., Polanowski, A. M., Clarke, L. J., Kitchener, J. A., & Deagle, B. E. (2021). Capturing open ocean biodiversity: Comparing environmental DNA metabarcoding to the continuous plankton recorder. *Molecular Ecology*, *30*(13), 3140–3157. DOI: 10.1111/mec.15587

Suthers, I. M., & Rissik, D. (2009). *Plankton: A guide to their ecology and monitoring for water quality* (2nd ed.). CSIRO. DOI: 10.1016/j.cub.2017.02.045

Svetlichny, L., Hubareva, E., Khanaychenko, A., & Uttieri, M. (2019). Response to salinity and temperature changes in the alien Asian copepod *Pseudodiaptomus marinus* introduced in the Black Sea. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *331*(8), 416–426. DOI: 10.1002/jez.2309

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., & Willerslev, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, *21*(8), 2045–2050. DOI: 10.1111/j.1365-294X.2012.05470.x

Taguchi, Y. H., & Oono, Y. (2005). Relational patterns of gene expression via non-metric multidimensional scaling analysis. *Bioinformatics*, *21*(6), 730–740. DOI: 10.1093/bioinformatics/bti067

Tait, L. W., South, P. M., Lilley, S. A., Thomsen, M. S., & Schiel, D. R. (2015). Assemblage and understory carbon production of native and invasive canopy-forming macroalgae. *Journal of Experimental Marine Biology and Ecology*, *469*, 10–17. DOI: 10.1016/j.jembe.2015.04.007

Tang, C. Q., Leasi, F., Obertegger, U., Kieneke, A., Barraclough, T. G., & Fontaneto, D. (2012). The widely used small subunit 18S rDNA molecule greatly underestimates true diversity in biodiversity surveys of the meiofauna. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(40), 16208–16212. DOI: 10.1073/pnas.1209160109

Teixeira, M. A. L., Vieira, P. E., Pleijel, F., Sampieri, B. R., Ravara, A., Costa, F. O., & Nygren, A. (2020). Molecular and morphometric analyses identify new lineages within a large *Eumida* (Annelida) species complex. *Zoologica Scripta*, *49*(2), 222–235. DOI: 10.1111/zsc.12397

Teixeira, M. A. M. L. (2013). Lançamento de uma biblioteca de referência de DNA barcodes para poliquetas de estuários de Portugal e do mar profundo da Peninsula Ibérica [Master's thesis, University of Minho]. RepositóriUM (repositorium.sdum.uminho.pt)

Teramoto, W., Sato-Okoshi, W., Abe, H., Nishitani, G., & Endo, Y. (2013). Morphology, 18S rRNA gene sequence and life history of a new *Polydora* species (Polychaeta: Spionidae) from northeastern Japan. *Aquatic Biology*, *18*(1), 31–45. DOI: 10.3354/ab00485

Thiyagarajan, V., Harder, T., & Qian, P. Y. (2003). Combined effects of temperature and salinity on larval development and attachment of the subtidal barnacle *Balanus trigonus* Darwin. *Journal of Experimental Marine Biology and Ecology*, *287*(2), 223–236. DOI: 10.1016/S0022-0981(02)00570-1

Torres, P., Costa, A. C., & Dionísio, M. A. (2012). New alien barnacles in the Azores and some remarks on the invasive potential of Balanidae. *Helgoland Marine Research*, *66*(4), 513–522. DOI: 10.1007/s10152-011-0287-7

Tyler-Walters, H., & Pizzolla, P. (2007). A hydroid (*Cordylophora caspia*). DOI: 10.17031/marlinsp.1511.2

Uttieri, M., Aguzzi, L., Aiese Cigliano, R., Amato, A., Bojanić, N., Brunetta, M., Camatti, E., Carotenuto, Y., Damjanović, T., Delpy, F., de Olazabal, A., Di Capua, I., Falcão, J., Fernandez de Puelles, M. L., Foti, G., Garbazey, O., Goruppi, A., Gubanova, A., Hubareva, E., Iriarte, A., Khanaychenko, A., Lučić, D., Marques, S. C., Mazzocchi, M. G., Mikuš, J., Minutoli, R., Pagano, M., Pansera, M., Percopo, I., Primo, A. L., Svetlichny, L., Rožić, S., Tirelli, V., Uriarte, I., Vidjak, O., Villate, F., Wootton, M., Zagami, G., & Zervoudaki, S. (2020). WGEUROBUS – Working Group "Towards a EURopean OBservatory of the non-indigenous calanoid copepod *Pseudodiaptomus marinUS*." *Biological Invasions*, *22*(3), 885–906. DOI: 10.1007/s10530-019-02174-8

Valentini, A., Pompanon, F., & Taberlet, P. (2009). DNA barcoding for ecologists. *Trends in Ecology and Evolution*, *24*(2), 110–117. DOI: 10.1016/j.tree.2008.09.011

van der Loos, L. M., & Nijland, R. (2021). Biases in bulk: DNA metabarcoding of marine communities and the methodology involved. *Molecular Ecology*, *30*(13), 3270–3288. DOI: 10.1111/mec.15592

Vieira, L., Azeiteiro, U., Ré, P., Pastorinho, R., Marques, J. C., & Morgado, F. (2003). Zooplankton distribution in a temperate estuary (Mondego estuary southern arm: Western Portugal). *Acta Oecologica*, *24*(SUPPL. 1). DOI: 10.1016/S1146-609X(03)00038-9

Vieira, L. R., Guilhermino, L., & Morgado, F. (2015). Zooplankton structure and dynamics in two estuaries from the Atlantic coast in relation to multi-stressors exposure. *Estuarine, Coastal and Shelf Science*, *167*, 347–367. DOI: 10.1016/j.ecss.2015.10.012

Vieira, P. E., Lavrador, A. S., Parente, M. I., Parretti, P., Costa, A. C., Costa, F. O., & Duarte, S. (2021). Gaps in DNA sequence libraries for Macaronesian marine macroinvertebrates imply decades till completion and robust monitoring. *Diversity and Distributions*, *27*(10), 2003–2015. DOI: 10.1111/ddi.13305

Vierna, J., Cuperus, J., Martínez-Lage, A., Jansen, J. M., Perina, A., Van Pelt, H., & González-Tizón, A. M. (2014). Species delimitation and DNA barcoding of Atlantic *Ensis* (Bivalvia, Pharidae). *Zoologica Scripta*, *43*(2), 161–171. DOI: 10.1111/zsc.12038

Vincent, A. T., Derome, N., Boyle, B., Culley, A. I., & Charette, S. J. (2017). Next-generation sequencing (NGS) in the microbiological world: How to make the most of your money. *Journal of Microbiological Methods*, *138*, 60–71. DOI: 10.1016/j.mimet.2016.02.016

von Ammon, U., Jeffs, A., Zaiko, A., van der Reis, A., Goodwin, D., Beckley, L. E., Malpot, E., & Pochon, X. (2020). A Portable Cruising Speed Net: Expanding Global Collection of Sea Surface Plankton

Data. *Frontiers in Marine Science*, *7*(December), 1–14. DOI: 10.3389/fmars.2020.615458

Walters, T. L., Lamboley, L. M., López-Figueroa, N. B., Rodríguez-Santiago, Á. E., Gibson, D. M., & Frischer, M. E. (2019). Diet and trophic interactions of a circumglobally significant gelatinous marine zooplankter, *Dolioletta gegenbauri* (Uljanin, 1884). *Molecular Ecology*, *28*(2), 176–189. DOI: 10.1111/mec.14926

Wangensteen, O. S., Palacín, C., Guardiola, M., & Turon, X. (2018). DNA metabarcoding of littoral hardbottom communities: High diversity and database gaps revealed by two molecular markers. *PeerJ*, *2018*(5), 1–30. DOI: 10.7717/peerj.4705

Weigand, H., Beermann, A. J., Čiampor, F., Costa, F. O., Csabai, Z., Duarte, S., Geiger, M. F., Grabowski, M., Rimet, F., Rulik, B., Strand, M., Szucsich, N., Weigand, A. M., Willassen, E., Wyler, S. A., Bouchez, A., Borja, A., Čiamporová-Zaťovičová, Z., Ferreira, S., Dijkstra, K. B., Eisendel, U., Freyhof, J., Gadawski, P., Graf, W., Haegerbaeumer, A., van der Hoorn, B. B., Japoshvili, B., Keresztes, L., Keskin, E., Leese, F., Macher, J. N., Mamos, T., Paz, G., Pešić, V., Teixeira, M. A. L., Várbíró, G., & Ekrem, T. (2019). DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. *Science of the Total Environment*, *678*, 499–524. DOI: 10.1016/j.scitotenv.2019.04.247

Westfall, K. M., Therriault, T. W., & Abbott, C. L. (2020). A new approach to molecular biosurveillance of invasive species using DNA metabarcoding. *Global Change Biology*, *26*(2), 1012–1022. DOI: 10.1111/gcb.14886

Westheide, W., & Schmidt, H. (2003). Cosmopolitan versus cryptic meiofaunal polychaete species: An approach to a molecular taxonomy. *Helgoland Marine Research*, *57*(1), 1–6. DOI: 10.1007/s10152-002-0114-2

Williams, C., Pontén, F., Moberg, C., Söderkvist, P., Uhlén, M., Pontén, J., Sitbon, G., & Lundeberg, J. (1999). A high frequency of sequence alterations is due to formalin fixation of archival specimens. *American Journal of Pathology*, *155*(5), 1467–1471. DOI: 10.1016/S0002-9440(10)65461-2

Wilson, J. G. (1988). The Biology of Estuarine Management. In *Environmental Pollution* (Vol. 57, Issue 2). DOI: 10.1016/0269-7491(89)90010-9

Wright, H. (2022). Abundance of Shell-Boring Polychaete Worms and Other Fouling Organisms in Aquacultured Oysters From Maine Used for Reef Restoration in Great Bay , NH [Undergraduate honors thesis, University of Maine]. Weblink: digitalcommons.library.umaine.edu/.

Wu, S., Xiong, J., & Yu, Y. Y. (2015). Taxonomic resolutions based on 18S rRNA Genes: A case study of subclass Copepoda. *PLoS ONE*, *10*(6), 1–19. DOI: 10.1371/journal.pone.0131498

Xiong, W., Li, H., & Zhan, A. (2016). Early detection of invasive species in marine ecosystems using high-throughput sequencing: technical challenges and possible solutions. *Marine Biology*, *163*(6), 1–12. DOI: 10.1007/s00227-016-2911-1

Xu, G., Jianghua, Y., & Xiaowei, Z. (2020). Study on the Selection of Marker Genes in Zooplankton DNA Metabarcoding Monitoring. *Asian Journal of Ecotoxicology*, *15*(2), 61–70. DOI: 10.7524/AJE.1673-5897.20190121001

Xu, H., Ding, H., Li, M., Qiang, S., Guo, J., Han, Z., Huang, Z., Sun, H., He, S., Wu, H., & Wan, F.

(2006). The distribution and economic losses of alien species invasion to China. *Biological Invasions*, *8*(7), 1495–1500. DOI: 10.1007/s10530-005-5841-2

Yang, J., Zhang, X., Zhang, W., Sun, J., Xie, Y., Zhang, Y., Burton, G. A., & Yu, H. (2017). Indigenous species barcode database improves the identification of zooplankton. *PLoS ONE*, *12*(10), 1–15. DOI: 10.1371/journal.pone.0185697

Yoder, M., Tandingan De Ley, I., King, I. W., Mundo-Ocampo, M., Mann, J., Blaxter, M., Poiras, L., & De Ley, P. (2006). DESS: A versatile solution for preserving morphology and extractable DNA of nematodes. *Nematology*, *8*(3), 367–376. DOI: 10.1163/156854106778493448

Zaiko, A., Martinez, J. L., Ardura, A., Clusa, L., Borrell, Y. J., Samuiloviene, A., Roca, A., & Garcia-Vazquez, E. (2015). Detecting nuisance species using NGST: Methodology shortcomings and possible application in ballast water monitoring. *Marine Environmental Research*, *112*, 64–72. DOI: 10.1016/j.marenvres.2015.07.002

Zar, J. H. (2010). *Biostatistical Analysis* (5th ed.). Prentice Hall.

Zhan, A., He, S., Brown, E. A., Chain, F. J. 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 in Ecology and Evolution*, *5*(9), 881–890. DOI: 10.1111/2041-210X.12230

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 in Ecology and Evolution*, *4*(6), 558–565. DOI: 10.1111/2041-210X.12037

Zhang, G. K., Chain, F. J. J., Abbott, C. L., & Cristescu, M. E. (2018). Metabarcoding using multiplexed markers increases species detection in complex zooplankton communities. *Evolutionary Applications*, *11*(10), 1901–1914. DOI: 10.1111/eva.12694

Zhao, L., Zhang, X., Xu, M., Mao, Y., & Huang, Y. (2021). DNA metabarcoding of zooplankton communities: species diversity and seasonal variation revealed by 18S rRNA and COI. *PeerJ Computer Science*, *9*. DOI: 10.7717/peerj.11057

Zhao, W., & Shen, H. (2016). A statistical analysis of China's fisheries in the 12th five-year period. *Aquaculture and Fisheries*, *1*, 41–49. DOI: 10.1016/j.aaf.2016.11.001

Zheng, L., He, J., Lin, Y., Cao, W., & Zhang, W. (2014). 16S rRNA is a better choice than COI for DNA barcoding hydrozoans in the coastal waters of China. *Acta Oecologica*, *33*(4), 55–76. DOI: 10.1007/s13131-014-0415-8

Supplementary Material

Table S1. DNA quantification and quality obtained via Nanodrop™ 1000 spectrophotometer.

Samples	ng/µL	A260/280	A260/230
SprA	111.1	1.85	2.33
SprB	71.8	1.84	2.16
SprC	49.6	1.86	2.20
AutA	7.6	1.89	1.27
AutB	10.0	1.88	1.40
AutC	6.5	1.95	0.75
WinA	15.6	1.96	1.73
WinB	10.6	2.03	1.75
WinC	11.8	1.90	1.62

Table S2. Settings and reference libraries used for the taxonomic assignment of reads in mBRAVE.

mBRAVE	
Trimming	Trim front: 0 bp Trim end: 0 bp Trim length: 313 bp Primer masking: off
Filtering	Min QV: 10 qv Min Length: 150 bp Max bases with low QV (<20): 25% Max bases with ultralow QV (<10): 25%
Other parameters	Pre-clustering threshold: none ID Distance threshold: 3% Exclude from OTU threshold: 3% Minimum OTU size: 0 OTU threshold: 3%
Pair End (Illumina instruments only)	Pair end merging: merge Assembler Min overlap: 20 bp Assembler Max substitution: 5 bp
Project Reference Libraries	SYS-CRLNONINSECTARTH SYS-CRLNONINSECTINVERT SYS-CRLINSECTA SYS-CRLCHORDATA SYS-CRLPROTISTA SYS-CRLBACTERIA DS-GAIMARIN DS-BIBLIO

Table S3. Settings used reads processing and the taxonomic assignment of the reads in SILVAngs.

SILVAngs	
Quantity	Max length (nucleotides): 4,000
	Max ambiguities (%): 1
	Max repetitives (%): 2
	Min alignment identity (%): 80
	Min alignment score: 40
	Min base pair score: 30
	Min length (nucleotides): 200
	Min quantity score: 30
Ngs	Classification similarity: 70
	SILVA release: 132
Cluster	Sequence identity: 0.97
Rarefaction	Datapoints: 100
Sina	Gap penalty: 5
Taxplot	Gap extension penalty: 2
	Max taxonomic depth: 20

Table S4. Compiled list of documented zooplankton (Z) and macrozoobenthos (M) species occurring in the Lima River estuary. Every taxon in the list was based on morphology-based reports. Species without sequenced records of both markers in BOLD Systems and GenBank databases are highlighted with (*). Underlined species correspond to non-indigenous species.

Phylum	Class	Order	Family	Species	Study group	Authority
Annelida	Polychaeta	Phyllodocida	Nereididae	*Alitta succinea*	M	(Leuckart, 1847)
Annelida	Polychaeta	Terebellida	Ampharetidae	*Alkmaria romijn**	M	Horst,1919
Annelida	Polychaeta	Terebellida	Ampharetidae	*Ampharete acutifrons*	M	(Grube, 1860)
Annelida	Polychaeta	Terebellida	Terebellidae	*Amphitritides gracilis*	M	(Grube, 1860)
Annelida	Polychaeta	Terebellida	Cirratulidae	*Aphelochaeta marioni*	M	(Saint-Joseph, 1894)

Phylum	Class	Order	Family	Species	Study group	Authority
Annelida	Polychaeta		Capitellidae	*Capitella capitata*	M	(Fabricius, 1780)
Annelida	Polychaeta		Chaetopteridae	*Chaetopterus variopedatus*	M	(Renier, 1804)
Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia tentaculata*	M	(Montagu, 1808)
Annelida	Polychaeta	Eunicida	Onuphidae	*Diopatra neapolitana*	M	Delle Chiaje, 1841
Annelida	Polychaeta	Phyllodocida	Phyllodocidae	*Eteone longa*	M	(Fabricius, 1780)
Annelida	Polychaeta		Maldanidae	*Euclymene lombricoides*	M	(Quatrefages, 1866)
Annelida	Polychaeta	Phyllodocida	Syllidae	*Exogone verugera**	M	(Claparède, 1868)
Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera tridactyla*	M	Schmarda, 1861
Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste diversicolor*	M	(O.F. Müller, 1776)
Annelida	Polychaeta		Capitellidae	*Heteromastus filiformis*	M	(Claparède, 1864)
Annelida	Polychaeta	Terebellida	Pectinariidae	*Lagis koreni*	M	Malmgren, 1866
Annelida	Polychaeta	Terebellida	Terebellidae	*Lanice conchilega*	Z and M	(Pallas, 1766)
Annelida	Polychaeta		Magelonidae	*Magelona mirabilis*	Z	(Johnston, 1865)
Annelida	Polychaeta	Spionida	Spionidae	*Malacoceros fuliginosus*	M	(Claparède, 1868)
Annelida	Polychaeta		Maldanidae	*Maldane sarsi*	M	Malmgren, 1865
Annelida	Polychaeta		Capitellidae	*Mediomastus fragilis**	M	Rasmussen, 1973
Annelida	Polychaeta	Terebellida	Melinnidae	*Melinna palmata*	M	Grube, 1870
Annelida	Polychaeta	Phyllodocida	Nereididae	*Micronereis variegata**	M	Claparède, 1863
Annelida	Polychaeta	Phyllodocida	Phyllodocidae	*Mysta picta**	M	(Quatrefages, 1866)
Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys cirrosa*	M	Ehlers, 1868
Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys hombergii*	M	Savigny in Lamarck, 1818
Annelida	Polychaeta		Oweniidae	*Owenia fusiformis*	M	Delle Chiaje, 1844
Annelida	Polychaeta	Phyllodocida	Nereididae	*Perinereis cultrifera*	M	(Grube, 1840)

Phylum	Class	Order	Family	Species	Study group	Authority
Annelida	Polychaeta	Phyllodocida	Sigalionidae	*Pholoe inornata*	M	Johnston, 1839
Annelida	Polychaeta	Phyllodocida	Phyllodocidae	*Phyllodoce longipes*	M	Kinberg, 1866
Annelida	Polychaeta	Spionida	Spionidae	*Polydora ciliata*	M	(Johnston, 1838)
Annelida	Polychaeta	Phyllodocida	Hesionidae	*Psamathe fusca*	M	Johnston, 1836
Annelida	Polychaeta	Sabellida	Sabellidae	*Pseudopotamilla reniformis*	M	(Bruguière, 1789)
Annelida	Polychaeta	Spionida	Spionidae	*Pygospio elegans*	M	Claparède, 1863
Annelida	Polychaeta	Spionida	Spionidae	*Scolelepis foliosa*	M	(Audouin & Milne Edwards, 1833)
Annelida	Polychaeta	Spionida	Spionidae	*Streblospio shrubsolii*	M	(Buchanan, 1890)
Arthropoda	Malacostraca	Mysida	Mysidae	*Acanthomysis longicornis*	M	(Milne Edwards, 1837)
Arthropoda	Hexanauplia	Calanoida	Acartiidae	*Acartia (Acanthacartia) tonsa*	Z	Giesbrecht, 1889
Arthropoda	Hexanauplia	Calanoida	Acartiidae	*Acartia (Acartiura) clausi*	Z	Dana, 1849
Arthropoda	Malacostraca	Amphipoda	Ampeliscidae	*Ampelisca serraticaudata**	M	Chevreux, 1888
Arthropoda	Malacostraca	Decapoda	Paguridae	*Anapagurus laevis*	M	(Bell, 1845 [in Bell, 1844-1853])
Arthropoda	Malacostraca	Decapoda	Atelecyclidae	*Atelecyclus rotundatus*	M	(Olivi, 1792)
Arthropoda	Malacostraca	Decapoda	Atyidae	*Atyaephyra desmarestii*	M	(Millet, 1831)
Arthropoda	Thecostraca	Balanomorpha	Elminiidae	*Austrominius modestus*	M	(Darwin, 1854)
Arthropoda	Branchiopoda	Anomopoda	Bosminidae	*Bosmina (Bosmina) longirostris*	Z	(O.F. Müller, 1785)
Arthropoda	Hexanauplia	Calanoida	Pseudodiaptomidae	*Calanipeda aquaedulcis*	Z	Krichagin, 1873
Arthropoda	Hexanauplia	Calanoida	Calanidae	*Calanus helgolandicus*	Z	(Claus, 1863)
Arthropoda	Hexanauplia	Siphonostomatoida	Caligidae	*Caligus coryphaenae**	Z	Steenstrup & Lütken, 1861
Arthropoda	Hexanauplia	Calanoida	Candaciidae	*Candacia armata*	Z	Boeck, 1872
Arthropoda	Malacostraca	Decapoda	Carcinidae	*Carcinus maenas*	Z and M	(Linnaeus, 1758)
Arthropoda	Hexanauplia	Calanoida	Centropagidae	*Centropages chierchiae*	Z	Giesbrecht, 1889

Phylum	Class	Order	Family	Species	Study group	Authority
Arthropoda	Hexanauplia	Calanoida	Centropagidae	*Centropages hamatus*	Z	(Lilljeborg, 1853)
Arthropoda	Hexanauplia	Calanoida	Centropagidae	*Centropages typicus*	Z	Krøyer, 1849
Arthropoda	Branchiopoda	Anomopoda	Daphniidae	*Ceriodaphnia reticulata**	Z	(Jurine, 1820)
Arthropoda	Hexanauplia	Calanoida	Clausocalanidae	*Clausocalanus arcuicornis*	Z	(Dana, 1849)
Arthropoda	Malacostraca	Amphipoda	Corophiidae	*Corophium multisetosum*	M	Stock, 1952
Arthropoda	Malacostraca	Amphipoda	Corophiidae	*Corophium volutator*	M	(Pallas, 1766)
Arthropoda	Malacostraca	Decapoda	Crangonidae	*Crangon crangon*	Z and M	(Linnaeus, 1758)
Arthropoda	Malacostraca	Isopoda	Anthuridae	*Cyathura carinata*	M	(Krøyer, 1847)
Arthropoda	Branchiopoda	Anomopoda	Daphniidae	*Daphnia longispina*	Z	(O.F. Müller, 1776)
Arthropoda	Branchiopoda	Anomopoda	Daphniidae	*Daphnia pulex*	Z	Leydig, 1860
Arthropoda	Malacostraca	Decapoda	Diogenidae	*Diogenes pugilator*	Z and M	(P. Roux, 1829)
Arthropoda	Hexanauplia	Cyclopoida	Corycaeidae	*Ditrichocorycaeus anglicus*	Z	(Lubbock, 1857)
Arthropoda	Malacostraca	Isopoda	Cirolanidae	*Eurydice pulchra*	M	Leach, 1815
Arthropoda	Hexanauplia	Harpacticoida	Tachidiidae	*Euterpina acutifrons*	Z	(Dana, 1847)
Arthropoda	Branchiopoda	Onychopoda	Podonidae	*Evadne nordmanni*	Z	Lovén, 1836
Arthropoda	Branchiopoda	Onychopoda	Podonidae	*Evadne spinifera*	Z	P.E. Müller, 1867
Arthropoda	Malacostraca	Mysida	Mysidae	*Gastrosaccus spinifer*	Z and M	(Goës, 1864)
Arthropoda	Malacostraca	Isopoda	Idoteidae	*Idotea chelipes*	Z and M	(Pallas, 1766)
Arthropoda	Malacostraca	Isopoda	Sphaeromatidae	*Lekanesphaera monodi**	M	(Arcangeli, 1934)
Arthropoda	Malacostraca	Decapoda	Polybiidae	*Liocarcinus holsatus*	M	(Fabricius, 1798)
Arthropoda	Malacostraca	Decapoda	Polybiidae	*Liocarcinus navigator*	M	(Herbst, 1794)
Arthropoda	Malacostraca	Amphipoda	Melitidae	*Melita palmata*	M	(Montagu, 1804)
Arthropoda	Malacostraca	Mysida	Mysidae	*Mesopodopsis slabberi*	Z	(Van Beneden, 1861)

Phylum	Class	Order	Family	Species	Study group	Authority
Arthropoda	Malacostraca	Decapoda	Polybiidae	*Necora puber*	M	(Linnaeus, 1767)
Arthropoda	Malacostraca	Mysida	Mysidae	*Neomysis integer*	Z and M	(Leach, 1814)
Arthropoda	Hexanauplia	Cyclopoida	Oithonidae	*Oithona nana*	Z	Giesbrecht, 1893
Arthropoda	Hexanauplia	Cyclopoida	Oithonidae	*Oithona plumifera*	Z	Baird, 1843
Arthropoda	Hexanauplia	Cyclopoida	Oithonidae	*Oithona similis*	Z	Claus, 1866
Arthropoda	Malacostraca	Decapoda	Grapsidae	*Pachygrapsus marmoratus*	Z	(J.C. Fabricius, 1787)
Arthropoda	Malacostraca	Decapoda	Paguridae	*Pagurus bernhardus*	Z	(Linnaeus, 1758)
Arthropoda	Malacostraca	Decapoda	Paguridae	*Pagurus pubescens*	M	Krøyer, 1838
Arthropoda	Malacostraca	Decapoda	Palaemonidae	*Palaemon elegans*	Z and M	Rathke, 1836
Arthropoda	Malacostraca	Decapoda	Palaemonidae	*Palaemon serratus*	Z and M	(Pennant, 1777)
Arthropoda	Hexanauplia	Calanoida	Paracalanidae	*Paracalanus parvus parvus*	Z	(Claus, 1863)
Arthropoda	Hexanauplia	Calanoida	Euchaetidae	*Paraeuchaeta hebes*	Z	(Giesbrecht, 1888)
Arthropoda	Malacostraca	Isopoda	Gnathiidae	*Paragnathia formica*	Z and M	(Hesse, 1864)
Arthropoda	Malacostraca	Amphipoda	Caprellidae	*Pariambus typicus**	M	(Krøyer, 1845)
Arthropoda	Branchiopoda	Ctenopoda	Sididae	*Penilia avirostris*	Z	Dana, 1849
Arthropoda	Malacostraca	Decapoda	Porcellanidae	*Pisidia longicornis*	Z	(Linnaeus, 1767)
Arthropoda	Branchiopoda	Onychopoda	Podonidae	*Pleopis polyphemoides*	Z	(Leuckart, 1859)
Arthropoda	Branchiopoda	Onychopoda	Podonidae	*Podon intermedius*	Z	Lilljeborg, 1853
Arthropoda	Branchiopoda	Onychopoda	Podonidae	*Podon leuckartii*	Z	(G.O. Sars, 1862)
Arthropoda	Thecostraca	Pollicipedomorpha	Pollicipedidae	*Pollicipes pollicipes*	M	(Gmelin, 1791 [in Gmelin, 1788-1792])
Arthropoda	Malacostraca	Decapoda	Porcellanidae	*Porcellana platycheles*	Z	(Pennant, 1777)
Arthropoda	Hexanauplia	Calanoida	Clausocalanidae	*Pseudocalanus elongatus*	Z	(Brady, 1865)

Phylum	Class	Order	Family	Species	Study group	Authority
Arthropoda	Malacostraca	Isopoda	Chaetiliidae	*Saduriella losadai**	M	Holthuis, 1964
Arthropoda	Malacostraca	Mysida	Mysidae	*Schistomysis spiritus*	Z	(Norman, 1860)
Arthropoda	Branchiopoda	Ctenopoda	Sididae	*Sida crystallina*	Z	(O.F. Müller, 1776)
Arthropoda	Malacostraca	Isopoda	Sphaeromatidae	*Sphaeroma serratum*	M	(J. C. Fabricius, 1787)
Arthropoda	Hexanauplia	Calanoida	Temoridae	*Temora longicornis*	Z	(Müller O.F., 1785)
Arthropoda	Hexanauplia	Calanoida	Temoridae	*Temora stylifera*	Z	(Dana, 1849)
Arthropoda	Malacostraca	Decapoda	Upogebiidae	*Upogebia pusilla*	Z	(Petagna, 1792)
Chaetognatha	Sagittoidea	Aphragmophora	Sagittidae	*Parasagitta friderici*	Z	(Ritter-Záhony, 1911)
Chaetognatha	Sagittoidea	Aphragmophora	Sagittidae	*Parasagitta setosa*	Z	(J. Müller, 1847)
Chordata	Actinopteri	Perciformes	Ammodytidae	*Ammodytes tobianus*	Z	Linnaeus, 1758
Chordata	Actinopteri	Atheriniformes	Atherinidae	*Atherina presbyter*	Z	Cuvier, 1829
Chordata	Actinopteri	Beloniformes	Belonidae	*Belone belone*	Z	(Linnaeus, 1760)
Chordata	Actinopteri	Blenniiformes	Blenniidae	*Blennius ocellaris*	Z	Linnaeus, 1758
Chordata	Actinopteri	Pleuronectiformes	Soleidae	*Buglossidium luteum*	Z	(Risso, 1810)
Chordata	Actinopteri	Callionymiformes	Callionymidae	*Callionymus lyra*	Z	Linnaeus, 1758
Chordata	Actinopteri	Eupercaria incertae sedis	Labridae	*Centrolabrus exoletus*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Gadiformes	Lotidae	*Ciliata mustela*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Blenniiformes	Blenniidae	*Coryphoblennius galerita*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Gobiiformes	Gobiidae	*Crystallogobius linearis*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Eupercaria incertae sedis	Labridae	*Ctenolabrus rupestris*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Eupercaria incertae sedis	Moronidae	*Dicentrarchus labrax*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Gobiesociformes	Gobiesocidae	*Diplecogaster bimaculata*	Z	(Bonnaterre, 1788)
Chordata	Actinopteri	Eupercaria incertae sedis	Sparidae	*Diplodus sargus*	Z	(Linnaeus, 1758)

Phylum	Class	Order	Family	Species	Study group	Authority
Chordata	Actinopteri	Perciformes	Trachinidae	*Echiichthys vipera*	Z	(Cuvier, 1829)
Chordata	Actinopteri	Clupeiformes	Engraulidae	*Engraulis encrasicolus*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Syngnathiformes	Syngnathidae	*Entelurus aequoreus*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Perciformes	Ammodytidae	*Hyperoplus lanceolatus*	Z	(Le Sauvage, 1824)
Chordata	Actinopteri	Eupercaria incertae sedis	Labridae	*Labrus bergylta*	Z	Ascanius, 1767
Chordata	Actinopteri	Eupercaria incertae sedis	Labridae	*Labrus merula*	Z	Linnaeus, 1758
Chordata	Actinopteri	Gobiesociformes	Gobiesocidae	*Lepadogaster lepadogaster*	Z	(Bonnaterre, 1788)
Chordata	Actinopteri	Perciformes	Liparidae	*Liparis montagui*	Z	(Donovan, 1804)
Chordata	Actinopteri	Blenniiformes	Blenniidae	*Lipophrys pholis*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Pleuronectiformes	Soleidae	*Microchirus variegatus*	Z	(Donovan, 1808)
Chordata	Actinopteri	Syngnathiformes	Syngnathidae	*Nerophis lumbriciformis*	Z	(Jenyns, 1835)
Chordata	Actinopteri	Syngnathiformes	Syngnathidae	*Nerophis ophidion*	Z	(Linnaeus, 1758)
Chordata	Appendicularia	Copelata	Oikopleuridae	*Oikopleura (Coecaria) longicauda*	Z	Fol, 1872
Chordata	Appendicularia	Copelata	Oikopleuridae	*Oikopleura (Vexillaria) dioica*	Z	(Vogt, 1854)
Chordata	Actinopteri	Blenniiformes	Blenniidae	*Parablennius gattorugine*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Pleuronectiformes	Soleidae	*Pegusa lascaris*	Z	(Risso, 1810)
Chordata	Actinopteri	Pleuronectiformes	Pleuronectidae	*Platichthys flesus*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Gobiiformes	Gobiidae	*Pomatoschistus microps*	Z	(Krøyer, 1838)
Chordata	Actinopteri	Gobiiformes	Gobiidae	*Pomatoschistus minutus*	Z	(Pallas, 1770)
Chordata	Actinopteri	Gobiiformes	Gobiidae	*Pomatoschistus pictus*	Z	(Malm, 1865)
Chordata	Actinopteri	Clupeiformes	Clupeidae	*Sardina pilchardus*	Z	(Walbaum, 1792)
Chordata	Actinopteri	Pleuronectiformes	Soleidae	*Solea senegalensis*	Z	Kaup, 1858

Phylum	Class	Order	Family	Species	Study group	Authority
Chordata	Actinopteri	Pleuronectiformes	Soleidae	*Solea solea*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Eupercaria incertae sedis	Sparidae	*Spondyliosoma cantharus*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Clupeiformes	Clupeidae	*Sprattus sprattus*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Eupercaria incertae sedis	Labridae	*Symphodus melops*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Syngnathiformes	Syngnathidae	*Syngnathus abaster*	Z	Risso, 1827
Chordata	Actinopteri	Syngnathiformes	Syngnathidae	*Syngnathus acus*	Z	Linnaeus, 1758
Chordata	Actinopteri	Perciformes	Trachinidae	*Trachinus draco*	Z	Linnaeus, 1758
Chordata	Actinopteri	Carangiformes	Carangidae	*Trachurus trachurus*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Gadiformes	Gadidae	*Trisopterus luscus*	Z	(Linnaeus, 1758)
Chordata	Actinopteri	Pleuronectiformes	Scophthalmidae	*Zeugopterus punctatus*	Z	(Bloch, 1787)
Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	*Clytia hemisphaerica*	Z	(Linnaeus, 1767)
Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	*Clytia islandica**	Z	(Kramp, 1919)
Cnidaria	Hydrozoa	Anthoathecata	Corynidae	*Codonium proliferum*	Z	(Forbes, 1848)
Cnidaria	Anthozoa	Actiniaria	Sagartiidae	*Cylista troglodytes**	M	(Price in Johnston, 1847)
Cnidaria	Hydrozoa	Siphonophorae	Diphyidae	*Diphyes dispar*	Z	Chamisso & Eysenhardt, 1821
Cnidaria	Hydrozoa	Limnomedusae	Geryoniidae	*Liriope tetraphylla*	Z	(Chamisso & Eysenhardt, 1821)
Cnidaria	Hydrozoa	Anthoathecata	Rathkeidae	*Lizzia blondina*	Z	Forbes, 1848
Cnidaria	Hydrozoa	Siphonophorae	Diphyidae	*Muggiaea atlantica*	Z	Cunningham, 1892
Cnidaria	Hydrozoa	Leptothecata	Phialellidae	*Phialella quadrata*	Z	(Forbes, 1848)
Cnidaria	Hydrozoa	Anthoathecata	Hydractiniidae	*Podocoryna carnea*	Z	M. Sars, 1846
Cnidaria	Hydrozoa	Anthoathecata	Hydractiniidae	*Podocorynoides minima*	Z	(Trinci, 1903)
Cnidaria	Hydrozoa	Anthoathecata	Corynidae	*Sarsia tubulosa*	Z	(M. Sars, 1835)
Cnidaria	Hydrozoa	Anthoathecata	Corynidae	*Stauridiosarsia gemmifera*	Z	(Forbes, 1848)

Phylum	Class	Order	Family	Species	Study group	Authority
Echinodermata	Ophiuroidea	Amphilepidida	Amphiuridae	*Amphipholis squamata*	M	(Delle Chiaje, 1828)
Echinodermata	Ophiuroidea	Amphilepidida	Amphiuridae	*Amphiura filiformis*	M	(O.F. Müller, 1776)
Echinodermata	Asteroidea	Forcipulatida	Asteriidae	*Asterias rubens*	M	Linnaeus, 1758
Echinodermata	Echinoidea	Spatangoida	Loveniidae	*Echinocardium cordatum*	Z and M	(Pennant, 1777)
Echinodermata	Ophiuroidea	Amphilepidida	Ophiotrichidae	*Ophiothrix fragilis*	Z	(Abildgaard in O.F. Müller, 1789)
Echinodermata	Echinoidea	Camarodonta	Parechinidae	*Paracentrotus lividus*	M	(Lamarck, 1816)
Mollusca	Bivalvia	Cardiida	Semelidae	*Abra alba*	M	(W. Wood, 1802)
Mollusca	Bivalvia	Cardiida	Semelidae	*Abra nitida*	M	(O. F. Müller, 1776)
Mollusca	Bivalvia	Cardiida	Semelidae	*Abra tenuis**	M	(Montagu, 1803)
Mollusca	Bivalvia	Cardiida	Cardiidae	*Acanthocardia tuberculata*	M	(Linnaeus, 1758)
Mollusca	Gastropoda	Nudibranchia	Aeolidiidae	*Aeolidia papillosa*	M	(Linnaeus, 1761)
Mollusca	Gastropoda	[unassigned] Caenogastropoda	Cerithiidae	*Bittium reticulatum*	M	(Linnaeus, 1758)
Mollusca	Gastropoda	Cephalaspidea	Bullidae	*Bulla striata*	M	(da Costa, 1778)
Mollusca	Gastropoda	Littorinimorpha	Bithyniidae	*Bythinia tentaculata*	M	Bruguière, 1792
Mollusca	Bivalvia	Cardiida	Cardiidae	*Cerastoderma edule*	M	(Linnaeus, 1758)
Mollusca	Bivalvia	Venerida	Cyrenidae	*Corbicula fluminea*	M	(O. F. Müller, 1774)
Mollusca	Gastropoda	Cephalaspidea	Cylichnidae	*Cylichna cylindracea*	M	(Pennant, 1777)
Mollusca	Bivalvia	Venerida	Mesodesmatidae	*Donacilla cornea*	M	(Poli, 1791)
Mollusca	Bivalvia	Venerida	Veneridae	*Dosinia exoleta*	M	(Linnaeus, 1758)
Mollusca	Bivalvia	Adapedonta	Pharidae	*Ensis ensis*	M	(Linnaeus, 1758)
Mollusca	Bivalvia	Adapedonta	Pharidae	*Ensis siliqua*	M	(Linnaeus, 1758)
Mollusca	Bivalvia	Adapedonta	Hiatellidae	*Hiatella arctica*	M	(Linnaeus, 1767)
Mollusca	Bivalvia	Galeommatida	Lasaeidae	*Kurtiella bidentata*	M	(Montagu, 1803)

Phylum	Class	Order	Family	Species	Study group	Authority
Mollusca	Gastropoda	Littorinimorpha	Littorinidae	*Littorina littorea*	Z	(Linnaeus, 1758)
Mollusca	Bivalvia	Lucinida	Lucinidae	*Loripes orbiculatus**	M	Poli, 1795
Mollusca	Bivalvia	Venerida	Mactridae	*Lutraria bruuni**	M	Powell, 1967
Mollusca	Bivalvia	Venerida	Mactridae	*Lutraria lutraria*	M	(Linnaeus, 1758)
Mollusca	Bivalvia	Cardiida	Tellinidae	*Macomangulus tenuis*	M	(da Costa, 1778)
Mollusca	Bivalvia	Venerida	Mactridae	*Mactra stultorum*	M	(Linnaeus, 1758)
Mollusca	Bivalvia	Cardiida	Tellinidae	*Moerella donacina*	M	(Linnaeus, 1758)
Mollusca	Bivalvia	Myida	Myidae	*Mya arenaria*	M	Linnaeus, 1758
Mollusca	Bivalvia	Mytilida	Mytilidae	*Mytilus galloprovincialis*	M	Lamarck, 1819
Mollusca	Bivalvia	Nuculida	Nuculidae	*Nucula nucleus*	M	(Linnaeus, 1758)
Mollusca	Bivalvia	Cardiida	Cardiidae	*Parvicardium pinnulatum*	M	(Conrad, 1831)
Mollusca	Gastropoda	Littorinimorpha	Hydrobiidae	*Peringia ulvae*	Z and M	(Pennant, 1777)
Mollusca	Bivalvia	Adapedonta	Pharidae	*Pharus legumen*	M	(Linnaeus, 1758)
Mollusca	Gastropoda		Physidae	*Physella acuta*	M	(Draparnaud, 1805)
Mollusca	Bivalvia	Venerida	Veneridae	*Polititapes rhomboides*	M	(Pennant, 1777)
Mollusca	Bivalvia	Venerida	Veneridae	*Ruditapes decussatus*	M	(Linnaeus, 1758)
Mollusca	Bivalvia	Cardiida	Semelidae	*Scrobicularia plana*	M	(da Costa, 1778)
Mollusca	Bivalvia	Adapedonta	Solenidae	*Solen capensis**	M	P. Fischer, 1881
Mollusca	Bivalvia	Venerida	Mactridae	*Spisula solida*	M	(Linnaeus, 1758)
Mollusca	Bivalvia	Venerida	Mactridae	*Spisula subtruncata*	M	(da Costa, 1778)
Mollusca	Bivalvia	Galeommatida	Lasaeidae	*Tellimya ferruginosa*	M	(Montagu, 1808)
Mollusca	Gastropoda	Nudibranchia	Trinchesiidae	*Tenellia adspersa*	M	(Nordmann, 1845)
Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Tritia incrassata*	M	(Strøm, 1768)

Phylum	Class	Order	Family	Species	Study group	Authority
Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Tritia nítida**	M	(Jeffreys, 1867)
Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Tritia reticulata*	M	(Linnaeus, 1758)
Mollusca	Gastropoda		Pyramidellidae	*Turbonilla lactea**	M	(Linnaeus, 1758)
Mollusca	Bivalvia	Myida	Corbulidae	*Varicorbula gibba*	M	(Olivi, 1792)
Mollusca	Bivalvia	Venerida	Veneridae	*Venerupis corrugata*	M	(Gmelin, 1791)
Platyhelminthes		Polycladida	Pleioplanidae	*Notoplana atomata**	M	(Müller OF, 1776)

Table S5. List of the 58 recovered species with COI and respective number of reads. The list only contains marine and brackish metazoans, according to WoRMS, and species with more than 8 reads in the dataset. Taxa underlined represent the non-indigenous species detected.

Phylum	Class	Order	Family	Species	Authority	No. of reads
Annelida	Polychaeta		Arenicolidae	Arenicola defodiens	Cadman & Nelson-Smith, 1993	11
Annelida	Polychaeta	Phyllodocida	Phyllodocidae	Eumida mackiei	Teixeira et al., 2020	1528
Annelida	Polychaeta	Phyllodocida	Glyceridae	Glycera alba	(Müller, 1776)	47
Annelida	Polychaeta	Terebellida	Terebellidae	Lanice conchilega	(Pallas, 1766)	178
Annelida	Polychaeta	Spionida	Spionidae	Laonice cirrata	(Sars, 1851)	42
Annelida	Polychaeta	Eunicida	Eunicidae	Lysidice ninetta	Audouin & Milne Edwards, 1833	90
Annelida	Polychaeta		Magelonidae	Magelona johnstoni	Fiege et al., 2000	36
Annelida	Polychaeta	Spionida	Spionidae	Malacoceros fuliginosus	(Claparède, 1868)	1524
Annelida	Polychaeta		Protodrilidae	Meiodrilus adhaerens	(Jägersten, 1952)	9
Annelida	Polychaeta	Phyllodocida	Nephtyidae	Nephtys hombergii	Savigny in Lamarck, 1818	420
Annelida	Clitellata	Tubificida	Naididae	Paranais botniensis	Sperber, 1948	19
Annelida	Polychaeta	Phyllodocida	Nereididae	Platynereis dumerilii	(Audouin & Milne Edwards, 1833)	9
Annelida	Polychaeta	Phyllodocida	Polynoidae	Polynoe scolopendrina	Savigny, 1822	12

Phylum	Class	Order	Family	Species	Authority	No. of reads
Annelida	Polychaeta		Protodrilidae	Protodrilus ciliatus	Jägersten, 1952	139
Annelida	Polychaeta	Phyllodocida	Hesionidae	Psamathe fusca	Johnston, 1836	18
Annelida	Polychaeta	Sabellida	Sabellidae	Pseudopotamilla reniformis	(Bruguière, 1789)	363
Annelida	Polychaeta		Sabellariidae	Sabellaria alveolata	(Linnaeus, 1767)	152
Annelida	Polychaeta		Sabellariidae	Sabellaria spinulosa	(Leuckart, 1849)	184
Annelida	Polychaeta	Spionida	Spionidae	Spio symphyta	Meißner et al., 2011	2055
Annelida	Polychaeta	Spionida	Spionidae	Spiophanes bombyx	(Claparède, 1870)	91
Annelida	Polychaeta	Terebellida	Cirratulidae	Tharyx setigera	Hartman, 1945	75
Arthropoda	Thecostraca	Balanomorpha	Balanidae	Amphibalanus eburneus	(Gould, 1841)	891
Arthropoda	Thecostraca	Balanomorpha	Balanidae	Balanus trigonus	Darwin, 1854	1175
Arthropoda	Thecostraca	Balanomorpha	Chthamalidae	Chthamalus montagui	Southward, 1976	71
Arthropoda	Insecta	Diptera	Chironomidae	Halocladius varians	(Staeger, 1839)	58
Arthropoda	Branchiopoda	Ctenopoda	Holopediidae	Holopedium gibberum	Zaddach, 1855	166
Arthropoda	Collembola		Hypogastruridae	Hypogastrura viatica	(Tullberg, 1872)	53
Arthropoda	Malacostraca	Amphipoda	Melitidae	Melita palmata	(Montagu, 1804)	9
Bryozoa	Gymnolaemata	Cheilostomatida	Electridae	Electra pilosa	(Linnaeus, 1767)	40
Bryozoa	Gymnolaemata	Cheilostomatida	Membraniporidae	Membranipora membranacea	(Linnaeus, 1767)	52
Chordata	Actinopteri	Mugiliformes	Mugilidae	Chelon labrosus	(Risso, 1827)	384
Chordata	Actinopteri	Gobiiformes	Gobiidae	Pomatoschistus microps	(Krøyer, 1838)	10
Chordata	Actinopteri	Gadiformes	Gadidae	Trisopterus luscus	(Linnaeus, 1758)	13
Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	Clytia hemisphaerica	(Linnaeus, 1767)	15
Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	Obelia dichotoma	(Linnaeus, 1758)	4152
Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	Obelia geniculata	(Linnaeus, 1758)	10

Phylum	Class	Order	Family	Species	Authority	No. of reads
Cnidaria	Scyphozoa	Rhizostomeae	Rhizostomatidae	Rhizostoma luteum	(Quoy & Gaimard, 1827)	15
Echinodermata	Ophiuroidea	Amphilepidida	Amphiuridae	Acrocnida brachiata	(Montagu, 1804)	11
Echinodermata	Ophiuroidea	Amphilepidida	Amphiuridae	Amphiura filiformis	(Müller, 1776)	35
Echinodermata	Ophiuroidea	Amphilepidida	Ophiotrichidae	Ophiothrix fragilis	(Abildgaard in Müller, 1789)	179
Echinodermata	Echinoidea	Camarodonta	Parechinidae	Psammechinus miliaris	(Müller, 1771)	144
Hemichordata	Enteropneusta	[unassigned] Enteropneusta	Ptychoderidae	Balanoglossus clavigerus	Delle Chiaje, 1829	873
Mollusca	Gastropoda	Nudibranchia	Goniodorididae	Ancula gibbosa	(Risso, 1818)	241
Mollusca	Gastropoda	[unassigned] Caenogastropoda	Cerithiidae	Bittium reticulatum	(da Costa, 1778)	219
Mollusca	Gastropoda	Littorinimorpha	Caecidae	Caecum trachea	(Montagu, 1803)	12
Mollusca	Gastropoda	Nudibranchia	Trinchesiidae	Catriona aurantia	(Alder & Hancock, 1842)	377
Mollusca	Gastropoda	Nudibranchia	Dotidae	Doto coronata	(Gmelin, 1791)	326
Mollusca	Gastropoda	Nudibranchia	Dotidae	Doto millbayana	Lemche, 1976	44
Mollusca	Gastropoda		Plakobranchidae	Elysia viridis	(Montagu, 1804)	25
Mollusca	Gastropoda	Nudibranchia	Facelinidae	Facelina bostoniensis	(Couthouy, 1838)	19
Mollusca	Gastropoda		Limapontiidae	Limapontia depressa	Alder & Hancock, 1862	1544
Mollusca	Bivalvia	Mytilida	Mytilidae	Mytilus edulis	Linnaeus, 1758	68
Mollusca	Gastropoda	Nudibranchia	Polyceridae	Palio nothus	(Johnston, 1838)	18
Mollusca	Gastropoda	Littorinimorpha	Hydrobiidae	Peringia ulvae	(Pennant, 1777)	880
Mollusca	Gastropoda	Nudibranchia	Tergipedidae	Tergipes tergipes	(Forsskål in Niebuhr, 1775)	36
Mollusca	Gastropoda	Trochida	Phasianellidae	Tricolia pullus	(Linnaeus, 1758)	986
Mollusca	Gastropoda	Nudibranchia	Trinchesiidae	Trinchesia foliata	(Forbes & Goodsir, 1839)	270
Porifera	Demospongiae	Suberitida	Halichondriidae	Hymeniacidon perlevis	(Montagu, 1814)	2965

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Table S6. List of the 104 recovered species with 18S, with the respective number of reads. The list only contains marine and brackish metazoans, according to WoRMS, and species with more than 8 reads. Taxa underlined represent the non-indigenous species detected.

Phylum	Class	Order	Family	Species	Authority	No. of reads
Annelida	Polychaeta		Capitellidae	*Capitella teleta*	Blake et al., 2009	25476
Annelida	Polychaeta		Capitellidae	*Dasybranchus caducus*	(Grube, 1846)	19
Annelida	Polychaeta	Phyllodocida	Phyllodocidae	*Eumida sanguinea*	(Örsted, 1843)	3262
Annelida	Polychaeta		Sabellariidae	*Gunnarea gaimardi*	(Quatrefages, 1848)	1403
Annelida	Polychaeta	Phyllodocida	Polynoidae	*Harmothoe imbricata*	(Linnaeus, 1767)	24
Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste diversicolor*	(Müller, 1776)	11
Annelida	Polychaeta		Capitellidae	*Heteromastus filiformis*	(Claparède, 1864)	41
Annelida	Polychaeta	Spionida	Spionidae	*Malacoceros fuliginosus*	(Claparède, 1868)	513
Annelida		Myzostomida	Myzostomatidae	*Myzostoma cirriferum*	Leuckart, 1836	60
Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys incisa*	Malmgren, 1865	117
Annelida	Polychaeta	Terebellida	Terebellidae	*Nicolea uspiana*	(Nogueira, 2003)	1637
Annelida	Polychaeta	Phyllodocida	Phyllodocidae	*Notophyllum foliosum*	(Sars, 1835)	23
Annelida	Polychaeta	Spionida	Poecilochaetidae	*Poecilochaetus serpens*	Allen, 1904	15
Annelida	Polychaeta	Terebellida	Terebellidae	*Polycirrus carolinensis*	Day, 1973	36
Annelida	Polychaeta		Spionidae	*Polydora onagawaensis*	Teramoto, et al., 2013	27
Annelida	Polychaeta	Phyllodocida	Goniadidae	*Progoniada regularis*	Hartman, 1965	14
Annelida	Polychaeta	Spionida	Spionidae	*Spiophanes bombyx*	(Claparède, 1870)	30
Annelida	Polychaeta	Terebellida	Terebellidae	*Thelepus cincinnatus*	(Fabricius, 1780)	42

Phylum	Class	Order	Family	Species	Authority	No. of reads
Arthropoda	Hexanauplia	Cyclopoida	Lichomolgidae	*Astericola clausii*	Rosoll, 1888	761
Arthropoda	Thecostraca	Lithoglyptida	Lithoglyptidae	*Auritoglyptes bicornis*	(Aurivillius, 1892)	22
Arthropoda	Thecostraca	Balanomorpha	Austrobalanidae	*Austrobalanus imperator*	(Darwin, 1854)	527
Arthropoda	Hexanauplia	Calanoida	Calanidae	*Calanoides carinatus*	(Krøyer, 1849)	61
Arthropoda	Hexanauplia	Calanoida	Calanidae	*Calanus finmarchicus*	(Gunnerus, 1770)	31
Arthropoda	Hexanauplia	Calanoida	Calanidae	*Calanus glacialis*	Jaschnov, 1955	2569
Arthropoda	Hexanauplia	Siphonostomatoida	Caligidae	*Caligus brevipedis*	Bassett-Smith, 1896	177
Arthropoda	Hexanauplia	Siphonostomatoida	Caligidae	*Caligus uniartus*	(Ho et al., 2004)	72
Arthropoda	Hexanauplia	Polyarthra	Canuellidae	*Canuella perplexa*	Scott & Scott, 1893	16
Arthropoda	Hexanauplia	Calanoida	Centropagidae	*Centropages typicus*	Krøyer, 1849	1998
Arthropoda	Branchiopoda	Anomopoda	Daphniidae	*Daphnia pulex*	Leydig, 1860	1567
Arthropoda	Malacostraca	Decapoda	Varunidae	*Eriocheir sinensis*	Milne Edwards, 1853	142
Arthropoda	Arachnida	Trombidiformes	Halacaridae	*Halacaroides antoniazziae*	Pepato et al., 2011	15
Arthropoda	Ostracoda	Podocopida	Cytheruridae	*Hemicytherura kajiyamai*	Hanai, 1957	11
Arthropoda	Hexanauplia	Calanoida	Centropagidae	*Isias clavipes*	Boeck, 1865	128
Arthropoda	Arachnida	Trombidiformes	Halacaridae	*Isobactrus uniscutatus*	(Viets, 1939)	17
Arthropoda	Ostracoda	Podocopida	Leptocytheridae	*Leptocythere lacertosa*	(Hirschmann, 1912)	39
Arthropoda	Hexanauplia	Cyclopoida	Lichomolgidae	*Lichomolgus canui*	Sars, 1917	13
Arthropoda	Hexanauplia	Calanoida	Metridinidae	*Metridia gerlachei*	Giesbrecht, 1902	15
Arthropoda	Hexanauplia	Cyclopoida	Lichomolgidae	*Modiolicola bifida*	Tanaka, 1961	130
Arthropoda	Hexanauplia	Cyclopoida	Mytilicolidae	*Mytilicola intestinalis*	Steuer, 1902	28
Arthropoda	Hexanauplia	Cyclopoida	Oithonidae	*Oithona davisae*	Ferrari & Orsi, 1984	4859

Phylum	Class	Order	Family	Species	Authority	No. of reads
Arthropoda	Hexanauplia	Harpacticoida	Laophontidae	*Paralaophonte (Paralaophonte) congenera*	(Sars, 1908)	286
Arthropoda	Hexanauplia	Harpacticoida	Miraciidae	*Paramphiascella fulvofasciata*	Rosenfield & Coull, 1974	52
Arthropoda	Thecostraca		Peltogasterellidae	*Peltogasterella sulcata*	(Lilljeborg, 1859)	31
Arthropoda	Thecostraca	Pollicipedomorpha	Pollicipedidae	*Pollicipes pollicipes*	(Gmelin, 1791 [in Gmelin, 1788-1792])	10
Arthropoda	Hexanauplia	Calanoida	Pseudodiaptomidae	*Pseudodiaptomus marinus*	Sato, 1913	961
Arthropoda	Thecostraca		Sacculinidae	*Sacculina carcini*	Thompson, 1836	375
Arthropoda	Ostracoda	Podocopida	Cytheruridae	*Semicytherura striata*	(Sars, 1866)	108
Arthropoda	Hexanauplia	Calanoida	Temoridae	*Temora longicornis*	(Müller, 1785)	2273
Bryozoa	Gymnolaemata	Cheilostomatida	Scruparіidae	*Scruparia chelata*	(Linnaeus, 1758)	10
Chaetognatha	Sagittoidea	Aphragmophora	Sagittidae	*Parasagitta friderici*	(Ritter-Záhony, 1911)	101
Chordata	Actinopteri	Acanthuriformes	Pomacanthidae	*Apolemichthys griffisi*	(Carlson & Taylor, 1981)	16
Chordata	Actinopteri	Gadiformes	Gadidae	*Gadus morhua*	Linnaeus, 1758	19
Cnidaria	Anthozoa	Actiniaria	Aiptasiidae	*Aiptasia insignis*	Carlgren, 1941	27
Cnidaria	Hydrozoa	Anthoathecata	Cordylophoridae	*Cordylophora caspia*	(Pallas, 1771)	109
Cnidaria	Hydrozoa	Anthoathecata	Rathkeidae	*Lizzia blondina*	Forbes, 1848	40
Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	*Obelia geniculata*	(Linnaeus, 1758)	166
Cnidaria	Scyphozoa	Rhizostomeae	Rhizostomatidae	*Rhopilema verrilli*	(Fewkes, 1887)	24
Echinodermata	Ophiuroidea	Amphilepidida	Amphiuridae	*Amphioplus cf. daleus (Silax daleus)*	*(Lyman, 1879)*	15
Echinodermata	Echinoidea	Aspidodiadematoida	Aspidodiadematidae	*Aspidodiadema jacobyi*	Agassiz, 1880	35

Phylum	Class	Order	Family	Species	Authority	No. of reads
Echinodermata	Ophiuroidea	Amphilepidida	Ophiotrichidae	*Ophiothrix (Ophiothrix) oerstedii*	Lütken, 1856	37
Echinodermata	Echinoidea	Camarodonta	Strongylocentrotidae	*Strongylocentrotus purpuratus*	(Stimpson, 1857)	19
Entoprocta			Barentsiidae	*Barentsia benedeni*	(Foettinger, 1887)	335
Mollusca	Gastropoda	Nudibranchia	Goniodorididae	*Ancula gibbosa*	(Risso, 1818)	438
Mollusca	Gastropoda	Nudibranchia	Discodorididae	*Asteronotus cespitosus*	(van Hasselt, 1824)	18
Mollusca	Bivalvia	Cardiida	Cardiidae	*Cerastoderma edule*	(Linnaeus, 1758)	33
Mollusca	Gastropoda	Nudibranchia	Cuthonidae	*Cuthona nana*	(Alder & Hancock, 1842)	29
Mollusca	Bivalvia	Cardiida	Donacidae	*Donax trunculus*	Linnaeus, 1758	76
Mollusca	Bivalvia	Adapedonta	Pharidae	*Ensis ensis*	(Linnaeus, 1758)	41
Mollusca	Gastropoda		Limapontiidae	*Ercolania felina*	(Hutton, 1882)	705
Mollusca	Bivalvia	Adapedonta	Hiatellidae	*Hiatella arctica*	(Linnaeus, 1767)	96
Mollusca	Gastropoda	Ellobiida	Ellobiidae	*Melampus fasciatus*	(Deshayes, 1830)	26
Mollusca	Gastropoda		Parhedylidae	*Microhedyle glandulifera*	(Kowalevsky, 1901)	90
Mollusca	Bivalvia	Myida	Myidae	*Mya arenaria*	Linnaeus, 1758	37
Mollusca	Bivalvia	Mytilida	Mytilidae	*Mytilus edulis*	Linnaeus, 1758	731
Mollusca	Gastropoda	Littorinimorpha	Hydrobiidae	*Peringia ulvae*	(Pennant, 1777)	54
Mollusca	Bivalvia	Venerida	Veneridae	*Petricola lapicida*	(Gmelin, 1791)	9
Mollusca	Bivalvia	Adapedonta	Pharidae	*Pharus legumen*	(Linnaeus, 1758)	109
Mollusca	Bivalvia	Adapedonta	Solenidae	*Solen strictus*	Gould, 1861	826
Mollusca	Bivalvia	Venerida	Mactridae	*Spisula solidissima*	(Dillwyn, 1817)	153

Phylum	Class	Order	Family	Species	Authority	No. of reads
Mollusca	Bivalvia	Cardiida	Solecurtidae	*Tagelus californianus*	(Conrad, 1837)	3420
Mollusca	Gastropoda	Nudibranchia	Tergipedidae	*Tergipes tergipes*	(Forsskål in Niebuhr, 1775)	38
Mollusca	Bivalvia	Venerida	Veneridae	*Venerupis aspera*	(Quoy & Gaimard, 1835)	47
Nematoda	Chromadorea	Monhysterida	Monhysteridae	*Diplolaimelloides meyli*	Timm, 1961	9
Nematoda	Chromadorea	Rhabditida	Anguinidae	*Halenchus fucicola*	(de Man, 1892) Cobb, 1933	9
Nematoda	Chromadorea	Rhabditida	Rhabditidae	*Litoditis marina*	(Bastian, 1865) Sudhaus, 2011	16
Nematoda	Chromadorea	Rhabditida	Rhabditidae	*Litoditis mediterranea*	(Sudhaus, 1974) Sudhaus, 2011	9
Nematoda	Chromadorea	Araeolaimida	Comesomatidae	*Sabatieria pulchra*	(Schneider, 1906)	68
Nematoda	Chromadorea	Desmodorida	Desmodoridae	*Spirinia parasitifera*	(Bastian, 1865) Gerlach, 1963	9
Nemertea	Hoplonemertea	Monostilifera	Tetrastemmatidae	*Tetrastemma peltatum*	Bürger, 1895	252
Nemertea	Hoplonemertea	Monostilifera	Tetrastemmatidae	*Tetrastemma vermiculus*	(Quatrefages, 1846)	64
Phoronida			Phoronidae	*Phoronis emigi*	Hirose et al., 2014	77
Platyhelminthes		Rhabdocoela	Solenopharyngidae	*Adenopharynx mitrabursalis*	Ehlers, 1972	80
Platyhelminthes		Proseriata	Coelogynoporidae	*Cirrifera dumosa*	Sopott, 1972	9
Platyhelminthes		Polycladida	Leptoplanidae	*Hoploplana californica*	Hyman, 1953	223
Platyhelminthes		Polycladida	Notoplanidae	*Notoplana australis*	(Schmarda, 1859)	538
Platyhelminthes		Polycladida	Eurylepidae	*Prostheceraeus vittatus*	(Montagu, 1815)	2800
Platyhelminthes		Rhabdocoela	Provorticidae	*Provortex balticus*	(Schultze, 1851) Graff, 1882	115
Platyhelminthes		Rhabdocoela		*Thalassoplanella collaris*	Luther, 1946	101
Platyhelminthes		Rhabdocoela	Gnathorhynchidae	*Uncinorhynchus flavidus*	Karling, 1947	211
Porifera	Demospongiae	Suberitida	Halichondriidae	*Hymeniacidon heliophila*	(Wilson, 1911)	8115
Rotifera	Eurotatoria	Ploima	Dicranophoridae	*Encentrum astridae*	Sørensen, 2001	10

Phylum	Class	Order	Family	Species	Authority	No. of reads
Rotifera	Eurotatoria	Ploima	Synchaetidae	*Synchaeta tremula*	(Muller, 1786)	70
Sipuncula	Phascolosomatidea	Phascolosomatida	Phascolosomatidae	*Phascolosoma (Phascolosoma) granulatum*	Leuckart, 1828	13
Xenacoelomorpha		Acoela	Mecynostomidae	*Mecynostomum auritum*	(Schultze, 1851)	9

Table S7. Characterization of the recovered taxa with COI and 18S, based on occurrence time in the plankton: holoplankton (HP), temporary benthos (TB), meroplankton eggs and larvae (MEL), meroplankton at least for the larvae (ML), meroplankton only for the larvae (MOL), non-planktonic (NP). NA, correspond to species for which no information concerning its occurrence in plankton was found.

Recovered species with DNA metabarcoding	In zooplankton	Details
Acrocnida brachiata	MOL	Brooding is common. Bursae is used as brood chambers where the embryos develop into juveniles and later crawl out from the bursal slits. (www.sealifebase.ca)
Adenopharynx mitrabursalis	NA	According to WoRMS is a benthic species. Could not find information about its reproduction
Aiptasia insignis	NA	According to WoRMS is a benthic species. Could not find information about its reproduction
Amphibalanus eburneus	MOL	Eggs stay in the mantle until hatched (Torres et al., 2012).
Amphioplus cf. daleus (Silax daleus)	NA	Could not find information about its reproduction

Recovered species with DNA metabarcoding	In zooplankton	Details
Amphiura filiformis	MOL	Brooding is common. Bursae is used as brood chambers where the embryos develop into juveniles and later crawl out from the bursal slits (www.sealifebase.ca).
Ancula gibbosa	MOL	Shelled veliger larvae live as plankton before transforming into adult form (Clark, 1975).
Apolemichthys griffisi	ML	The eggs, released and fecundated in the surface, are dispersed by the currents (Sapolu, 2005).
Arenicola defodiens	MOL	The larvae dispersed from the adult grounds (around 3 setigers and 0.5 mm in length) to a sediment bare location (Pires et al., 2015; Cubber, 2019).
Aspidodiadema jacobyi	MOL	Eggs are held either on the peristome around the periproct, or deep into the concavities on the petaloids. Embryos develop into planktotrophic larvae (www.sealifebase.ca).
Astericola clausii	NA	Could not find information about its reproduction.
Asteronotus cespitosus	MOL	Eggs are deposited on a substratum where they develop and hatch into (planktonic) vestigial veliger larval stage (www.sealifebase.ca).
Auritoglyptes bicornis	ML	There is no specific information about the larval development of *Auritoglyptes*, but such a widespread distribution may be evidence of the presence of planktonic nauplii (Chan et al., 2013).
Austrobalanus imperator	ML	(Egan & Anderson, 1988).
Balanoglossus clavigerus	MEL	Egg-mass are discharged by the female, from its burrow, and then the sperms are discharged by the male, from its burrow. The tornaria larva swims freely, leading a planktonic life feeding on minute organisms (species-identification.org).

Recovered species with DNA metabarcoding	In zooplankton	Details
Balanus trigonus	MOL	Eggs hatch into planktonic nauplii and leave the mantle cavity (www.sealifebase.ca).
Barentsia benedeni	MOL	The eggs are brooded externally in the vestibule (inside the ring of tentacles) and hatch into lecithotrophic larvae which spend a few hours in the plankton (invasions.si.edu/nemesis/).
Bittium reticulatum	MOL	The fertilized eggs are brooded in the mantle cavity, sometimes for several months, and are released as nauplius larvae (Chukhchin, 1969).
Caecum trachea	ML	In the plankton, larvae are found with shells of from 1 1/2 whorls and diameter of 230 up to shells with 2 whorls and diameter of 290 (Chukhchin, 1969).
Calanoides carinatus	HP	
Calanus finmarchicus	HP	
Calanus glacialis	HP	
Caligus brevipedis	MOL	Caligidae typically have direct life-cycles and hence the infection of new susceptible hosts is horizontal from an infected host to other susceptible hosts. Dispersal of these parasites is achieved through non-feeding planktonic nauplii and the free-living, infective planktonic copepodid stage which locates and attaches to a new host (Hayes et al., 2021).
Caligus uniartus	MOL	Caligidae typically have direct life-cycles and hence the infection of new susceptible hosts is horizontal from an infected host to other susceptible hosts. Dispersal of these parasites is achieved through non-feeding planktonic nauplii and the free-living, infective planktonic copepodid stage which locates and attaches to a new host (Hayes et al., 2021).
Canuella perplexa	NA	According to WoRMS is a benthic species. Could not find information about its reproduction.

Recovered species with DNA metabarcoding	In zooplankton	Details
Capitella teleta	MOL	When female worms are ready to lay their eggs, they create an encasement called a brood tube. The worm remains inside the brood tube along with her offspring until they become larval stages (thenode.biologists.com).
Catriona aurantia	MOL	The "nest" area is delineated by a zone of silt and fecal material in which the adults and their egg masses may be found (Clark, 1975).
Centropages typicus	HP	
Cerastoderma edule	MEL	Fertilization is external (meaning eggs develop in the water column) (www.marlin.ac.uk).
Chelon labrosus	MEL	Eggs and larvae pelagic (www.fishbase.se).
Chthamalus montagui	MOL	Egg masses (egg lamellae) are brooded in the mantle cavity (Burrows, 1999).
Cirrifera dumosa	NA	Could not find information about its reproduction nor its relation to zooplankton.
Clytia hemisphaerica	TB	Some larvae stages are benthic, but overall are planktonic whole life (Houliston, 2021).
Cordylophora caspia	MOL	Planula larvae swim or crawl for short periods (e.g., <24hrs) so that dispersal away from the parent colony (www.marlin.ac.uk).
Cuthona nana	MOL	Reproductively typical of opisthobranchs in that it is a reciprocally copulating hermaphrodite that deposits eggs within a gelatinous stroma (Rivest, 1978).
Daphnia pulex	HP	
Dasybranchus caducus	MEL	After fertilization, most eggs become planktonic (www.sealifebase.ca).
Diplolaimelloides meyli	NP	See Conway (2015)

Recovered species with DNA metabarcoding	In zooplankton	Details
Donax trunculus	MEL	
Doto coronata	MOL	Eggs are deposited on a substratum where they develop and hatch into (planktonic) vestigial veliger larval stage (www.sealifebase.ca).
Doto millbayana	MOL	Eggs are deposited on a substratum where they develop and hatch into (planktonic) vestigial veliger larval stage (www.sealifebase.ca).
Electra pilosa	MOL	Sexually produced embryos develop into larvae which are released into the plankton (britishbryozoans.myspecies.info)
Elysia viridis	MOL	The species has a life span of 12^15 months, and sexually mature slugs /512 mm) produce benthic egg masses (Trowbridge, 2000)
Encentrum astridae	NA	
Ensis ensis	ML	Embryos develop into free-swimming trocophore larvae (www.sealifebase.ca).
Ercolania felina	MOL	Hypodermic insemination can be unilateral (meaning fecundation is internal) (Baur, 1998).
Eriocheir sinensis	MEL	The adults are semelparous in that they mate once and then die (meaning they release the eggs/embryo before they die) (www.cabi.org/isc/).
Eumida mackiei	ML	Recent identification by Teixeira et al. (2020). At least planktonic larvae, according to WoRMS.
Eumida sanguinea	MEL	Most eggs become planktonic (www.sealifebase.ca).

Recovered species with DNA metabarcoding	In zooplankton	Details
Facelina bostoniensis	MOL	Eggs are deposited on a substratum where they develop and hatch into (planktonic) vestigial veliger larval stage (www.sealifebase.ca).
Gadus morhua	MEL	Pelagic eggs. Larvae are pelagic up to 2.5 months before settling on the bottom (www.fishbase.se).
Glycera alba	MEL	Most eggs become planktonic (www.sealifebase.ca).
Gunnarea gaimardi	ML	According to WoRMS.
Halacaroides antoniazziae	NA	Can't find information about its life cycle.
Halenchus fucicola	NP	See Conway (2015).
Halocladius varians	ML	Information about its life cycle not found. Although larvae seem to be mostly benthonic, but under certain conditions they can migrate, entering temporarily in the plankton. Their migrations are mostly due to water currents (Britton & Johnson, 1987).
Harmothoe imbricata	MEL	Most eggs become planktonic (www.sealifebase.ca).
Hediste diversicolor	MEL	Most eggs become planktonic (www.sealifebase.ca).
Hemicytherura kajiyamai	MOL	Eggs may either be attached to a substratum or brooded, where they grow and hatch as nauplii (planktonic stage) (www.sealifebase.ca).
Heteromastus filiformis	MEL	Most eggs become planktonic (www.sealifebase.ca).

Recovered species with DNA metabarcoding	In zooplankton	Details
Hiatella arctica	MEL	External fecundization. Embryos develop into free-swimming trocophore larvae (Hiebert et al., 2015).
Holopedium gibberum	HP	
Hoploplana californica	NA	Couldn't find any information relating this species to zooplankton.
Hymeniacidon heliophila	ML	The zygote develops into parenchymella larva (free-swimming) (www.sealifebase.ca).
Hymeniacidon perlevis	ML	Ova are fertilised in the sponge body, where they give rise to ciliated larvae (Gaino et al., 2010).
Hypogastrura viatica	HP	
Isias clavipes	HP	
Isobactrus uniscutatus	NA	Couldn't find any information relating this species to zooplankton.
Lanice conchilega	MEL	Most eggs become planktonic (www.sealifebase.ca).
Laonice cirrata	MEL	Most eggs become planktonic (www.sealifebase.ca).
Leptocythere lacertosa	MOL	Eggs may either be attached to a substratum or brooded, where they grow and hatch as nauplii (planktonic stage) (www.sealifebase.ca).
Lichomolgus canui	NA	Couldn't find any information relating this species to zooplankton.
Limapontia depressa	ML	Limapontia has a pelagic larval stage (Den Hartog, 1959).
Litoditis marina	NP	
Litoditis mediterranea	NP	

Recovered species with DNA metabarcoding	In zooplankton	Details
Lizzia blondina	TB	Very common in the plankton. However, it has a polyp stage that is benthic (species-identification.org).
Lysidice ninetta	MEL	Most eggs become planktonic (www.sealifebase.ca).
Magelona johnstoni	MEL	Most eggs become planktonic (www.sealifebase.ca).
Malacoceros fuliginosus	MEL	Most eggs become planktonic (www.sealifebase.ca).
Mecynostomum auritum	NA	Couldn't find any information relating this species to zooplankton
Meiodrilus adhaerens	ML	According to WoRMS. No information was found about eggs.
Melampus fasciatus	MOL	Due to the geographic its distribution, the occurrence of free-swimming larvae became probable. Produce egg masses in substratum (Marcus & Marcus, 1965).
Melita palmata	NP	Seems like this species is not planktonic at any stage (not even larvae)
Membranipora membranacea	MEL	Eggs are fertilized then released, and quickly develop into cyphonautes larvae which may feed and develop as plankton for several months (inverts.wallawalla.edu)
Metridia gerlachei	HP	
Microhedyle glandulifera	ML	Owing to the low number (maximum 35) of yolk-rich eggs which indi-cates lecithotrophic development, it is unlikely that larvae play a major role in long-distance dispersal (Eder, 2011).
Modiolicola bifida	NA	Scarce available information about this species
Mya arenaria	MEL	Fertilization is external and the eggs can be carried many miles by the current (www.marlin.ac.uk).

Recovered species with DNA metabarcoding	In zooplankton	Details
Mytilicola intestinalis	MEL	Eggs detached and grown in the laboratory hatched after 7 days at 18° C. There are a nauplius and a metanauplius larval stages, which are free-swimming (www.nobanis.org).
Mytilus edulis	MEL	Gametes are shed into the water where fertilization occurs. After the egg is fertilized it turns into a ciliated trocophore larva (animaldiversity.org).
Myzostoma cirriferum	MEL	Embryogenesis starts after egg laying and takes place in the water column. Ciliated protrochophores and trochophores are free-swimming (Euckhaut & Jangoux, 1993).
Nephtys hombergii	ML	The pelagic life cycle lasts seven to eight weeks at the end of which larvae metamorphose into benthic juveniles (www.marlin.ac.uk).
Nephtys incisa	MEL	Most eggs become planktonic (Zajac & Whitlatch, 1988; see also www.sealifebase.ca).
Nicolea uspiana	ML	Reproductive and developmental traits of free spawning with lecithotrophic development (Garraffoni et al., 2014).
Notophyllum foliosum	MEL	Most eggs become planktonic (www.sealifebase.ca).
Notoplana australis	MOL	Prolonged breeding season and would deposit its eggs in a coiled, gelatinous egg string (Anderson, 1997).
Obelia dichotoma	MEL	Fertilization is external with both eggs and sperm being released into the sea. Fertilization results in an embryo that develops into a typical planula larva (www.marlin.ac.uk).
Obelia geniculata	MEL	External fertilization, by releasing gametes into the sea, resulting into lecithotrophic planula larvae (Govindarajan et al., 2005).
Oithona davisae	HP	

Recovered species with DNA metabarcoding	In zooplankton	Details
Ophiothrix (Ophiothrix) oerstedii	MEL	The mode of reproduction is pluteus larvae. Eggs would be emitted into seawater with high sperm densities and fertilization success would be enhanced (Hendler, 1982; Mladenov, 1983).
Ophiothrix fragilis	MEL	The eggs are fertilized and develop in the water column. Larvae develop in the water column; strong currents may cause a passive migration (animaldiversity.org).
Palio nothus	MOL	Eggs are deposited on a substratum where they develop and hatch into (planktonic) vestigial veliger larval stage (www.sealifebase.ca).
Paralaophonte (Paralaophonte) congenera	NA	Was not able to find any information relating to its life cycle
Paramphiascella fulvofasciata	MOL	See Dahms (1986).
Paranais botniensis	NA	Information about its life cycle not found
Parasagitta friderici	HP	
Peltogasterella sulcata	MOL	These eggs are fertilized inside the maternal mantle cavity by spermatozoa from the cypris-cell receptacles. The zygotes develop into large nauplii. The nauplii leave the maternal mantle cavity and become a part of surface plankton (Yanagimachi, 1961).
Peringia ulvae	MOL	There is considerable conflicting evidence over the developmental mechanism of the larvae of this species. Snails producing planktotrophic forms have several (7-22) smaller eggs that hatch into veliger larvae at around 150 microns. (...) Age at maturity the eggs are laid preferentially on the shells of live individuals of this species but also on empty shells and grains of sand (www.marlin.ac.uk).

Recovered species with DNA metabarcoding	In zooplankton	Details
Petricola lapicida	ML	Embryos develop into free-swimming trochophore larvae (www.sealifebase.ca).
Pharus legumen	ML	Embryos develop into free-swimming trochophore larvae (www.sealifebase.ca).
Phascolosoma (Phascolosoma) granulatum	ML	According WoRMS.
Phoronis emigi	MOL	The eggs are brooded within the adults' tube, then are released only when they have hatched. They do not feed and spend only about 4 days in the plankton as larvae (earthlife.net)
Platynereis dumerilii	MEL	External fertilization induces the formation of thousands of small zygotes and ultimately implies the death of the reproducing males and females (Schenkelaars & Gazave, 2021)-
Poecilochaetus serpens	MEL	The type of egg and sperm suggest that the entire development takes place in the plankton (Blake et al., 1996).
Pollicipes pollicipes	MOL	See Cruz & Araújo (1999).
Polycirrus carolinensis	ML	According to WoRMS.
Polydora onagawaensis	MOL	The larvae developed inside the egg capsules for 2 weeks (10°C, laboratory conditions), until the 3-chaetiger stage, before being released as planktonic larvae (Teramoto et al., 2013).
Polynoe scolopendrina	MEL	Most eggs become planktonic (www.sealifebase.ca).
Pomatoschistus microps	MOL	Adhesive eggs are deposited under or between stones, shells and aquatic plants (www.fishbase.se).

Recovered species with DNA metabarcoding	In zooplankton	Details
Progoniada regularis	ML	According to WoRMS
Prostheceraeus vittatus	NA	Couldn't find any information relating this species to zooplankton
Protodrilus ciliatus	ML	Possesses planktonic developmental stages (Martínez et al., at Handbook of Zoology Online).
Provortex balticus	NA	Couldn't find any information relating this species to zooplankton
Psamathe fusca	ML	According to WoRMS.
Psammechinus miliaris	MOL	Embryos develop into planktotrophic larvae (www.sealifebase.ca).
Pseudodiaptomus marinus	HP	
Pseudopotamilla reniformis	MEL	Most eggs become planktonic (www.sealifebase.ca).
Rhizostoma luteum	TB	Free-swimming medusa and bottom-dwelling polyp. The life cycle of *R. luteum* follows the general pattern of metagenesis of scyphozoans (Kienberger et al., 2018)
Rhopilema verrilli	TB	Egg is laid by the adult medusa which later develops into a free-living planula, then to a scyphistoma to a strobila, and lastly to a free-living young medusa (Kienberger et al., 2018).
Sabatieria pulchra	NP	See Conway (2015).
Sabellaria alveolata	ML	The larvae probably spend anything between 6 weeks and 6 months in the plankton (ukmpa.marinebiodiversity.org)
Sabellaria spinulosa	ML	According to Natural Resources Wales (cdn.naturalresources.wales)

Recovered species with DNA metabarcoding	In zooplankton	Details
Sacculina carcini	MOL	The female releases fertilized eggs into the abdominal cavity of the host, where the eggs are incubated and develop into free-living larvae (Lützen et al., 2018).
Scruparia chelata	MOL	Larvae are released from adults (not the embryos) (Woollacott & Zimmer, 2013).
Semicytherura striata	NA	Couldn't find any information relating this species to zooplankton
Solen strictus	ML	Embryos develop into free-swimming trocophore larvae
Spio symphyta	ML	According to WoRMS.
Spiophanes bombyx	MEL	Most eggs become planktonic (www.sealifebase.ca).
Spirinia parasitifera	NP	See Conway (2015).
Spisula solidissima	MEL	Gametes are broadcast into the water column (fecundization occurs in the water column) (www.fisheries.noaa.gov).
Strongylocentrotus purpuratus	MOL	Embryos develop into planktotrophic larvae (www.sealifebase.ca).
Synchaeta tremula	HP	
Tagelus californianus	MEL	Eggs and larvae are pelagic (Wolotira Jr, 1989).
Temora longicornis	HP	
Tergipes tergipes	MOL	Eggs are deposited on a substratum where they develop and hatch into (planktonic) vestigial veliger larval stage (www.sealifebase.ca).

Recovered species with DNA metabarcoding	In zooplankton	Details
Tetrastemma peltatum	ML	According to WoRMS.
Tetrastemma vermiculus	ML	According to WoRMS.
Thalassoplanella collaris	NA	Couldn't find any information relating this species to zooplankton
Tharyx setigera	MOL	According to WoRMS.
Thelepus cincinnatus	NP	The eggs are very large and development is therefore believed to be non-pelagic. Sexual reproduction occurs all year and it is assumed that the larvae are not pelagic (www.iopan.gda.pl).
Tricolia pullus	MEL	External fertilization occurs when orange ova are released singly from the mantle cavity into the plankton. Free living trochophore larvae hatch after ten hours (Manly, 1976; Smith, 2021).
Trinchesia foliata	MOL	Eggs are deposited on a substratum where they develop and hatch into (planktonic) vestigial veliger larval stage (www.sealifebase.ca).
Trisopterus luscus	MEL	Pouting eggs are pelagic (Alonso-Fernández et al., 2011).
Uncinorhynchus flavidus	NA	Couldn't find any information relating this species to zooplankton
Venerupis aspera	ML	See Ota & Tokeshi (2000).