NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF
ENVIRONMENTAL SCIENCES AND
ENGINEERING

The Function Of The Gut Microbiome Of Macrobenthic Species Under Different Sediment Contamination

Joana Filipa Marujo Neves

Master in Environmental Engineering

DOCTORATE IN ENVIRONMENT AND SUSTAINABILITY

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Adviser: Jorge Lobo Arteaga
Assistant Researcher – IPMA I.P. – Portuguese Institute of Sea and Atmosphere

Co-advisers: Marta Susana Silvestre Gouveia Martins
Associate Professor, NOVA University Lisbon

Maria Helena Soares Martins Adão
Associate Professor, University of Évora

Examination Committee:

Chair: Alexandra de Jesus Branco Ribeiro,
Full Professor, NOVA University Lisbon

Rapporteurs: Filipe José Oliveira Costa,
Associate Professor, University of Minho

Paula Maria Chainho de Oliveira,
Assistant Professor, Faculty of Sciences of the University of Lisbon

Adviser: Jorge Lobo Arteaga,
Assistant Researcher, IPMA I.P., Portuguese Institute of Sea and At mosphere

Members: Ana Filipa Ferreira do Vale,
Assistant Professor, Faculty of Sciences of the University of Lisbon

Alexandra de Jesus Branco Ribeiro,
Full Professor, NOVA University Lisbon

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Reconhecimento do uso de tecnologias e ferramentas de Inteligência Artificial (IA) generativa, softwares e outras ferramentas de apoio.

Reconheço a utilização do ChatGPT 3.5 (Open AI, https://chat.openai.com), Grammarly (https://app.grammarly.com/), e Deepl (https://www.deepl.com/en/translator) para correção de escrita em inglês, e a utilização do Office365 (Microsoft, https://www.office.com) para a elaboração do documento e tratamento de dados. Reconheço a utilização de linguagem de código Python (https://www.python.org/) e R (https://www.r-project.org/about.html) para processamento, visualização e análise estatística de dados. Reconheço a utilização do software QGIS 3.22.1 (https://www.qgis.org/en/site/) para produção de mapas. Reconheço a utilização do software Zotero (https://www.zotero.org/) para produção de citações e referências ao longo do documento.

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"*All we have to decide is what to do with the time that is given us.*"

(J.R.R. Tolkien).

Abstract

Estuarine ecosystems are dynamic and productive environments, yet they are particularly vulnerable to anthropogenic pressures, including inorganic contamination associated with sediment. These contaminants, particularly trace metals, can disrupt sediment biogeochemistry and affect benthic organisms. In this context, the gut microbiome of sediment-dwelling invertebrates has emerged as a promising tool for environmental assessment due to its sensitivity to local conditions and its role in host physiology, immunity and ecological function.

This PhD thesis focused on evaluating how sedimentary inorganic contamination affects the structure and predicted function of the gut microbiome in the common cockle *Cerastoderma edule* (Linnaeus, 1758), a widespread and ecologically relevant estuarine bivalve species. An integrative approach was employed in this study, combining (i) field characterization of macrobenthic communities and sediment geochemistry across a contamination gradient in the Sado Estuary, (ii) *in situ* analysis of bacterial and fungal gut microbiomes through 16S rRNA and ITS metabarcoding, and (iii) a controlled *in vivo* bioassay assessing microbiome responses to mercury-enriched sediments. Environmental characterization revealed variation in both sediment characteristics and ecological status across sites, with areas located near anthropogenic sources exhibiting higher trace metal enrichment, particularly mercury, and lower ecological quality based on macrobenthic indices, while more distant or less exposed sites showed lower contamination and healthier benthic communities. These environmental gradients were also reflected in the gut microbiome structure and function of *C. edule*, with more diverse and functionally distinct bacterial communities in impacted areas, and site-specific fungal responses. Functional inference highlighted shifts in pathways related to sulfur and nitrogen metabolism, stress, and microbial resilience. The bioassay confirmed that short-term mercury spiked-sediment exposure induces significant perturbations in bacterial dysbiosis and functional restructuring, while fungal communities remained taxonomically stable but functionally altered.

The findings support the concept of the gut microbiome as an integrative bioindicator of environmental quality that can detect early ecological perturbations. The results contributed to the understanding of host-microbiome-environment interactions, addressed a knowledge gap in microbiome-aware ecotoxicology, and contributed to broader efforts in environmental health assessment.

Keywords: Gut Microbiome; Estuarine Ecology; Trace Metal Enrichment; Metabarcoding; *Cerastoderma edule*; Sentinel species

Resumo

Os ecossistemas estuarinos são ambientes dinâmicos e produtivos, mas particularmente vulneráveis a pressões de origem antropogénica, incluindo a contaminação inorgânica associada ao sedimento. Estes contaminantes, em especial os metais traço, podem perturbar a biogeoquímica sedimentar e afetar os organismos bentónicos. Neste contexto, o microbioma intestinal de invertebrados que habitam o sedimento tem-se revelado uma ferramenta promissora para a avaliação ambiental, devido à sua sensibilidade às condições locais e ao seu papel na fisiologia, imunidade e função ecológica do hospedeiro.

Esta tese de doutoramento centrou-se na avaliação do impacto da contaminação inorgânica sedimentar na estrutura e função predita do microbioma intestinal do berbigão *Cerastoderma edule* (Linnaeus, 1758), uma espécie bivalve estuarina de ampla distribuição e relevante do ponto de vista ecológico. Foi adotada uma abordagem integrativa que combinou: (i) a caracterização in situ das comunidades macrobentónicas e da geoquímica sedimentar ao longo de um gradiente de contaminação no estuário do Sado; (ii) a análise *in situ* dos microbiomas intestinais bacteriano e fúngico através de metabarcoding dos marcadores 16S rRNA e ITS; e (iii) um bioensaio controlado *in vivo* para avaliar as respostas do microbioma à exposição a sedimentos enriquecidos com mercúrio. A caracterização ambiental revelou variações tanto nas características sedimentares como no estado ecológico entre os locais estudados, sendo que as áreas próximas de fontes antropogénicas apresentaram maior enriquecimento em metais traço, particularmente mercúrio, e menor qualidade ecológica com base em índices macrobentónicos. Por outro lado, locais mais distantes ou menos expostos evidenciaram níveis de contaminação mais baixos e comunidades bentónicas mais saudáveis. Estes gradientes ambientais refletiram-se também na estrutura e função do microbioma intestinal de *C. edule*, com comunidades bacterianas mais diversas e funcionalmente distintas nas áreas impactadas, e respostas fúngicas específicas por local. A inferência funcional destacou alterações em vias metabólicas relacionadas com o enxofre e o azoto, mecanismos de stress e resiliência microbiana. O bioensaio confirmou que a exposição de curto prazo a sedimentos enriquecidos com mercúrio induz perturbações na estrutura bacteriana significativa e reestruturação funcional, enquanto as comunidades fúngicas permaneceram estáveis do ponto de vista taxonómico, mas com alterações funcionais.

Os resultados obtidos apoiam o conceito do microbioma intestinal como um bioindicador integrador da qualidade ambiental, capaz de detetar perturbações ecológicas precoces. Este trabalho contribuiu para aprofundar a compreensão das interações entre hospedeiro, microbioma e ambiente, preencher as lacunas no conhecimento na área da ecotoxicologia sensível ao microbioma e reforçar os esforços para uma avaliação mais ampla da saúde ambiental.

Palavras-chave: Microbioma Intestinal; Ecologia Estuarina; Enriquecimento em Metais Traço; Metabarcoding; *Cerastoderma edule*; Espécie Sentinela

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ACRONYMS

AMBI	AZTI's Marine Biotic Index
BOLD	Barcode of Life Data Systems
COI	Cytochrome c Oxidase subunit
eDNA	Environmental DNA
EG	Ecological groups
EF	Enrichment Factor
HTS	High throughput sequencing
ITS	Internal Transcribed Spacer
MSFD	Marine Strategy framework directive
PCR	Polymerase Chain Reaction
WoRMS	World Register of Marine Species

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INTRODUCTION

1.1 General Introduction

Marine sediments, including those in estuarine environments, form the largest single ecosystem on Earth. Most of the processes that take place in these sediments are mediated by a variety of micro-, meio- and macro-organisms that inhabit them (Snelgrove, 1999). These organisms are deeply interconnected and play an essential role in biogeochemical processes, and as such, are fundamental to the functioning of estuarine ecosystems (Schratzberger & Ingels, 2018). In addition to their ecological relevance, estuarine sediments support important economic activities, such as artisanal fishing and aquaculture of benthic species, contributing significantly to the livelihoods of coastal communities (Barbier, 2017; Costanza et al., 1997). One of the most important processes for the functioning of shallow coastal and estuarine ecosystems is the flow of organic matter and the exchange of particulate and dissolved nutrients between sediments and water, a process known as benthic-pelagic coupling (Brady et al., 2013; Brigolin et al., 2011; Hochard et al., 2012; Kristensen & Kostka, 2005).

In this context, macrobenthic invertebrates represent a particularly important group due to their role in promoting decomposition, nutrient cycling and energy transfer (Brown et al., 2000). Among these, filter-feeding bivalves such as *Cerastoderma edule* (Linnaeus, 1758) and *Ruditapes decussatus* (Linnaeus, 1758) play key roles in removing suspended particles, modifying the sediment by bioturbation and promoting nutrient cycling, as well as being recognized as bioindicators sensitive to inorganic contaminants (Esposito et al., 2018; Lobo et al., 2010; Lopes et al., 2018; Santos et al., 2022). Their biological activity modifies the geochemical conditions at the sediment-water interface and interacts intensely with microbial communities, making these interactions crucial in aquatic ecosystems.

Macrobenthic invertebrates play a functional role in estuaries and aquatic ecosystems, not only by modifying the structure of sediments and improving microbial processes, but also by promoting the decomposition of organic matter (Kristensen et al., 2014). Their activity facilitates microbial access to organic matter through processes such as particle disaggregation (Rice, 1986) and grazing or "gardening", which allow bacteria to grow exponentially (Kemp, 1987). However, feeding activities can also compete with microbial communities, removing organic matter that would otherwise be available for degradation (Heilskov & Holmer, 2001). Thus, feeding strategy can influence the dynamics of pollution in sediments (Gaston & Young, 1992). For instance, the biological activities of polychaetes, such as *Capitella teleta* Blake, Grassle & Eckelbarger, 2009, have been demonstrated to enhance bacterial proliferation and facilitate the decomposition of organic matter within sediments, probably by stimulating the activity of α-Proteobacteria (Kunihiro et al., 2011). Recent studies show that the relationship between the microbiome and the host can be modulated by chemical exposure (Bahrndorff et al., 2016; Jin et al., 2017). For example, *Capitella teleta* has been shown to increase sediment metabolism and reduce sulfate reduction, suggesting enhanced oxygenation and stimulation of aerobic bacteria (Jang et al., 2021). Another study showed that exposure to hydrocarbons in *C. teleta* led to an increase in the expression of genes associated with chemoheterotrophy and the degradation of aromatic compounds (Hochstein et al., 2019). More broadly, increased microbial growth is often linked to invertebrate activities, such as bioturbation (Palomo & Iribarne, 2000). Polychaetes and bivalves are known to play a key role in the bioturbation of sediments, allowing them to be oxygenated (Widdicombe et al., 2000). Benthic macrofauna, such as polychaetes and bivalves, are a key element in the functioning of the marine ecosystem. They not only deliver oxygen to anoxic areas of sediments, but periodic irrigation in their burrows creates frequent redox oscillations from adjacent sediments (Norling et al., 2007).

The health of aquatic systems can be assessed by evaluating macroinvertebrate communities, as they are key bioindicators of water pollution. (Goulding et al., 2021). Water and sediment pollution is one of the most serious environmental problems (Couto & Ribeiro, 2022) and represents a significant threat to both humans and ecosystems (Gammal et al., 2023; Sharma et al., 2020). One of the main environmental problems in estuarine ecosystems is associated with the release of contaminants, which are often related to the anthropogenic activities nearby (Caeiro et al., 2005; Mil-Homens et al., 2014). Trace metals have the potential to impact the benthic macroinvertebrate species (Dauvin, 2008; Luoma, 1983; Mucha et al., 2003), due to the fact that the estuarine sediments can act as both a sink and a potential source of pollutants that affect benthic organisms.

Contaminants such as trace metals can enter and accumulate in sediments through various routes, including liquid effluent discharges, surface runoff and leachate from urban, industrial and agricultural activities, as well as atmospheric deposition (Caeiro et al., 2005; Couto & Ribeiro, 2022; Ribeiro et al., 2018). This accumulation generally results from the initial introduction of metals into the environment in a dissolved state. Subsequently, they tend to accumulate in suspended particles, inorganic and organic colloids, mainly due to the high affinity of these metals with surfaces of large specific area. In addition to anthropogenic sources, trace metals can also have natural origins (Loring, 1991), which makes it difficult to distinguish the impacts caused by anthropogenic disturbances on the integrity of ecosystems (Loring, n.d.; Mil-Homens et al., 2006; Pastorinho et al., 2012).

Macrobenthic invertebrates are generally used as bioindicators of ecosystem integrity when assessing the impacts of human activities, since their sedentary lifestyle and relatively long life cycle allow them to reflect the conditions of contamination and disturbance over time at a given site (Chiarelli & Roccheri, 2014; Dauer, 1993; Lobo et al., 2010; Mucha et al., 2003; Neira & Höpner, 1994; P. Veiga et al., 2016). Ecological indices, which are widely used to assess environmental quality, rely on species-level identification of macrobenthic communities (Cardoso et al., 2012; Pinto et al., 2009; Rehitha et al., 2022). Traditionally this has been based on morphological taxonomy However, in recent years, DNA metabarcoding has emerged as a powerful and complementary tool for biodiversity assessment (Aylagas et al., 2014, 2016, 2018; Beentjes et al., 2019). This molecular approach utilizes two key markers: the nuclear 18S rRNA gene, which provides broad coverage across eukaryotes, and the mitochondrial COI gene, which provides higher resolution at the species level. When used together, these markers serve to enhance species detection, including cryptic and morphologically indistinguishable taxa, thereby strengthening complementing morphological identification (Duarte et al., 2021, 2023; Lobo et al., 2017).

Furthermore, these organisms exhibit varying degrees of sensitivity and tolerance to diverse categories of disturbance (Borja et al., 2000). The AMBI index (AZTI Marine Biotic Index)(Borja et al., 2000) captures this variability by classifying benthic species into ecological groups according to their tolerance to organic enrichment. Building on this, the M-AMBI index (Muxika et al., 2007) integrates AMBI with species richness and diversity, offering a multimetric approach that aligns with the European Water Framework Directive. Increasing anthropogenic pressures have profoundly altered benthic habitats, contributing to the global expansion of anoxic zones with serious consequences for benthic biodiversity, species abundance and distribution (Diaz & Rosenberg, 2008). Since the loss of species can disrupt the functioning of ecosystems, understanding the variability and mechanisms underlying ecosystem processes, as well as the conditions under which species affect these processes, is one of the main focuses of ecological research (Solan et al., 2004).

Interactions between macrobenthic infauna and microbial communities in soft sediments are therefore complex and ecologically significant, with wider implications for sediment chemistry, nutrient fluxes and microbial community structure at all scales. Some microorganisms can also inhibit the activity of invertebrates, acting as parasites or pathogens (Harris, 1993). This complex dynamic can be observed in marine polychaetes such as *Hediste diversicolor* (O.F. Müller, 1776) and *Marenzelleria viridis* (Verrill, 1873), which alter the activity of chemoautotrophic bacteria in the sediments, in particular by increasing the populations of aerobic, sulphur-oxidizing and nitrifying bacteria (Vasquez-Cardenas et al., 2016). By stimulating microbial activity, invertebrates can also increase the permeability of sediments to dissolved matter and oxygen and facilitate the removal of toxic metabolites from sediments (Kristensen & Mikkelsen, 2003).

Microorganisms form complex symbiotic communities. These communities include bacteria, archaea, fungi, and viruses, that are present in a specific environment, they are collectively known as microbiomes. This encompasses not only the microorganisms themselves, but also the environmental conditions that shape their composition (Marchesi & Ravel, 2015). In recent years, it has become evident that microbiomes play critical roles in host nutrition, defense, immunity, development, physiology and behavior (Duperron et al., 2020; El-Son et al., 2025; Evariste et al., 2019). The presence of microbial communities in host tissues is essential for the processing of nutrients and the detoxification of chemical compounds (Claus et al., 2016; Hollister et al., 2014). Examples such as mussels from deep-sea hydrothermal vents illustrate this, as they harbor bacteria in their gill epithelium capable of oxidizing toxic hydrogen sulfide and fixing carbon, thus directly contributing to the nutrition of the host (Halary et al., 2008).The functions of bivalve are facilitated by the gut microbiota, which plays a crucial role in the digestion of dissolved organic compounds and the enhancement of the immune response, a phenomenon that is particularly evident in aquaculture environments or environments that have been subjected to anthropogenic impacts. (Claus et al., 2016; Timmins-Schiffman et al., 2021). Most microbiota are located in the epithelium surfaces that separate the internal from the external environment which acts as a buffer and first line of defense against pollutants and stressors (Barr et al., 2013).

The gut microbiome is a dynamic and complex biological system composed of commensal, symbiotic and pathogenic microbial communities, mainly anaerobic (Sommer & Bäckhed, 2013; Wüst et al., 2011). These communities often confer metabolic capabilities on the host that it would not otherwise have, such as the degradation of complex polysaccharides (B. Gao, Runying Zeng, et al., 2017). In all metazoans, the microbiome consists of microbial communities living on the internal and external surfaces (Nicholson et al., 2012). This distinction may result from the presence of a resident microbiome or from the selective absorption and/or differential survival of ingested microbes (Harris, 1993). This differentiation implies that the gut microbiome is not merely a passive reflection of the environment but a functionally adapted community, potentially responding differently to environmental disturbances (Kunselman et al., 2022; McFall-Ngai et al., 2013; Pierce & Ward, 2018). Also, studies on sponges, corals and crustaceans have shown that the microbial communities associated with the host differ from those found in the surrounding environment (Fan et al., 2012; Harris, 1993; Kimes et al., 2013; Meyer et al., 2014; Rosenberg et al., 2007).

Invertebrates can act as reservoirs and vectors for microorganisms, both through their internal microbiota, such as the gut, which often remains relatively stable even when the host moves between locations, and through microbes attached to external surfaces like shells or appendages. For example, crabs and benthic shrimps have been shown to transport gut bacteria over distances of up to 25 km (Troussellier et al., 2017). The study by Foshtomi et al., (2015) provides evidence of a link between microbial biodiversity and ecosystem function in marine sediments, which can be influenced by macrofauna density and functional diversity. This type of relationship is particularly relevant in estuarine zones, where the structure of the benthic community and variable environmental conditions can strongly influence microbial profiles (Gammal et al., 2023).

Some studies confirm species-specific gut microbiomes, while others document variation within the same species or between sites, as in Mengoni et al.,(2013), oysters (King et al., 2012), and Brachidontes mussels (Cleary et al., 2015). Taken together, these data reinforce that both environmental factors, such as contamination, and intrinsic host traits shape gut microbiome composition and function (Harris, 1993; Pierce & Ward, 2018). However, mechanisms and interspecific variability remain unclear: oysters and mussels may share core taxa yet differ functionally and seasonally, reflecting host influences and environmental interactions (Akter et al., 2023; Pierce & Ward, 2018). Environmental factors further modulate microbial structure, with studies in *Mytilus* spp. and *Crassostrea* spp. showing seasonal and habitat-related variation, demonstrating that contaminant responses occur against a dynamic ecological background (Khan & Malik, 2021; Santibáñez et al., 2022; Vezzulli et al., 2018). Estimating the functional capacity of bacterial and fungal communities can elucidate ecological roles under stress, revealing adaptation or dysfunction along contamination gradients (Evariste et al., 2019; Ramette, 2007)

In general, invertebrates are assumed to host a core gut microbiome, consistently found in individuals of the same species (Lau et al., 2002), as reported in zebrafish (Roeselers et al., 2011), tunicates (Dishaw et al., 2014), and shrimp (Rungrassamee et al., 2016). Yet, host–microbiome interactions can be context-dependent (Buffie & Pamer, 2013), varying between geographically distinct populations (H. Liu et al., 2016; Wong et al., 2013), reflecting the interplay between intrinsic and extrinsic factors, the exposome, which shapes unique microbiome traits linked to evolution and life history (Simon et al., 2019), consistent with the holobiont concept of host and microbial communities as integrated ecological units (O'Brien et al., 2019).

Microbial communities have the ability to mediate the biotransformation of a wide variety of chemical contaminants, directly affecting their bioavailability, toxicity and persistence in the environment (Adamovsky et al., 2018; Claus et al., 2016). This metabolic capacity makes the microbiome a promising target for applications in ecotoxicology and biomonitoring. As with transcriptomic markers used in environmental studies, the composition of the microbial community can sensitively reflect exposure to pollutants, acting as a functional bioindicator of chemical stress (Feswick et al., 2017; L. Liu et al., 2021). For example, research with terrestrial isopods has shown that changes in gut bacterial composition are associated with short- and long-term responses to trace metal exposure (Lapanje et al., 2010). Similarly, in marine environments, Neave et al., (2012) observed that individuals of *Ophelina* sp. collected from sites contaminated with copper and zinc showed marked changes in their gut microbiota, with an increase in the order *Alteromonadales*. These results reinforce the potential of the gut microbiome as an ecological diagnostic tool in contexts of inorganic contamination (X. Li et al., 2019; Šrut et al., 2019). Additionally, recent reviews of marine host-associated microbiomes in the Southern Hemisphere highlight growing recognition of surface microbiomes, including invertebrates, macroalgae, and vertebrates, as sensitive indicators of environmental change (Ochoa-Sánchez et al., 2023).

Understanding how pollutants disrupt the host-microbiome relationship particularly in macrobenthic infauna is a growing challenge in ecological and toxicological studies (Adamovsky et al., 2018; Brown et al., 2000; Evariste et al., 2019; Robinson et al., 2010). Interspecific differences in microbial composition at a given site, along with intraspecific similarities between individuals from distant sites, point to potential species-specific microbial associations (Dishaw et al., 2012, 2014; Fraune et al., 2016; Pierce et al., 2016; Zurel et al., 2011). In addition to contamination, the composition and function of the bivalve gut microbiome can be modulated by both intrinsic and extrinsic factors, such as host species, habitat, season, and feeding regime (Akter, 2022; Guo et al., 2023; Murphy et al., 2019; Pierce et al., 2016). Among the most promising groups for this type of research are filter-feeding bivalves, whose direct exposure to sediments and the water column makes them effective sentinels for microbial changes associated with contamination (Masanja et al., 2023; Pierce & Ward, 2018). These patterns suggest that studying bivalve populations in estuarine zones with different levels of contamination can reveal both central components and local adaptations of the gut microbiome. The potential of microbiome-based indicators for environmental monitoring has been increasingly highlighted in recent literature, with growing emphasis on the integration of molecular tools such as environmental genomics into marine assessment programs (Cordier et al., 2021).

Metagenomics makes it possible to identify functional potential based on the present genes (Adamovsky et al., 2018; Bao et al., 2024), while metatranscriptomics and metaproteomics are better suited to capturing genes or proteins in activity (Gouveia et al., 2020). Metabolomics, in turn, provides a real-time functional picture, although it is still limited by the quality of the available databases (Gertsman & Barshop, 2018). However, taxonomic profiles alone are insufficient, as functional redundancy, i.e. the ability of different taxa to perform similar functions, can mask relevant functional changes (Puente-Sánchez et al., 2024). The integration of these approaches into multi-omics strategies, although challenging, is promising for uncovering causal relationships between the microbiome and contaminant metabolism, as well as the underlying toxicological mechanisms (Akter et al., 2023; Masanja et al., 2023; Timmins-Schiffman et al., 2021). This concept is essential for understanding the resilience of the microbial community (Allison & Martiny, 2008; Moya & Ferrer, 2016), implying that different sets of species can perform the same essential ecological functions, even under different environmental conditions. This reinforces the need to investigate the function of the microbiome alongside taxonomic composition (Burke et al., 2011).

However, the exclusive use of taxonomic markers, such as 16S rRNA, offers a limited view of functional diversity, since a single OTU can encompass multiple bacterial genotypes with distinct metabolic capacities and variable environmental responses (Andam, 2019; Pan et al., 2023; Rowan-Nash et al., 2019). This limitation becomes particularly relevant in estuarine environments, where high physicochemical heterogeneity can modulate the functional expression of apparently similar communities. However, recognize the potential and the integration of microbiome-based approaches into environmental assessment frameworks align with emerging scientific recommendations and support more sensitive detection of ecosystem change (Adamovsky et al., 2018; Benjamino et al., 2018; Duperron et al., 2020).

Despite their functional importance, fungi remain largely neglected in marine microbiome studies (Amend et al., 2019). Their identification is complicated by the limitations of current genetic databases, especially with markers such as ITS(Internal Transcribed Spacer), and the difficulty of distinguishing obligate from (Burgaud et al., 2013; Su et al., 2021) facultative marine fungi (Gladfelter et al., 2019; Richards et al., 2012). This underrepresentation compromises the full characterization of fungal diversity and their ecological functions, particularly in marine and estuarine environments where distinguishing obligate marine fungi from terrestrial-derived species is challenging (Amend et al., 2019; Gladfelter et al., 2019; Richards et al., 2012). The systematic inclusion of the fungal component in microbiome studies may, however, reveal previously unknown symbiotic interactions, with significant implications for the stability of microbial networks in estuarine environments (Burgaud 2013; Su 2021)

The relevance of the gut microbiome as an indicator of environmental change is well-established. However, research has identified specific bacterial and fungal taxa that have been consistently linked to particular types of contamination and human activities. These microbial signatures not only provide insights into ecosystem stressors but also illustrate how community composition can serve as a sensitive indicator of environmental pressures. Bacteria such as *Desulfobacterota* (e.g., sulfate-reducing *Desulfovibrio* spp.) are associated with anoxic, metal-contaminated environments, contributing to sulfur cycling and mercury methylation (Demin et al., 2024; Figueiredo et al., 2018; Muyzer & Stams, 2008). *Campylobacterota,* adapted to microaerophilic conditions, occurs in areas affected by wastewater and agricultural runoff, indicating fecal pollution and nutrient enrichment (Costa & Iraola, 2019; Figueiredo et al., 2018; Waite et al., 2019). *Pseudomonas* spp. and *Shewanella* spp., linked to human and industrial activities, degrade hydrocarbons and reduce metals such as Arsenic (Colwell & Sparks, 1967; Darma et al., 2022). In fungi, *Candida, Aspergillus, Penicillium*, and *Fusarium* are common in contaminated sediments, where they decompose organic matter, degrade pollutants, and mediate trophic and chemical interactions (Blaylock et al., 2001; M. Gao et al., 2024; Pang et al., 2021; Richards et al., 2012).

Microbiomes play a central role in ecosystem functioning, resilience, and restoration, and their integration into environmental management is increasingly advocated. Recognizing microbiomes as part of the "holobiont" framework can inform sustainable coastal strategies (Trevathan-Tackett et al., 2019). The gut microbiome of benthic bivalves such as *Cerastoderma edule*, *Ruditapes decussatus*, and *Scrobicularia plana* (da Costa, 1778) offers a sensitive, integrative indicator of environmental quality, as these species bioaccumulate metals like cadmium, lead, and arsenic (Anajjar et al., 2008; Cheggour et al., 2000; Rodrigues et al., 2017). This approach complements the (2000/60/EC) and the Marine Strategy Framework Directive (2008/56/EC), which encourage the adoption of functional and integrative indicators but have yet to fully incorporate microbial metrics. Examples include shifts in *Anemonia viridis* (Forsskål, 1775) microbiota under varying anthropogenic pressures (Palladino et al., 2022) and the emerging concept of "microbiome-assisted restoration" to enhance resilience in degraded habitats (Corinaldesi et al., 2023).

Research into the gut microbiome of estuarine bivalves is an innovative and ecologically relevant approach to monitoring environmental quality, with the potential to complement national and European regulatory frameworks. This line of research is linked to instruments like international commitments such as the United Nations Sustainable Development Goals (SDGs), such as:

SDG 14 - Life in Water, by enabling the detection of microbial changes associated with diffuse pollution.

SDG6 - Drinking Water and Sanitation, by assessing the impact of urban and agricultural effluents.

SDG12 - Sustainable Production and Consumption, through its applicability in aquaculture practices.

SDG3 - Health and Well-being, contributing to microbiological traceability and food safety.

Studies carried out in Portuguese waters (Braga et al., 2022; Pires et al., 2022) show alterations in bacterial communities and histopathological lesions in bivalves exposed to parasites, trace metals and biotoxins. Although they do not focus directly on the gut microbiome, the results suggest that the microbial response may reflect early environmental stress in widely distributed benthic organisms. In addition to their direct effects on benthic organisms, inorganic contaminants have been shown to disrupt host-associated microbiomes, linking sediment contamination to shifts in gut microbial communities (Dale et al., 2019; Kakade et al., 2020; X. Li et al., 2019). The available evidence suggests a link between gut dysbiosis in fish and exposure to trace metals (Kakade et al., 2020), reviews on toxic metal impacts on microbiome diversity and function (Assefa & Köhler, 2020), and studies identifying altered microbiome structure as early indicators of metal stress.

In the case of infaunal bivalves, such as *Cerastoderma edule*, burial and filtration activities directly influence the sedimentary environment, promoting local oxygenation and organic enrichment, with impacts on the associated microbial diversity (Rossi et al., 2008; Wiesebron et al., 2021). In many aquatic organisms, microbiota contributes to a wide range of metabolic functions, producing metabolites, peptides and proteins that interact with the host and complement its metabolic capacity (B. Gao, Chi, et al., 2017; Harris, 1993; Nicholson et al., 2012).

The common cockle (*Cerastoderma edule* (Linnaeus, 1758)) is of considerable economic and ecological importance. In Portugal, it is a commercially valuable species, widely harvested for human consumption and an important component of estuarine ecosystems. Cockles act as suspension feeders and filter organisms, contributing to nutrient cycling and serving as prey for birds and other animals (Jensen, 1985; Kang et al., 1999; Rossi et al., 2008). Their recognized status as bioindicators, their ecological role and their interactions with parasites, sediment microbiota, and environmental factors, suggest a complex microbial association (Albuixech-Martí et al., 2021; Lobo et al., 2010; Paul-Pont et al., 2010; Richard et al., 2022; K. Veiga et al., 2019). Although studies specifically on the gut microbiome of *C. edule* are still lacking, its biological and ecological characteristics make it a highly relevant model species for investigating host-microbiome interactions in estuarine environments. This is further supported by research on other bivalves in aquaculture, which highlights the importance of studying gut microbiomes in natural populations to better understand resilience to environmental stressors (dos Santos et al., 2020; Dubert et al., 2017; Fahim Ullah Khan et al., 2021; Murphy et al., 2019; Pierce & Ward, 2018; Pimentel et al., 2021; Santibáñez et al., 2022; Timmins-Schiffman et al., 2021; Vaughn et al., 2024). In this context, the gut microbiome of bivalves such as *Cerastoderma edule* can offer a complementary metric to traditional water and sediment quality analyses.

In the context of aquaculture, it is essential to understand the shellfish microbiome. Studies have shown that shellfish microbiota play a vital role in host health, supporting nutrient processing, immunity and disease resistance (Diwan et al., 2022; Paillard et al., 2022). Disturbances in these microbial communities, such as those induced by hypoxia, can lead to dysbiosis, affecting the overall health and survival of the host (Montúfar-Romero et al., 2025). In some cases, the relationships between the host and the microbiome have commercial and public health relevance, particularly when decomposition and the transmission of pathogens are involved. The microbiomes of marine invertebrates have only been described for a few species, mainly those with commercial value, such as oysters and mussels (Akter et al., 2023; Jurinović et al., 2022; King et al., 2012; Offret et al., 2020; Pierce & Ward, 2018). For example, research on bivalve mollusks has often focused on their ability to accumulate and excrete bacteria pathogenic to humans, such as *Escherichia coli* (Colwell & Sparks, 1967; DePaola et al., 1990; Froelich & Oliver, 2013; Harris, 1993; Valley et al., 2009).

Recent studies have highlighted the importance of bivalves, such as oysters and mussels, in understanding host–microbiome interactions. For example, research has evaluated the effect of temperature on the mussel *Mytilus coruscus* (Y.-F. Li et al., 2019), seasonal influences on *Mytilus edulis* (Manduzio et al., 2004), the influence of natural versus aquaculture environments on *M. galloprovincialis* and *M. edulis* (Santibáñez et al., 2022; Vezzulli et al., 2018), and the effects of ocean acidification on *M. coruscus* (Khan & Malik, 2021). Collectively, these studies demonstrate that shellfish microbiomes are closely linked to environmental factors and can provide insights into nutrient cycling and ecosystem health.

The microbiome's response to trace metals should be interpreted within the broader context of responses to multiple ecological stressors. Research across aquatic organisms has shown that microbial alterations can be induced by temperature gradients, seasonal variation, ocean acidification, and organic pollution, among other factors. This perspective recognizes metals as one of several stress agents capable of modulating microbiome structure and function, reinforcing the importance of integrated approaches in ecotoxicology (Adamovsky et al., 2018; Evariste et al., 2019; Feswick et al., 2017).

Technological advances have significantly facilitated the characterization of the composition and function of microbiomes. These elements play a central role in the metabolism of carbohydrates, amino acids, and lipids, as well as the sulfur and nitrogen cycles and the breakdown of organic compounds, including hydrocarbons (Claus et al., 2016; Halary et al., 2008; Harris, 1993; Hiergeist et al., 2015). Metabarcoding, especially through the sequencing of genes such as 16S rRNA for bacteria and ITS for fungi, has become a widely used method for assessing microbial biodiversity, offering expedient and comparable characterization in complex environments such as estuarine sediments (Hiergeist et al., 2015). While taxonomic markers make it possible to identify microbial composition, functional approaches, such as metagenomics, offer a window into the potential functions performed by the communities detected (Bao et al., 2024; Gouveia et al., 2020; Z. Liu et al., 2024; Wemheuer et al., 2020).

Despite the existence of national environmental monitoring programs in line with European directives, the application of the microbiome concept, particularly the gut microbiome of benthic invertebrates, has not yet been integrated into routine protocols (C. Li et al., 2020; Licciano et al., 2007; Zhu et al., 2023). Current surveillance remains focused on detecting pathogenic microorganisms with public health implications, which limits the exploration of the microbiome as a functional community with ecological relevance (Desdouits et al., 2023; Lattos et al., 2021; Lopez-Joven et al., 2018; Ramos et al., 2024; Sousa et al., 2022).

1.2 Context and Motivation

In recent years, the concept of the microbiome has gained prominence as a key element in understanding host–environment interactions, particularly under environmental stress. While research on terrestrial microbiomes, especially in humans, has grown rapidly, studies on marine invertebrates remain comparatively scarce, despite their critical ecological roles. In estuarine macrobenthic species, the gut microbiome not only mirrors environmental conditions but can also influence host physiology, detoxification capacity, and broader ecological functions (Harris 1993; Hollister et al., 2014; Claus et al., 2016).

Benthic invertebrates such as *Cerastoderma edule* are of particular interest due to their essential role in sediment biogeochemistry (Rossi et al., 2008; Wiesebron et al., 2021) and their continuous exposure to contaminants (Anajjar et al., 2008; Cheggour et al., 2000; Rodrigues et al., 2017; Braga et al., 2022; Pires et al., 2022). Unlike the extensively characterized human gut microbiome, microbial assemblages in these invertebrates are shaped by sediment properties, feeding strategies (Pierce & Ward, 2018; Masanja et al., 2023), and anthropogenic stressors (Anajjar et al., 2008; Cheggour et al., 2000; Rodrigues et al., 2017; Braga et al., 2022; Pires et al., 2022; Li et al., 2019; Kakade et al., 2020). Importantly, gut-associated microbial communities often differ markedly from those in surrounding sediments, suggesting selective recruitment or symbiotic associations (Pierce & Ward, 2018; Harris, 1993).

Investigating the effects of inorganic contamination on the gut microbiome of benthic invertebrates in estuarine sediments can provide early indicators of environmental disturbance and reveal adaptive or functional microbial responses. Such insights are particularly valuable for ecological risk assessment and environmental monitoring, especially in transitional waters affected by sediment-bound pollutants.

1.3 Problems and Objectives

Estuarine environments are dynamic and vulnerable ecosystems where benthic communities are often exposed to complex mixtures of contaminants. Inorganic pollutants, such as metals, accumulate in sediments and can alter microbial community structures (Anajjar et al., 2008; Cheggour et al., 2000; Rodrigues et al., 2017; Dale et al., 2019; Li et al., 2019; Šrut et al., 2019) and disrupt host-associated microbiomes (Adamovsky et al., 2018; Evariste et al., 2019; Kakade et al., 2020; Jin et al., 2017). Although research has shown that the gut microbiome plays a central role in host nutrition, detoxification, and immune response, its functional dynamics in estuarine macrobenthic invertebrates, particularly under contamination gradients, remain poorly understood (Adamovsky et al., 2018; Evariste et al., 2019).

A major challenge lies in disentangling the effects of environmental stressors on gut microbial communities from natural variability, and in establishing reliable indicators of ecological health based on microbial responses. Furthermore, the ecological consequence of microbiome shifts at the organism and population levels remain underexplored, especially for sediment-dwelling species like *Cerastoderma edule*, which are ecologically and economically relevant.

This PhD project aims to address these gaps by investigating the structure and potential functions of the gut microbiome in macrobenthic invertebrates across a contamination

gradient in the Sado estuary. The study combines field sampling, sediment and biological analyses, and an in vivo bioassay to assess microbial responses to inorganic contamination.
The specific objectives are to:

1. Evaluate the ecological status of estuarine sites based on macrobenthic community composition and structure.
2. Characterize *in situ* the gut microbiome of *a macrobenthic species* collected from estuarine sites along a contamination gradient, assessing diversity, taxonomic composition, and predicted functional potential in relation to sediment characteristics and adjacent anthropogenic activities.
3. Evaluate shifts in gut microbial community structure and functional potential through a controlled laboratory bioassay, exposing *Cerastoderma edule* to a selected inorganic contaminant in sediment, to infer microbiome responses under standardized conditions.

1.4 Approach and Contributions

This PhD project employs an interdisciplinary approach combining benthic ecology, microbial ecotoxicology, and molecular biology to explore how sedimentary inorganic contamination affects the gut microbiome of estuarine macrobenthic invertebrates. The study is centered on the Sado estuary in Portugal, a system subject to multiple anthropogenic pressures and well suited for investigating contamination gradients.

The methodology integrates:

- **Field assessments**, including sediment biogeochemical characterization (e.g., granulometry, organic matter content, trace metal concentrations) and macrobenthic fauna assessments to establish ecological status across sampling sites.
- **Molecular analysis** of the gut microbiome in *Cerastoderma edule*, using 16S rRNA gene sequencing for bacteria and ITS (Internal Transcribed Spacer) sequencing for fungi, enabling the assessment of microbial diversity, taxonomic composition, and predicted functional profiles through bioinformatic tools. In parallel, COI (cytochrome c oxidase I) and 18S rRNA metabarcoding were applied to characterize macrobenthic communities, allowing the evaluation of biodiversity patterns and their response to environmental gradients.
- **A controlled laboratory bioassay**, exposing *C. edule* to a selected contaminant under standardized conditions to identify specific microbial shifts and infer microbial resilience or vulnerability.

This research is expected to contribute significantly to current knowledge by:

- Providing an integrated ecological assessment of estuarine quality through the analysis of macrobenthic communities, using AMBI and M-AMBI indices in combination with morphological and molecular (COI and 18S rRNA metabarcoding) identification, thereby linking species composition with environmental factors and sediment contamination.
- Providing one of the first detailed characterizations of the gut microbiome (bacterial and fungal) of *C. edule* in relation to sedimentary contamination.
- Demonstrating the ecological relevance of gut microbial responses to inorganic pollutants.
- Contributing empirical evidence towards the potential use of gut microbiota as bioindicators in estuarine environmental monitoring.

- Establishing the basis for future applications in microbial ecotoxicology and environmental risk assessment, with potential implications for conservation and management of estuarine systems.

1.5 Document Organization

This thesis is organized into six chapters, structured according to the progression of the research objectives and corresponding scientific outputs:

- **Chapter 1 – Introduction:** Provides the general background, context, and motivation for the study. It presents the scientific problem, defines the research questions and objectives, and outlines the methodological approach. This chapter also introduces the relevance of investigating the gut microbiome of estuarine macrobenthic invertebrates under sedimentary inorganic contamination.
- **Chapter 2 – Ecological Quality Assessment of Estuarine Macrobenthic Communities Using an Integrative Approach (First Research Paper):** Focuses on the environmental characterization of selected sites within the Sado estuary. It presents the results of sediment geochemistry, including granulometry, organic matter, and metal contamination, and characterizes the macrobenthic communities present. The ecological status of each site is assessed using the AZTI Marine Biotic Index (AMBI), establishing a baseline for understanding environmental gradients and selecting relevant sites for microbiome analysis.
- **Chapter 3 – Gut Microbiome Composition and Functional Prediction in *Cerastoderma edule* Along a Contamination Gradient (Second Research Paper):** Examines the diversity and structure of the gut microbiome of the bivalve *Cerastoderma edule* collected from sites with varying contamination levels in Sado Estuary. The chapter integrates bacterial (16S rRNA) and fungal (ITS) community profiling with predicted functional inference to explore host-microbe-environment interactions.
- **Chapter 4 – Experimental Assessment of Microbiome Response to Metal Exposure in *Cerastoderma edule* (Third Research Paper):** Describes a controlled bioassay designed to evaluate the effects of a specific inorganic contaminant (mercury) on the gut microbiome of *C. edule*. Shifts in microbiome community composition and predicted functional potential are analyzed to assess microbial resilience or sensitivity to pollution.
- **Chapter 5 – General Discussion:** Integrates and discusses the findings from the previous chapters in the broader context of benthic ecology, microbiome research, and environmental stress. It addresses the ecological implications of microbial shifts and evaluates the potential of gut microbiota as early indicators of sediment quality and environmental disturbance.

- **Chapter 6 – Conclusions and Future Perspectives:** Summarizes the key findings and scientific contributions of the thesis. It highlights the limitations of the current work and proposes future research directions, particularly regarding the application of microbiome-based indicators in environmental monitoring and multi-omics integration.

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2

ECOLOGICAL QUALITY ASSESSMENT OF ESTUARINE MACROBENTHIC COMMUNITIES USING AN INTEGRATIVE APPROACH

This Chapter Is a published Manuscript

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2.1 Abstract

Macrobenthic communities play a crucial role in the functioning of estuarine ecosystems and serve as bio- indicators of environmental quality. This study assessed the ecological quality of an estuarine system using the AMBI and M-AMBI indices. The following parameters were considered: (i) environmental factors (total organic matter, organic carbon, grain size, calcium carbonate), (ii) sediment trace metals (Pb, Zn, Cu, Cr, Co, Ni, Hg, Li, As), (iii) species composition (morphological and molecular identification), and (iv) anthropogenic activities. The results demonstrated notable differences between study areas, reflecting hydrodynamic processes and human activities. The AMBI index indicated that all areas exhibited conditions classified as "slightly disturbed." However, the composition of the ecological groups and M-AMBI results differed according to the identification method. This approach allowed for a more complete understanding of communities, by integrating the influence of anthropogenic activities on the sediment and macrobenthic communities, highlighting the importance of using both identification methodologies.

Keywords: Metabarcoding; Marine biodiversity; Trace metals; Anthropogenic activities; AMBI index; Monitoring.

2.2 Introduction

Anthropogenic activities have increasingly degraded water and marine ecosystems, presenting a major challenge for our society. Estuaries represent complex environments, harboring biodiversity while supporting human activities, such as housing, manufacturing, maritime trade and fishing (Kennish, 2016; Snelgrove, 1999; Snelgrove et al., 1997). These activities often conflict with biodiversity conservation, leading to environmental degradation (Kennish, 1992; Gaston et al., 1998; Chapman and Wang, 2001; Cardoso et al., 2012; Mil-Homens et al., 2014; Cuevas et al., 2018). Monitoring these activities impacts is essential for diagnosing ecosystem degradation and implementing effective environmental management measures (Caeiro et al., 2017; Freitas et al., 2008; Goulding et al., 2021).

The Sado Estuary, located on the west coast of Portugal, is one of the largest estuaries areas in Europe and the second largest in Portugal. The northern part of the estuary contains an industry belt with pollution sources such as pulp and paper production, pesticides, fertilizers, aquacultures, and shipyards (Caeiro et al., 2005). This industrial activity subjects the northern area to significant environmental pressures, affecting local biodiversity (Freitas et al., 2008). Conversely, the southern part is characterized by intensive agriculture, such as rice fields and traditional salt pans, alongside aquaculture (Caeiro et al., 2005; Pereira et al., 2000). The hydrodynamics of the Sado estuary promotes sediment accumulation, leading to the persistence of contaminants such as trace metals, acting as both a sink and a source of pollution (Mil- Homens et al., 2014; Pilo´ et al., 2016). These contaminants originate from effluents, runoff, leachates, industrial and agricultural activities, as well as atmospheric deposition (Caeiro et al., 2005; Couto and Ribeiro, 2022; Ribeiro et al., 2018). Although previous studies have shown that anthropogenic sources influence the elemental composition (e.g., Cd, Cu, Zn and Pb) of estuarine sediments (Cortesão and Vale, 1995; Moreira et al., 2009), limited research has focused on the impacts of contamination on specific species (Costa et al., 2008; Garcês and Costa, 2009) rather than on macrobenthic communities. Studies of macrobenthic communities in the Sado estuary have demonstrated that environmental factors such as hydrodynamics, salinity, grain size, calcium carbonate, sediment structure and organic enrichment, are key determinants of community distribution and diversity (Amaral and Costa, 1999; Caeiro et al., 2005; Lillebø et al., 2011; Lobo et al., 2017; Mucha and Costa, 1999; Vale et al., 2010).

Trace metals tend to accumulate in fine-grained sediments, and colloidal particles due to their high affinity for large surface areas. Differentiating the contributions of anthropogenic sources of trace metals remains challenging, complicating the assessment of anthropogenic impacts on the integrity of ecosystems (Mil-Homens et al., 2006; Pastorinho et al., 2012). Sediments heterogeneity (Lobo et al., 2017; Mil-Homens et al., 2014) and the different methodological approaches to the study of trace metals conducted in the Sado Estuary make it challenge the establishment of reference values for results comparison purposes and for estimating anthropogenic enrichment in the estuary (Couto and Ribeiro, 2022). To address this, a geochemical baseline was developed, considering the natural variability of trace metal concentrations and the relationships between trace metals and normalizing elements to establish regional elemental baseline variability (e.g., Herbert L. Windom et al., 1989). Normalizing elements (e.g., Al, Fe, Li) are not commonly associated with anthropogenic inputs, and enrichment factors help to determine whether concentrations are natural or anthropogenic (Pastorinho et al., 2012).

Macrobenthic invertebrates are frequently employed as bioindicators of ecosystem integrity (Chiarelli and Roccheri, 2014; Dauer, 1993; Lobo et al., 2010; Mucha et al., 2003; Pini et al., 2015; Veiga et al., 2016); these organisms exhibit distinct sensitivities and tolerances to environmental disturbances, making them effective indicators of ecological quality (Borja et al., 2000). Metabarcoding has improved macrobenthic community assessments, providing precise species composition data, while morphological approaches remain essential for taxonomic validation, both increasing the accuracy of ecological assessments (Aylagas et al., 2018; Hering et al., 2018; Hollatz et al., 2017; Lejzerowicz et al., 2015; Pawlowski et al., 2022). The AMBI-AZTI Marine Biotic Index (Borja et al., 2000) and M-AMBI index (Muxika et al., 2007) were developed to assess the ecological status of marine ecosystems in accordance with the requirements of the European Water Framework Directive (WFD). These indices have been applied globally demonstrating efficacy in assessing environmental quality and identifying habitats affected by degradation, including eutrophication and pollution (Muxika et al., 2005; Carvalho et al., 2006; Sivaraj et al., 2014; Munari et al., 2022; Onyena et al., 2023).

The complexity and vulnerability of estuarine systems to natural and anthropogenic changes call for an integrative approach to assess their ecological quality (Chapman et al., 2013; Chapman and Wang, 2001). For this reason, his study aimed to assess the ecological quality status of macrobenthic communities of the Sado estuary using an integrative approach that considered: (i) environmental parameters, (ii) the trace metals in sediments, (iii) species composition using molecular, namely metabarcoding (18S and COI makers) and morphological identifications, (iv) local anthropogenic activities, and (v) the AMBI and M-AMBI indices.

2.3 Materials and methods

2.3.1 Sampling survey

The intertidal zones of the Sado estuary encompass approximately 78 km^2, of which 30 % is comprised of salt marshes and intertidal flats (Freitas et al., 2008). The estuary exhibits a warm temperate, semi- diurnal mesotidal regime, with tidal amplitudes ranging from 0.6 m to 1.6 m during neap and spring tides (Martins et al., 2001), It is connected to the ocean by a narrow, deep channel (maximum depth of 50 m), and it is partially divided by intertidal sand-banks, which contribute significantly to the general pattern of estuarine circulation (Carvalho et al., 2001). Riverine input demonstrates pronounced seasonal fluctuations, influencing salinity and temperature conditions. Salinity gradient ranges between 0.75 upstream to 35.34 down-stream (Sroczynska et al., 2021), primarily influenced by the Sado River flow (annual mean of 40 m^3. s^{-1}) with most of freshwater discharges occurring during winter months (Gonçalves et al., 2015). Temperature also displays considerable seasonal and interannual variability, ranging from 10 to 26 ◦C. This work was conducted as part of the project D4ss (ALT20-03- 0145-FEDER-029400), which aimed to better understand the trophic chains of the Sado estuary. Sampling was conducted in autumn and spring to capture seasonal variations in environmental factors, particularly freshwater inflow, which can influence trophic dynamics within the estuary. Three areas were selected based on their hydrodynamic characteristics, biogeochemical properties, and proximity to anthropogenic activities (Caeiro et al., 2005) (Fig. 1): "Tróia" (TR) in the South Channel, "Gâmbia" (GAM) and "Navigator" (NAV) in the North Channel. The TR area, is located near the Tróia peninsula and adjacent to the Sado Nature Reserve, is highly influenced by tidal hydrodynamics and has a short water residence time, making it less likely to be exposed to direct contamination sources from anthropogenic activities (Caeiro et al., 2005; Lobo et al., 2017; Vale et al., 2010). In contrast, GAM and NAV areas present low hydrodynamics, facilitating the retention of fine-grained particles and consequently the accumulation of contaminants and organic matter (Caeiro et al., 2005; Freitas et al., 2008; Quevauviller et al., 1989). NAV is situated near the industrial zone and the Port of Setúbal. GAM is the only location that is placed inside the Sado Estuary Nature Reserve, close to oyster and aquaculture production (Cabrita et al., 2020).

Seasonal and interannual variations in environmental factors, including freshwater in-flow, play a key role in shaping trophic dynamics within the estuary, which justified the seasonal approach of this study. However, previous research indicates that macrobenthic community composition in this estuary is more influenced by spatial factors, such as hydrodynamics, organic matter content, sediment granulometry, and calcium carbonate levels, than by seasonal changes. Therefore, the design of this study prioritized spatial heterogeneities, which are primary drivers of estuarine community structure.

Sediment samples were collected in the lower littoral zone, during spring tide, using a hand corer (11 cm inner diameter and 20 cm depth), during four sampling campaigns, two in Autumn (Aut) and two in Spring (Spr), in the three previously described areas (Figure 1): i) November to December 2019 (Aut19), ii) May to June 2020 (Spr20), iii) November to December 2020 (Aut20), and iv) May to June 2021 (Spr21). In each area, three sampling sites were selected, each site being 10 m apart from each other. During the Aut19 and Spr20 campaigns five sediment cores were collected at each site: four for macrobenthic analysis and one for sediment characterization. The Aut20 and Spr21 campaigns were conducted to assess the geochemical variability of the sediment. During these campaigns, three samples per area were collected solely for sediment characterization. In total, 108 sediment cores were collected from which 72 for macrobenthic analysis (36 for morphological and 36 for molecular analysis) and 36 for sediment characterization. The number of samples collected for macrobenthic analysis was determined based on previous studies in similar estuarine environments, which indicate that this level of replication is sufficient to capture the diversity and structure of macrobenthic communities (Lobo et al., 2017; Muxika et al., 2007). The samples for macrobenthic invertebrates identification were sieved in situ over a 0.5 mm mesh using estuarine water to sort all the organisms. This sieving process is crucial for separating macrobenthic specimens from the sediment and is fundamental for accurate subsequent identification and analysis. All material retained on the sieve was stored in plastic containers and transported to the laboratory under refrigeration. In the laboratory, the organisms were sorted. The samples used for morphological identification were fixed in 4 % buffered formalin solution for one week, then washed and preserved in 70 % ethanol at room temperature. The remaining samples were preserved in 96 % ethanol and stored at 4 ◦C until molecular identification was carried out. The homogenized sediment samples for organic carbon (C_{org}) and trace metal analysis were sieved through a 2 mm square mesh sieve to remove coarse material (shells and gravel) and then milled, using agate pots, in a Fritsch Pulverisette 7 Classic Line planetary mill.

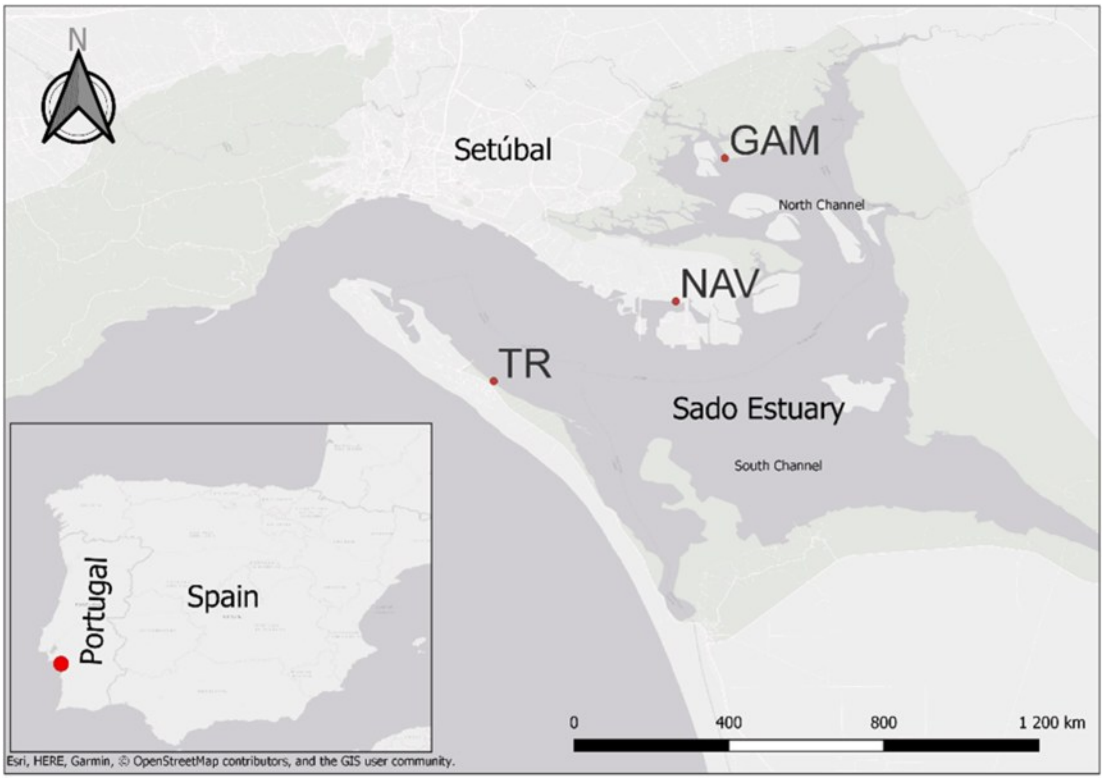

Figure 1 Location of the 3 areas selected for the collection of sediment samples in the Sado Estuary: Tróia (TR); Navigator (NAV); Gâmbia (GAM).

2.3.2 Sediment characterization

2.3.2.1 Determination of total organic matter(TOM), organic carbon (C_{org}), $CaCO_3$ and grain size

In order to characterize the sedimentary habitat, the following variables were considered: (i) organic carbon (C_{org}) content of sediments, that was determined by subtracting the inorganic carbon content from the total carbon content, using high-temperature combustion on a Leco Truspec micro-analyzer CHNS. Firstly, the samples were incinerated in a muffle furnace for 3 h at 400 ◦C to destroy the organic matter. Three replicate subsamples of 2 mg of each dried and homogenized sediment sample were measured, before and after combustion. The precision of repeated measurements was equal to or lower than 0.03 % (dry weight); (ii) calcium carbonate (CaCO3) content (%), determined by multiplying the percentage weight of inorganic carbon by 8.332 if it is predominantly present as calcite or aragonite; (iii) total organic matter content (% TOM), determined by multiplying the C_{org} (%) by the conventional 'Van Bemmelen factor' of 2.5 adapted for marine sediments (Pribyl, 2010; Shamrikova et al., 2022), which assumes that 40 % of the organic matter is organic carbon (Craft et al., 1991; Ouyang and Lee, 2020); (iv) sediment granulometry, considering the following granulometric fractions: gravel (> 2 mm), sand (2–0.063 mm), and the fine fraction(<0.063 mm), which were determined by hydraulic sieving after treating the samples with H_2O_2 and following disaggregation with pyrophosphate (Caeiro et al., 2005). These sediment characteristics were selected due to their significant influence on benthic community structure (e.g., Amaral and Costa, 1999; Vale et al., 2010). Water quality parameters, including salinity, were not prioritized in this study due to their relative stability in the sampled areas and their lesser impact on macrobenthic communities compared to sediment variables

2.3.2.2 Determination of trace metals

For simplicity, this work uses "metals" to refer to trace metals Lead (Pb), Zinc (Zn), Copper (Cu), Chromium (Cr), Cobalt (Co), Nickel (Ni), Mercury (Hg), Lithium (Li), and the metalloid Arsenic (As). The metals were quantified by Inductively Coupled Plasma – Mass Spectrometry (Perkin Elmer NEXIon 2000C) after a total decomposition carried out in a closed Teflon vessel microwave-assisted system (CEM MARS 5), using approximately 100 mg of homogenized sediment sample (granulometric fraction <2 mm) with 3 mL of aqua-regia (36 % hydrochloric acid and 60 % nitric acid; 3:1 v/v) and 1 mL of hydrofluoric acid. The heating of the Teflon vessels was carried out in two steps according to the following procedure: 1) 25 min temperature increase to 180 ◦C and held for 20 min and, 2) in a second digestion step, hydrofluoric acid (HF) was neutralized with 10 mL of a 4 % boric acid solution at 170 ◦C and held for 10 min. After being removed from the microwave and cooled, the final solutions were transferred to 50 mL polypropylene bottles and diluted with Type 1 ultrapure water to 50 mL. Before analysis, the obtained digested solutions were diluted by 0.5:10 using a 2 % nitric acid (HNO_3) solution. Analytical results indicated a good agreement between the certified and measured values with metals recovery between 90 and 116% (Appendix A - Table 2). Total Hg in dried sediment samples (average 15 mg weight) was quantified by thermal pyrolysis atomic absorption analysis (LECO 254 Advanced Mercury Analyzer, AMA). This technique involves sample pyrolysis in a combustion tube at 750 ◦C in an oxygen atmosphere. The sample is collected on a gold amalgamator and subsequently detected by atomic absorption spectrometry (AAS) using a silicon UV diode detector (Costley et al., 2000). Quality assurance was evaluated using the MESS-4 certified reference material (CRM) (National Research Council of Canada), daily procedural blanks and duplicate samples. The value obtained was determined and processed by software AMA 254 using an internal calibration curve, and the Hg concentration is expressed in mg/kg dry weight.

2.3.2.3 Definition of trace metals baseline for the Sado estuary

Normalization uses one of the most abundant elements in the Earth's crust, whose concentration is less affected by anthropogenic inputs, namely aluminum, iron, and lithium (Windom et al., 1989; Kersten and Smedes, 2002; Loring, 1991; Mil-Homens et al., 2006, 2007). In the present study, the trace metals contents were normalized to Li to identify and estimate anthropogenic inputs in estuarine and coastal sediments (Loring, 1990). Lithium is considered a normalizing element due to its critical role in one or more major trace metals carriers (e.g., clay minerals), its ability to reflect granulometric variability in sediments, and its rare association with human influence. Therefore, Li was used to help understand which part of that metal concentration is natural and which is influenced by human activities. For this purpose, a linear regression was conducted, assuming that values above the 95 % confidence interval may indicate metal enrichment in the sediment.

2.3.2.4 Determination of Enrichment Factors (EF)

The enrichment factor (EF) is a useful indicator that reflects the status and degree of environmental contamination (Mil-Homens et al., 2006). EF is usually applied to reduce the variation generated by mineralogy, helping to discriminate between natural and anthropogenic sources and to deduce the degree of trace metal pollution in sediments. In light of this, trace metal enrichment of the sediments was assessed using enrichment factors normalized by the reference metal (Li) (Metal EF = (metal/ref. element)sample/(metal/ref. element)ref. material. An EF of 1 indicates that enrichment is primarily of natural origin; between 1 and 1.5 indicates low enrichment, however it may indicate some anthropo- genic influence on trace metal concentrations, while EFs above 1.5 may indicate that the sediment it has been enriched by natural processes (e. g., biota) or human activity (Loring, 1990; Mil-Homens et al., 2006).

2.3.3 Macrobenthic species identification

Species identification was carried out by morphological and molecular methods. Species names were checked against the World Register of Marine Species (WoRMS) database (WoRMS Editorial Board, 2024).

2.3.3.1 Morphological-based identification

Morphological identifications were performed to the lowest possible taxon level employing a stereomicroscope using identification keys (Ryland and Hayward, 1995; Ryland and Hayward, 2017; Pierre Fauvel, 1927). All samples were re-examined in order to ensure identification accuracy.

2.3.3.2 Molecular-based identification

2.3.3.2.1 Metabarcoding

The bulk samples were homogenized following the protocol used by Aylagas et al. (2016b). Total DNA from each bulk sample was extracted using five replicates with approximately 30 mg each, using Omega Biotek's E.Z.N.A.® Tissue DNA Kit, according to the manufacturer's protocols. High-throughput sequencing (HTS) was per- formed by Genoinseq (Cantanhede, Portugal). Samples were sequenced using Illumina Sequencing by amplification of 18S rRNA and COI genes, using the KAPA HiFi HotStart PCR Kit according to the recommendations of the manufacturer, with 0.3 μM of each PCR primer. For 18S V4 region, 2.5 μL of template DNA were used in a total volume of 25 μL, the forward primer TAReuk454FWD1 5'-CCAGCASCYGCGGGTAATTCC -3' and the reverse primer TAReukREV3 5'- ACTTTCGTTCTTGATYRA -3' (Lejzerowicz et al., 2015). The PCR conditions consider a 3 min denaturation at 95 ◦C, followed by 10 cycles of 98 ◦C for 20 s, 57 ◦C for 30 sand 72 ◦C for 30 s, 25 cycles of 98 ◦C for 20 s, 47 ◦C for 30 s and 72 ◦C for 30s and a final extension at 72 ◦C for 5 min. For COI region, 5 μL of template DNA in a total volume of 25 μL was used, and the primer pairs mICOIintF 5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′ (Leray et al., 2013) and LoboR1 5′-TAAACYTCWGGRTGWCCRAARAAYCA-3′ (Lobo et al., 2013). The PCR conditions were a 3 min denaturation at 95 ◦C, followed by 35 cycles of 98 ◦C for 20 s, 60 ◦C for 30', and 72 ◦C for 30', and a final extension at 72 ◦C for 5 min. Negative PCR controls were included in all amplification procedures. A limited-cycle PCR reaction was used to add sequencing adapters and dual indexes to the DNA amplified for hypervariable regions with specific primers, according to the manufacturer's recommendations The SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017) was used to purify and normalize the PCR products which were then pooled, and pair-end sequenced using Illumina MiSeq® sequencer with MiSeq® reagent Kit v3 (600 cycles), according to manufacturer's instructions (Illumina, San Diego, CA, USA).

2.3.3.2.2 Bioinformatics pipelines

Raw reads were extracted from the Illumina MiSeq® System in fastq format and quality-filtered using PRINSEQ version 0.20.4 (Schmieder and Edwards, 2011). This process removed sequencing adapters, trimmed bases with an average quality lower than Q25 in a window of 5 bases and eliminated reads with <100bp for the 18S region and 150 bp for the COI gene. Reads provided by the sequencing facility, filtered forward and reverse, were merged by overlapping paired-end reads in mothur (make.contigs function, default alignment) (version 1.39.5), and the primer sequences were also removed (trim.seqs function, default) (Duarte et al., 2023; Kozich et al., 2013; Schloss et al., 2009). After these steps, the reads were ready to be introduced in the chosen two public platforms: COI reads were sub- mitted into the Multiplex Barcode Research and Visualization Environment – MBrave (www.mbrave.net; Ratnasingham (2019)), which is linked with BOLD (Ratnasingham and Hebert, 2007) and SILVAngs database for 18S reads (https://ngs. arb-silva.de/silvangs/; Quast et al. (2013)).

The fasta files created for COI primer were uploaded in mBRAVE by using the sample batch function. Since the primers were previously removed in mothur, the initial and final parts of the obtained sequences were kept at 0 bp, and only trimming length was applied (≤ 313 bp). In the filtering parameter reads with lower quality were then removed if the average quality value (QV) was <10 or sequences shorter than 150 bp. This step allows 2 % of nucleotides with a > QV and a maximum of 1% nucleotides with >10 QV. The reads that fulfilled the previous criteria were further de-replicated and arranged in Operational Taxonomic Units (OTUs) using a distance limit of 3 %. The fusion of the paired ends of the reads MiSeq required a minimal overlap of 20 bp, allowing 5 substitutions of nucleotides. The obtained OTUs were identified as taxonomic using a 97 % similarity threshold against the BOLD database that includes several publicly available reference libraries (Hollatz et al., 2017; Lobo et al., 2017). For 18S, reads were processed by the amplicon analysis pipeline of the SILVA project (SILVAngs 1.4) (Quast et al., 2013). Each read was aligned using the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21,008) (Pruesse et al., 2012), against the SILVA SSU rRNA SEED and quality controlled (Quast et al., 2013). In this analysis, reads with fewer than 200 nucleotides aligned and reads with >1 % ambiguity, or 2 % homopolymers were excluded. Reads with low alignment quality (80 alignment identity, 40 alignment score reported by SINA) were identified and excluded. Following these initial quality control steps, identical reads were identified (dereplication), unique reads were clustered (OTUs) on a per-sample basis, and the reference read of each OTU was classified. In order to dereplicate data and cluster it, VSEARCH was used (version 2.17.0; https: //github.com/torognes/vsearch) (Rognes et al., 2016), and applied identity criteria of 1.00 and 0.99 to the data, respectively. In order to classify the data, BLASTn was used (2.11.0+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Camacho et al., 2009), with standard settings using the non-redundant version of the SILVA SSU Ref dataset (release 138.1; http://www.arb-silva.de). OTU reference reads were mapped onto all reads that were assigned to the respective OTU. The function "(% sequence identity + % alignment coverage)/2" was applied and whenever the value did not exceed 93, the reads without any or weak classifications remained unclassified. Only OTUs that could be identified to a taxonomic level with a similarity threshold of 99 % were considered for further analysis. Reads were excluded when matched to non-metazoan and non-invertebrate metazoan groups.

To maximize the results, full databases (i.e., BOLD for COI and SILVA for 18S) were used for taxonomic assignments, and each species match was individually evaluated for reliability. Taxonomic assignments were carefully checked using BOLDSYSTEMS (https://www.boldsystems. org/) for COI and BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for 18S to resolve discrepancies and keep the correct taxonomic identification (e.g., synonyms and clear cases of misidentification). When incongruences could not be solved, the following criteria were applied: i) when the percentage of similarity could not discriminate species within a genus, the genus was assigned and indicated with beta (β); ii) when the percentage of similarity could not discriminate genera within the family, the family was assigned and indicated with alpha (α). Also, putative non-indigenous species (NIS) have been marked with an asterisk (*), based on their native distribution.

2.3.3.3 AMBI and M-AMBI indices

AZTI Marine Biotic Index - AMBI (Borja et al., 2000), and the Multivariate M-AMBI index (Muxika et al., 2007) are common indices used to assess the ecological quality status of marine and transitional ecosystems by exploring the sensitivity/tolerance of soft bottom macrobenthos to disturbance. The indices provide a pollution or disturbance classification of a particular site, representing the "health" of the benthic community. AMBI (http://www.azti.es) is based on the relative distribution of sensitive/tolerant species groups and (Borja et al., 2000). It considers five Ecological Groups (EG): EG I, species highly sensitive to organic enrichment and present in unpolluted conditions; EG II, species indifferent to enrichment, usually exhibiting low temporal variability; EG III, species tolerant to organic enrichment; EG IV, second-order opportunistic species that emerge in slightly to pronounced disturbance conditions; and EG V, first-order opportunistic species that proliferate in reduced conditions. The AMBI index and the M-AMBI index require the use of species abundance. However, due to the results of the metabarcoding approach, it was necessary to use species presence (p) and absence (a) as an alternative to species abundance for the meta- barcoding methodology (Aylagas et al., 2014, 2018). Both abundance and presence/absence of species were used to determine the AMBI and M-AMBI of the morphological-based identification methodology. For molecular identification, the presence or absence of species (p/a) AMBI) was used. In addition, to determine the AMBI and M-AMBI of the aggregation of the two methodologies (morphological- and molecular- based), the presence and absence of species was used ((p/a) AMBI). The AMBI values vary from 0 to 7, indicating different ecosystem status according to pollution tolerances of the species present: 0 to 1.2, un- polluted; 1.3 to 3.3, slightly polluted; 3.4 to 5, moderately polluted; 5.1 to 6, heavily polluted and 6.1 to 7, extremely polluted. M-AMBI was designed to comply with the Water Framework Directive (WFD) requirements. It uses a multivariate approach that integrates the response of three selected metrics, i.e., species richness, Shannon's diversity index and the biotic index AMBI. A high status indicates that the reference condition can be considered as an optimum where the M-AMBI is close to 1. If the status is 'poor', the M-AMBI approaches zero. The M-AMBI classification is based on the European intercalibration exercise for the implementation of the WFD and has thresholds for distinct levels of quality. Specifically, for 'high' quality, the M-AMBI value should be>0.77; for 'good' quality, the value should be between 0.53 and 0.77; for 'moderate' quality, the value should be between 0.38 and 0.53; for 'poor' quality, the value should be between 0.20 and 0.38; and for 'bad' quality, the value should be <0.20.

2.3.4 Statistical analysis

Given that molecular data do not support abundance measures, both molecular and morphological datasets were converted to a presence/ absence format to ensure analytical consistency and comparability of results across methodologies. The normality tests and non-parametric tests were conducted using the Statistica software for Windows (Statsoft, Inc., USA; version 14.0.015), applying significance level of $p > 0.05$ for all statistical analyses. Sediment characterization graphs were performed using Grapher(version 12). The reduced number of sediment samples per sampling period and per study area does not guarantee the three assumptions necessary for the parametric criteria, so a non-parametric test was applied. Therefore, differences among sampling periods in each study area were primarily tested using a Kruskal-Wallis test. Following this step, the Mann-Whitney U test was employed to assess differences in the content of studied variables between the study areas. Box-and-whisker plots were computed for each study area, showing the minimum, maximum, median, outliers, and lower and upper quartiles for each variable.

In this study, benthic macrofaunal species were investigated across three locations (TR, NAV, and GAM) during two seasonal periods (Aut19 and Spr20), using both morphological and molecular methodologies. To ensure a robust comparative approach, morphological and molecular data were transformed to presence/absence (0/1) format. Differences in macrobenthic species across seasons and study areas were evaluated using PERMANOVA test in R ("vegan" package; Jari Oksanen et al., 2023) based on a similarity matrix constructed with the Bray-Curtis coefficient. PERMANOVA was applied separately to morphological and molecular data, followed by a final analysis on the combined dataset to assess spatial and temporal variations in community structure. Post hoc comparisons identified specific group differences within and among study areas and seasons.

The R software (R Core Team, 2022) was used for data cleaning, organization, analysis, and graphic production. Venn diagrams were created with VennDiagram package (Version 1. 7. 3) to visualize category intersections (Hanbo Chen, 2022). In order to verify the existence of a relationship between the biotic and abiotic parameters of the sediment, a canonical correspondence analysis (CCA) was conducted using presence/absence of combined data (molecular and morphological) in the software PAleontological STatistics (PAST) version 4.09. The species represented in the CCA were selected according to their proportions in each AMBI ecological group, and for simplicity, a maximum of 5 species were considered for visualization on the plot.

2.4 Results

2.4.1 Sediment characterization

Since the samples collected during both autumn (Aut) and spring (Spr) periods showed similar variation ranges (in appendix A - Figure 8 and 9), they were integrated in the same group for each area. Further analysis using the Kruskal-Wallis test showed no significant differences between the sampling periods in each study area for all variables examined (examples of which can be seen in appendix A Figure 10). However, the Mann-Whitney U Test indicated statistically significant differences in the contents of fine fraction, sand, organic carbon, calcium carbonate, and in “trace metal” concentrations among the three studied areas (GAM, NAV, and TR) (see Figure. 2 and 3 and Appendix A - Table 3). A few exceptions are sand and Co in GAM and NAV, and gravel in NAV and TR.

The sediments in the GAM area showed a high variability in grain size, with the highest median gravel content of around 20 %. GAM also had the highest concentrations of Ni, Cr, and Co, as well as a high sand content (78 %). In the NAV area, there was a high median content of fine fraction, organic carbon (C_{org}), and Li, As, Pb, Zn, Cu and Hg. The sediments in the TR area are characterized by higher median sand and calcium carbonate ($CaCO_3$) contents. Furthermore, when compared to all other areas, TR had the lowest concentrations of trace metals.

Pearson's correlation coefficients were determined considering all datasets, that Li exhibits a negative correlation with sand and a significant positive correlation with fine fraction and organic carbon contents (Appendix A,Table 4). In addition, there were positive and significant ($p < 0.05$) correlation coefficients of all elements (excluding Ni and Cr) with fine-grained sediment components, and between CaCO3 and sand contents. Chromium and Ni are only significantly correlated between them and to a lesser degree with Co. This association reflects a lithological influence further explained in the discussion section (more details of the coefficients of Pearson's correlation and p values on Appendix A: Table 4). To compensate for the effect of sediment and grain-size composition, metal concentrations were normalized to Li content. Most of the metals are considered to have a significant positive linear correlation with Li (Table 4). The regression analysis models between metals and Li are shown in Figure 11. Based on these models it is possible to evaluate the occurrence of possible metal enrichments (derived from natural and or anthropogenic sources). All points that fall inside the 95% confidence band of metal versus Li concentrations are characterized as natural sediments, whereas the sampling points that fall above the 95% confidence limit are considered to belong to a metal-enriched population. For most of the metals studied it is possible to observe the occurrence of samples which present a greater dispersion compared to the others. These may represent samples that are enriched in relation to natural variability. In the studied samples these enrichments can be associated with natural sources (e.g., Ni and Cr) and anthropogenic sources (e.g., Hg, Cu, Zn, Pb, As). In general, the latter are from the Navigator area, which is highly impacted by different industries. If these samples are excluded from the regression model, a regional baseline model is obtained, and the metal enrichment factor can be obtained from the regression equation. Table 5 shows the samples with EF > 1.5.

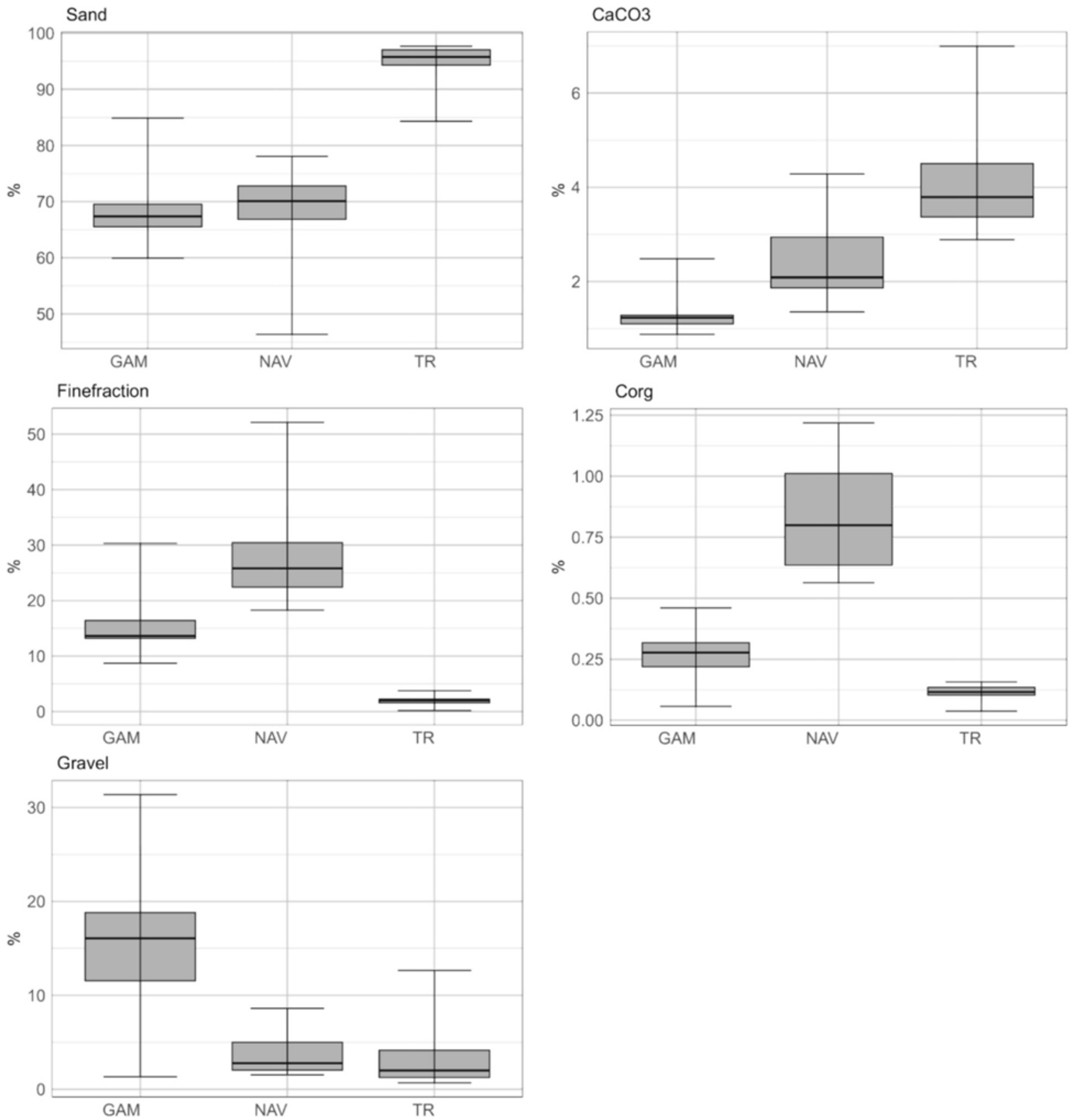

Figure 2 Box-whisker plots for the sediment composition parameters in the three studied areas of the Sado Estuary

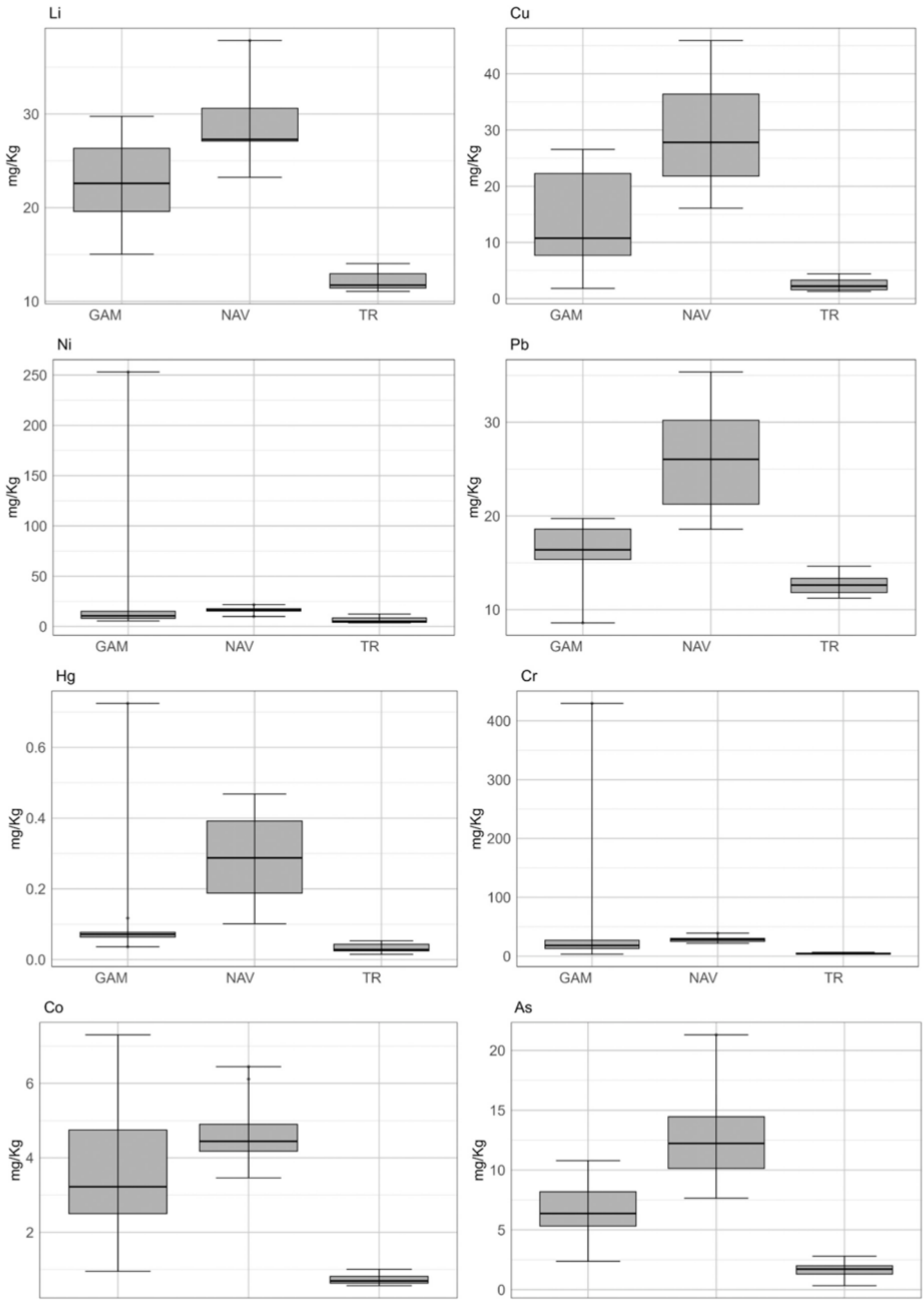

Figure 3 Box-whisker plots for the "metals" concentration in the three studied areas of the Sado Estuary.

2.4.2 Macrobenthic composition characterization

A total of 95 taxa were identified to species level combining both morphology- and molecular-based identification methods (Appendix A - Table 6). The taxa identified belonged to 9 phyla, 16 classes, 39 orders, 78 families, and 107 genera (Table 6; Figure 4). The dominant groups in the three areas of study were the Annelida and Mollusca phyla, followed by the Arthropoda phylum. Only four species were found to be present across the three study areas: *Cerastoderma edule* (Linnaeus, 1758), *Cyathura carinata* (Krøyer, 1847), *Hediste diversicolor* (O.F. Müller, 1776), and *Ruditapes philippinarum* (A. Adams & Reeve, 1850). NAV, GAM and TR have 8, 16 and 48 exclusive species, respectively (Table 6). These findings highlight the complexity of the macrobenthic communities and set the stage for a detailed analysis of the methodologies used for their identification, which is further discussed in the discussion.

Thirty-seven species were identified exclusively by morphological identification, and 44 species were identified exclusively by metabarcoding, using two genetic markers (Figure 5). Using both methods, only 18 species were identified in common, only 4 species were identified using both markers, 9 species exclusively using 18S, and 31 species were only identified using COI. In some instances, sequences obtained with the 18S marker could not be 100 % accurately identified. In cases where the similarity percentage was equal for several species of the same genus, the genus was considered (β): the polychaeta *Diopatra* sp. β, the hermit crab *Clibanarius* sp. β and the nematode *Tetrastemma* sp. β. When the results matched with more than two genera within the same family, the family was considered (α): the cnidarian Hydractiniidae α, the molluscs Psammobiidae α, Solecurtidae α, Tellinidae α, Veneridae α and Nassariidae α. With the COI marker, no incongruences were found. Additionally, the results show that 21 species, identified exclusively by molecular methods (COI and 18S markers), are putative non-indigenous species. Following the identification of these putative non-indigenous species (NIS), a review of the morphological data was conducted to determine whether any of these species had been undetected or misidentified. However, no corresponding specimens were found within the morphological samples.

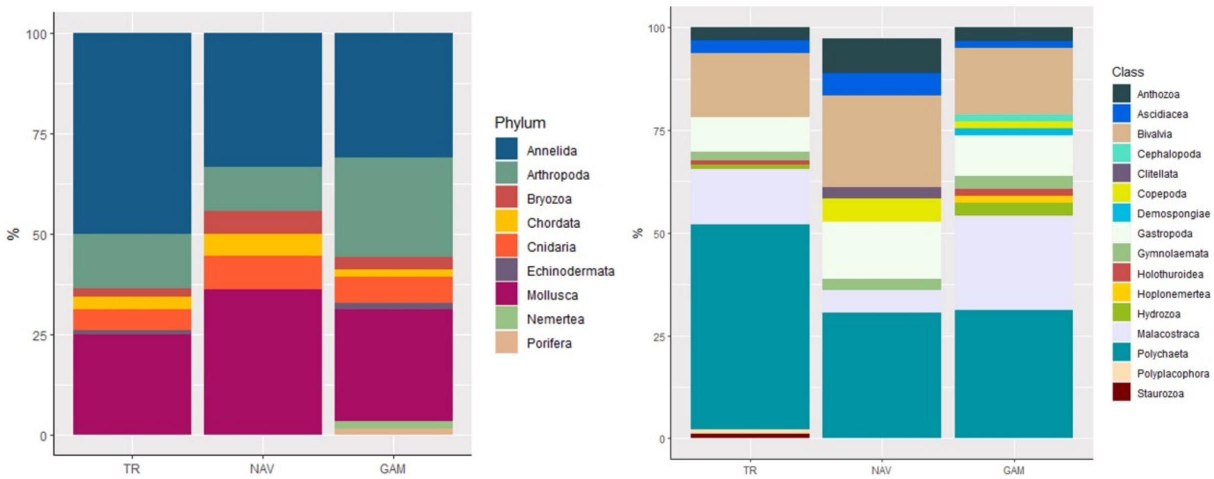

Figure 4 Taxonomic profiles for phylum and class, combining morphological and metabarcoding (COI and 18S markers) identifications, based on the proportion of invertebrate taxa.

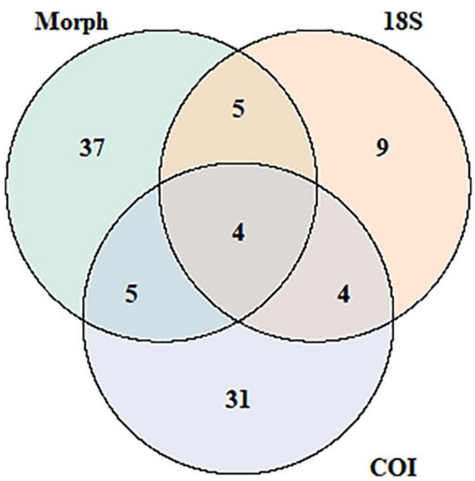

Figure 5 Venn's Diagram showing the number of invertebrate species detected through morphological characters (Morph) and by metabarcoding using two genetic markers (18S and COI).

The PERMANOVA results revealed no statistically significant differences between seasons (Aut19 and Spr20) at any of the locations for morphological, molecular, or combined data ($p > 0.1$). However, statistically significant differences were identified between the locations, particularly involving TR area compared to NAV and GAM areas. For the morphological data, significant differences were found between NAV and GAM ($p = 0.02$) and between TR and both locations ($p < 0.005$). This pattern was consistent for molecular and combined data, with TR differing significantly from NAV and GAM, and additional significant differences observed between NAV and GAM ($p < 0.015$). The results are presented in Appendix A -Table 7.

To verify the relationship between the abiotic and biotic factors analyzed in the samples, a canonical correspondence analysis (Figure 6) was conducted. This analysis explains the relational variability of the abiotic and biotic factors with the two primary ordering axes, CCA1

(57,37 %) and CCA2 (42,63 %). The CCA1 axis correlates positively with TR and negatively with NAV and GAM, while the CC2 axis correlates positively with TR and NAV but negatively with GAM. Furthermore, the CCA indicates that metals have a stronger relationship with NAV than with TR and GAM. The distinct positioning of the three areas (TR, GAM and NAV) shows the differences in environmental characteristics, which in turn influence species composition. Specifically, TR exhibits a positive correlation with sand and CaCo3, while GAM is positively correlated with gravel fraction. On the other hand, NAV is positively correlated with all the studied metals, as well as the fine fraction, TOM, and C_{org}. CCA revealed distinct relationships between species and environmental parameters across the three study areas. In TR, characterized by sandy sediments and high $CaCO_3$ content, the predominance of EG I species such as *Spisula subtruncata* (da Costa, 1778) and *Urothoe grimaldii* Chevreux, 1895, indicated a good environmental quality. In GAM, where gravel predominates, the prevalence of EG III species like *Notomastus latericeus* Sars, 1851, and *Abra nitida* (O. F. Müller, 1776), suggested a moderate level of disturbance. Conversely, NAV, characterized by fine sediments and high metal concentrations, was dominated by EG III species such as *Heteromastus filiformis* (Claparède, 1864), and *Carcinus maenas* (Linnaeus, 1758), reflecting some disturbance higher than in GAM.

It should be noted that, due to the nature of the data used (presence/ absence), many species share identical scores, resulting in overlap in their positions on the ordination plot. Consequently, it was not possible to explicitly represent the species vectors in the figure. Instead, species positions were added manually to improve visualization. The specific coordinates of species along the CCA axes are provided in Appendix A - Table 8.

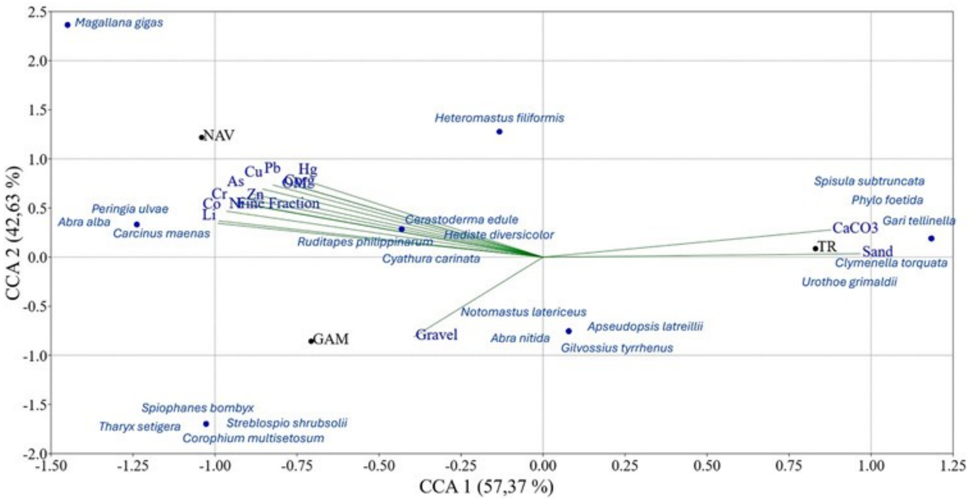

Figure 6 . Biplot of the Canonical Correspondence Analysis (CCA) performed to examine the relationship between abiotic parameters and species (blue dots) in TR, NAV, and GAM areas. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4.3 Morphological- and molecular-based AMBI and M-AMBI indices

For the AMBI index, species that are not assigned to an ecological group so far have been considered 'not assigned'. Hard substrate species, such as *Bugula neritina* (Linnaeus, 1758), *Tricellaria inopinata* d'Hondt & Occhipinti Ambrogi, 1985, *Tricellaria ternata* (Ellis & Solander, 1786), were not considered, as well as *Acanthosepion pharaonic* (Ehrenberg, 1831) which not a macrobenthic species. As there were no statistically significant differences between the seasons within each area, the AMBI index was applied by the aggregation of seasonal data for each location. Ecological group III dominates in both NAV and GAM locations across all methodological approaches (Figure 7, Table 1). The morphological method indicates that TR, GAM, and NAV have high percentages of EG III, with values of 66.3 %, 90.5 %, and 98.9 %, respectively. However, when AMBI was recalculated using presence/absence transformed data, EG III remained dominant in GAM (71 %) and NAV (80.8%). In contrast, EG I showed a slight dominance (41.7 %) over EG III (31,5 %) in TR.

The results of (p/a) AMBI molecular data analysis indicate that the dominant group for TR, GAM and NAV is EG III, with percentages of 34.7 %, 55.6 % and 52.4 %, respectively. However, the other ecological groups are differently represented when compared to morphological data (Table 1). The (p/a) AMBI of the aggregated methodologies indicates that for the TR, the dominant group was EG I (36.5 %), while the dominant groups for NAV and GAM remained unchanged, EG III (54.9 % and 56.7 %, respectively). The mean AMBI values decreased further when compared to the values obtained from separate methodologies, however, the disturbance classification remained 'slightly disturbed'.

M-AMBI, Shannon-Wiener index (H'), and Richness indicated status changes based on different methodologies used in this study (Table 1). The M-AMBI results of the morphological data indicate that the GAM and NAV have a status of 'Good' and 'Moderate', respectively. Following, the presence/absence transformation of the morphological data, the M- AMBI results for GAM and NAV remained 'Good' and 'Moderate', respectively. In TR, both the original and transformed data classified the status as 'High'. The metabarcoding results show a different status for the same areas 'High' and 'Good', respectively. Nevertheless, the aggregation of methodologies demonstrates the same status of meta- barcoding. TR maintained a 'High' status across all methodologies, also had the highest H' values and richness for both separated and aggregated methodologies. In contrast, the NAV exhibited low H' values and species richness for both separated and aggregated methodologies.

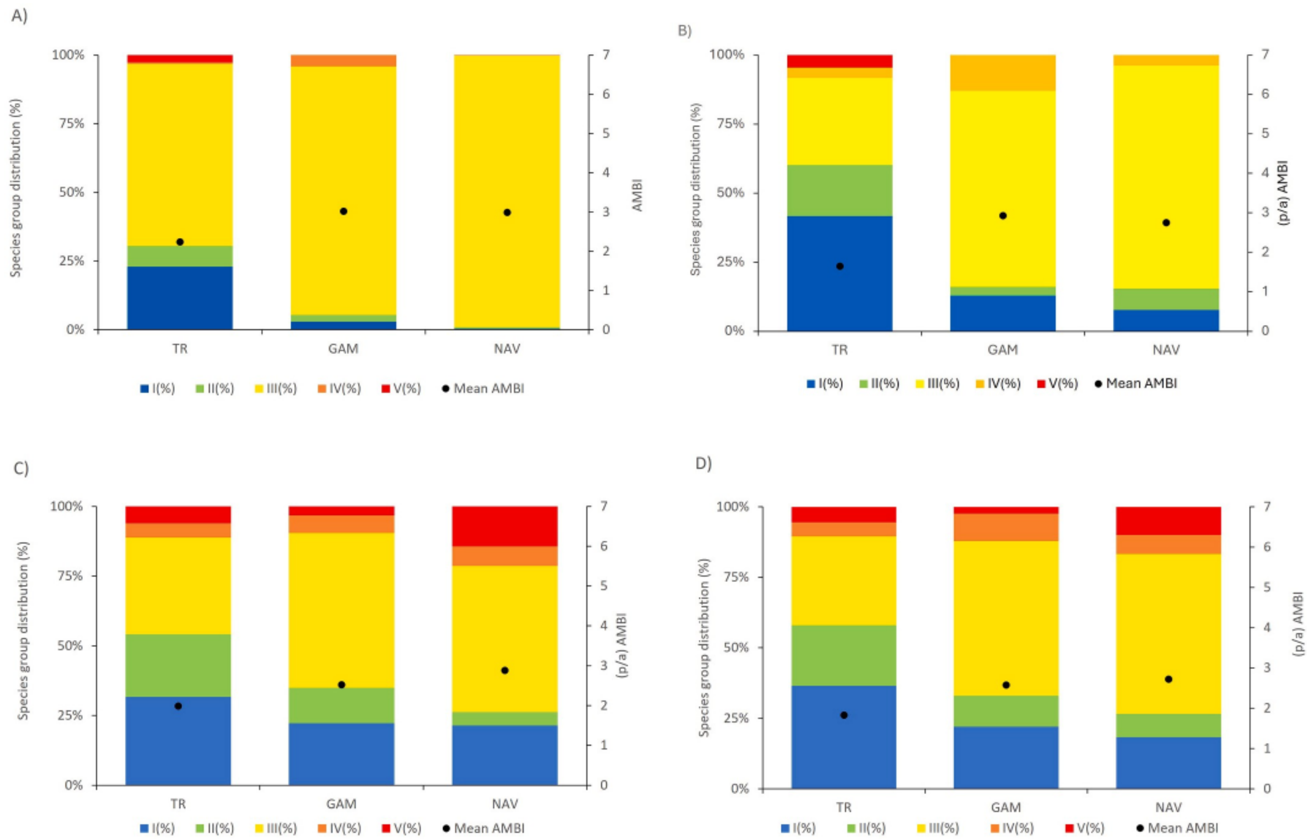

Figure 7 The contribution of each ecological group and mean AMBI values were determined based on the abundance of species using morphology (A), and on presence/absence of species using morphology (B), metabarcoding (C) and both methodologies (D).

Table 1 Summary table of results exported from AMBI and M-AMBI index (AZTI Marine Biotic Index).

Methodology	***Locations***	***EG I (%)***	***EG II (%)***	***EG III (%)***	***EG IV (%)***	***EG V (%)***	***AMBI***	***Disturbance Classification***	***Diversity (Shannon-Wiener index H')***	***Richness***	***M-AMBI***	***Status***	***Not assigned (%)***
Morphology	***TR***	23	7,5	66,3	0,5	2,7	2,241	Slightly disturbed	3,726	65	0.999	High	7,1
		41,7*	18,5*	31,5*	3,7*	4,6*	1,646*		5,7448*	65 *	10.006 *	High *	14,3*
	GAM	3	2,4	90,5	4,1	0	3,021		2,564	17	0,601	Good	1,2
		12,9*	3,2*	71*	12,9*	0*	2,932*		3,6314*	17 *	0.55118 *	Good *	6,1*
	NAV	0,4	0,4	98,9	0,2	0	2,987		1,691	8	0,479	Moderate	0
		7,7*	7,7*	80,8*	3,8*	0*	2,75*		2,6874*	8 *	0.46684 *	Moderate *	0*
Metabarcoding	***TR***	31,6	22,4	34,7	5,1	6,1	1,985	Slightly disturbed	5,114	43	0,974	High	8,4
	GAM	22,2	12,7	55,6	6,3	3,2	2,52		5,186	46	0,955	High	18,2
	NAV	21,4	4,8	52,4	7,1	14,3	2,883		4,537	29	0,760	Good	28,8
Morphology + Metabarcoding	***TR***	36,5	21,5	31,5	5	5,5	1,824	Slightly disturbed	6,273	96	1,000	High	13
	GAM	22	11	54,9	9,8	2,4	2,574		5,519	59	0,775	High	16,3
	NAV	18,3	8,3	56,7	6,7	10	2,719		4,771	35	0,642	Good	22,1

*Indicates results from presence/absence transformed morphological data

2.5 Discussion

2.5.1 Sediment physicochemical characterization across the estuary

The known biotic and abiotic heterogeneity of bottom sediments in the Sado estuary (Lobo et al., 2017; Mil-Homens et al., 2014; Sroczynska et al., 2021), is corroborated by this study. The sediments exhibit a heterogeneous distribution in the study areas, displaying a gradient of clayey to sandy compositions. Higher concentration of trace metals such as Ni, Co, and Cr was observed in GAM, which may be due to the presence of heavy minerals (e.g., pyroxenes and amphiboles) in the sand fraction. These minerals may be derived from the weathering of basic and intermediate igneous rocks from the Sines Massif Complex. They enter the estuary by littoral drift from south to north during high-energy events from the southwest (Jesus et al., 2007; Miranda et al., 2007). This nickel and chromium enrichment in sandy sediments was also observed in a previous study of sediment samples from the Sado estuary (Mil-Homens et al., 2013). The organic matter content was found to be higher in areas where the percentage of fine fraction was higher. NAV presents the highest levels of total organic matter, organic carbon, and trace metals. This can be attributed to a long residence time which may be at the origin of the large surface area of fine-grained sediment particles, which in turn provides more potential binding sites for C_{org}. Additionally, the high median concentrations of metals suggest their binding to organic matter and fine-grained particles, which may play a role in the distribution of metals in the sediments. In comparison, TR, characterized by low residence time, has a higher percentage of sand and a higher $CaCO_3$ content, indicating the presence of shell debris. The relatively low total metal concentrations observed in the TR sediments may be associated with the high sand content, which can act as an inert diluent of elemental concentrations. It is known that sediment characteristics such as organic matter and trace metal concentration are dependent on grain size distribution (Krumgalz et al., 1992; Mil-Homens et al., 2014). The marked variation in the granulometry of the study areas along the estuary explains the observed results. The accumulation of trace metals in sediments is influenced by physicochemical variables, with hydro- dynamics representing the major factor (Darr et al., 2014; Mucha et al., 2003; Pitacco et al., 2021; Verdelhos et al., 2015).

Trace metal enrichment refers to the process of certain environmental conditions causing trace metal concentrations to exceed expected reference levels, since most metals naturally occur in the environment (Loring, 1991). This natural presence complicates the analysis of the influence of anthropogenic activities on sediments, as the presence of trace metals does not automatically allow us to infer an enrichment of anthropogenic origin (Arisekar et al., 2022; Mucha et al., 2003). In most sediments, there is a linear relationship between inert elements (such as iron and aluminum) and the size of the sediment particles (Mil-Homens et al., 2007; Pastorinho et al., 2012), since the levels of these metals essentially depend on the erosion/transport/ sedimentation process. The positive correlation of Li with the fine fraction and organic carbon contents suggests an association with fine- grained particles (Loring, 1990). Normalization makes it possible to compensate sediment particle size variability when assessing the present elements in the environment (Kersten and Smedes, 2002; Loring, 1991). The metals Hg, Cu, Pb and Zn obtained enrichment factors above 1.5 at the NAV, indicating enrichment sources other than natural ones. This enrichment at the NAV may be related to the fact that this area is close to a shipyard and chemical industries such as pulp and fertilizer production which can be carried into bodies of water through runoff (Caeiro et al., 2005; Monteiro et al., 1995). The hydrodynamic and the high content of fine particles of the sediments at the NAV also influence these results. GAM obtained an EF higher than >20 for Cr and Ni, suggesting a high enrichment of these metals in the sediments.

This study used a total dissolution technique to extract the trace metals, which did not assess their bioavailability in sediments. Yet, the evaluation of Cr and Ni for bioavailability could provide crucial insights into their potential ecological risks (Griscom and Fisher, 2004). The presence of anthropogenic activities in the area such as aquaculture (fish and oysters), may use metal structures that could influence these values (Costa et al., 2008). While EF indicates the source of metal enrichment, bioavailability assesses how accessible these metals are to organisms (Costa et al., 2008). This information is crucial for developing management measures and environmental policies specific for each ecosystem (Amiard, 1992; Arisekar et al., 2022; Bryan and Langston, 1992). The results Cr and Ni enrichment factor for TR were above 1.5, which could be attributed to a small anthropogenic influence from port activities.

2.5.2 Macrobenthos composition characterization using an integrative approach

The characterization of macrobenthic composition was conducted using two methodologies: metabarcoding and morphological characters. In this study, the metabarcoding results were carefully verified due to the presence of sequences identifying putative NIS in mainland Portugal, which exposed inconsistencies in taxonomic identifications within SILVAngs database. Consequently, some identifications were limited to the family (α) or genus level (β). These limitations are due to challenges specific to the metabarcoding technique, such as low taxonomic of the 18S marker (Wu et al., 2015), and incomplete reference data in data- bases (Duarte et al., 2021, 2023; Keck et al., 2022; Leite et al., 2021).

Metabarcoding identified 44 species, of which 21 were classified as putative NIS for mainland Portugal, based on their native distribution (Table 6). However, morphological confirmation is needed to validate the presence of these species. This confirmation is crucial because it provides direct visual evidence of the organism, reducing the possibility of false positives due to degraded DNA or contamination, and ensuring that the identified sequences correspond to physically present individuals (Duarte et al., 2021; Zaiko et al., 2018). This morphological verification may be challenging due to factors such as DNA remnants from degraded organisms, microscopic life stages, low abundance, specific distribution, or cryptic species.

In contrast, the morphological method did not identify any putative NIS, highlighting the sensitivity of molecular techniques, especially metabarcoding, for early detection in monitoring programs (Amaral et al., 2022; Brown et al., 2016; Duarte et al., 2021; Zaiko et al., 2016). Molecular techniques, in particular metabarcoding, can detect environmental DNA (eDNA) present in the environment, making them more effective at identifying species that are present in low abundances or are difficult to detect visually (Dully et al., 2021; Ruppert et al., 2019; Zaiko et al., 2016). These findings demonstrate the potential of metabarcoding for early detection and effective monitoring in ecosystems like the Sado Estuary, where intense maritime traffic (Cabral et al., 2020) increases the risk of NIS introductions (Castro et al., 2020), with potential ecological and economic impacts (Grosholz, 2002; Molnar et al., 2008). Nevertheless, while metabarcoding offers robust detection accuracy (Hering et al., 2018), morphological identification remains a crucial step for validating species presence and ensuring reliable ecological assessments. The sampling design provided sufficient replication to capture the diversity and structure of macrobenthic communities, aligning with previous findings that similar sample sizes are adequate for estuarine biodiversity assessments (Duarte et al., 2023; Lobo et al., 2017; Muxika et al., 2007).

The species spatial distribution in the three study areas is controlled by abiotic factors, as vastly shown (e.g., Martins et al. (2013)) found that the spatial distribution of benthic Polychaeta along the Portuguese mainland coast was influenced by organic matter and sediment granulometry. Other factors, such as anthropogenic pollution also influence species distribution (Chapman and Wang, 2001; Vale et al., 2010). In this study the CCA results also show that species distribution is affected by trace metals concentrations over the three areas.

The CCA results as well as the absence of statistically significant differences between Autumn and Spring suggest community stability across seasons, likely influenced by the adaptive characteristics of species inhabiting this estuary. Our results align with previous studies (e.g., (Amaral and Costa, 1999; Vale et al., 2010) indicating that spatial fac- tors such as sediment grain size and trace metal concentrations are the primary structuring elements of macrobenthic communities in the Sado Estuary, with limited influence from seasonal variations. The dominance of the Annelida, Mollusca, and Arthropoda phyla suggests that these taxa are well-adapted to the estuarine conditions. The combination of both methodologies yielded a total of 95 taxa, thereby demonstrating complementarity of both methods in biodiversity assessments especially in coastal and estuarine ecosystems where community diversity and structure are complex (Aylagas et al., 2016a; Duarte et al., 2023).

Furthermore, AMBI results of combined methodologies show that tolerant species (EG III) are consistently dominant at GAM and NAV, and slightly in TR, highlighting that the presence of species tolerant to environmental stress may contribute to the observed seasonal stability. The TR location is characterized by sandy sediments with a high $CaCO_3$ content, which provide a stable habitat (Vale et al., 2010) favoring sensitive species (EG I), such as *Atylus* sp.. These species are indicators of high ecological quality due to their sensitivity to disturbance, such as pollution (Borja et al., 2000). Despite the prevalence of sensitive species, TR exhibited slight enrichment in chromium and nickel, which may be associated to the nature of the sediments (Mil-Homens et al., 2013), with a possible contribution from nearby maritime activities (Caeiro et al., 2005). These human activities can disturb the ecosystem, potentially leading to the emergence of opportunistic species such as Capitella capitata (Fabricius, 1780) (EG V) and *Glycera alba* (O.F. Müller, 1776) (EG IV), leading to an AMBI classification of 'slightly disturbed', while the M-AMBI index indicated "High" ecological quality (Borja
et al., 2000), which is likely due to the slight dominance of sensitive species to disturbance in TR.

The GAM is characterized by the predominance of tolerant species to disturbance (Fig. 7; Table 1), potentially linked to the enrichment in trace metals, such as Cu, Cr and Ni. However, the detection of a higher number of sensitive and indifferent species when using the combined data elevated the ecological classification of GAM from "good" to "high". It is worth mentioning that some of these species, such as *Urothoe grimaldii*, *Ampelisca* sp., *Atylus* sp. and *Processa parva Holthuis*, 1951, were exclusive detection through morphological analysis (Table 1). The impact of proximate aquaculture activities, such as oyster farming, may contribute to these community patterns, as suggested by studies indicating that proximity to oyster culture can influence community composition (Bouchet and Sauriau, 2008).

The presence in finer sediments of high metal concentrations in NAV area is indicative of a significant influence from nearby anthropogenic activities, including industrial operations and shipyards, which contribute to metal enrichment (Caeiro et al., 2005; Lobo et al., 2010; Moreira et al., 2009; Cortesão and Vale, 1995; Freitas et al., 2008). The macrobenthic species at NAV were dominated by tolerant species, such as *Hediste diversicolor* (Moreira et al., 2006) and *Cerastoderma edule* (Lobo et al., 2010; Veiga et al., 2019), which are resilient to organic enrichment (Borja et al., 2000). However, metabarcoding enabled the detection of sensitive species, such as *Rissoa parva* (da Costa, 1778). This resulted in an improvement in the ecological quality classification from "Moderate" to "Good", as indicated by M-AMBI index (Table 1). The detection of sensitive species at NAV, under conditions of trace metal enrichment (Hg, Pb, Cu and Zn), indicates that the concentrations of these trace metals are not yet at levels that are toxic to the macrobenthic species. Further research regarding the bioavailability of these metals is required to provide a more comprehensive understanding of their potential impacts on macrobenthic communities.

The application of both methods allowed for the identification of a greater number of species across the various ecological groups represented in the AMBI index, resulting in different proportions of the five ecological groups for each method. Nevertheless, the AMBI index results indicated that, independently of the identification method employed, all locations were classified as "slightly disturbed". The transformation of morphological data into a presence/absence format has been demonstrated to be a viable approach in this and other studies (Checon et al., 2018; Warwick et al., 2010). However, it was observed that such transformation had a slight influence on both the AMBI and M-AMBI indices. Therefore, the discrepancies observed in the combined data set (morphological and molecular) can be attributed not only to the molecular (p/a) AMBI, as previously noted by Duarte et al. (2023), but also to this transformation applied to the morphological data. The sensitivity of these indices was also affected by the identification method employed, thereby demonstrating the limitations of each approach. Consequently, the combined use of metabarcoding and morphological analysis addresses these individual limitations and provides a more comprehensive evaluation of the ecological quality in complex estuarine habitats.

2.6 Conclusions

This study offers a clearer understanding of the ecological quality of the macrobenthic communities in the Sado estuary. Our results indicate that seasonality does not affect variability in sediment composition, trace metal concentrations, and species composition.

Despite trace metals originating from different industrial sources, their significant positive correlations suggest that mixing processes driven by the local hydrodynamic conditions play a crucial role in their distribution. Although the sediments are enriched in trace metals, the concentrations may not yet be at toxic levels for species, which may explain the occurrence of sensitive species in locations with higher metal enrichment. However, the bioavailability of these trace metals was not assessed, which is crucial for understanding their potential impact on ecosystems and should be addressed in future research.

Molecular techniques, such as metabarcoding, which can detect eDNA, are capable of identifying cryptic species and putative non- indigenous species. However, these methods require rigorous curation of results to avoid inappropriate environmental management measures, showing that morphological identification is essential for reliable environmental assessments.

Using both methods to identify macrobenthic organisms, the AMBI results consistently suggest "slight disturbances" across all locations. According to the M-AMBI index, ecological quality was rated as "High" for both TR and GAM, while NAV was rated as "Good". However, NAV appears more affected, potentially due to its proximity to industrial effluent discharge and a shipyard, whereas TR, located near a nature reserve with minimal anthropogenic activity, reflects a higher ecological quality.

Macrobenthic organisms effectively indicate ecological quality, reflecting the impact of human activities and the success of environmental management measures. However, new challenges, such as the spread of non-indigenous species, in particular those that could become invasive, are an overly complex topic. The inevitable appearance of these species, due to the lack of physical barriers and the intensive maritime activities, may be a cause for concern. For this reason, a comprehensive approach contributing to management measures targeting current relevant issues is required in order to avoid major or even irreversible problems.

In conclusion, the integrated approach adopted in this study demonstrates the advantages of both morphological and metabarcoding techniques to be combined, thereby addressing the limitations of each method and providing a more comprehensive understanding of the biodiversity and ecological quality of estuarine macrobenthic communities.

CRediT authorship contribution statement

Joana Marujo Neves: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Marta Martins: Writing – review & editing, Validation, Supervision, Resources, Investigation, Conceptualization. Helena Adão: Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. Mário Mil-Homens: Writing – review & editing, Visualization, Validation, Resources, Formal analysis. Maria Helena Costa: Writing – review & editing, Validation. Jorge Lobo-Arteaga: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Joana Marujo Neves reports financial support was provided by Foundation for Science and Technology. Joana Marujo Neves reports equipment, drugs, or supplies, statistical analysis, and writing assistance were provided by Portuguese Institute for the Sea and Atmosphere. Joana Marujo Neves reports equipment, drugs, or supplies was provided by Marine and Environmental Sciences Centre. Joana Marujo Neves reports a relationship with Foundation for Science and Technology that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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A Appendix

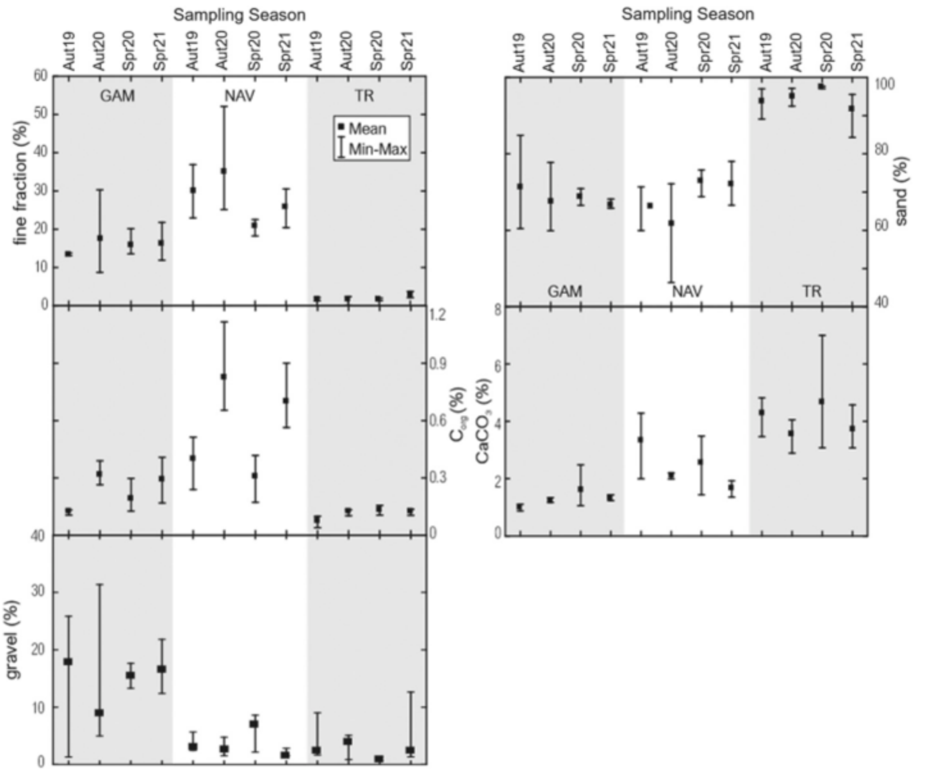

Figure 8 Grain-size composition, organic carbon (C_{org}), and calcium carbonate ($CaCO_3$) contents in the studied surface sediments.

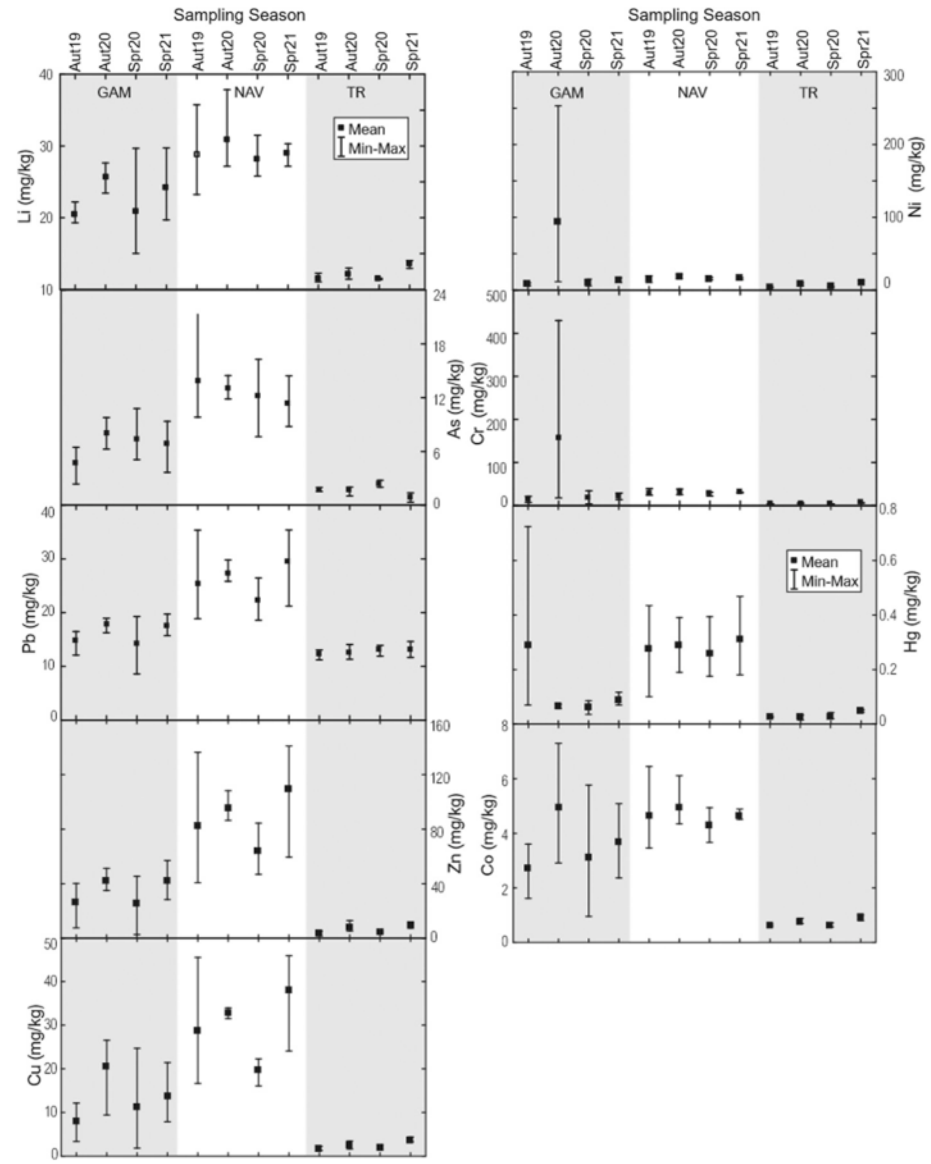

Figure 9 Trace metal concentrations in the studied surface sediments

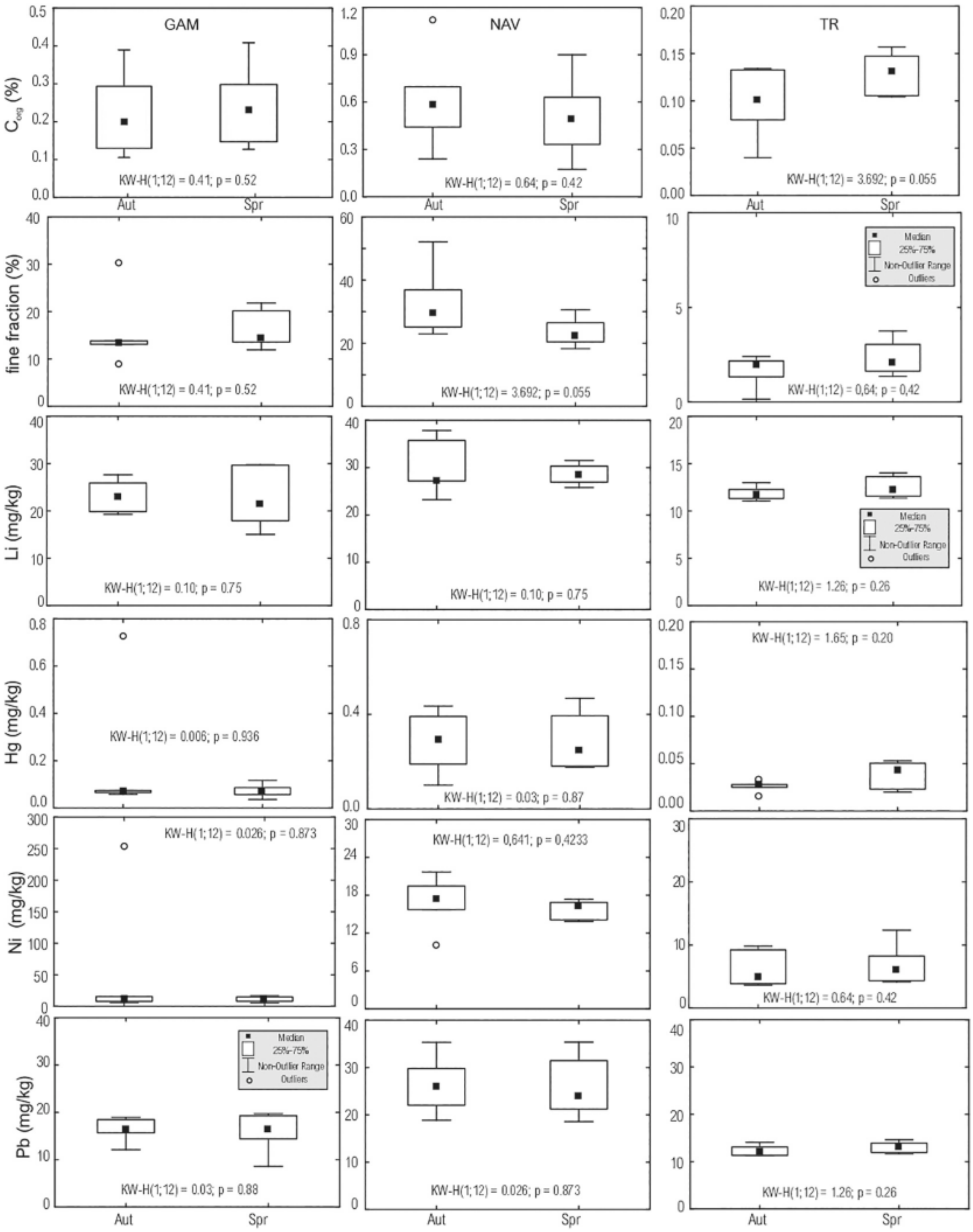

Figure 10 Bow-whisker plots for selected studied variables and Median, Kruskal-Wallis test (KW-H) and p-values in the three studied areas of the Sado estuary

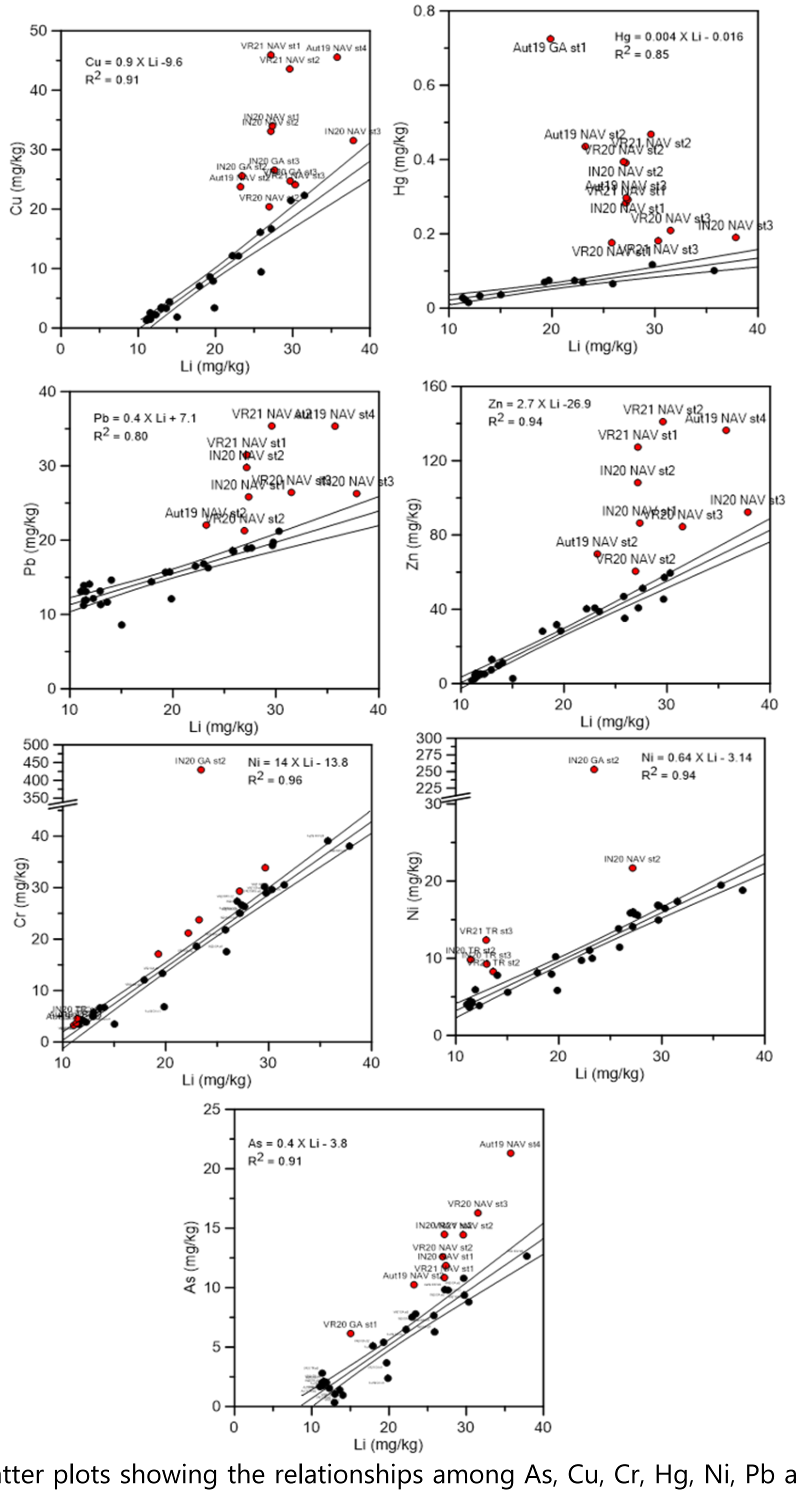

Figure 11 Scatter plots showing the relationships among As, Cu, Cr, Hg, Ni, Pb and Zn concentrations (mg/kg) and Li (mg/kg)) in surface sediments from the three studied areas of the Sado estuary. Black dots represent the samples that have 95 % confidence in representing the natural sediments. Red dots are sediment samples that fall above the 95 % confidence limit and are considered to belong to a metal-enriched population. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2 Certified and obtained As, Pb, Zn, Cu, Cr, Co, Ni, Hg, and Li concentrations (mg/kg) in the certified reference material MESS-4 (National Research Council of Canada); mean (x) and standard deviation (sd) of 7 and 4 replicates for Hg and the rest of the studied "metals", respectively.

Element	Certified values (x ± sd)	Obtained values (x ± sd)	Recovery (%)
Hg	0.090± 0.040	0.105 ± 0.011	116
Li	65.3 ± 6.8	61.5 ± 2.5	94
As	21.7 ± 2.8	20.1 ± 0.8	93
Co	13.0 ± 0.8	12.2 ± 0.1	94
Cr	94.3 ± 1.8	91.2 ± 3.8	97
Cu	32.9 ± 1.8	29.8 ± 0.3	91
Ni	42.8 ± 1.6	42.7 ± 3.2	100
Pb	21.5 ± 1.2	19.6 ± 0.5	91
Zn	147 ± 6	141 ± 3	96

Table 3. Summary of results from the application of the Mann-Whitney test. (*) indicates no statistically significant differences.

	GAM (n=12) vs NAV(n=12)			GAM (n=12) vs TR (n=12)			NAV (n=12) vs TR (n=12)		
Variable	U	Z	*p*	U	Z	*p*	U	Z	*p*
fine fraction	11	-3,493	0,0005	0	4,1281	0,00004	0	4,12805	0,00004
sand	56	-0,8949	0,3708*	1	-4,0703	0,00005	0	-4,12805	0,00004
gravel	15	3,262	0,0011	15	3,262	0,00111	52	1,12583	0,26024*
Corg	14	-3,3198	0,0009	23	2,8001	0,00511	0	4,12805	0,00004
CaCO3	9	-3,6084	0,0003	0	-4,1281	0,00004	17	-3,14656	0,00165
Li	26	-2,6269	0,0086	0	4,1281	0,00004	0	4,12805	0,00004
Ni	28	-2,5115	0,012	25	2,6847	0,00726	1	4,07032	0,00005
Cr	32	-2,2805	0,0226	10	3,5507	0,00038	0	4,12805	0,00004
Co	42	-1,7032	0,0885*	1	4,0703	0,00005	0	4,12805	0,00004
Cu	21	-2,9156	0,0036	9	3,6084	0,00031	0	4,12805	0,00004
Zn	5	-3,8394	0,0001	13	3,3775	0,00073	0	4,12805	0,00004
As	9	-3,6084	0,0003	1	4,0703	0,00005	0	4,12805	0,00004
Pb	6	-3,7816	0,0002	20	2,9734	0,00295	0	4,12805	0,00004
Hg	13	-3,3775	0,0007	4	3,8971	0,0001	0	4,12805	0,00004

Table 4 Pearson's correlation coefficients (r) for the studied variables of the three areas studied in the Sado Estuary. Correlation is significant at the 0.05 level (two-tailed) in italic.

	Li	Ni	Cr	Co	Cu	Zn	As	Pb	Hg	ff	C_{org}	$CaCO_3$	sand
Li	*1*												
Ni	*0,16*	*1*											
Cr	*0,21*	*1*	*1*										
Co	*0,93*	*0,47*	*0,52*	*1*									
Cu	*0,86*	*0,23*	*0,28*	*0,87*	*1*								
Zn	*0,84*	*0,09*	*0,13*	*0,79*	*0,96*	*1*							
As	*0,9*	*0,13*	*0,18*	*0,86*	*0,89*	*0,91*	*1*						
Pb	*0,82*	*0,05*	*0,09*	*0,76*	*0,94*	*0,99*	*0,9*	*1*					
Hg	*0,46*	*-0,04*	*-0,02*	*0,34*	*0,46*	*0,5*	*0,44*	*0,49*	*1*				
ff	*0,88*	*0,07*	*0,12*	*0,8*	*0,8*	*0,76*	*0,8*	*0,72*	*0,5*	*1*			
C_{org}	*0,75*	*0,09*	*0,12*	*0,66*	*0,76*	*0,76*	*0,68*	*0,74*	*0,44*	*0,85*	*1*		
$CaCO_3$	*-0,51*	*-0,2*	*-0,22*	*-0,51*	*-0,35*	*-0,3*	*-0,32*	*-0,23*	*-0,25*	*-0,4*	*-0,22*	*1*	
sand	*-0,8*	*-0,07*	*-0,11*	*-0,7*	*-0,58*	*-0,55*	*-0,64*	*-0,49*	*-0,49*	*-0,83*	*-0,62*	*0,61*	*1*

Table 5 Sediment samples with EF > 1.5 .

Season/Location/Station	Hg	Cr	Ni	Pb	Cu	Zn
Aut19 NAV st1	2			1,6	1,7	1,7
Aut19 NAV st2	1,9				1,7	1,7
Aut19 TR st1		1,9				
Aut19 TR st3		2				
Aut20 NAV st1	1,8				1,9	1,8
Aut20 NAV st2	2,3			1,6	1,8	1,9
Aut20 TR st2		2,12	2,3			
Aut20 TR st3			1,8			
Aut20 GAM st2		22,7	21,3		1,8	
Spr21 NAV st1	2,7			1,7	2,5	2,1
Spr21 NAV st2	2,7			1,8	2,1	2,5
Spr21 TR st3			2,4			

Table 6 List of species composition of the macrobenthic communities in this study, through morphological (Morph) and molecular (COI and 18S) methodologies. Number of specimens (Morph) and reads (COI and 18S).

								Season	**Aut19**								
								Location	**TR**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Clitellata	Tubificida	Naididae	*Nais*	*Nais elinguis*	Müller, 1774	IV									
Animalia	Annelida	Polychaeta	Amphinomida	Amphinomidae				not assigned				5					
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae	*Eunice*	*Eunice oerstedii*	Stimpson, 1853	II							1		
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae				II				2					
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Aponuphis*	*Aponuphis brementi*	(Fauvel, 1916)	II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Diopatra* β	*Diopatra* sp.		II			7 β						8 β
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Onuphis*	*Onuphis farensis*	Gil & Machado, 2014	II				2					
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera alba*	(O.F. Müller, 1776)	IV		24			117			70	
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera* sp.		II				3					
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tesselata*	Grube, 1863	II	1								
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tridactyla*	Schmarda, 1861	II			10						16
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae				II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys cirrosa*	Ehlers, 1868	II				1			2		
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys* sp.		II				11					
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste diversicolor*	(O.F. Müller, 1776)	III					135			326	
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste* sp.		III							1		
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Perinereis*	*Perinereis* sp.		III									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae				not assigned		68			855			1146	
Animalia	Annelida	Polychaeta	Phyllodocida	Phyllodocidae	*Phyllodoce*	*Phyllodoce mucosa*	Örsted, 1843	III									

								Season	Aut19								
								Location	**TR**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta	Phyllodocida	Sigalionidae	*Sigalion*	*Sigalion mathildae*	Audouin & Milne Edwards, 1832	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Syllidae	*Myrianida*	*Myrianida pachycera**	(Augener, 1913)	II									
Animalia	Annelida	Polychaeta	Sabellida	Sabellariidae	*Sabellaria*	*Sabellaria spinulosa*	(Leuckart, 1849)	I									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Pseudopolydora*	*Pseudopolydora paucibranchiata**	(Okuda, 1937)	IV									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Scolelepis*	*Scolelepis neglecta**	Surugiu, 2016	III		1753							
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spio*	*Spio symphyta**	Meißner, Bick & Bastrop, 2011	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spiophanes*	*Spiophanes bombyx*	(Claparède, 1870)	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Streblospio*	*Streblospio shrubsolii*	(Buchanan, 1890)	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae				III				4					
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Caulleriella*	*Caulleriella* sp.		not assigned									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirratulus*	*Cirratulus* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia tentaculata*	(Montagu, 1808)	IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx acutus**	Webster & Benedict, 1887	III									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae	*Terebella*	*Terebella lapidaria*	Linnaeus, 1767	I									
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae				I									
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella capitata*	(Fabricius, 1780)	V	1						8		
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella* sp.		V							1		

								Season	Aut19								
								Location	**TR**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Capitellidae	*Dasybranchus*	*Dasybranchus caducus*	(Grube, 1846)	III									242
Animalia	Annelida	Polychaeta		Capitellidae	*Heteromastus*	*Heteromastus filiformis*	(Claparède, 1864)	IV									
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus latericeus*	Sars, 1851	III	6		198	11		4	16		84
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus profondus*	Eisig, 1887	III					187				
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus* sp.		not assigned	3								
Animalia	Annelida	Polychaeta		Capitellidae				V		90			1746		10	1121	
Animalia	Annelida	Polychaeta		Chaetopteridae	*Phyllochaetopterus*	*Phyllochaetopterus* sp.		I									45
Animalia	Annelida	Polychaeta		Chaetopteridae	*Spiochaetopterus*	*Spiochaetopterus costarum*	(Claparède, 1869)	I								233	
Animalia	Annelida	Polychaeta		Maldanidae	*Axiothella*	*Axiothella* sp.		I									
Animalia	Annelida	Polychaeta		Maldanidae	*Clymenella*	*Clymenella torquata*	(Leidy, 1855)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene oerstedii*	(Claparède, 1863)	I			18						128
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene* sp.		not assigned	7			4			8		
Animalia	Annelida	Polychaeta		Maldanidae	*Heteroclymene*	*Heteroclymene robusta*	Arwidsson, 1906	V									
Animalia	Annelida	Polychaeta		Maldanidae	*Johnstonia*	*Johnstonia clymenoides*	Quatrefages, 1866	not assigned							1		
Animalia	Annelida	Polychaeta		Maldanidae	*Leiochone*	*Leiochone leiopygos*	(Grube, 1860)	III		1910			9431			19287	
Animalia	Annelida	Polychaeta		Maldanidae	*Maldane*	*Maldane sarsi*	Malmgren, 1865	II									
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella affinis*	(M. Sars in G.O. Sars, 1872)	III	2						4		

								Season	Aut19								
								Location	TR								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella* sp.		III									4
Animalia	Annelida	Polychaeta		Maldanidae				I	7						10		
Animalia	Annelida	Polychaeta		Orbiniidae	*Orbinia*	*Orbinia* sp.		I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo foetida*	(Claparède, 1868)	I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo* sp.		I					687				
Animalia	Annelida	Polychaeta		Orbiniidae	*Scoloplos*	*Scoloplos armiger*	(Müller, 1776)	III	1						1		
Animalia	Annelida	Polychaeta		Oweniidae	*Galathowenia*	*Galathowenia oculata*	(Zachs, 1923)	II									
Animalia	Annelida	Polychaeta		Oweniidae	*Myriochele*	*Myriochele* sp.		III							1		
Animalia	Annelida	Polychaeta		Oweniidae	*Owenia*	*Owenia fusiformis*	Delle Chiaje, 1844	II									
Animalia	Annelida	Polychaeta		Oweniidae				not assigned							3		
Animalia	Arthropoda	Copepoda	Harpacticoida	Harpacticidae	*Harpacticus*	*Harpacticus* sp.		not assigned									
Animalia	Arthropoda	Copepoda	Harpacticoida	Thalestridae				not assigned									
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae	*Ampelisca*	*Ampelisca* sp.		I	1			1	17		2	180	
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae				I							18		
Animalia	Arthropoda	Malacostraca	Amphipoda	Atylidae	*Atylus*	*Atylus* sp.		I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Corophiidae	*Corophium*	*Corophium multisetosum*	Stock, 1952	III									
Animalia	Arthropoda	Malacostraca	Amphipoda	Dexaminidae	*Dexamine*	*Dexamine* sp.		III									
Animalia	Arthropoda	Malacostraca	Amphipoda	Oedicerotidae	*Perioculodes*	*Perioculodes* sp.		II							1		
Animalia	Arthropoda	Malacostraca	Amphipoda	Phoxocephalidae				I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe grimaldii*	Chevreux, 1895	I							1	92	

								Season	**Aut19**								
								Location	**TR**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Arthrop-oda	Malacos-traca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe* sp.		I				1					
Animalia	Arthrop-oda	Malacos-traca	Amphipoda					not assigned	1			1			4		
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Alpheidae	*Athanas*	*Athanas nitescens*	(Leach, 1814)	I									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Benthesicymidae	*Benthesicy-mus*	*Benthesicymus laciniatus*	Rathbun, 1906	not assigned									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Callianassidae	*Gilvossius*	*Gilvossius tyrrhenus*	(Petagna, 1792)	III	1								
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Carcinidae	*Carcinus*	*Carcinus maenas*	(Linnaeus, 1758)	III									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Clibanarius* β	*Clibanarius* sp.		II									11 β
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Diogenes*	*Diogenes armatus**	Almón, Cuesta, Schubart & García Raso in Almón, Cuesta, Schubart, Armenia & García Raso, 2021	II								1879	
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Diogenes*	*Diogenes costatus**	Henderson, 1893	II									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Grapsidae	*Planes*	*Planes* sp.		not assigned									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Processidae	*Processa*	*Processa parva*	Holthuis, 1951	I									
Animalia	Arthrop-oda	Malacos-traca	Isopoda	Anthuridae	*Cyathura*	*Cyathura carinata*	(Krøyer, 1847)	III				6			2		
Animalia	Arthrop-oda	Malacos-traca	Isopoda	Cirolanidae	*Cirolana*	*Cirolana cranchii*	Leach, 1818	II							2		
Animalia	Arthrop-oda	Malacos-traca	Tanaidacea	Apseudidae	*Apseudopsis*	*Apseudopsis latreillii*	(Milne Edwards, 1828)	III	34			4			29		
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Bugulidae	*Bugula*	*Bugula neritina**	(Linnaeus, 1758)	ignored									
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Candidae	*Tricellaria*	*Tricellaria inopinata**	d'Hondt & Occhipinti Ambrogi, 1985	ignored									
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Candidae	*Tricellaria*	*Tricellaria ternata**	(Ellis & Solander, 1786)	ignored									

								Season	Aut19								
								Location	TR								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Watersiporidae	*Watersipora*	*Watersipora subtorquata**	(d'Orbigny, 1852)	ignored									
Animalia	Bryozoa							II									
Animalia	Chordata	Ascidiacea	Aplousobran-chia	Clavelinidae	*Clavelina*	*Clavelina lepa-diformis*	(Müller, 1776)	not assigned									
Animalia	Chordata	Ascidiacea	Phlebobran-chia	Corellidae	*Corella*	*Corella eumyota**	Traustedt, 1882	III									
Animalia	Chordata	Ascidiacea	Stolidobran-chia	Pyuridae	*Microcos-mus*	*Microcosmus squamiger**	Michaelsen, 1927	II		7			3			21	
Animalia	Chordata	Ascidiacea	Stolidobran-chia	Styelidae	*Styela*	*Styela plicata**	(Lesueur, 1823)	II					357			426	
Animalia	Cnidaria	Anthozoa	Actiniaria		*Paraiptasia*	*Paraiptasia* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Alcyonacea	Coralliidae	*Corallium*	*Corallium* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Malacalcyo-nacea	Isididae				not assigned			21						
Animalia	Cnidaria	Anthozoa	Scleralcyo-nacea	Helioporidae	*Heliopora*	*Heliopora* sp.		not assigned			284						
Animalia	Cnidaria	Anthozoa	Scleralcyo-nacea	Veretillidae	*Veretillum*	*Veretillum cynomorium*	(Pallas, 1766)	I	1	29473						127	
Animalia	Cnidaria	Hydrozoa	Anthoathecata	Hydractiniidae α				I			6 α			3 α			114 α
Animalia	Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	*Obelia*	*Obelia dicho-toma**	(Linnaeus, 1758)	II									
Animalia	Cnidaria	Staurozoa	Stauromedusae	Haliclystidae	*Haliclystus*	*Haliclystus tenuis**	Kishinouye, 1910	not assigned								169	
Animalia	Echino-dermata	Holothu-roidea	Apodida	Synaptidae	*Lep-tosynapta*	*Leptosynapta inhaerens*	(O.F. Müller, 1776)	I				1					
Animalia	Mollusca	Bivalvia	Adapedonta	Solenidae	*Solen*	*Solen margina-tus*	Pulteney, 1799	I									
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Cerasto-derma*	*Cerastoderma edule*	(Linnaeus, 1758)	III				2	1364	358	1		5
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Parvicar-dium*	*Parvicardium exiguum*	(Gmelin, 1791)	I	1			1					

								Season	Aut19								
								Location	**TR**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae	*Gari*	*Gari tellinella*	(Lamarck, 1818)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae α				I									
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra alba*	(W. Wood, 1802)	III									
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra nitida*	(O. F. Müller, 1776)	III							1		
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Scrobicularia*	*Scrobicularia plana*	(da Costa, 1778)	III									
Animalia	Mollusca	Bivalvia	Cardiida	Solecurtidae α				not assigned									
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Asbjornsenia*	*Asbjornsenia pygmaea*	(Lovén, 1846)	I				4			1		
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Macomangulus*	*Macomangulus tenuis*	(da Costa, 1778)	I				5			1		
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae α				I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Loripes*	*Loripes orbiculatus*	Poli, 1795	I				4					
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinella*	*Lucinella divaricata*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinoma*	*Lucinoma borealis*	(Linnaeus, 1767)	I									
Animalia	Mollusca	Bivalvia	Myida	Corbulidae	*Varicorbula*	*Varicorbula gibba*	(Olivi, 1792)	IV									
Animalia	Mollusca	Bivalvia	Nuculida	Nuculidae	*Nucula*	*Nucula hanleyi*	Winckworth, 1931	I	2								
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Lutraria*	*Lutraria Lutraria*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula solida*	(Linnaeus, 1758)	I	2						1		
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula subtruncata*	(da Costa, 1778)	I				2					
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Petricola*	*Petricola lapicida*	(Gmelin, 1791)	not assigned									
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Ruditapes*	*Ruditapes philippinarum**	(A. Adams & Reeve, 1850)	III								683	

								Season	Aut19								
								Location	**TR**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Venerida	Veneridae α				I									
Animalia	Mollusca	Bivalvia		Ostreidae	*Magallana*	*Magallana gigas**	(Thunberg, 1793)	III									
Animalia	Mollusca	Cephalopoda	Sepiida	Sepiidae	*Acanthosepion*	*Acanthosepion pharaonis**	(Ehrenberg, 1831)	ignored									
Animalia	Mollusca	Gastropoda	Cephalaspidea	Philinidae	*Philine*	*Philine* sp.		II									
Animalia	Mollusca	Gastropoda	LittoriniMa	Hydrobiidae	*Peringia*	*Peringia ulvae*	(Pennant, 1777)	III									
Animalia	Mollusca	Gastropoda	LittoriniMa	Rissoidae	*Rissoa*	*Rissoa parva*	(da Costa, 1778)	I									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Nassarius*	*Nassarius argenteus**	(Marrat, 1877)	not assigned									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Tritia*	*Tritia reticulata*	(Linnaeus, 1758)	II				4	28		2	93	
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae α				II			11 α						7 α
Animalia	Mollusca	Gastropoda	Neogastropoda	Raphitomidae	*Leufroyia*	*Leufroyia leufroyi*	(Michaud, 1828)	not assigned							3		
Animalia	Mollusca	Gastropoda	Nudibranchia	Goniodorididae	*Ancula*	*Ancula gibbosa*	(Risso, 1818)	I									
Animalia	Mollusca	Gastropoda	Trochida	Calliostomatidae	*Calliostoma*	*Calliostoma zizyphinum*	(Linnaeus, 1758)	I				1					
Animalia	Mollusca	Gastropoda	Trochida	Trochidae	*Jujubinus*	*Jujubinus striatus*	(Linnaeus, 1758)	I				4			4		
Animalia	Mollusca	Gastropoda	Trochida	Trochidae				not assigned				1					
Animalia	Mollusca	Gastropoda		Limapontiidae	*Limapontia*	*Limapontia depressa**	Alder & Hancock, 1862	I									
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella depressa*	Pennant, 1777	I					343				
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella pellucida*	Linnaeus, 1758	I									
Animalia	Mollusca	Polyplacophora	Callochitonida	Callochitonidae	*Callochiton*	*Callochiton septemvalvis*	(Montagu, 1803)	I							1		
Animalia	Nemertea	Hoplonemertea	Monostilifera	Tetrastemmatidae	*Tetrastemma* β	*Tetrastemma* sp.		III									
Animalia	Porifera	Demospongiae	Verongiida					not assigned									

								Season	Aut19								
								Location	GAM								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Clitellata	Tubificida	Naididae	*Nais*	*Nais elinguis*	Müller, 1774	IV									
Animalia	Annelida	Polychaeta	Amphinomida	Amphinomidae				not assigned									
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae	*Eunice*	*Eunice oerstedii*	Stimpson, 1853	II									
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae				II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Aponuphis*	*Aponuphis bre-menti*	(Fauvel, 1916)	II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Diopatra* β	*Diopatra* sp.		II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Onuphis*	*Onuphis farensis*	Gil & Machado, 2014	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera alba*	(O.F. Müller, 1776)	IV									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera* sp.		II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tesselata*	Grube, 1863	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tridactyla*	Schmarda, 1861	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae				II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys cirrosa*	Ehlers, 1868	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys* sp.		II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste diversicolor*	(O.F. Müller, 1776)	III	12	4273	136	9	10932	192	29	64	887
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste* sp.		III									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Perinereis*	*Perinereis* sp.		III									4
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae				not assigned		807							
Animalia	Annelida	Polychaeta	Phyllodocida	Phyllodocidae	*Phyllodoce*	*Phyllodoce mucosa*	Örsted, 1843	III									

								Season	Aut19								
								Location	**GAM**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta	Phyllodocida	Sigalionidae	*Sigalion*	*Sigalion mathildae*	Audouin & Milne Edwards, 1832	II	4								
Animalia	Annelida	Polychaeta	Phyllodocida	Syllidae	*Myrianida*	*Myrianida pachycera**	(Augener, 1913)	II									
Animalia	Annelida	Polychaeta	Sabellida	Sabellariidae	*Sabellaria*	*Sabellaria spinulosa*	(Leuckart, 1849)	I									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Pseudopolydora*	*Pseudopolydora paucibranchiata**	(Okuda, 1937)	IV									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Scolelepis*	*Scolelepis neglecta**	Surugiu, 2016	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spio*	*Spio symphyta**	Meißner, Bick & Bastrop, 2011	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spiophanes*	*Spiophanes bombyx*	(Claparède, 1870)	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Streblospio*	*Streblospio shrubsolii*	(Buchanan, 1890)	III								312	
Animalia	Annelida	Polychaeta	Spionida	Spionidae				III									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Caulleriella*	*Caulleriella* sp.		not assigned	1								
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirratulus*	*Cirratulus* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia* sp.		IV				3					
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia tentaculata*	(Montagu, 1808)	IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx acutus**	Webster & Benedict, 1887	III		5			6621				
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx* sp.		IV						4			
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae	*Terebella*	*Terebella lapidaria*	Linnaeus, 1767	I									
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae				I									
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella capitata*	(Fabricius, 1780)	V									
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella* sp.		V									

								Season	Aut19								
								Location	GAM								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Capitellidae	*Dasybranchus*	*Dasybranchus caducus*	(Grube, 1846)	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Heteromastus*	*Heteromastus filiformis*	(Claparède, 1864)	IV									
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus latericeus*	Sars, 1851	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus profondus*	Eisig, 1887	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus* sp.		not assigned									
Animalia	Annelida	Polychaeta		Capitellidae				V		1415							
Animalia	Annelida	Polychaeta		Chaetopteridae	*Phyllochaetopterus*	*Phyllochaetopterus* sp.		I									
Animalia	Annelida	Polychaeta		Chaetopteridae	*Spiochaetopterus*	*Spiochaetopterus costarum*	(Claparède, 1869)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Axiothella*	*Axiothella* sp.		I									
Animalia	Annelida	Polychaeta		Maldanidae	*Clymenella*	*Clymenella torquata*	(Leidy, 1855)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene oerstedii*	(Claparède, 1863)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene* sp.		not assigned									
Animalia	Annelida	Polychaeta		Maldanidae	*Heteroclymene*	*Heteroclymene robusta*	Arwidsson, 1906	V									
Animalia	Annelida	Polychaeta		Maldanidae	*Johnstonia*	*Johnstonia clymenoides*	Quatrefages, 1866	not assigned									
Animalia	Annelida	Polychaeta		Maldanidae	*Leiochone*	*Leiochone leiopygos*	(Grube, 1860)	III									
Animalia	Annelida	Polychaeta		Maldanidae	*Maldane*	*Maldane sarsi*	Malmgren, 1865	II									
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella affinis*	(M. Sars in G.O. Sars, 1872)	III									

								Season	Aut19								
								Location	GAM								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella* sp.		III									
Animalia	Annelida	Polychaeta		Maldanidae				I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Orbinia*	*Orbinia* sp.		I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo foetida*	(Claparède, 1868)	I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo* sp.		I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Scoloplos*	*Scoloplos armiger*	(Müller, 1776)	III									
Animalia	Annelida	Polychaeta		Oweniidae	*Galathowenia*	*Galathowenia oculata*	(Zachs, 1923)	II									
Animalia	Annelida	Polychaeta		Oweniidae	*Myriochele*	*Myriochele* sp.		III									
Animalia	Annelida	Polychaeta		Oweniidae	*Owenia*	*Owenia fusiformis*	Delle Chiaje, 1844	II									
Animalia	Annelida	Polychaeta		Oweniidae				not assigned									
Animalia	Arthropoda	Copepoda	Harpacticoida	Harpacticidae	*Harpacticus*	*Harpacticus* sp.		not assigned									11
Animalia	Arthropoda	Copepoda	Harpacticoida	Thalestridae				not assigned									
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae	*Ampelisca*	*Ampelisca* sp.		I	2								
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae				I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Atylidae	*Atylus*	*Atylus* sp.		I	1								
Animalia	Arthropoda	Malacostraca	Amphipoda	Corophiidae	*Corophium*	*Corophium multisetosum*	Stock, 1952	III		1126							
Animalia	Arthropoda	Malacostraca	Amphipoda	Dexaminidae	*Dexamine*	*Dexamine* sp.		III									
Animalia	Arthropoda	Malacostraca	Amphipoda	Oedicerotidae	*Perioculodes*	*Perioculodes* sp.		II									
Animalia	Arthropoda	Malacostraca	Amphipoda	Phoxocephalidae				I	1								
Animalia	Arthropoda	Malacostraca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe grimaldii*	Chevreux, 1895	I									

								Season	Aut19								
								Location	**GAM**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Arthrop-oda	Malacos-traca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe* sp.		I									
Animalia	Arthrop-oda	Malacos-traca	Amphipoda					not assigned	1								
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Alpheidae	*Athanas*	*Athanas nitescens*	(Leach, 1814)	I									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Benthesicymidae	*Benthesicymus*	*Benthesicymus laciniatus*	Rathbun, 1906	not assigned									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Callianassidae	*Gilvossius*	*Gilvossius tyrrhenus*	(Petagna, 1792)	III									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Carcinidae	*Carcinus*	*Carcinus maenas*	(Linnaeus, 1758)	III									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Clibanarius* β	*Clibanarius* sp.		II									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Diogenes*	*Diogenes armatus**	Almón, Cuesta, Schubart & García Raso in Almón, Cuesta, Schubart, Armenia & García Raso, 2021	II									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Diogenes*	*Diogenes costatus**	Henderson, 1893	II									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Grapsidae	*Planes*	*Planes* sp.		not assigned									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Processidae	*Processa*	*Processa parva*	Holthuis, 1951	I	1								
Animalia	Arthrop-oda	Malacos-traca	Isopoda	Anthuridae	*Cyathura*	*Cyathura carinata*	(Krøyer, 1847)	III	6			16			14		
Animalia	Arthrop-oda	Malacos-traca	Isopoda	Cirolanidae	*Cirolana*	*Cirolana cranchii*	Leach, 1818	II									
Animalia	Arthrop-oda	Malacos-traca	Tanaidacea	Apseudidae	*Apseudopsis*	*Apseudopsis latreillii*	(Milne Edwards, 1828)	III	19								
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Bugulidae	*Bugula*	*Bugula neritina**	(Linnaeus, 1758)	ignored									
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Candidae	*Tricellaria*	*Tricellaria inopinata**	d'Hondt & Occhipinti Ambrogi, 1985	ignored		43							
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Candidae	*Tricellaria*	*Tricellaria ternata**	(Ellis & Solander, 1786)	ignored									

								Season	Aut19								
								Location	**GAM**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Bryozoa	Gymnolaemata	Cheilostomatida	Watersiporidae	*Watersipora*	*Watersipora subtorquata**	(d'Orbigny, 1852)	ignored									
Animalia	Bryozoa							II									
Animalia	Chordata	Ascidiacea	Aplousobranchia	Clavelinidae	*Clavelina*	*Clavelina lepadiformis*	(Müller, 1776)	not assigned									
Animalia	Chordata	Ascidiacea	Phlebobranchia	Corellidae	*Corella*	*Corella eumyota**	Traustedt, 1882	III									
Animalia	Chordata	Ascidiacea	Stolidobranchia	Pyuridae	*Microcosmus*	*Microcosmus squamiger**	Michaelsen, 1927	II									
Animalia	Chordata	Ascidiacea	Stolidobranchia	Styelidae	*Styela*	*Styela plicata**	(Lesueur, 1823)	II		132						76	
Animalia	Cnidaria	Anthozoa	Actiniaria		*Paraiptasia*	*Paraiptasia* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Alcyonacea	Coralliidae	*Corallium*	*Corallium* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Malacalcyonacea	Isididae				not assigned									
Animalia	Cnidaria	Anthozoa	Scleralcyonacea	Helioporidae	*Heliopora*	*Heliopora* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Scleralcyonacea	Veretillidae	*Veretillum*	*Veretillum cynomorium*	(Pallas, 1766)	I		17							
Animalia	Cnidaria	Hydrozoa	Anthoathecata	Hydractiniidae α				I									
Animalia	Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	*Obelia*	*Obelia dichotoma**	(Linnaeus, 1758)	II								177	
Animalia	Cnidaria	Staurozoa	Stauromedusae	Haliclystidae	*Haliclystus*	*Haliclystus tenuis**	Kishinouye, 1910	not assigned									
Animalia	Echinodermata	Holothuroidea	Apodida	Synaptidae	*Leptosynapta*	*Leptosynapta inhaerens*	(O.F. Müller, 1776)	I									
Animalia	Mollusca	Bivalvia	Adapedonta	Solenidae	*Solen*	*Solen marginatus*	Pulteney, 1799	I									
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Cerastoderma*	*Cerastoderma edule*	(Linnaeus, 1758)	III				1	183	263			6
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Parvicardium*	*Parvicardium exiguum*	(Gmelin, 1791)	I									

								Season	Aut19								
								Location	GAM								
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Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae	*Gari*	*Gari tellinella*	(Lamarck, 1818)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae α				I									4 α
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra alba*	(W. Wood, 1802)	III							1		
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra nitida*	(O. F. Müller, 1776)	III			5						4
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Scrobicularia*	*Scrobicularia plana*	(da Costa, 1778)	III	1	46							
Animalia	Mollusca	Bivalvia	Cardiida	Solecurtidae α				not assigned			250 α			59 α			237 α
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Asbjornsenia*	*Asbjornsenia pygmaea*	(Lovén, 1846)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Macomangulus*	*Macomangulus tenuis*	(da Costa, 1778)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae α				I									6 α
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Loripes*	*Loripes orbiculatus*	Poli, 1795	I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinella*	*Lucinella divaricata*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinoma*	*Lucinoma borealis*	(Linnaeus, 1767)	I									
Animalia	Mollusca	Bivalvia	Myida	Corbulidae	*Varicorbula*	*Varicorbula gibba*	(Olivi, 1792)	IV									
Animalia	Mollusca	Bivalvia	Nuculida	Nuculidae	*Nucula*	*Nucula hanleyi*	Winckworth, 1931	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Lutraria*	*Lutraria Lutraria*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula solida*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula subtruncata*	(da Costa, 1778)	I									
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Petricola*	*Petricola lapicida*	(Gmelin, 1791)	not assigned									
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Ruditapes*	*Ruditapes philippinarum**	(A. Adams & Reeve, 1850)	III								18993	

								Season	Aut19								
								Location	GAM								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Venerida	Veneridae α				I									47 α
Animalia	Mollusca	Bivalvia		Ostreidae	*Magallana*	*Magallana gigas**	(Thunberg, 1793)	III									
Animalia	Mollusca	Cephalopoda	Sepiida	Sepiidae	*Acanthosepion*	*Acanthosepion pharaonis**	(Ehrenberg, 1831)	ignored									
Animalia	Mollusca	Gastropoda	Cephalaspidea	Philinidae	*Philine*	*Philine* sp.		II									
Animalia	Mollusca	Gastropoda	LittoriniMa	Hydrobiidae	*Peringia*	*Peringia ulvae*	(Pennant, 1777)	III	10	3429	27	3			2	1030	14
Animalia	Mollusca	Gastropoda	LittoriniMa	Rissoidae	*Rissoa*	*Rissoa parva*	(da Costa, 1778)	I									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Nassarius*	*Nassarius argenteus**	(Marrat, 1877)	not assigned									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Tritia*	*Tritia reticulata*	(Linnaeus, 1758)	II									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae α				II									
Animalia	Mollusca	Gastropoda	Neogastropoda	Raphitomidae	*Leufroyia*	*Leufroyia leufroyi*	(Michaud, 1828)	not assigned									
Animalia	Mollusca	Gastropoda	Nudibranchia	Goniodorididae	*Ancula*	*Ancula gibbosa*	(Risso, 1818)	I									
Animalia	Mollusca	Gastropoda	Trochida	Calliostomatidae	*Calliostoma*	*Calliostoma zizyphinum*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Gastropoda	Trochida	Trochidae	*Jujubinus*	*Jujubinus striatus*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Gastropoda	Trochida	Trochidae				not assigned									
Animalia	Mollusca	Gastropoda		Limapontiidae	*Limapontia*	*Limapontia depressa**	Alder & Hancock, 1862	I									
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella depressa*	Pennant, 1777	I		221			12				
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella pellucida*	Linnaeus, 1758	I									
Animalia	Mollusca	Polyplacophora	Callochitonida	Callochitonidae	*Callochiton*	*Callochiton septemvalvis*	(Montagu, 1803)	I									
Animalia	Nemertea	Hoplonemertea	Monostilifera	Tetrastemmatidae	*Tetrastemma* β	*Tetrastemma* sp.		III			24 β						
Animalia	Porifera	Demospongiae	Verongiida					not assigned		26							

								Season	Aut19								
								Location	NAV								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Clitellata	Tubificida	Naididae	*Nais*	*Nais elinguis*	Müller, 1774	IV		93							
Animalia	Annelida	Polychaeta	Amphinomida	Amphinomidae				not assigned									
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae	*Eunice*	*Eunice oerstedii*	Stimpson, 1853	II									
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae				II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Aponuphis*	*Aponuphis brementi*	(Fauvel, 1916)	II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Diopatra* β	*Diopatra* sp.		II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Onuphis*	*Onuphis farensis*	Gil & Machado, 2014	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera alba*	(O.F. Müller, 1776)	IV									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera* sp.		II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tesselata*	Grube, 1863	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tridactyla*	Schmarda, 1861	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae				II				1					
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys cirrosa*	Ehlers, 1868	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys* sp.		II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste diversicolor*	(O.F. Müller, 1776)	III	12	1	121		149	81	3	121	152
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste* sp.		III									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Perinereis*	*Perinereis* sp.		III			4						
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae				not assigned									
Animalia	Annelida	Polychaeta	Phyllodocida	Phyllodocidae	*Phyllodoce*	*Phyllodoce mucosa*	Örsted, 1843	III									

								Season	Aut19								
								Location	NAV								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta	Phyllodocida	Sigalionidae	*Sigalion*	*Sigalion mathildae*	Audouin & Milne Edwards, 1832	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Syllidae	*Myrianida*	*Myrianida pachycera**	(Augener, 1913)	II									
Animalia	Annelida	Polychaeta	Sabellida	Sabellariidae	*Sabellaria*	*Sabellaria spinulosa*	(Leuckart, 1849)	I		354							
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Pseudopolydora*	*Pseudopolydora paucibranchiata**	(Okuda, 1937)	IV									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Scolelepis*	*Scolelepis neglecta**	Surugiu, 2016	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spio*	*Spio symphyta**	Meißner, Bick & Bastrop, 2011	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spiophanes*	*Spiophanes bombyx*	(Claparède, 1870)	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Streblospio*	*Streblospio shrubsolii*	(Buchanan, 1890)	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae				III									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Caulleriella*	*Caulleriella* sp.		not assigned									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirratulus*	*Cirratulus* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia tentaculata*	(Montagu, 1808)	IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx acutus**	Webster & Benedict, 1887	III									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae	*Terebella*	*Terebella lapidaria*	Linnaeus, 1767	I									
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae				I	1			1					
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella capitata*	(Fabricius, 1780)	V									
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella* sp.		V					224			189	

								Season	Aut19								
								Location	**NAV**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Capitellidae	*Dasybranchus*	*Dasybranchus caducus*	(Grube, 1846)	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Heteromastus*	*Heteromastus filiformis*	(Claparède, 1864)	IV						8			
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus latericeus*	Sars, 1851	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus profondus*	Eisig, 1887	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus* sp.		not assigned									
Animalia	Annelida	Polychaeta		Capitellidae				V		9			251				
Animalia	Annelida	Polychaeta		Chaetopteridae	*Phyllochaetopterus*	*Phyllochaetopterus* sp.		I									
Animalia	Annelida	Polychaeta		Chaetopteridae	*Spiochaetopterus*	*Spiochaetopterus costarum*	(Claparède, 1869)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Axiothella*	*Axiothella* sp.		I									
Animalia	Annelida	Polychaeta		Maldanidae	*Clymenella*	*Clymenella torquata*	(Leidy, 1855)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene oerstedii*	(Claparède, 1863)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene* sp.		not assigned									
Animalia	Annelida	Polychaeta		Maldanidae	*Heteroclymene*	*Heteroclymene robusta*	Arwidsson, 1906	V									
Animalia	Annelida	Polychaeta		Maldanidae	*Johnstonia*	*Johnstonia clymenoides*	Quatrefages, 1866	not assigned									
Animalia	Annelida	Polychaeta		Maldanidae	*Leiochone*	*Leiochone leiopygos*	(Grube, 1860)	III		3							
Animalia	Annelida	Polychaeta		Maldanidae	*Maldane*	*Maldane sarsi*	Malmgren, 1865	II									
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella affinis*	(M. Sars in G.O. Sars, 1872)	III									

								Season	Aut19								
								Location	NAV								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella* sp.		III									
Animalia	Annelida	Polychaeta		Maldanidae				I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Orbinia*	*Orbinia* sp.		I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo foetida*	(Claparède, 1868)	I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo* sp.		I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Scoloplos*	*Scoloplos armiger*	(Müller, 1776)	III									
Animalia	Annelida	Polychaeta		Oweniidae	*Galathowenia*	*Galathowenia oculata*	(Zachs, 1923)	II									
Animalia	Annelida	Polychaeta		Oweniidae	*Myriochele*	*Myriochele* sp.		III									
Animalia	Annelida	Polychaeta		Oweniidae	*Owenia*	*Owenia fusiformis*	Delle Chiaje, 1844	II									
Animalia	Annelida	Polychaeta		Oweniidae				not assigned									
Animalia	Arthropoda	Copepoda	Harpacticoida	Harpacticidae	*Harpacticus*	*Harpacticus* sp.		not assigned									17
Animalia	Arthropoda	Copepoda	Harpacticoida	Thalestridae				not assigned			4						
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae	*Ampelisca*	*Ampelisca* sp.		I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae				I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Atylidae	*Atylus*	*Atylus* sp.		I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Corophiidae	*Corophium*	*Corophium multisetosum*	Stock, 1952	III									
Animalia	Arthropoda	Malacostraca	Amphipoda	Dexaminidae	*Dexamine*	*Dexamine* sp.		III									
Animalia	Arthropoda	Malacostraca	Amphipoda	Oedicerotidae	*Perioculodes*	*Perioculodes* sp.		II									
Animalia	Arthropoda	Malacostraca	Amphipoda	Phoxocephalidae				I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe grimaldii*	Chevreux, 1895	I									

								Season	Aut19								
								Location	NAV								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Arthropoda	Malacostraca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe* sp.		I									
Animalia	Arthropoda	Malacostraca	Amphipoda					not assigned									
Animalia	Arthropoda	Malacostraca	Decapoda	Alpheidae	*Athanas*	*Athanas nitescens*	(Leach, 1814)	I									
Animalia	Arthropoda	Malacostraca	Decapoda	Benthesicymidae	*Benthesicymus*	*Benthesicymus laciniatus*	Rathbun, 1906	not assigned									
Animalia	Arthropoda	Malacostraca	Decapoda	Callianassidae	*Gilvossius*	*Gilvossius tyrrhenus*	(Petagna, 1792)	III									
Animalia	Arthropoda	Malacostraca	Decapoda	Carcinidae	*Carcinus*	*Carcinus maenas*	(Linnaeus, 1758)	III									
Animalia	Arthropoda	Malacostraca	Decapoda	Diogenidae	*Clibanarius* β	*Clibanarius* sp.		II									
Animalia	Arthropoda	Malacostraca	Decapoda	Diogenidae	*Diogenes*	*Diogenes armatus**	Almón, Cuesta, Schubart & García Raso in Almón, Cuesta, Schubart, Armenia & García Raso, 2021	II									
Animalia	Arthropoda	Malacostraca	Decapoda	Diogenidae	*Diogenes*	*Diogenes costatus**	Henderson, 1893	II									
Animalia	Arthropoda	Malacostraca	Decapoda	Grapsidae	*Planes*	*Planes* sp.		not assigned									
Animalia	Arthropoda	Malacostraca	Decapoda	Processidae	*Processa*	*Processa parva*	Holthuis, 1951	I									
Animalia	Arthropoda	Malacostraca	Isopoda	Anthuridae	*Cyathura*	*Cyathura carinata*	(Krøyer, 1847)	III	14			7			2		
Animalia	Arthropoda	Malacostraca	Isopoda	Cirolanidae	*Cirolana*	*Cirolana cranchii*	Leach, 1818	II									
Animalia	Arthropoda	Malacostraca	Tanaidacea	Apseudidae	*Apseudopsis*	*Apseudopsis latreillii*	(Milne Edwards, 1828)	III									
Animalia	Bryozoa	Gymnolaemata	Cheilostomatida	Bugulidae	*Bugula*	*Bugula neritina**	(Linnaeus, 1758)	ignored									
Animalia	Bryozoa	Gymnolaemata	Cheilostomatida	Candidae	*Tricellaria*	*Tricellaria inopinata**	d'Hondt & Occhipinti Ambrogi, 1985	ignored									
Animalia	Bryozoa	Gymnolaemata	Cheilostomatida	Candidae	*Tricellaria*	*Tricellaria ternata**	(Ellis & Solander, 1786)	ignored									

								Season	Aut19								
								Location	**NAV**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Watersiporidae	*Watersipora*	*Watersipora subtorquata**	(d'Orbigny, 1852)	ignored									
Animalia	Bryozoa							II									
Animalia	Chordata	Ascidiacea	Aplousobran-chia	Clavelinidae	*Clavelina*	*Clavelina lepa-diformis*	(Müller, 1776)	not assigned						4			
Animalia	Chordata	Ascidiacea	Phlebobran-chia	Corellidae	*Corella*	*Corella eumyota**	Traustedt, 1882	III									
Animalia	Chordata	Ascidiacea	Stolidobran-chia	Pyuridae	*Microcos-mus*	*Microcosmus squamiger**	Michaelsen, 1927	II		136							
Animalia	Chordata	Ascidiacea	Stolidobran-chia	Styelidae	*Styela*	*Styela plicata**	(Lesueur, 1823)	II									
Animalia	Cnidaria	Anthozoa	Actiniaria		*Paraiptasia*	*Paraiptasia* sp.		not assigned						6			
Animalia	Cnidaria	Anthozoa	Alcyonacea	Coralliidae	*Corallium*	*Corallium* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Malacalcyo-nacea	Isididae				not assigned									
Animalia	Cnidaria	Anthozoa	Scleralcyo-nacea	Helioporidae	*Heliopora*	*Heliopora* sp.		not assigned			7			47			
Animalia	Cnidaria	Anthozoa	Scleralcyo-nacea	Veretillidae	*Veretillum*	*Veretillum cynomorium*	(Pallas, 1766)	I									
Animalia	Cnidaria	Hydrozoa	Anthoathecata	Hydractiniidae α				I									
Animalia	Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	*Obelia*	*Obelia dicho-toma**	(Linnaeus, 1758)	II									
Animalia	Cnidaria	Staurozoa	Stauromedusae	Haliclystidae	*Haliclystus*	*Haliclystus tenuis**	Kishinouye, 1910	not assigned									
Animalia	Echino-dermata	Holothu-roidea	Apodida	Synaptidae	*Lep-tosynapta*	*Leptosynapta inhaerens*	(O.F. Müller, 1776)	I									
Animalia	Mollusca	Bivalvia	Adapedonta	Solenidae	*Solen*	*Solen margina-tus*	Pulteney, 1799	I									
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Cerasto-derma*	*Cerastoderma edule*	(Linnaeus, 1758)	III			8			10			21
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Parvicar-dium*	*Parvicardium exiguum*	(Gmelin, 1791)	I									

								Season	Aut19								
								Location	NAV								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae	*Gari*	*Gari tellinella*	(Lamarck, 1818)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae α				I									
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra alba*	(W. Wood, 1802)	III	46			62			145		
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra nitida*	(O. F. Müller, 1776)	III									
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Scrobicularia*	*Scrobicularia plana*	(da Costa, 1778)	III									
Animalia	Mollusca	Bivalvia	Cardiida	Solecurtidae α				not assigned			129 α			50 α			119 α
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Asbjornsenia*	*Asbjornsenia pygmaea*	(Lovén, 1846)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Macomangulus*	*Macomangulus tenuis*	(da Costa, 1778)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae α				I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Loripes*	*Loripes orbiculatus*	Poli, 1795	I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinella*	*Lucinella divaricata*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinoma*	*Lucinoma borealis*	(Linnaeus, 1767)	I									
Animalia	Mollusca	Bivalvia	Myida	Corbulidae	*Varicorbula*	*Varicorbula gibba*	(Olivi, 1792)	IV									
Animalia	Mollusca	Bivalvia	Nuculida	Nuculidae	*Nucula*	*Nucula hanleyi*	Winckworth, 1931	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Lutraria*	*Lutraria Lutraria*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula solida*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula subtruncata*	(da Costa, 1778)	I									
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Petricola*	*Petricola lapicida*	(Gmelin, 1791)	not assigned			9						
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Ruditapes*	*Ruditapes philippinarum**	(A. Adams & Reeve, 1850)	III					915				

								Season	Aut19								
								Location	**NAV**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Venerida	Veneridae α				I									
Animalia	Mollusca	Bivalvia		Ostreidae	*Magallana*	*Magallana gigas**	(Thunberg, 1793)	III						15			
Animalia	Mollusca	Cephalopoda	Sepiida	Sepiidae	*Acanthosepion*	*Acanthosepion pharaonis**	(Ehrenberg, 1831)	ignored									
Animalia	Mollusca	Gastropoda	Cephalaspidea	Philinidae	*Philine*	*Philine* sp.		II									
Animalia	Mollusca	Gastropoda	LittoriniMa	Hydrobiidae	*Peringia*	*Peringia ulvae*	(Pennant, 1777)	III	34			21	883		36	7336	7
Animalia	Mollusca	Gastropoda	LittoriniMa	Rissoidae	*Rissoa*	*Rissoa parva*	(da Costa, 1778)	I					689				
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Nassarius*	*Nassarius argenteus**	(Marrat, 1877)	not assigned									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Tritia*	*Tritia reticulata*	(Linnaeus, 1758)	II									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae α				II									
Animalia	Mollusca	Gastropoda	Neogastropoda	Raphitomidae	*Leufroyia*	*Leufroyia leufroyi*	(Michaud, 1828)	not assigned									
Animalia	Mollusca	Gastropoda	Nudibranchia	Goniodorididae	*Ancula*	*Ancula gibbosa*	(Risso, 1818)	I									
Animalia	Mollusca	Gastropoda	Trochida	Calliostomatidae	*Calliostoma*	*Calliostoma zizyphinum*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Gastropoda	Trochida	Trochidae	*Jujubinus*	*Jujubinus striatus*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Gastropoda	Trochida	Trochidae				not assigned									
Animalia	Mollusca	Gastropoda		Limapontiidae	*Limapontia*	*Limapontia depressa**	Alder & Hancock, 1862	I									
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella depressa*	Pennant, 1777	I									
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella pellucida*	Linnaeus, 1758	I								38	
Animalia	Mollusca	Polyplacophora	Callochitonida	Callochitonidae	*Callochiton*	*Callochiton septemvalvis*	(Montagu, 1803)	I									
Animalia	Nemertea	Hoplonemertea	Monostilifera	Tetrastemmatidae	*Tetrastemma* β	*Tetrastemma* sp.		III									
Animalia	Porifera	Demospongiae	Verongiida					not assigned									

								Season	Spr20								
								Location	TR								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Clitellata	Tubificida	Naididae	*Nais*	*Nais elinguis*	Müller, 1774	IV									
Animalia	Annelida	Polychaeta	Amphinomida	Amphinomidae				not assigned									
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae	*Eunice*	*Eunice oerstedii*	Stimpson, 1853	II									
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae				II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Aponuphis*	*Aponuphis bre-menti*	(Fauvel, 1916)	II	1						4		
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Diopatra* β	*Diopatra* sp.		II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Onuphis*	*Onuphis farensis*	Gil & Machado, 2014	II							2		
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera alba*	(O.F. Müller, 1776)	IV	1			1					
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera* sp.		II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tesselata*	Grube, 1863	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tridactyla*	Schmarda, 1861	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae				II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys cirrosa*	Ehlers, 1868	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys* sp.		II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste diversicolor*	(O.F. Müller, 1776)	III								101	
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste* sp.		III									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Perinereis*	*Perinereis* sp.		III									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae				not assigned									
Animalia	Annelida	Polychaeta	Phyllodocida	Phyllodocidae	*Phyllodoce*	*Phyllodoce mucosa*	Örsted, 1843	III		2	1						

								Season	Spr20								
								Location	**TR**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta	Phyllodocida	Sigalionidae	*Sigalion*	*Sigalion mathildae*	Audouin & Milne Edwards, 1832	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Syllidae	*Myrianida*	*Myrianida pachycera**	(Augener, 1913)	II									
Animalia	Annelida	Polychaeta	Sabellida	Sabellariidae	*Sabellaria*	*Sabellaria spinulosa*	(Leuckart, 1849)	I									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Pseudopolydora*	*Pseudopolydora paucibranchiata**	(Okuda, 1937)	IV								3	
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Scolelepis*	*Scolelepis neglecta**	Surugiu, 2016	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spio*	*Spio symphyta**	Meißner, Bick & Bastrop, 2011	III								330	
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spiophanes*	*Spiophanes bombyx*	(Claparède, 1870)	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Streblospio*	*Streblospio shrubsolii*	(Buchanan, 1890)	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae				III							3		
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Caulleriella*	*Caulleriella* sp.		not assigned									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirratulus*	*Cirratulus* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia tentaculata*	(Montagu, 1808)	IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx acutus**	Webster & Benedict, 1887	III									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae	*Terebella*	*Terebella lapidaria*	Linnaeus, 1767	I									
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae				I									
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella capitata*	(Fabricius, 1780)	V									
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella* sp.		V									

								Season	Spr20								
								Location	**TR**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Capitellidae	*Dasybranchus*	*Dasybranchus caducus*	(Grube, 1846)	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Heteromastus*	*Heteromastus filiformis*	(Claparède, 1864)	IV						4			
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus latericeus*	Sars, 1851	III	17		327	26		99	16		241
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus profondus*	Eisig, 1887	III		187			82			755	
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus* sp.		not assigned				5			4		
Animalia	Annelida	Polychaeta		Capitellidae				V		4328			175			121	
Animalia	Annelida	Polychaeta		Chaetopteridae	*Phyllochaetopterus*	*Phyllochaetopterus* sp.		I									
Animalia	Annelida	Polychaeta		Chaetopteridae	*Spiochaetopterus*	*Spiochaetopterus costarum*	(Claparède, 1869)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Axiothella*	*Axiothella* sp.		I				4					
Animalia	Annelida	Polychaeta		Maldanidae	*Clymenella*	*Clymenella torquata*	(Leidy, 1855)	I				21					
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene oerstedii*	(Claparède, 1863)	I			4	12		79			33
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene* sp.		not assigned				3			5		
Animalia	Annelida	Polychaeta		Maldanidae	*Heteroclymene*	*Heteroclymene robusta*	Arwidsson, 1906	V				1					
Animalia	Annelida	Polychaeta		Maldanidae	*Johnstonia*	*Johnstonia clymenoides*	Quatrefages, 1866	not assigned									
Animalia	Annelida	Polychaeta		Maldanidae	*Leiochone*	*Leiochone leiopygos*	(Grube, 1860)	III		13818			27457			12588	
Animalia	Annelida	Polychaeta		Maldanidae	*Maldane*	*Maldane sarsi*	Malmgren, 1865	II				11					
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella affinis*	(M. Sars in G.O. Sars, 1872)	III				14					

								Season	Spr20								
								Location	TR								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella* sp.		III				2		23			
Animalia	Annelida	Polychaeta		Maldanidae				I							2		
Animalia	Annelida	Polychaeta		Orbiniidae	*Orbinia*	*Orbinia* sp.		I				1					
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo foetida*	(Claparède, 1868)	I			3			3			12
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo* sp.		I		10878			89			2278	
Animalia	Annelida	Polychaeta		Orbiniidae	*Scoloplos*	*Scoloplos armiger*	(Müller, 1776)	III									
Animalia	Annelida	Polychaeta		Oweniidae	*Galathowenia*	*Galathowenia oculata*	(Zachs, 1923)	II									
Animalia	Annelida	Polychaeta		Oweniidae	*Myriochele*	*Myriochele* sp.		III									
Animalia	Annelida	Polychaeta		Oweniidae	*Owenia*	*Owenia fusiformis*	Delle Chiaje, 1844	II							1		
Animalia	Annelida	Polychaeta		Oweniidae				not assigned				1					
Animalia	Arthropoda	Copepoda	Harpacticoida	Harpacticidae	*Harpacticus*	*Harpacticus* sp.		not assigned									
Animalia	Arthropoda	Copepoda	Harpacticoida	Thalestridae				not assigned									
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae	*Ampelisca*	*Ampelisca* sp.		I	13	1001		19	91		11	171	
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae				I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Atylidae	*Atylus*	*Atylus* sp.		I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Corophiidae	*Corophium*	*Corophium multisetosum*	Stock, 1952	III									
Animalia	Arthropoda	Malacostraca	Amphipoda	Dexaminidae	*Dexamine*	*Dexamine* sp.		III								853	
Animalia	Arthropoda	Malacostraca	Amphipoda	Oedicerotidae	*Perioculodes*	*Perioculodes* sp.		II									
Animalia	Arthropoda	Malacostraca	Amphipoda	Phoxocephalidae				I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe grimaldii*	Chevreux, 1895	I									

								Season	Spr20								
								Location	TR								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Arthropoda	Malacostraca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe* sp.		I									
Animalia	Arthropoda	Malacostraca	Amphipoda					not assigned									
Animalia	Arthropoda	Malacostraca	Decapoda	Alpheidae	*Athanas*	*Athanas nitescens*	(Leach, 1814)	I									
Animalia	Arthropoda	Malacostraca	Decapoda	Benthesicymidae	*Benthesicymus*	*Benthesicymus laciniatus*	Rathbun, 1906	not assigned									
Animalia	Arthropoda	Malacostraca	Decapoda	Callianassidae	*Gilvossius*	*Gilvossius tyrrhenus*	(Petagna, 1792)	III				1	155	26		17	6
Animalia	Arthropoda	Malacostraca	Decapoda	Carcinidae	*Carcinus*	*Carcinus maenas*	(Linnaeus, 1758)	III									
Animalia	Arthropoda	Malacostraca	Decapoda	Diogenidae	*Clibanarius* β	*Clibanarius* sp.		II									
Animalia	Arthropoda	Malacostraca	Decapoda	Diogenidae	*Diogenes*	*Diogenes armatus**	Almón, Cuesta, Schubart & García Raso in Almón, Cuesta, Schubart, Armenia & García Raso, 2021	II									
Animalia	Arthropoda	Malacostraca	Decapoda	Diogenidae	*Diogenes*	*Diogenes costatus**	Henderson, 1893	II									
Animalia	Arthropoda	Malacostraca	Decapoda	Grapsidae	*Planes*	*Planes* sp.		not assigned									
Animalia	Arthropoda	Malacostraca	Decapoda	Processidae	*Processa*	*Processa parva*	Holthuis, 1951	I									
Animalia	Arthropoda	Malacostraca	Isopoda	Anthuridae	*Cyathura*	*Cyathura carinata*	(Krøyer, 1847)	III				6					
Animalia	Arthropoda	Malacostraca	Isopoda	Cirolanidae	*Cirolana*	*Cirolana cranchii*	Leach, 1818	II									
Animalia	Arthropoda	Malacostraca	Tanaidacea	Apseudidae	*Apseudopsis*	*Apseudopsis latreillii*	(Milne Edwards, 1828)	III	93			67			143		
Animalia	Bryozoa	Gymnolaemata	Cheilostomatida	Bugulidae	*Bugula*	*Bugula neritina**	(Linnaeus, 1758)	ignored					6				
Animalia	Bryozoa	Gymnolaemata	Cheilostomatida	Candidae	*Tricellaria*	*Tricellaria inopinata**	d'Hondt & Occhipinti Ambrogi, 1985	ignored									
Animalia	Bryozoa	Gymnolaemata	Cheilostomatida	Candidae	*Tricellaria*	*Tricellaria ternata**	(Ellis & Solander, 1786)	ignored					6				

								Season	Spr20								
								Location	TR								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Bryozoa	Gymnolaemata	Cheilostomatida	Watersiporidae	*Watersipora*	*Watersipora subtorquata**	(d'Orbigny, 1852)	ignored									
Animalia	Bryozoa							II									
Animalia	Chordata	Ascidiacea	Aplousobranchia	Clavelinidae	*Clavelina*	*Clavelina lepadiformis*	(Müller, 1776)	not assigned									
Animalia	Chordata	Ascidiacea	Phlebobranchia	Corellidae	*Corella*	*Corella eumyota**	Traustedt, 1882	III						5			
Animalia	Chordata	Ascidiacea	Stolidobranchia	Pyuridae	*Microcosmus*	*Microcosmus squamiger**	Michaelsen, 1927	II		45			11			17	
Animalia	Chordata	Ascidiacea	Stolidobranchia	Styelidae	*Styela*	*Styela plicata**	(Lesueur, 1823)	II									
Animalia	Cnidaria	Anthozoa	Actiniaria		*Paraiptasia*	*Paraiptasia* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Alcyonacea	Coralliidae	*Corallium*	*Corallium* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Malacalcyonacea	Isididae				not assigned									
Animalia	Cnidaria	Anthozoa	Scleralcyonacea	Helioporidae	*Heliopora*	*Heliopora* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Scleralcyonacea	Veretillidae	*Veretillum*	*Veretillum cynomorium*	(Pallas, 1766)	I		62							
Animalia	Cnidaria	Hydrozoa	Anthoathecata	Hydractiniidae α				I						14 α			
Animalia	Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	*Obelia*	*Obelia dichotoma**	(Linnaeus, 1758)	II									
Animalia	Cnidaria	Staurozoa	Stauromedusae	Haliclystidae	*Haliclystus*	*Haliclystus tenuis**	Kishinouye, 1910	not assigned									
Animalia	Echinodermata	Holothuroidea	Apodida	Synaptidae	*Leptosynapta*	*Leptosynapta inhaerens*	(O.F. Müller, 1776)	I	1		2			118	1		96
Animalia	Mollusca	Bivalvia	Adapedonta	Solenidae	*Solen*	*Solen marginatus*	Pulteney, 1799	I							1		
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Cerastoderma*	*Cerastoderma edule*	(Linnaeus, 1758)	III			6	1		296			8
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Parvicardium*	*Parvicardium exiguum*	(Gmelin, 1791)	I									

								Season	Spr20								
								Location	TR								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae	*Gari*	*Gari tellinella*	(Lamarck, 1818)	I	1			2			3		
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae α				I									
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra alba*	(W. Wood, 1802)	III									
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra nitida*	(O. F. Müller, 1776)	III	1			2					
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Scrobicularia*	*Scrobicularia plana*	(da Costa, 1778)	III	2								
Animalia	Mollusca	Bivalvia	Cardiida	Solecurtidae α				not assigned									
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Asbjornsenia*	*Asbjornsenia pygmaea*	(Lovén, 1846)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Macomangulus*	*Macomangulus tenuis*	(da Costa, 1778)	I							1		
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae α				I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Loripes*	*Loripes orbiculatus*	Poli, 1795	I				4					
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinella*	*Lucinella divaricata*	(Linnaeus, 1758)	I	2								
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinoma*	*Lucinoma borealis*	(Linnaeus, 1767)	I									
Animalia	Mollusca	Bivalvia	Myida	Corbulidae	*Varicorbula*	*Varicorbula gibba*	(Olivi, 1792)	IV				1			1		
Animalia	Mollusca	Bivalvia	Nuculida	Nuculidae	*Nucula*	*Nucula hanleyi*	Winckworth, 1931	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Lutraria*	*Lutraria Lutraria*	(Linnaeus, 1758)	I				1					
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula solida*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula subtruncata*	(da Costa, 1778)	I									
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Petricola*	*Petricola lapicida*	(Gmelin, 1791)	not assigned									
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Ruditapes*	*Ruditapes philippinarum**	(A. Adams & Reeve, 1850)	III									

								Season	Spr20								
								Location	TR								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Venerida	Veneridae α				I									
Animalia	Mollusca	Bivalvia		Ostreidae	*Magallana*	*Magallana gigas**	(Thunberg, 1793)	III									
Animalia	Mollusca	Cephalopoda	Sepiida	Sepiidae	*Acanthosepion*	*Acanthosepion pharaonis**	(Ehrenberg, 1831)	ignored									
Animalia	Mollusca	Gastropoda	Cephalaspidea	Philinidae	*Philine*	*Philine* sp.		II									
Animalia	Mollusca	Gastropoda	LittoriniMa	Hydrobiidae	*Peringia*	*Peringia ulvae*	(Pennant, 1777)	III									
Animalia	Mollusca	Gastropoda	LittoriniMa	Rissoidae	*Rissoa*	*Rissoa parva*	(da Costa, 1778)	I									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Nassarius*	*Nassarius argenteus**	(Marrat, 1877)	not assigned					20			841	
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Tritia*	*Tritia reticulata*	(Linnaeus, 1758)	II	2			3	8		3		
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae α				II						5 α			5 α
Animalia	Mollusca	Gastropoda	Neogastropoda	Raphitomidae	*Leufroyia*	*Leufroyia leufroyi*	(Michaud, 1828)	not assigned							1		
Animalia	Mollusca	Gastropoda	Nudibranchia	Goniodorididae	*Ancula*	*Ancula gibbosa*	(Risso, 1818)	I									
Animalia	Mollusca	Gastropoda	Trochida	Calliostomatidae	*Calliostoma*	*Calliostoma zizyphinum*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Gastropoda	Trochida	Trochidae	*Jujubinus*	*Jujubinus striatus*	(Linnaeus, 1758)	I				3			1		
Animalia	Mollusca	Gastropoda	Trochida	Trochidae				not assigned									
Animalia	Mollusca	Gastropoda		Limapontiidae	*Limapontia*	*Limapontia depressa**	Alder & Hancock, 1862	I									
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella depressa*	Pennant, 1777	I									
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella pellucida*	Linnaeus, 1758	I									
Animalia	Mollusca	Polyplacophora	Callochitonida	Callochitonidae	*Callochiton*	*Callochiton septemvalvis*	(Montagu, 1803)	I									
Animalia	Nemertea	Hoplonemertea	Monostilifera	Tetrastemmatidae	*Tetrastemma* β	*Tetrastemma* sp.		III									
Animalia	Porifera	Demospongiae	Verongiida					not assigned									

								Season	Spr20								
								Location	GAM								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Clitellata	Tubificida	Naididae	*Nais*	*Nais elinguis*	Müller, 1774	IV									
Animalia	Annelida	Polychaeta	Amphinomida	Amphinomidae				not assigned									
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae	*Eunice*	*Eunice oerstedii*	Stimpson, 1853	II									
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae				II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Aponuphis*	*Aponuphis brementi*	(Fauvel, 1916)	II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Diopatra* β	*Diopatra* sp.		II						5 β			
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Onuphis*	*Onuphis farensis*	Gil & Machado, 2014	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera alba*	(O.F. Müller, 1776)	IV									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera* sp.		II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tesselata*	Grube, 1863	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tridactyla*	Schmarda, 1861	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae				II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys cirrosa*	Ehlers, 1868	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys* sp.		II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste diversicolor*	(O.F. Müller, 1776)	III	3	107	95	9	596	67	2	142	245
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste* sp.		III									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Perinereis*	*Perinereis* sp.		III									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae				not assigned									
Animalia	Annelida	Polychaeta	Phyllodocida	Phyllodocidae	*Phyllodoce*	*Phyllodoce mucosa*	Örsted, 1843	III									

								Season	Spr20								
								Location	GAM								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta	Phyllodocida	Sigalionidae	*Sigalion*	*Sigalion mathildae*	Audouin & Milne Edwards, 1832	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Syllidae	*Myrianida*	*Myrianida pachycera**	(Augener, 1913)	II					214				
Animalia	Annelida	Polychaeta	Sabellida	Sabellariidae	*Sabellaria*	*Sabellaria spinulosa*	(Leuckart, 1849)	I									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Pseudopolydora*	*Pseudopolydora paucibranchiata**	(Okuda, 1937)	IV									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Scolelepis*	*Scolelepis neglecta**	Surugiu, 2016	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spio*	*Spio symphyta**	Meißner, Bick & Bastrop, 2011	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spiophanes*	*Spiophanes bombyx*	(Claparède, 1870)	III									3
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Streblospio*	*Streblospio shrubsolii*	(Buchanan, 1890)	III		2454			90				
Animalia	Annelida	Polychaeta	Spionida	Spionidae				III									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Caulleriella*	*Caulleriella* sp.		not assigned									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirratulus*	*Cirratulus* sp.		IV	1								
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia* sp.		IV	2								
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia tentaculata*	(Montagu, 1808)	IV	1								
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx acutus**	Webster & Benedict, 1887	III									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx* sp.		IV		1046							
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae	*Terebella*	*Terebella lapidaria*	Linnaeus, 1767	I									
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae				I									
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella capitata*	(Fabricius, 1780)	V									
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella* sp.		V					8274				

								Season	**Spr20**								
								Location	**GAM**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Capitellidae	*Dasybranchus*	*Dasybranchus caducus*	(Grube, 1846)	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Heteromastus*	*Heteromastus filiformis*	(Claparède, 1864)	IV									
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus latericeus*	Sars, 1851	III						212			
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus profondus*	Eisig, 1887	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus* sp.		not assigned									
Animalia	Annelida	Polychaeta		Capitellidae				V									
Animalia	Annelida	Polychaeta		Chaetopteridae	*Phyllochaetopterus*	*Phyllochaetopterus* sp.		I									
Animalia	Annelida	Polychaeta		Chaetopteridae	*Spiochaetopterus*	*Spiochaetopterus costarum*	(Claparède, 1869)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Axiothella*	*Axiothella* sp.		I									
Animalia	Annelida	Polychaeta		Maldanidae	*Clymenella*	*Clymenella torquata*	(Leidy, 1855)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene oerstedii*	(Claparède, 1863)	I						177			
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene* sp.		not assigned									
Animalia	Annelida	Polychaeta		Maldanidae	*Heteroclymene*	*Heteroclymene robusta*	Arwidsson, 1906	V									
Animalia	Annelida	Polychaeta		Maldanidae	*Johnstonia*	*Johnstonia clymenoides*	Quatrefages, 1866	not assigned									
Animalia	Annelida	Polychaeta		Maldanidae	*Leiochone*	*Leiochone leiopygos*	(Grube, 1860)	III									
Animalia	Annelida	Polychaeta		Maldanidae	*Maldane*	*Maldane sarsi*	Malmgren, 1865	II									
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella affinis*	(M. Sars in G.O. Sars, 1872)	III									

								Season	Spr20								
								Location	**GAM**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella* sp.		III						8			
Animalia	Annelida	Polychaeta		Maldanidae				I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Orbinia*	*Orbinia* sp.		I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo foetida*	(Claparède, 1868)	I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo* sp.		I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Scoloplos*	*Scoloplos armiger*	(Müller, 1776)	III									
Animalia	Annelida	Polychaeta		Oweniidae	*Galathowenia*	*Galathowenia oculata*	(Zachs, 1923)	II									
Animalia	Annelida	Polychaeta		Oweniidae	*Myriochele*	*Myriochele* sp.		III									
Animalia	Annelida	Polychaeta		Oweniidae	*Owenia*	*Owenia fusiformis*	Delle Chiaje, 1844	II									
Animalia	Annelida	Polychaeta		Oweniidae				not assigned									
Animalia	Arthropoda	Copepoda	Harpacticoida	Harpacticidae	*Harpacticus*	*Harpacticus* sp.		not assigned									
Animalia	Arthropoda	Copepoda	Harpacticoida	Thalestridae				not assigned									
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae	*Ampelisca*	*Ampelisca* sp.		I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae				I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Atylidae	*Atylus*	*Atylus* sp.		I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Corophiidae	*Corophium*	*Corophium multisetosum*	Stock, 1952	III									
Animalia	Arthropoda	Malacostraca	Amphipoda	Dexaminidae	*Dexamine*	*Dexamine* sp.		III									
Animalia	Arthropoda	Malacostraca	Amphipoda	Oedicerotidae	*Perioculodes*	*Perioculodes* sp.		II									
Animalia	Arthropoda	Malacostraca	Amphipoda	Phoxocephalidae				I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe grimaldii*	Chevreux, 1895	I									

								Season	**Spr20**								
								Location	**GAM**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Arthrop-oda	Malacos-traca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe* sp.		I									
Animalia	Arthrop-oda	Malacos-traca	Amphipoda					not assigned									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Alpheidae	*Athanas*	*Athanas ni-tescens*	(Leach, 1814)	I					1324				
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Benthesicymidae	*Benthesicy-mus*	*Benthesicymus laciniatus*	Rathbun, 1906	not assigned								36	
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Callianassidae	*Gilvossius*	*Gilvossius tyr-rhenus*	(Petagna, 1792)	III						50			
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Carcinidae	*Carcinus*	*Carcinus mae-nas*	(Linnaeus, 1758)	III					41712	58			
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Clibanarius* β	*Clibanarius* sp.		II									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Diogenes*	*Diogenes ar-matus**	Almón, Cuesta, Schu-bart & García Raso in Almón, Cuesta, Schu-bart, Armenia & García Raso, 2021	II									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Diogenes*	*Diogenes cos-tatus**	Henderson, 1893	II					3				
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Grapsidae	*Planes*	*Planes* sp.		not assigned					5				
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Processidae	*Processa*	*Processa parva*	Holthuis, 1951	I									
Animalia	Arthrop-oda	Malacos-traca	Isopoda	Anthuridae	*Cyathura*	*Cyathura cari-nata*	(Krøyer, 1847)	III	6			4			1		
Animalia	Arthrop-oda	Malacos-traca	Isopoda	Cirolanidae	*Cirolana*	*Cirolana cranchii*	Leach, 1818	II									
Animalia	Arthrop-oda	Malacos-traca	Tanaidacea	Apseudidae	*Apseudopsis*	*Apseudopsis latreillii*	(Milne Edwards, 1828)	III									
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Bugulidae	*Bugula*	*Bugula ne-ritina**	(Linnaeus, 1758)	ignored									
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Candidae	*Tricellaria*	*Tricellaria in-opinata**	d'Hondt & Occhipinti Ambrogi, 1985	ignored									
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Candidae	*Tricellaria*	*Tricellaria ter-nata**	(Ellis & Solander, 1786)	ignored									

								Season	Spr20								
								Location	GAM								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Watersiporidae	*Watersipora*	*Watersipora subtorquata**	(d'Orbigny, 1852)	ignored					87				
Animalia	Bryozoa							II									
Animalia	Chordata	Ascidiacea	Aplousobran-chia	Clavelinidae	*Clavelina*	*Clavelina lepa-diformis*	(Müller, 1776)	not assigned									
Animalia	Chordata	Ascidiacea	Phlebobran-chia	Corellidae	*Corella*	*Corella eumyota**	Traustedt, 1882	III									
Animalia	Chordata	Ascidiacea	Stolidobran-chia	Pyuridae	*Microcos-mus*	*Microcosmus squamiger**	Michaelsen, 1927	II									
Animalia	Chordata	Ascidiacea	Stolidobran-chia	Styelidae	*Styela*	*Styela plicata**	(Lesueur, 1823)	II			5						
Animalia	Cnidaria	Anthozoa	Actiniaria		*Paraiptasia*	*Paraiptasia* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Alcyonacea	Coralliidae	*Corallium*	*Corallium* sp.		not assigned					189				
Animalia	Cnidaria	Anthozoa	Malacalcyo-nacea	Isididae				not assigned									
Animalia	Cnidaria	Anthozoa	Scleralcyo-nacea	Helioporidae	*Heliopora*	*Heliopora* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Scleralcyo-nacea	Veretillidae	*Veretillum*	*Veretillum cynomorium*	(Pallas, 1766)	I									
Animalia	Cnidaria	Hydrozoa	Anthoathecata	Hydractiniidae α				I						26 α			
Animalia	Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	*Obelia*	*Obelia dicho-toma**	(Linnaeus, 1758)	II									
Animalia	Cnidaria	Staurozoa	Stauromedusae	Haliclystidae	*Haliclystus*	*Haliclystus tenuis**	Kishinouye, 1910	not assigned									
Animalia	Echino-dermata	Holothu-roidea	Apodida	Synaptidae	*Lep-tosynapta*	*Leptosynapta inhaerens*	(O.F. Müller, 1776)	I						149			
Animalia	Mollusca	Bivalvia	Adapedonta	Solenidae	*Solen*	*Solen margina-tus*	Pulteney, 1799	I									
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Cerasto-derma*	*Cerastoderma edule*	(Linnaeus, 1758)	III			364			137			211
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Parvicar-dium*	*Parvicardium exiguum*	(Gmelin, 1791)	I									

								Season	Spr20								
								Location	GAM								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae	*Gari*	*Gari tellinella*	(Lamarck, 1818)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae α				I									
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra alba*	(W. Wood, 1802)	III									
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra nitida*	(O. F. Müller, 1776)	III									6
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Scrobicularia*	*Scrobicularia plana*	(da Costa, 1778)	III	1							45	
Animalia	Mollusca	Bivalvia	Cardiida	Solecurtidae α				not assigned			35 α			9 α			384 α
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Asbjornsenia*	*Asbjornsenia pygmaea*	(Lovén, 1846)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Macomangulus*	*Macomangulus tenuis*	(da Costa, 1778)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae α				I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Loripes*	*Loripes orbiculatus*	Poli, 1795	I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinella*	*Lucinella divaricata*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinoma*	*Lucinoma borealis*	(Linnaeus, 1767)	I						8			
Animalia	Mollusca	Bivalvia	Myida	Corbulidae	*Varicorbula*	*Varicorbula gibba*	(Olivi, 1792)	IV									
Animalia	Mollusca	Bivalvia	Nuculida	Nuculidae	*Nucula*	*Nucula hanleyi*	Winckworth, 1931	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Lutraria*	*Lutraria Lutraria*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula solida*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula subtruncata*	(da Costa, 1778)	I									
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Petricola*	*Petricola lapicida*	(Gmelin, 1791)	not assigned									
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Ruditapes*	*Ruditapes philippinarum**	(A. Adams & Reeve, 1850)	III									

								Season	Spr20								
								Location	GAM								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Venerida	Veneridae α				I									
Animalia	Mollusca	Bivalvia		Ostreidae	*Magallana*	*Magallana gigas**	(Thunberg, 1793)	III									
Animalia	Mollusca	Cephalopoda	Sepiida	Sepiidae	*Acanthosepion*	*Acanthosepion pharaonis**	(Ehrenberg, 1831)	ignored		3							
Animalia	Mollusca	Gastropoda	Cephalaspidea	Philinidae	*Philine*	*Philine* sp.		II									
Animalia	Mollusca	Gastropoda	LittoriniMa	Hydrobiidae	*Peringia*	*Peringia ulvae*	(Pennant, 1777)	III	3	7001		1	182			1976	3
Animalia	Mollusca	Gastropoda	LittoriniMa	Rissoidae	*Rissoa*	*Rissoa parva*	(da Costa, 1778)	I					967				
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Nassarius*	*Nassarius argenteus**	(Marrat, 1877)	not assigned									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Tritia*	*Tritia reticulata*	(Linnaeus, 1758)	II									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae α				II						9 α			
Animalia	Mollusca	Gastropoda	Neogastropoda	Raphitomidae	*Leufroyia*	*Leufroyia leufroyi*	(Michaud, 1828)	not assigned									
Animalia	Mollusca	Gastropoda	Nudibranchia	Goniodorididae	*Ancula*	*Ancula gibbosa*	(Risso, 1818)	I		4							
Animalia	Mollusca	Gastropoda	Trochida	Calliostomatidae	*Calliostoma*	*Calliostoma zizyphinum*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Gastropoda	Trochida	Trochidae	*Jujubinus*	*Jujubinus striatus*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Gastropoda	Trochida	Trochidae				not assigned									
Animalia	Mollusca	Gastropoda		Limapontiidae	*Limapontia*	*Limapontia depressa**	Alder & Hancock, 1862	I					5470				
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella depressa*	Pennant, 1777	I									
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella pellucida*	Linnaeus, 1758	I									
Animalia	Mollusca	Polyplacophora	Callochitonida	Callochitonidae	*Callochiton*	*Callochiton septemvalvis*	(Montagu, 1803)	I									
Animalia	Nemertea	Hoplonemertea	Monostilifera	Tetrastemmatidae	*Tetrastemma* β	*Tetrastemma* sp.		III									4 β
Animalia	Porifera	Demospongiae	Verongiida					not assigned		4						3	

								Season	Spr20								
								Location	NAV								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Clitellata	Tubificida	Naididae	*Nais*	*Nais elinguis*	Müller, 1774	IV									
Animalia	Annelida	Polychaeta	Amphinomida	Amphinomidae				not assigned									
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae	*Eunice*	*Eunice oerstedii*	Stimpson, 1853	II									
Animalia	Annelida	Polychaeta	Eunicida	Eunicidae				II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Aponuphis*	*Aponuphis brementi*	(Fauvel, 1916)	II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Diopatra* β	*Diopatra* sp.		II									
Animalia	Annelida	Polychaeta	Eunicida	Onuphidae	*Onuphis*	*Onuphis farensis*	Gil & Machado, 2014	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera alba*	(O.F. Müller, 1776)	IV									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera* sp.		II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tesselata*	Grube, 1863	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae	*Glycera*	*Glycera tridactyla*	Schmarda, 1861	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Glyceridae				II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys cirrosa*	Ehlers, 1868	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nephtyidae	*Nephtys*	*Nephtys* sp.		II									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste diversicolor*	(O.F. Müller, 1776)	III	10	143	47	6	1210	368	3	106	7
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Hediste*	*Hediste* sp.		III									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae	*Perinereis*	*Perinereis* sp.		III									
Animalia	Annelida	Polychaeta	Phyllodocida	Nereididae				not assigned									
Animalia	Annelida	Polychaeta	Phyllodocida	Phyllodocidae	*Phyllodoce*	*Phyllodoce mucosa*	Örsted, 1843	III									

								Season	Spr20								
								Location	NAV								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta	Phyllodocida	Sigalionidae	*Sigalion*	*Sigalion mathildae*	Audouin & Milne Edwards, 1832	II									
Animalia	Annelida	Polychaeta	Phyllodocida	Syllidae	*Myrianida*	*Myrianida pachycera**	(Augener, 1913)	II									
Animalia	Annelida	Polychaeta	Sabellida	Sabellariidae	*Sabellaria*	*Sabellaria spinulosa*	(Leuckart, 1849)	I									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Pseudopolydora*	*Pseudopolydora paucibranchiata**	(Okuda, 1937)	IV									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Scolelepis*	*Scolelepis neglecta**	Surugiu, 2016	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spio*	*Spio symphyta**	Meißner, Bick & Bastrop, 2011	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Spiophanes*	*Spiophanes bombyx*	(Claparède, 1870)	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae	*Streblospio*	*Streblospio shrubsolii*	(Buchanan, 1890)	III									
Animalia	Annelida	Polychaeta	Spionida	Spionidae				III									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Caulleriella*	*Caulleriella* sp.		not assigned									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirratulus*	*Cirratulus* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Cirriformia*	*Cirriformia tentaculata*	(Montagu, 1808)	IV									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx acutus**	Webster & Benedict, 1887	III									
Animalia	Annelida	Polychaeta	Terebellida	Cirratulidae	*Tharyx*	*Tharyx* sp.		IV									
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae	*Terebella*	*Terebella lapidaria*	Linnaeus, 1767	I		9449	432		8147	132			
Animalia	Annelida	Polychaeta	Terebellida	Terebellidae				I									
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella capitata*	(Fabricius, 1780)	V									
Animalia	Annelida	Polychaeta		Capitellidae	*Capitella*	*Capitella* sp.		V									

								Season	Spr20								
								Location	**NAV**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Capitellidae	*Dasybranchus*	*Dasybranchus caducus*	(Grube, 1846)	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Heteromastus*	*Heteromastus filiformis*	(Claparède, 1864)	IV						32	1		
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus latericeus*	Sars, 1851	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus profondus*	Eisig, 1887	III									
Animalia	Annelida	Polychaeta		Capitellidae	*Notomastus*	*Notomastus* sp.		not assigned									
Animalia	Annelida	Polychaeta		Capitellidae				V					147			44	
Animalia	Annelida	Polychaeta		Chaetopteridae	*Phyllochaetopterus*	*Phyllochaetopterus* sp.		I									
Animalia	Annelida	Polychaeta		Chaetopteridae	*Spiochaetopterus*	*Spiochaetopterus costarum*	(Claparède, 1869)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Axiothella*	*Axiothella* sp.		I									
Animalia	Annelida	Polychaeta		Maldanidae	*Clymenella*	*Clymenella torquata*	(Leidy, 1855)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene oerstedii*	(Claparède, 1863)	I									
Animalia	Annelida	Polychaeta		Maldanidae	*Euclymene*	*Euclymene* sp.		not assigned									
Animalia	Annelida	Polychaeta		Maldanidae	*Heteroclymene*	*Heteroclymene robusta*	Arwidsson, 1906	V									
Animalia	Annelida	Polychaeta		Maldanidae	*Johnstonia*	*Johnstonia clymenoides*	Quatrefages, 1866	not assigned									
Animalia	Annelida	Polychaeta		Maldanidae	*Leiochone*	*Leiochone leiopygos*	(Grube, 1860)	III									
Animalia	Annelida	Polychaeta		Maldanidae	*Maldane*	*Maldane sarsi*	Malmgren, 1865	II									
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella affinis*	(M. Sars in G.O. Sars, 1872)	III									

								Season	Spr20								
								Location	NAV								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Annelida	Polychaeta		Maldanidae	*Praxillella*	*Praxillella* sp.		III									
Animalia	Annelida	Polychaeta		Maldanidae				I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Orbinia*	*Orbinia* sp.		I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo foetida*	(Claparède, 1868)	I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Phylo*	*Phylo* sp.		I									
Animalia	Annelida	Polychaeta		Orbiniidae	*Scoloplos*	*Scoloplos armiger*	(Müller, 1776)	III									
Animalia	Annelida	Polychaeta		Oweniidae	*Galathowenia*	*Galathowenia oculata*	(Zachs, 1923)	II							1		
Animalia	Annelida	Polychaeta		Oweniidae	*Myriochele*	*Myriochele* sp.		III									
Animalia	Annelida	Polychaeta		Oweniidae	*Owenia*	*Owenia fusiformis*	Delle Chiaje, 1844	II									
Animalia	Annelida	Polychaeta		Oweniidae				not assigned									
Animalia	Arthropoda	Copepoda	Harpacticoida	Harpacticidae	*Harpacticus*	*Harpacticus* sp.		not assigned			5						4
Animalia	Arthropoda	Copepoda	Harpacticoida	Thalestridae				not assigned									
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae	*Ampelisca*	*Ampelisca* sp.		I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Ampeliscidae				I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Atylidae	*Atylus*	*Atylus* sp.		I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Corophiidae	*Corophium*	*Corophium multisetosum*	Stock, 1952	III									
Animalia	Arthropoda	Malacostraca	Amphipoda	Dexaminidae	*Dexamine*	*Dexamine* sp.		III									
Animalia	Arthropoda	Malacostraca	Amphipoda	Oedicerotidae	*Perioculodes*	*Perioculodes* sp.		II									
Animalia	Arthropoda	Malacostraca	Amphipoda	Phoxocephalidae				I									
Animalia	Arthropoda	Malacostraca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe grimaldii*	Chevreux, 1895	I									

								Season	Spr20								
								Location	NAV								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Arthrop-oda	Malacos-traca	Amphipoda	Urothoidae	*Urothoe*	*Urothoe* sp.		I									
Animalia	Arthrop-oda	Malacos-traca	Amphipoda					not assigned									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Alpheidae	*Athanas*	*Athanas nitescens*	(Leach, 1814)	I									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Benthesicymidae	*Benthesicymus*	*Benthesicymus laciniatus*	Rathbun, 1906	not assigned									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Callianassidae	*Gilvossius*	*Gilvossius tyrrhenus*	(Petagna, 1792)	III									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Carcinidae	*Carcinus*	*Carcinus maenas*	(Linnaeus, 1758)	III					3963	7			
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Clibanarius* β	*Clibanarius* sp.		II									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Diogenes*	*Diogenes armatus**	Almón, Cuesta, Schubart & García Raso in Almón, Cuesta, Schubart, Armenia & García Raso, 2021	II									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Diogenidae	*Diogenes*	*Diogenes costatus**	Henderson, 1893	II									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Grapsidae	*Planes*	*Planes* sp.		not assigned									
Animalia	Arthrop-oda	Malacos-traca	Decapoda	Processidae	*Processa*	*Processa parva*	Holthuis, 1951	I									
Animalia	Arthrop-oda	Malacos-traca	Isopoda	Anthuridae	*Cyathura*	*Cyathura carinata*	(Krøyer, 1847)	III	25			3			7		
Animalia	Arthrop-oda	Malacos-traca	Isopoda	Cirolanidae	*Cirolana*	*Cirolana cranchii*	Leach, 1818	II									
Animalia	Arthrop-oda	Malacos-traca	Tanaidacea	Apseudidae	*Apseudopsis*	*Apseudopsis latreillii*	(Milne Edwards, 1828)	III									
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Bugulidae	*Bugula*	*Bugula neritina**	(Linnaeus, 1758)	ignored									
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Candidae	*Tricellaria*	*Tricellaria inopinata**	d'Hondt & Occhipinti Ambrogi, 1985	ignored									
Animalia	Bryozoa	Gymnolae-mata	Cheilosto-matida	Candidae	*Tricellaria*	*Tricellaria ternata**	(Ellis & Solander, 1786)	ignored									

								Season	Spr20								
								Location	NAV								
								Station	1			2			3		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Bryozoa	Gymnolaemata	Cheilostomatida	Watersiporidae	*Watersipora*	*Watersipora subtorquata**	(d'Orbigny, 1852)	ignored					53			6	
Animalia	Bryozoa							II								1053	
Animalia	Chordata	Ascidiacea	Aplousobranchia	Clavelinidae	*Clavelina*	*Clavelina lepadiformis*	(Müller, 1776)	not assigned									
Animalia	Chordata	Ascidiacea	Phlebobranchia	Corellidae	*Corella*	*Corella eumyota**	Traustedt, 1882	III									
Animalia	Chordata	Ascidiacea	Stolidobranchia	Pyuridae	*Microcosmus*	*Microcosmus squamiger**	Michaelsen, 1927	II									
Animalia	Chordata	Ascidiacea	Stolidobranchia	Styelidae	*Styela*	*Styela plicata**	(Lesueur, 1823)	II									
Animalia	Cnidaria	Anthozoa	Actiniaria		*Paraiptasia*	*Paraiptasia* sp.		not assigned									5
Animalia	Cnidaria	Anthozoa	Alcyonacea	Coralliidae	*Corallium*	*Corallium* sp.		not assigned								45	
Animalia	Cnidaria	Anthozoa	Malacalcyonacea	Isididae				not assigned									
Animalia	Cnidaria	Anthozoa	Scleralcyonacea	Helioporidae	*Heliopora*	*Heliopora* sp.		not assigned									
Animalia	Cnidaria	Anthozoa	Scleralcyonacea	Veretillidae	*Veretillum*	*Veretillum cynomorium*	(Pallas, 1766)	I									
Animalia	Cnidaria	Hydrozoa	Anthoathecata	Hydractiniidae α				I									
Animalia	Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	*Obelia*	*Obelia dichotoma**	(Linnaeus, 1758)	II									
Animalia	Cnidaria	Staurozoa	Stauromedusae	Haliclystidae	*Haliclystus*	*Haliclystus tenuis**	Kishinouye, 1910	not assigned									
Animalia	Echinodermata	Holothuroidea	Apodida	Synaptidae	*Leptosynapta*	*Leptosynapta inhaerens*	(O.F. Müller, 1776)	I									
Animalia	Mollusca	Bivalvia	Adapedonta	Solenidae	*Solen*	*Solen marginatus*	Pulteney, 1799	I									
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Cerastoderma*	*Cerastoderma edule*	(Linnaeus, 1758)	III			145						
Animalia	Mollusca	Bivalvia	Cardiida	Cardiidae	*Parvicardium*	*Parvicardium exiguum*	(Gmelin, 1791)	I									

								Season	Spr20								
								Location	**NAV**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae	*Gari*	*Gari tellinella*	(Lamarck, 1818)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Psammobiidae α				I									
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra alba*	(W. Wood, 1802)	III	6						3		
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Abra*	*Abra nitida*	(O. F. Müller, 1776)	III									
Animalia	Mollusca	Bivalvia	Cardiida	Semelidae	*Scrobicularia*	*Scrobicularia plana*	(da Costa, 1778)	III								8	
Animalia	Mollusca	Bivalvia	Cardiida	Solecurtidae α				not assigned			7 α			5 α			12 α
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Asbjornsenia*	*Asbjornsenia pygmaea*	(Lovén, 1846)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae	*Macomangulus*	*Macomangulus tenuis*	(da Costa, 1778)	I									
Animalia	Mollusca	Bivalvia	Cardiida	Tellinidae α				I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Loripes*	*Loripes orbiculatus*	Poli, 1795	I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinella*	*Lucinella divaricata*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Lucinida	Lucinidae	*Lucinoma*	*Lucinoma borealis*	(Linnaeus, 1767)	I									
Animalia	Mollusca	Bivalvia	Myida	Corbulidae	*Varicorbula*	*Varicorbula gibba*	(Olivi, 1792)	IV									
Animalia	Mollusca	Bivalvia	Nuculida	Nuculidae	*Nucula*	*Nucula hanleyi*	Winckworth, 1931	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Lutraria*	*Lutraria Lutraria*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula solida*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Bivalvia	Venerida	Mactridae	*Spisula*	*Spisula subtruncata*	(da Costa, 1778)	I									
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Petricola*	*Petricola lapicida*	(Gmelin, 1791)	not assigned									
Animalia	Mollusca	Bivalvia	Venerida	Veneridae	*Ruditapes*	*Ruditapes philippinarum**	(A. Adams & Reeve, 1850)	III									

								Season	Spr20								
								Location	**GAM**								
								Station	**1**			**2**			**3**		
Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Authority**	**AMBI**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**	**Morph**	**COI**	**18S**
Animalia	Mollusca	Bivalvia	Venerida	Veneridae α				I			5 α			8 α			
Animalia	Mollusca	Bivalvia		Ostreidae	*Magallana*	*Magallana gigas**	(Thunberg, 1793)	III			4						
Animalia	Mollusca	Cephalopoda	Sepiida	Sepiidae	*Acanthosepion*	*Acanthosepion pharaonis**	(Ehrenberg, 1831)	ignored									
Animalia	Mollusca	Gastropoda	Cephalaspidea	Philinidae	*Philine*	*Philine* sp.		II					219				
Animalia	Mollusca	Gastropoda	LittoriniMa	Hydrobiidae	*Peringia*	*Peringia ulvae*	(Pennant, 1777)	III	7	425		5	65			447	
Animalia	Mollusca	Gastropoda	LittoriniMa	Rissoidae	*Rissoa*	*Rissoa parva*	(da Costa, 1778)	I					2827				
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Nassarius*	*Nassarius argenteus**	(Marrat, 1877)	not assigned									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae	*Tritia*	*Tritia reticulata*	(Linnaeus, 1758)	II									
Animalia	Mollusca	Gastropoda	Neogastropoda	Nassariidae α				II									
Animalia	Mollusca	Gastropoda	Neogastropoda	Raphitomidae	*Leufroyia*	*Leufroyia leufroyi*	(Michaud, 1828)	not assigned									
Animalia	Mollusca	Gastropoda	Nudibranchia	Goniodorididae	*Ancula*	*Ancula gibbosa*	(Risso, 1818)	I									
Animalia	Mollusca	Gastropoda	Trochida	Calliostomatidae	*Calliostoma*	*Calliostoma zizyphinum*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Gastropoda	Trochida	Trochidae	*Jujubinus*	*Jujubinus striatus*	(Linnaeus, 1758)	I									
Animalia	Mollusca	Gastropoda	Trochida	Trochidae				not assigned									
Animalia	Mollusca	Gastropoda		Limapontiidae	*Limapontia*	*Limapontia depressa**	Alder & Hancock, 1862	I		389							
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella depressa*	Pennant, 1777	I									
Animalia	Mollusca	Gastropoda		Patellidae	*Patella*	*Patella pellucida*	Linnaeus, 1758	I									
Animalia	Mollusca	Polyplacophora	Callochitonida	Callochitonidae	*Callochiton*	*Callochiton septemvalvis*	(Montagu, 1803)	I									
Animalia	Nemertea	Hoplonemertea	Monostilifera	Tetrastemmatidae	*Tetrastemma* β	*Tetrastemma* sp.		III									
Animalia	Porifera	Demospongiae	Verongiida					not assigned									

Putative non-indigenous species (*). β: similarity could not discriminate species within a genus; α: similarity could not discriminate genera within a family.

Table 7 PERMANOVA analysis results for assessing seasonal and spatial variations in macrobenthic community composition across three study locations (TR, NAV, and GAM) and two seasonal periods (Aut19 and Spr20). Analyses were conducted separately for morphological, molecular, and combined datasets, with significance levels indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), and NS representing non-significant results.

Group 1	Group 2	Methodology	*p* value	Significance
Aut19.TR	Spr20.TR	Morphological	0.1	NS
Aut19.NAV	Spr20.NAV		0.2	NS
Aut19.GAM	Spr20.GAM		0.8	NS
Aut19	Spr20		0.514	NS
NAV	GAM		0.02	*
TR	NAV		0.004	**
TR	GAM		0.003	**
Aut19.TR	Spr20.TR	Molecular	0.1	NS
Aut19.NAV	Spr20.NAV		0.2	NS
Aut19.GAM	Spr20.GAM		0.8	NS
Aut19	Spr20		0.305	NS
NAV	GAM		0.012	*
TR	NAV		0.003	**
TR	GAM		0.004	**
Aut19.TR	Spr20.TR	Combined	0.1	NS
Aut19.NAV	Spr20.NAV		0.2	NS
Aut19.GAM	Spr20.GAM		0.9	NS
Aut19	Spr20		0.365	NS
NAV	GAM		0.008	**
TR	NAV		0.003	**
TR	GAM		0.004	**

Table 8 Scores of individual taxa along Canonical Correspondence Analysis (CCA) Axis 1 and Axis 2. Scores represent the positioning of taxa along the environmental gradients identified in the CCA, indicating the strength and direction of their correlation with key environmental parameters, such as sediment type and metal concentrations, across the study areas.

Taxa	Axis 1	Axis 2	AMBI group
Bryozoa	-1,4495	2,3640	II
Clavelina lepadiformis	-1,4495	2,3640	not assigned
Galathowenia oculata	-1,4495	2,3640	II
Glyceridae	-1,4495	2,3640	II
Magallana gigas	-1,4495	2,3640	III
Nais elinguis	-1,4495	2,3640	IV
Paraiptasia sp.	-1,4495	2,3640	not assigned
Patella pellucida	-1,4495	2,3640	I
Petricola lapicida	-1,4495	2,3640	not assigned
Philine sp.	-1,4495	2,3640	II
Sabellaria spinulosa	-1,4495	2,3640	I
Terebella lapidaria	-1,4495	2,3640	I
Terebellidae	-1,4495	2,3640	I
Thalestridae	-1,4495	2,3640	not assigned
Abra alba	-1,2384	0,3330	III
Carcinus maenas	-1,2384	0,3330	III
Corallium sp.	-1,2384	0,3330	not assigned
Harpacticus sp.	-1,2384	0,3330	not assigned
Limapontia depressa	-1,2384	0,3330	I
Perinereis sp.	-1,2384	0,3330	III
Peringia ulvae	-1,2384	0,3330	III
Rissoa parva	-1,2384	0,3330	I
Solecurtidae	-1,2384	0,3330	not assigned
Veneridae	-1,2384	0,3330	I
Watersipora subtorquata	-1,2384	0,3330	ignored
NAV	-1,0406	1,2185	-
Acanthosepion pharaonis	-1,0272	-1,6980	ignored
Ancula gibbosa	-1,0272	-1,6980	I
Athanas nitescens	-1,0272	-1,6980	I
Atylus sp.	-1,0272	-1,6980	I
Benthesicymus laciniatus	-1,0272	-1,6980	not assigned
Caulleriella sp.	-1,0272	-1,6980	not assigned
Cirratulus sp.	-1,0272	-1,6980	IV
Cirriformia sp.	-1,0272	-1,6980	IV
Cirriformia tentaculata	-1,0272	-1,6980	IV
Corophium multisetosum	-1,0272	-1,6980	III
Diogenes costatus	-1,0272	-1,6980	II
Lucinoma borealis	-1,0272	-1,6980	I

Taxa	Axis 1	Axis 2	AMBI group
Myrianida pachycera	-1,0272	-1,6980	II
Nemertea	-1,0272	-1,69796	III
Obelia dichotoma	-1,0272	-1,69796	II
Phoxocephalidae	-1,0272	-1,69796	I
Planes sp.	-1,0272	-1,69796	not assigned
Processa parva	-1,0272	-1,69796	I
Psammobiidae	-1,0272	-1,69796	I
Sigalion mathildae	-1,0272	-1,69796	II
Spiophanes bombyx	-1,0272	-1,69796	III
Streblospio shrubsolii	-1,0272	-1,69796	III
Tellinidae	-1,0272	-1,69796	I
Tharyx setigera	-1,0272	-1,69796	III
Tharyx sp.	-1,0272	-1,69796	IV
Tricellaria inopinata	-1,0272	-1,69796	ignored
Verongiida	-1,0272	-1,69796	not assigned
Li (mg/kg)	-0,9915	0,3441	-
Cr (mg/kg)	-0,9650	0,4671	-
Ni (mg/kg)	-0,9343	0,5523	-
fine fraction	-0,9325	0,5564	-
As (mg/kg)	-0,9123	0,5992	-
Zn (mg/kg)	-0,8882	0,6427	-
Cu (mg/kg)	-0,8548	0,6934	-
Pb (mg/kg)	-0,8233	0,7340	-
Corg	-0,7957	0,7651	-
OM	-0,7954	0,7654	-
Hg (mg/kg)	-0,7515	0,8082	-
GAM	-0,7069	-0,8565	-
Capitella sp.	-0,4309	0,2855	V
Capitellidae	-0,4309	0,2855	V
Cerastoderma edule	-0,4309	0,2855	III
Cyathura carinata	-0,4309	0,2855	III
Hediste diversicolor	-0,4309	0,2855	III
Ruditapes philippinarum	-0,4309	0,2855	III
Scrobicularia plana	-0,4309	0,2855	III
gravel	-0,3936	-0,8109	-
Heliopora sp.	-0,1328	1,2772	not assigned
Heteromastus filiformis	-0,1328	1,2772	IV
Leiochone leiopygos	-0,1328	1,2772	III
Microcosmus squamiger	-0,1328	1,2772	II
Abra nitida	0,0784	-0,7538	III
Ampelisca sp.	0,0784	-0,7538	I
Amphipoda	0,0784	-0,7538	not assigned
Apseudopsis latreillii	0,0784	-0,7538	III

Taxa	**Axis 1**	**Axis 2**	**AMBI group**
Diopatra sp.	0,0784	-0,7538	II
Euclymene oerstedii	0,0784	-0,7538	I
Gilvossius tyrrhenus	0,0784	-0,7538	III
Hydractiniidae	0,0784	-0,7538	I
Leptosynapta inhaerens	0,0784	-0,7538	I
Nassariidae	0,0784	-0,7538	II
Nereididae	0,0784	-0,7538	not assigned
Notomastus latericeus	0,0784	-0,7538	III
Patella depressa	0,0784	-0,7538	I
Praxillella sp.	0,0784	-0,7538	III
Styela plicata	0,0784	-0,7538	II
Veretillum cynomorium	0,0784	-0,7538	I
TR	0,8307	0,0864	-
CaCO3	0,8768	0,2773	-
sand	0,9676	0,0345	-
Ampeliscidae	1,1839	0,1904	I
Amphinomidae	1,1839	0,1904	not assigned
Aponuphis brementi	1,1839	0,1904	II
Asbjornsenia pygmaea	1,1839	0,1904	I
Axiothella sp.	1,1839	0,1904	I
Bugula neritina	1,1839	0,1904	ignored
Calliostoma zizyphinum	1,1839	0,1904	I
Callochiton septemvalvis	1,1839	0,1904	I
Capitella capitata	1,1839	0,1904	V
Cirolana cranchii	1,1839	0,1904	II
Clibanarius sp.	1,1839	0,1904	II
Clymenella torquata	1,1839	0,1904	I
Corella eumyota	1,1839	0,1904	III
Dasybranchus caducus	1,1839	0,1904	III
Dexamine sp.	1,1839	0,1904	III
Diogenes armatus	1,1839	0,1904	II
Euclymene sp.	1,1839	0,1904	not assigned
Eunice oerstedii	1,1839	0,1904	II
Eunicidae	1,1839	0,1904	II
Gari tellinella	1,1839	0,1904	I
Glycera alba	1,1839	0,1904	IV
Glycera sp.	1,1839	0,1904	II
Glycera tesselata	1,1839	0,1904	II
Glycera tridactyla	1,1839	0,1904	II
Haliclystus tenuis	1,1839	0,1904	not assigned
Hediste sp.	1,1839	0,1904	III
Heteroclymene robusta	1,1839	0,1904	V
Isididae	1,1839	0,1904	not assigned

Taxa	Axis 1	Axis 2	AMBI group
Johnstonia clymenoides	1,1839	0,1904	not assigned
Jujubinus striatus	1,1839	0,1904	I
Leufroyia leufroyi	1,1839	0,1904	not assigned
Loripes orbiculatus	1,1839	0,1904	I
Lucinella divaricata	1,1839	0,1904	I
Lutraria Lutraria	1,1839	0,1904	I
Macomangulus tenuis	1,1839	0,1904	I
Maldane sarsi	1,1839	0,1904	II
Maldanidae	1,1839	0,1904	I
Myriochele sp.	1,1839	0,1904	III
Nassarius argenteus	1,1839	0,1904	not assigned
Nephtys cirrosa	1,1839	0,1904	II
Nephtys sp.	1,1839	0,1904	II
Notomastus profondus	1,1839	0,1904	III
Notomastus sp.	1,1839	0,1904	not assigned
Nucula hanleyi	1,1839	0,1904	I
Onuphis farensis	1,1839	0,1904	II
Orbinia sp.	1,1839	0,1904	I
Owenia fusiformis	1,1839	0,1904	II
Oweniidae	1,1839	0,1904	not assigned
Parvicardium exiguum	1,1839	0,1904	I
Perioculodes sp.	1,1839	0,1904	II
Phyllochaetopterus sp.	1,1839	0,1904	I
Phyllodoce mucosa	1,1839	0,1904	III
Phylo foetida	1,1839	0,1904	I
Phylo sp.	1,1839	0,1904	I
Praxillella affinis	1,1839	0,1904	III
Pseudopolydora paucibranchiata	1,1839	0,1904	IV
Scolelepis neglecta	1,1839	0,1904	III
Scoloplos armiger	1,1839	0,1904	III
Solen marginatus	1,1839	0,1904	I
Spio symphyta	1,1839	0,1904	III
Spiochaetopterus costarum	1,1839	0,1904	I
Spionidae	1,1839	0,1904	III
Spisula solida	1,1839	0,1904	I
Spisula subtruncata	1,1839	0,1904	I
Tricellaria ternata	1,1839	0,1904	ignored
Tritia reticulata	1,1839	0,1904	II
Trochidae	1,1839	0,1904	not assigned
Urothoe grimaldii	1,1839	0,1904	I
Urothoe sp.	1,1839	0,1904	I
Varicorbula gibba	1,1839	0,1904	IV

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3

BIVALVE GUT MICROBIOME RESPONSES TO SEDIMENT CHARACTERISTICS AND ANTHROPOGENIC ACTIVITIES IN *CERASTODERMA EDULE* (LINNAEUS, 1758)

This Chapter is Manuscript Submitted

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3.1 Abstract

Estuaries are highly dynamic ecosystems that are influenced by fluctuating environmental conditions and sediment-associated contamination. These factors can significantly affect microbiomes associated with their hosts. In this study, we investigated the gut microbiome of the bivalve *Cerastoderma edule* by integrating bacteria (16S rRNA) and fungal (ITS) diversity data with sediment physicochemical parameters across three estuarine sites subjected to varying degrees of anthropogenic pressure. Microbial diversity and composition were assessed on a seasonal basis using next-generation sequencing and multivariate analyses. Alpha diversity metrics revealed differences in bacterial communities between sites and seasons, whereas fungal diversity remained relatively stable across locations. This suggests that gut-associated fungi are more resilient to environmental pressures, although specific compositional shifts were observed in the impacted areas, with some taxa showing correlations with trace metal concentrations. Principal Component Analysis (PCA) indicated strong associations between gut microbial composition and sediment parameters, including organic matter, salinity, and trace metals. In particular, the bacterial communities exhibited strong responses to trace metals, such as Hg, Zn, and Pb. Functional inference (FAPROTAX) highlighted the microbial involvement in nitrogen, sulfur, and carbon cycling, indicating potential adaptive responses to contamination. Together, these findings support the utility of the *C. edule* gut microbiome as a sensitive indicator of estuarine environmental status and host–environment interactions. Moreover, they underscored the importance of including both bacterial and fungal components to obtain a more holistic understanding of microbiome-mediated ecosystem functions.

Keywords: Environmental disturbance; 16S rRNA; ITS sequencing; Estuarine ecotoxicology; Host–microbe interactions; Functional prediction

3.2 Introduction

Microbiomes, communities of microorganisms, such as bacteria, viruses, archaea, and fungi that inhabit a given environment, are recognized as indicators of the host's health, playing crucial roles in digestion, metabolism, and immune system regulation (Adamovsky et al., 2018; Appiah et al., 2023; Benjamino et al., 2018; Flint et al., 2012; Glasl et al., 2016; G. M. King et al., 2012; Liu et al., 2016; Pass et al., 2015). Also, microbiome studies in water and soil have provided critical insights into ecosystem health and environmental quality (Karimi et al., 2017; Pass et al., 2015; Qi et al., 2022; Su et al., 2021). Despite the focus on bacterial communities, the fungal community remains an under-explored group, even though they play essential roles in nutrient cycling, organic matter decomposition and host health (Amend et al., 2019; Bahram & Netheway, 2021; Pérez, 2021; Wijayawardene et al., 2022).

Marine benthic macro- and micro-organisms are intrinsically linked and work together to facilitate essential physical and chemical processes, including the carbon and nitrogen cycles, which directly influence sediment quality and the associated infaunal communities (Bertics & Ziebis, 2009; Braeckman et al., 2008; Pennifold & Davis, 2001; Widdicombe et al., 2000; Yazdani Foshtomi et al., 2015; W. Zhang et al., 2019). Each organism hosts its microbiome, shaped by combining intrinsic and extrinsic factors (Akter et al., 2023; Benson et al., 2010; Boto, 2014; Lawson et al., 2022, 2022; Tabrett & Horton, 2020) . The microbiota of bivalves, for instance, is characterized by its diversity and sensitivity to environmental factors, including water temperature, salinity, temporal, seasonality, and contamination (Balbi et al., 2021; Masanja et al., 2023; Pierce & Ward, 2018, 2019; Timmins-Schiffman et al., 2021). These characteristics underscore the microbiome's potential for serving as a valuable sentinel in ecological monitoring (Adamovsky et al., 2018; Benjamino et al., 2018; Milan et al., 2018; Pierce & Ward, 2019). Although bacterial communities have been the focus of extensive research, there is also growing recognition of the importance of fungal communities, particularly within the context of host health and ecosystem function (Bahram & Netherway, 2021; S. Li et al., 2022; Oliveira et al., 2024; Yurgel et al., 2022). However, research into the role of fungi as part of the microbiome in bivalves remains limited. Recent studies have highlighted the importance of fungi in marine microbiomes, suggesting potential interactions with bacterial communities and influences on host physiology (Li et al., 2022). A more comprehensive and fundamental understanding of how the synergistic interactions between benthic macro- and micro-organism communities respond to environmental stressors is essential for better assessment of the anthropogenic impacts on marine ecosystems.

Environmental conditions, including salinity, temperature, and organic and inorganic contaminants, profoundly influence the microbiome's communities structure and functionality (Britt et al., 2020; Gastaldi et al., 2024; Goto & Wallace, 2007; Hochstein et al., 2019; Jeanine Brantschen et al., 2020; Jin et al., 2017; Li et al., 2019; Sun et al., 2012). Research focusing on oysters (*Crassostera* sp.) and mussels (*Mytilus* sp.) has demonstrated the impact of these parameters on microbiome composition in different organs, influencing not only the health of the host organism but also the mortality rates and profitability in aquaculture systems (Arfken et al., 2017; Horodesky et al., 2020; Li et al., 2019; Pierce & Ward, 2019; Schoinas et al., 2023; Timmins-Schiffman et al., 2021). Several studies have demonstrated the impact of inorganic contaminants, particularly trace metals and oil derivatives, on gut microbiome communities, highlighting their sensitivity to anthropogenic pressures (Diaz-Bone & Van De Wiele, 2009, 2010; Walter et al., 2019).

The common cockle (*Cerastoderma edule* (Linnaeus, 1758)) is of considerable economic and ecological importance. In Portugal, it is a commercially valuable species, widely harvested for human consumption and an important component of estuarine ecosystems. Cockles act as suspension feeders and filter organisms, contributing to nutrient cycling and serving as prey for birds and other animals (Jensen, 1985; Kang et al., 1999; Rossi et al., 2008). Their recognized status as bioindicators, their ecological role and their interactions with parasites, sediment microbiota, and environmental factors, suggest a complex microbial association (Albuixech-Martí et al., 2021; Lobo et al., 2010; Paul-Pont et al., 2010; Richard et al., 2022.; Veiga et al., 2019). Although studies specifically on the gut microbiome of *C. edule* are still lacking, its biological and ecological characteristics make it a highly relevant model species for investigating host–microbiome interactions in estuarine environments. This is further supported by research on other bivalves in aquaculture, which highlights the importance of studying gut microbiomes in natural populations to better understand resilience to environmental stressors (Claus et al., 2016; Santibáñez et al., 2022; Timmins-Schiffman et al., 2021).

This study aims to comprehend the effects of anthropogenic activities on the gut microbiome of the species *C. edule*. Employing Next-Generation Sequencing (NGS), the bacterial and fungal composition was assessed concerning sedimentary and environmental parameters across locations characterized by different human activities. The study specifically focused on evaluating the influence of trace metals, and sediment physicochemical characteristics on gut microbiome diversity and function across two different seasons.

3.3 Materials and methods

3.3.1 Study area

The Sado estuary, located in the southwestern Portuguese coast, follows a warm-temperate, semi-diurnal mesotidal regime, with tidal ranges between 0.6 m during neap and 1.6 m during spring tides (Martins et al., 2001). The intertidal zones of the estuary cover approximately ~78 km^2, with salt marshes and intertidal plains making up about 30% of the area (Freitas et al., 2008). It connects to the ocean through a narrow, deep channel (up to 50 m deep) and is partially divided by intertidal sandbanks, which influence estuarine circulation (Carvalho et al., 2001). The river inflow fluctuates seasonally, with most freshwater discharges occurring in winter (Gonçalves et al., 2015).

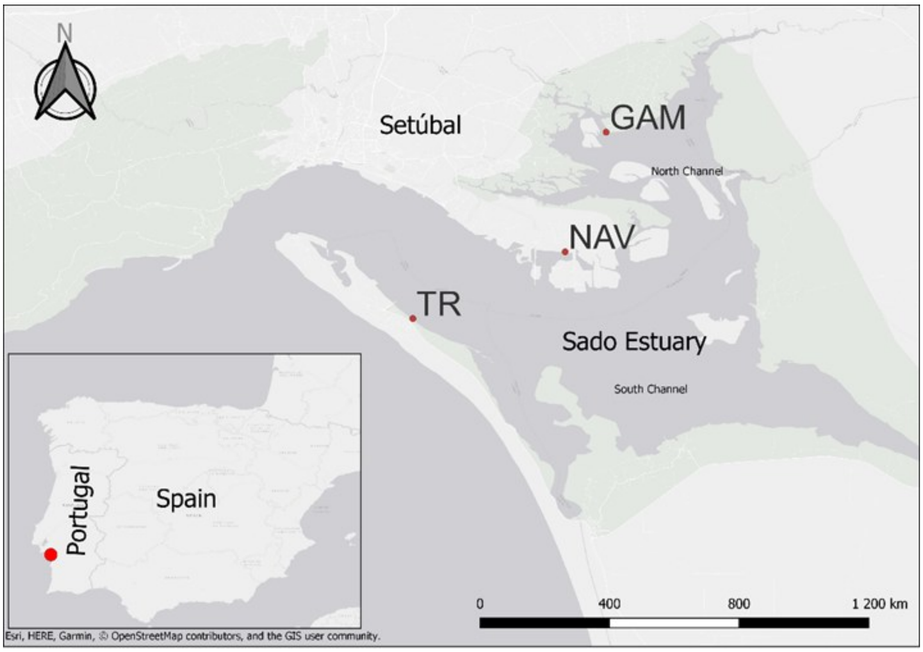

Figure 12 Location of the 3 sampling sites in Sado Estuary: Tróia (TR); Navigator (NAV); Gâmbia (GAM).

Three distant areas were selected, based on their biogeochemistry and adjacent anthropogenic activities (Caeiro et al., 2005): Tróia (TR) in the South Channel, and Gâmbia (GAM) and Navigator (NAV) in the North Channel (Figure 12). The TR area, located on the Tróia peninsula near the Sado Nature Reserve, is highly influenced by tidal hydrodynamics, leading to a shorter water residence time and reduced exposure to contamination from anthropogenic activities (Caeiro et al., 2005; Lobo et al., 2017; Vale et al., 2010). On the other hand, GAM and NAV have lower hydrodynamics, allowing for the retention of fine-grained particles and the accumulation of contaminants and organic matter (Caeiro et al., 2005; Freitas et al., 2008; Quevauviller et al., 1989) . NAV is located near an industrial area and the port of Setúbal. The GAM area is located within the Sado Nature Reserve, close to oyster and aquaculture farms (Cabrita et al., 2020).

3.3.2 Sampling survey

A total of 18 sediment samples were collected from the intertidal zone (three from each study area) during two sampling campaigns using a hand core (20 cm deep 11 cm diameter): November to December 2020 (Aut20) and May to June 2021 (Spr21). These samples were used for the physicochemical characterization of the sediment. Simultaneously, 42 common cockles (*C. edule*) were collected from each area during both campaigns. Specimens were collected by hand at low tide, transported to the laboratory in sterilized, refrigerated boxes, and preserved in 70% ethanol at 4ºC until further processing.

3.3.3 Sediment characterization

Sediment physicochemical characterization followed validated methodologies previously applied in related studies (Marujo Neves et al., 2025; Vieira et al., 2024).

3.3.4 Determination of Organic Matter, Organic Carbon, CaCO3, Grain Size and Environmental Parameters

The organic carbon (C_{org}) content was determined by subtracting the inorganic carbon fraction, following high-temperature combustion using a Leco Truspec micro-analyzer CHNS (Mil-Homens et al., 2023). Before analysis, sediment samples were combusted in a muffle furnace at 400 °C for 3 hours to remove organic matter. Three replicate subsamples (2 mg each) of dried, homogenized sediment were measured before and after combustion, with a precision of 0.03% (dry weight).

The calcium carbonate ($CaCO_3$) content was estimated by multiplying the inorganic carbon percentage by 8.332, assuming the predominant presence of calcite or aragonite. Total organic matter (TOM) content was determined by multiplying the organic carbon (C_{org}) content by the conventional 'Van Bemmelen factor' of 2.5, as adapted for marine sediments (Pribyl, 2010; Shamrikova et al., 2022), which assumes that 40% of the organic matter is organic carbon (Craft et al., 1991; Ouyang & Lee, 2020). Granulometric analysis was conducted to classify sediment fractions into gravel (> 2mm), sand (2 - 0.063 mm), and fine fraction (<0.063 mm), following hydraulic sieving after H_2O_2 treatment and disaggregation with pyrophosphate (Caeiro et al., 2005).

Additionally, environmental parameters were recorded directly from the sediment surface at each sampling site using a VWR pHenomenal® MU 600H multi-parameter probe: temperature, pH, salinity and dissolved oxygen, providing insights into the physico-chemical conditions at the time of collection.

3.3.5 Determination of Trace Metals

Mercury (Hg) concentration was determined using thermal pyrolysis atomic absorption analysis (LECO 254 Advanced Mercury Analyser, AMA). The method involved pyrolyzing 15 mg of freeze-dried and ground sediment fraction lower than 2mm at 750°C in an oxygen atmosphere, capturing the sample on a gold amalgamator, and detecting it via atomic absorption spectrometry (AAS) using a silicon UV diode detector (Costley et al., 2000). Quality control was ensured using the MESS-4 certified reference material (CRM) (National Research Council of Canada), procedural blanks and duplicate samples. Hg concentrations were expressed in mg kg^{-1} of dry weight.

Other trace metals (As, Li, Mn, Ni, Cr, U, Ba, Co, Cu, Zn, Pb and Mo) were analyzed using Inductively Coupled Plasma – Mass Spectrometry (Perkin Elmer NEXIon 2000C) after total digestion in a closed Teflon vessel microwave system (CEM MARS 5). Approximately 100 mg of homogenized freeze-dried and ground sediment (<2 mm fraction) was digested with a mixture of Aqua Regia (36% hydrochloric acid and 60% nitric acid (HNO_3); 3:1 v/v) and hydrofluoric acid. The digestion process consisted of two steps: (1) heating to 180ºC for 25 minutes, followed by a 20-minute hold; (2) neutralization of HF with 10 ml of a 4% boric acid solution at 170°C for 10 minutes. After cooling, the solutions were transferred to 50 mL bottles and diluted with Type 1 ultrapure water. Before analysis, samples were diluted with HNO_3 (2%). Trace metal recovery ranged from 85-115%, ensuring analytical accuracy.

3.3.6 Sample preparation, DNA extraction, amplification and sequencing

The complete intestinal tracts of 42 *C. edule* specimens, seven individuals per site and season, were dissected and immediately stored at -80 °C until further processing. For each site-season combination, the intestinal tracts of the seven individuals were pooled by homogenizing equal amounts of tissue from each specimen. Pooling was employed to obtain a representative profile of the gut microbiome associated with each environmental condition (site and season), minimizing the influence of individual variability and emphasizing broader ecological patterns. The pooled material was then used for total DNA extraction and subsequent sequencing, resulting in a total of six composite samples for DNA-based analysis.

Total genomic DNA from cockles was extracted from 250 mg of each composite sample using the DNeasy PowerLyzer PowerSoil Kit, according to the manufacturer´s protocol (Qiagen). The quality and quantity of the extracted DNA were assessed using a Qubit 4.0 Fluorometer (Invitrogen). The extracted DNA was sequenced to characterize the bacterial and fungal communities present in the cockle gut using NGS. The sequencing targeted the V3-V4 hypervariable regions of the 16S rRNA gene for prokaryotes and the internal transcribed spacer regions (ITS1-ITS5F) of rDNA for fungi. Amplicons were sequenced on the Illumina MiSeq paired-end platform, generating 250 bp paired-end raw reads, according to Novogene (China).

3.3.7 Metabarcoding data analysis

Raw reads were processed using QIIIME1 version 1.9.1 (Bolyen et al., 2019; Caporaso et al., 2010). Chimeras were removed from all the sequences using USEARCH, version 2.16 (Edgar, 2010). The remaining sequences were classified using the Silva database release 132 (Quast et al., 2012; Yilmaz et al., 2014) and assigned to Operational Taxonomic Units (OTUs) with a 97% similarity threshold. In addition, Chao1, Shannon, and Simpson indices were calculated to quantify the microbial diversity within each sample. To compare shared and unique genera among samples, Venn diagrams were generated with the Venny 2.1 tool (Oliveros, 2007).

The Functional Annotation of Prokaryotic Taxa (FAPROTAX) (Louca et al., 2016) was used to analyze the potential functionality of the prokaryotic communities, focusing on key pathways involved in some of the major biogeochemical cycles. Raw reads were deposited into the NCBI Sequence Read Archive (SRA) database under accession number PRJNA1216054.

3.3.8 Statistical analysis

Principal Component Analysis (PCA) was used to compare taxonomic abundances with sediment characteristics across the samples employing OriginPro V.2024 (OriginLab Corporation, Nothampton, MA, USA).

3.4 Results and Discussion

3.4.1 Sediment characterization

Sediment characterization revealed distinct physicochemical profiles across the study sites (Appendix B - Table 10). TR was predominantly sandy (>90%) with high $CaCO_3$ content, low organic matter (OM), low total organic carbon (C_{org}), and lower trace metal concentrations, reflecting limited contaminant retention capacity. The NAV area exhibited the finest grained sediments with higher organic matter, C_{org} values, and Zn, Cu, Pb, and Ni concentrations. Mercury (Hg) levels were also elevated in this area, with several samples showing enrichment factors (EF > 1.5) indicative of anthropogenic input, likely linked to industrial activities in the vicinity (Marujo Neves et al., 2025). These conditions are typical of areas with low hydrodynamic energy, a long residence time of the sediment and a high degree of accumulation (Krumgalz et al., 1992; Mil-Homens et al., 2014). GAM had more heterogeneous grain size, including higher gravel content, and variable metal concentrations, particularly Cr and Ni. In addition, NAV had the lowest spring temperatures and oxygen levels, while TR had the overall pH and oxygen saturation. These sediment characteristics are influenced by the estuarine hydrodynamics and sediment transport regime are consistent with previously described studies of spatial heterogeneity in the Sado estuary (Lobo et al., 2017; Mil-Homens et al., 2013; Mucha et al., 2003; Sroczyńska et al., 2021; Vieira et al., 2024).

3.4.2 Gut microbiome diversity and composition

A total of 580.412 OTUs were obtained from prokaryotic 16S rRNA gene sequencing of the intestinal tracts of *C. edule* collected from the three areas during two seasons (Aut20GAM, Spr21GAM, Aut20NAV Spr21NAV, Aut20TR, and Spr21TR). Alpha diversity indices, including Shannon and Simpson, showed clear differences among areas and seasons (Table 9). In autumn (Aut20), the highest bacterial diversity was observed in NAV (Shannon index:7.689; Simpson index: 0.983), while the lowest was found in TR (2.544 and 0.668). During spring (Spr21), bacterial diversity was lower but followed a similar trend, with GAM and NAV gut samples showing higher diversity than TR.

Table 9 Alpha diversity of the gut microbiome of *Cerastoderma edule* for prokaryotes (16S rRNA gene) and fungi (ITS regions).

Season	Location	16S / ITS	Observed sp.	Shannon	Simpson	Chao1
Aut20	GAM	16S	754	7.162	0.980	764.043
	NAV		1821	7.689	0.983	1.834.735
	TR		844	2.544	0.668	946.047
	GAM	ITS	191	3.649	0.868	194.462
	NAV		230	2.145	0.640	244.500
	TR		242	2.830	0.723	244.786
Spr21	GAM	16S	1497	6.361	0.955	1.585.444
	NAV		1612	5.975	0.898	1.717.547
	TR		781	2.738	0.603	888.201
	GAM	ITS	110	2.882	0.782	116.111
	NAV		128	2.174	0.594	221.500
	TR		240	3.530	0.822	226.833

Overall, samples collected in autumn from NAV and GAM showed greater bacterial diversity than those collected in spring, whereas TR samples were less diverse in autumn than in spring. The Chao1 estimator indicated higher richness in the NAV sample in both seasons (autumn: 1.834.735 and spring: 1.717.547), suggesting that these two samples contained a greater number of species. In contrast, the GAM and TR samples had lower richness, supporting the trend observed in the Shannon and Simpson indices.

A total of 666.771 OTUs were analyzed from ITS sequencing. The richness and evenness of the fungal communities were estimated using Chao1, Shannon and Simpson (Table 9). The most diverse samples were Aut20GAM and Spr21TR, with Shannon and Simpson indices of 3.649 and 0.868 and 3.530 and 0.822, respectively. In contrast, the NAV sample showed a lower diversity of these indices in both seasons.

The main bacterial phyla represented in all the intestinal tracts of the studied cockles were *Mycoplasmatota*, *Pseudomonadota*, *Bacillota* and *Campylobacterota* across all sites and seasons (Figure 13A), possibly related to adjacent human activities. The *Mycoplasmatota* phylum was present in all samples, with an increase in relative abundance during spring. This phylum was more abundant in the gut of *C. edule* samples from TR in both spring and autumn, with relative abundances of 63.62% and 48.31%, respectively. The most abundant genus was *Mycoplasma*, especially in the TR sample during the spring months. This genus is commonly observed in bivalve microbiomes and is often associated with undisturbed environments characterized by low trace metal concentrations, which are consistent with the characteristics of this area, as also noted by Milan et al. (2018) . The sandy sediments and low organic matter content in TR samples may favor the high abundance of this phylum by reducing pollutant retention, thereby supporting a microbial community that is less affected by anthropogenic pressures.

The low abundance (~5%) and near absence of *Mycoplasmatota* in the Aut20NAV and GAM gut samples, respectively, suggests that this phylum may be more sensitive to environmental changes (Cleary et al., 2015; G. M. King et al., 2012). Conversely, the high relative abundance of the *Pseudomonadota*, *Bacillota* and *Actinomycetota* phyla at these sites may indicate a shift in the microbial community to adapt to changing environmental conditions. These phyla include genera known for their metabolic versatility and resilience, which may explain their predominance in areas more affected by human activities.

The phylum *Pseudomonadota* was mainly found in the GAM and NAV samples in autumn, with relative abundances of 42.62% and 40.83%, respectively. In contrast, it was more abundant in the TR sample in spring (24.99%). This phylum often contains metabolically versatile bacteria able to thrive in distinct environments. Its abundance in certain samples suggests favorable growth conditions, possibly related to the availability of resources or less competition (Marín, 2015). The most abundant members of this phylum were *Woeseia* and *Vibrio*. *Woeseia* was particularly abundant in the GAM and NAV samples in autumn, especially in Aut20GAM (24.59%), while in the TR sample, its abundance was less than 1% in both seasons. This genus is typically associated with deep-sea, coastal, or continental environments and may play a role in the cycling of detrital proteins in marine benthic ecosystems (Hoffmann et al., 2020).

On the other hand, *Vibrio* was more abundant in the TR sample, especially during the spring months (Fig. 13B). This genus, often associated with pathogenicity, showed a reduced abundance in disturbed sites such as NAV and GAM sites. However, certain *Vibrio* species may contribute positively by facilitating processes related to host nutrition and biogeochemical cycling (Ceccarelli & Colwell, 2014; Grimes, 2020; Thompson et al., 2004; Vezzulli et al., 2018). This suggests that environmental conditions, such as salinity, influence the role of *Vibrio* in the gut microbiome of *C. edule* (Schmidt et al., 2014). The prevalence of *Vibrio* in the gut microbiome of the TR area suggests a potential role in digestion and nutrient assimilation (Vezzulli et al., 2018). Moreover, *Vibrio* is part of the natural microbiota of marine bivalves and crustaceans and may help to compensate for the limited organic matter present in the environment (W. L. King et al., 2019).

Other genera associated with marine environments include *Halioglobus*, mainly found in the NAV and GAM samples, indicating that these nitrogen-reducing bacteria can thrive in metal-enriched sediments (Han et al., 2019; Zeng et al., 2023). *Candidatus Endoecteinascidia*, identified in the Aut20NAV sample, is associated with the ascidian *Ecteinascidia turbinata* Herdman, 1880, commonly found in industrial settings (Moss et al., 2003). *Candidatus Thiobios*, also observed in this study, is known to have an ectosymbiotic relationship with marine ciliates (Rinke et al., 2009). While their role in the gut of *C. edule* remains unclear, their presence may indicate environmental acquisition or potential functional associations.

The predominance of the phylum *Bacillota* was observed in the GAM and NAV sites during both seasons, while its relative abundance was lower in TR (2.09% and 1.87%, respectively). It is shown that the abundance of *Pseudomonadota* and *Bacillota* in the gut could be associated with organic matter and contaminants, including trace metals. These are typical characteristics of disturbed environments, such as the GAM and NAV sites, which are subject to higher levels of human activities, including aquaculture and industries (Chen et al., 2016; G. M. King et al., 2012).

The phylum *Campylobacterota* showed a similar relative abundance in GAM in both seasons (autumn: 6.34%, spring: 6.83%). However, a significant increase in relative abundance was observed in the spring NAV sample, (32,58%). This increase could be attributed to fine sediments rich in organic matter, which provide ideal conditions for chemoautotrophic bacteria belonging to this phylum. In contrast, the abundance in TR was lower. In addition, studies have linked genera from this phylum, such as *Arcobacter* and *Campylobacter,* to polluted environments (Costa & Iraola, 2019; Cui et al., 2019). Campylobacter species are well-known pathogens and have been frequently detected in edible bivalves such as oysters and mussels, particularly when consumed raw (Jurinović et al., 2022; Lattos et al., 2021; Wilson & Moore, 1996). Although *C. edule* is typically cooked before consumption, the presence of these bacteria in its gut microbiome suggests potential environmental exposure to fecal contamination and highlights the role of filter-feeding bivalves as reservoirs or vectors within estuarine food webs.

The phyla *Actinomycetota* and *Bacteroidota* were predominantly found in NAV samples, while *Verrucomicrobiota* and *Desulfobacterota* were particularly abundant in the gut of *C. edule* from GAM. This pattern may be due to the increased presence of organic matter and contaminants associated with industrial and aquaculture activities. Other, less abundant phyla, including *Fusobacteriota* and *Acidobacteriota*, were present in the spring. *Fusobacteriota* have been associated with the gut microbiota of wild mussel and may reflect adaptation to anaerobic or sediment-rich conditions (Santibáñez et al., 2022). In contrast, *Acidobacteriota* are rarely reported in bivalve guts and are more commonly associated with marine sediments, suggesting a likely environmental or transient origin (Akter et al., 2023; Pierce & Ward, 2019).

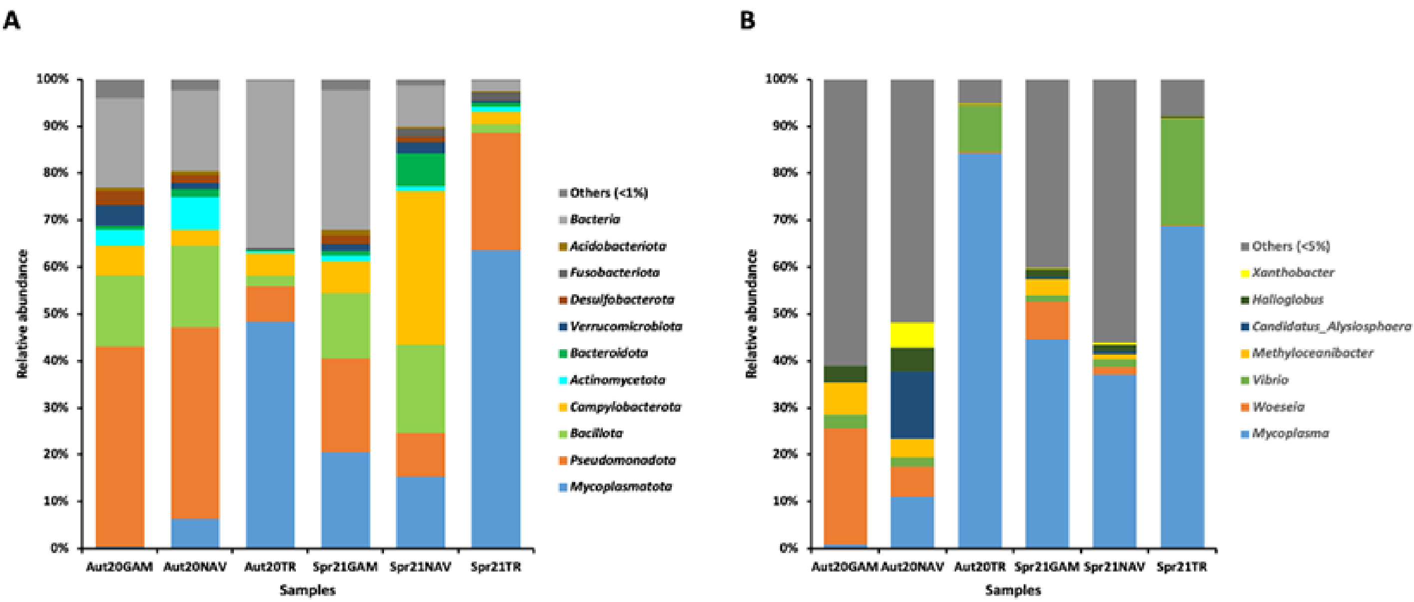

Figure 13 Relative abundances of prokaryotic (16S rRNA gene) phyla (A) and genera (B) in the gut microbiome of *Cerastoderma edule* from three different areas (NAV, GAM and TR) across two different seasons (Aut20 and Spr21).

The Venn diagrams for the bacterial communities showed significant differences in genera between sites and seasons (Figure 14). In spring (Spr21), the percentage of common genera in the gut of *C. edule* increased to 41.2% across all sites, whereas in autumn it decreased to 27.8%. This seasonal variation suggests a shift in bacterial communities, likely influenced by environmental changes such as temperature and salinity, as well as the ongoing influence of human activities. Similar patterns have been reported in a study of the bivalves *Crassostrea virginica* (Gmelin, 1791) and *Mytilus edulis* Linnaeus, 1758, where a high number of shared OTUs were found between the two species (Pierce & Ward, 2019).

In both seasons, cockles from NAV had the highest number of unique genera (autumn: 27.7% and spring: 17.5%), indicating a greater diversity in its bacterial community. This increased diversity is probably due to the industrial activities present at this site. In contrast, the TR sample had fewer unique genera, suggesting a more stable bacterial community. The most distinct samples were GAM and TR, which shared less than 3% of their genera, highlighting significant differences between their bacterial communities.

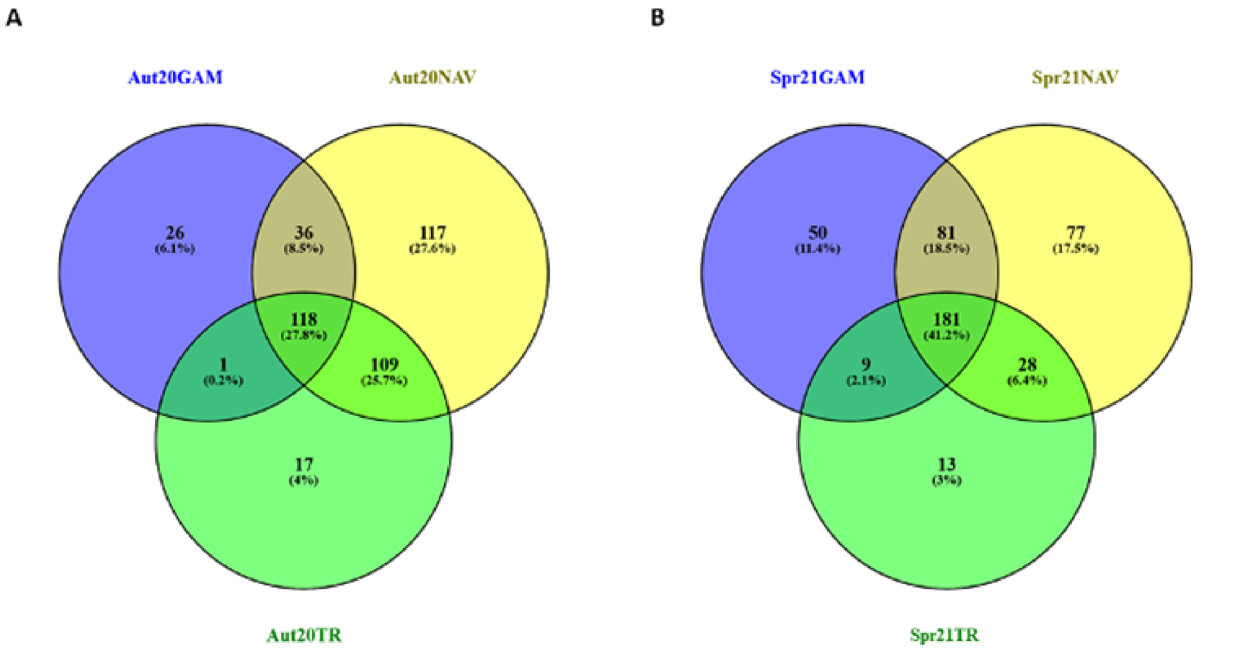

Figure 14 Venn diagram of the genera in the gut microbiome of *C. edule* from three different areas (NAV, GAM and TR) across two different seasons (Aut20 and Spr21). (A) corresponds to Autumn, and (B) to Spring.

The fungal communities present in the gastrointestinal tracts of the cockles from the three sites sampled in different seasons showed similar diversity (Figure 15). However, the abundance of these fungal communities differed between the guts of the cockles from each site, which may indicate the existence of a core microbiome for *C. edule*, although confirmation would require comparison with environmental microbiomes (sediment and water), which were not analyzed in this study.

Most of the fungal sequences showed a dominance of the *Mucoromycota* phylum in gut samples from all sites. This phylum showed adaptability, with an increase in relative abundance observed in gut samples from GAM and NAV from autumn to spring, while a decrease was observed in TR samples during this period. Furthermore, this phylum is recognized for its role in decomposition of organic matter, which is essential for the microbial growth within the trophic networks (Pawłowska et al., 2019). The most abundant genus in all samples was the oleaginous fungus *Umbelopsis,* with relative abundances ranging from 96.54% to 68.12%.

Another abundant phylum, Ascomycota, showed site- and season-specific variations. From autumn to spring a decline was observed in the GAM and NAV samples. In contrast, an increase was observed in TR digestive samples during the same period, suggesting a positive response to environmental stability. This phylum was mainly represented by the genus *Tetracladium* in TR samples and by members of the order *Helotiales*, mainly in GAM samples in autumn and in TR samples in spring.

On the other hand, a higher prevalence of *Basidiomycota* was observed in the NAV gut samples in autumn (Aut20), mainly represented by the genus *Inocybe*. This could be attributed to the increased availability of organic matter and trace metals, which are known to influence the fungal composition in soils (Campos & Tejera, 2011). Similar patterns have also been reported in estuarine sediments, where *Basidiomycota* contributes to organic matter decomposition and nutrient cycling (Amend et al., 2019; Qu et al., 2024). These fungi have also been shown to play roles in microbial interactions and overall ecosystem stability (Amend et al., 2019; Burgaud et al., 2013). Although their role in the gut of *C. edule* has yet to be fully established, the detection of *Basidiomycota* taxa in this study suggests potential involvement in digestive or ecological functions. These findings highlight the importance of including fungal diversity in assessments of estuarine ecosystem health and host resilience.

The phylum *Rozellomycota* includes intracellular parasitic and symbiont fungi, which are mainly found in aquatic environments and sediments (Galand et al., 2016; Ng et al., 2022; Z. Zhang et al., 2021). Furthermore, this group has been associated with environments contaminated by trace metals, suggesting a potential tolerance or adaptation to trace metal-contaminated conditions (Lin et al., 2019). In this study, *Rozellomycota* was predominantly represented in cockles from GAM and NAV during autumn, corresponding to the most affected areas identified. These results demonstrate the susceptibility of fungal communities to both environmental variables and anthropogenic pressures, with the cockle gut from TR exhibiting characteristics of a less disturbed state, providing a baseline for comparison.

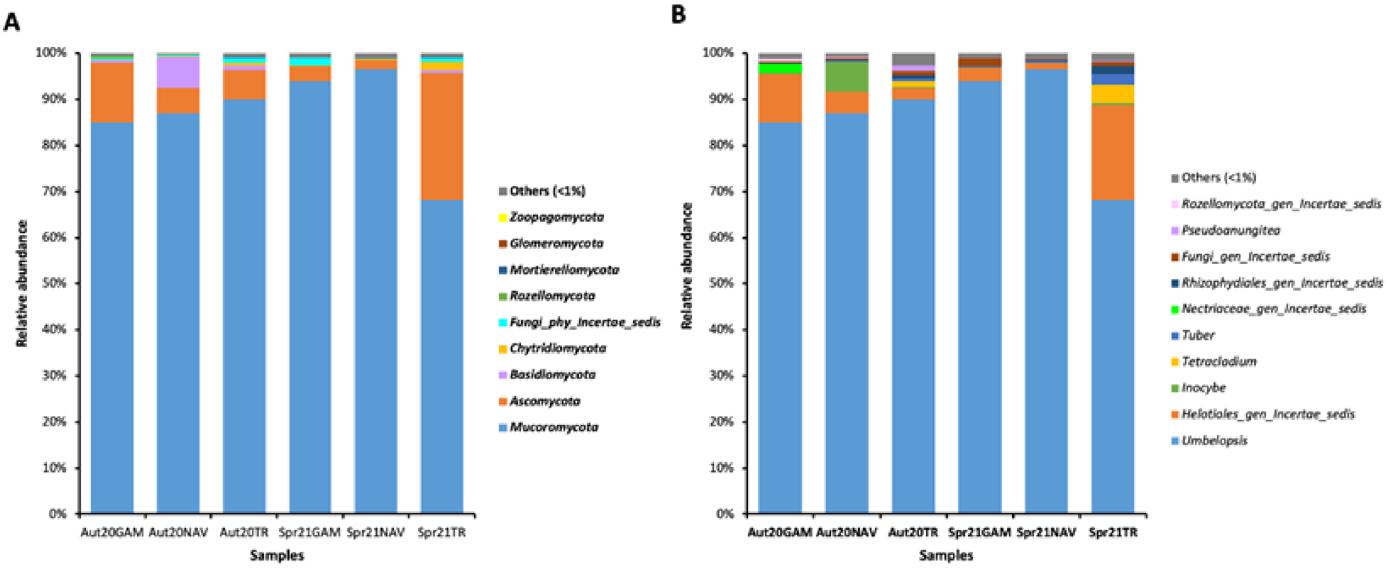

Figure 15 Relative abundances of fungal (ITS) phyla (A) and genera (B) in the gut microbiome of *Cerastoderma edule* from three different areas (NAV, GAM and TR) during two different seasons (Aut20 and Spr21).

Principal component analysis (PCA) was performed at the phylum and genus levels to determine associations between the samples and the microbial composition (Figure 16). At the bacterial phylum level, the TR samples formed a consistent cluster regardless of season and were influenced by the abundance of *Mycoplasmatota*. The remaining samples showed no clustering trend based on site or season and appear to be associated with specific phyla. The Spr21NAV sample is influenced by the abundance of *Bacteroidota* and *Campylobacterota*, the Aut20GAM sample by *Pseudomonadota,* and the Spr21GAM and Aut20NAV samples by *Verrucomicrobiota, Acidobacteriota*, and *Chloroflexota*. A similar pattern was observed at the genus level (Figure 16B), where the samples from the TR site are influenced by the genera *Mycoplasma* and *Vibrio*. On the other hand, the samples tend to cluster more by site, with each site being determined by a specific group of genera.

In contrast to bacterial communities, fungal profiles showed different patterns of structuring across sites and seasons. Fungal communities showed a lower variability in PCA, suggesting greater stability in fungal composition across samples despite environmental variation. This observation may suggest a more conserved ecological role of fungi in the gut or a slower response to environmental changes compared to bacteria. As shown in previous studies, fungal communities in marine invertebrates often show high individual variability and are less influenced by spatial factors (Amend et al., 2019; Gladfelter et al., 2019; Ng et al., 2022), which may explain the patterns observed in this work. The samples from the GAM area, together with the Spr21NAV and Aut20TR samples, cluster closely together, mainly influenced by the phylum

Mucoromycota, which includes saprotrophic fungi capable of degrading complex organic matter (Naranjo-Ortiz & Gabaldón, 2019; Spatafora et al., 2016), which may explain their prevalence in these samples.

On the other hand, the Aut20NAV and Spr21TR samples were more distinct, influenced by the abundance of *Basidiomycota* and *Ascomycota*, respectively, two fungal groups widely recognized for their role in organic matter degradation and diverse ecological interactions. At the genus level*, Umbelopsis* was the most abundant genus across all samples, suggesting a potential role as a core taxon within the gut fungal community. However, specific genera (Figure 16D) from the phylum *Rozellomycota* and the family *Rhizophydiales* influenced the distribution of the Aut20GAM and Spr21TR samples, respectively. These results suggest that fungal communities respond to localized environmental conditions.

PCA analysis showed that the gut samples from TR had the most consistent bacterial communities across seasons and a relatively stable fungal composition compared to other sites. This stability suggests the presence of a core microbiome, as observed in other bivalves such as oysters and mussels (Pierce & Ward, 2019), where certain microbial taxa persist despite environmental fluctuations. This pattern may indicate some degree of host regulation of microbial composition, maintaining functional stability in the gut ecosystem.

In contrast, gut samples from NAV and GAM showed greater shifts, due to fluctuating environmental conditions and human activities. Similar findings have been reported in oysters, where the gut microbial diversity is influenced by transient microbial populations, i.e. microorganisms temporarily introduced into the gut through ingested food particles or environmental exposures that do not establish permanent associations with the host (G. M. King et al., 2012). The observed variation in NAV and GAM may therefore reflect both dietary changes and site-specific environmental stressors.

Fungal communities from NAV and GAM exhibited compositional characteristics that differed from TR, suggesting that environmental stressors may be shaped by their structure in similar ways. Although anthropogenic activities at the NAV and GAM sites differ, they are likely to result in analogous environmental conditions that influence fungal community structure.

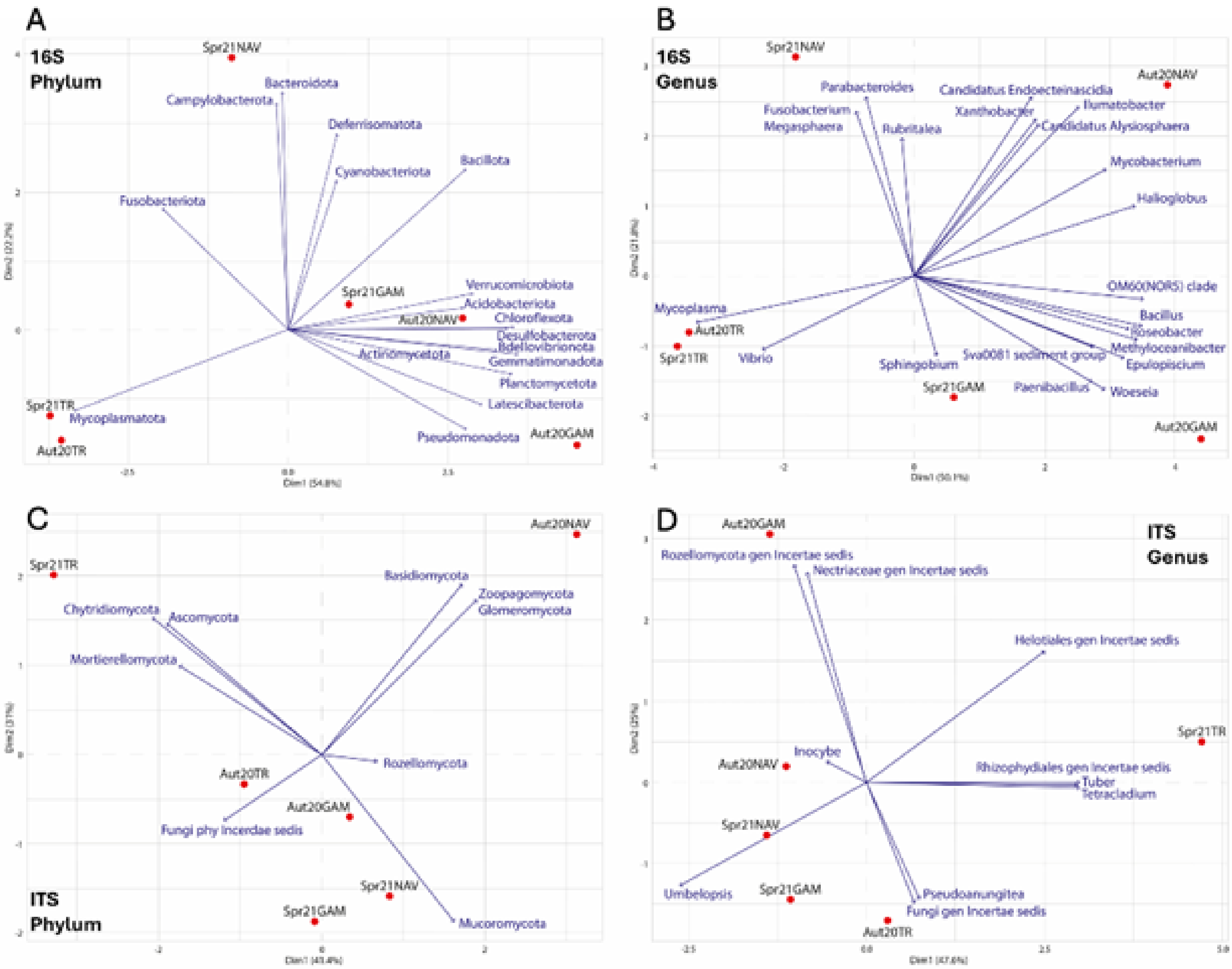

Figure 16 Principal Component Analysis (PCA) of the *C. edule* gut microorganisms at the (A) bacterial phylum (B) bacterial genus (C) fungal phylum and (D) fungal genus taxonomic levels.

3.4.3 Predicted bacterial functional profiles of the Gut microbiome

The predicted functional profile of the *C. edule* gut microbiome, as determined by FAPROTAX, revealed metabolic activities particularly related to nitrogen, sulfur, and carbon cycling (Figure 17), which varied between sampling sites (GAM, NAV and TR) and seasons (Autumn 2020, Spring 2021). Sulfur cycling functions were prominent in all samples, suggesting the presence of sulfur-reducing bacteria, which play a key role in estuarine sediment metabolism. Sulfate-reducing bacteria are widely recognized as key players in sulfur cycling in marine sediments, where they contribute to organic matter degradation and elemental transformations

(Wasmund et al., 2017). Arsenate respiration was particularly high in Aut20GAM and Spr21NAV where arsenic was present in the sediment samples. Although arsenic was not identified as being enriched at these sites, its presence may still influence microbial functional responses. Nitrogen fixation showed the highest relative abundance in TR samples (Figure 17), indicating that nitrogen-fixing bacteria may contribute to nitrogen inputs in this estuarine system. Nitrification and denitrification were enriched in Spr21GAM and Spr21NAV, highlighting nitrogen turnover as a key function in these samples. Ammonification was enriched in Aut20GAM, suggesting increased nitrogen remineralization activity. The variability in nitrogen-related functions suggests that sediment properties and contamination levels strongly influence microbial nitrogen transformations in the gut of *C. edule*. The enhanced denitrification in the Spr21NAV sample may indicate a response to anthropogenic nutrient inputs, probably related to industrial activities in the NAV area. This aligns with findings from estuarine systems where denitrification has been shown to be influenced by trace metal contamination (Magalhães et al., 2007).

Carbon cycling and degradation pathways also showed remarkable site-specific variation. Hydrocarbon and aromatic compound degradation were relatively more active in Spr21NAV and Aut20NAV, respectively, suggesting that the microbial communities in these samples are influenced by organic pollutants, possibly derived from industrial effluents or harbor activities. The enrichment of hydrocarbon degradation pathways in NAV samples suggests that microbial communities in the gut of *C. edule* have genetic potential for hydrocarbon degradation. However, as direct measurements of organic contamination were not performed in this study, further studies using metagenomics or functional assays would be required to confirm this metabolic capability. The presence of hydrocarbon-degrading taxa such as *Pseudomonas*, may indicate an adaptive response to environmental stressors, as similarly observed in fish gut microbiomes exposed to oil contamination (Walter et al., 2019). *Alcanivorax*, a well-known hydrocarbonoclastic bacterium often associated with oil-contaminated environments, has been identified in similar contexts (Cao et al., 2022; Golyshin et al., 2003; Krolicka et al., 2019), highlighting the importance of these microbial assemblages in assessing anthropogenic pressures.

The dominance of oxygenic photoautotrophs in the Spr21TR sample indicates a possible shift of the microbial community towards primary production in these areas. The functional diversity observed in *C. edule* gut microbiomes is consistent with the degree of anthropogenic influence at each site. Samples from the industrially impacted sites NAV (industrial influence) and GAM (aquaculture influence) showed higher abundances of pollutant degradation pathways, suggesting that gut microbiota respond to contamination levels and adapt to environmental stressors. In contrast, TR samples showed greater nitrogen fixation and photoautotrophic activity, suggesting that microbial communities at this site are associated with more stable and less contaminated estuarine conditions. It is important to note that FAPROTAX-based functional predictions rely on taxonomic inference and may not fully capture the metabolic potential of the gut microbiome. Future studies using metagenomics or transcriptomics may provide direct evidence of microbial functional activity. These findings highlight the importance of microbial communities in estuarine health and resilience.

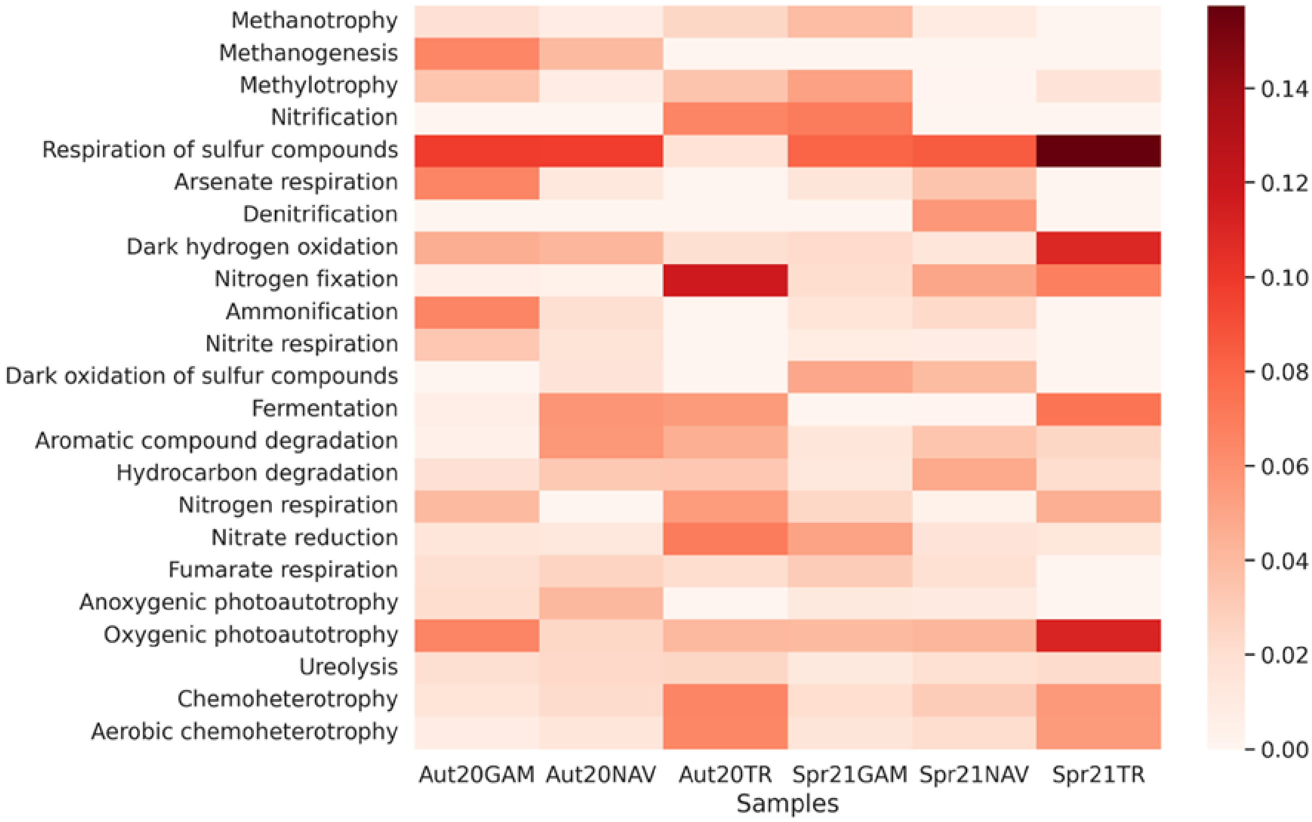

Figure 17 Predicted functional profiles of the *C. edule* gut microbiome from the Sado Estuary. Functional annotations were inferred from 16S rRNA gene sequences using the FAPROTAX database. The color scale represents the relative abundance of each predicted metabolic function, ranging from 0.00 (white) to 0.15 (dark red). Darker shades indicate a higher predicted functional potential for the corresponding metabolic pathway in each sample.

3.4.4 Correlation between sediment properties and *Cerastoderma edule* gut microbial composition

Using sediment characteristics and composition and microbial taxonomy, a PCA analysis revealed a clear separation of *C. edule* gut microbial and fungal communities influenced by environmental variables, sediment characteristics, and trace metals, from different sampling sites (Figure 18). The analysis consistently explained over 70% of the variation at the phylum level, highlighting how site-specific environmental conditions and anthropogenic activities shape the gut microbial diversity.

Bacterial taxa from the gut of cockles collected at NAV were associated with the high concentrations of Hg, Zn and Pb (Figure 18A), consistent with previously reported sediment Enrichment Factors (EF > 1.5) at this site (Marujo Neves et al., 2025). These trace metals, characteristic of industrial activities, may create specific environmental stresses that favor the proliferation of taxa adapted to contaminated conditions, such as members of the phylum *Campylobacterota* (Figure 18A). Thus, our results suggest that the gut microbial communities in cockles from NAV are shaped, at least in part, by the influence of industrial pollution, probably from nearby shipyards and chemical industries (Caeiro et al., 2005; Monteiro et al., 1995). Similarly, fungal taxa identified in the gut microbiome of *C. edule* from the GAM were associated with elevated levels of Ni and Cr (Figure 18B), both of which showed enrichment factors (EFs) exceeding 20. The strong correlation between the fungal phylum *Rozellomycota* with the trace metals is consistent with studies suggesting that this fungal group thrives in metal-enriched sediments (Lu et al., 2022; Rosenqvist et al., 2025; Z. Zhang et al., 2021). This correlation might indicate an ecological preference or tolerance to metal-enriched conditions, although the specific mechanisms, whether intrinsic or host-mediated, remain to be elucidated.

Conversely, the stability observed in the gut microbiome of cockles from TR suggests an adaptation to the natural, less impacted conditions at this site. Sediment samples from the TR area showed a lower metal enrichment for Cr and Ni, reinforcing its classification as a less impacted site. In addition to sediment metal composition, anthropogenic activities such as industrial discharges and aquaculture practices also contribute to shaping the gut microbiome of *C. edule*, by influencing microbial diversity and functional potential. The gut microbiome from TR exhibited lower abundance and diversity of both bacterial and fungal taxa, reflecting minimal anthropogenic disturbance, and was characterized by a higher relative abundance of nitrogen-fixing bacteria and photoautotrophs, suggesting microbial communities adapted to more stable estuarine conditions. This observation is consistent with studies showing that less impacted estuaries tend to maintain more stable and less diverse microbial communities because they are not exposed to the same levels of environmental stressors (Bao et al., 2024; Crump & Bowen, 2024). Studies on the dynamics of microbial community structure in urbanized estuaries highlight the importance of monitoring microbial responses to environmental change over time (Dai et al., 2018; Jeffries et al., 2016; Zhou et al., 2021).

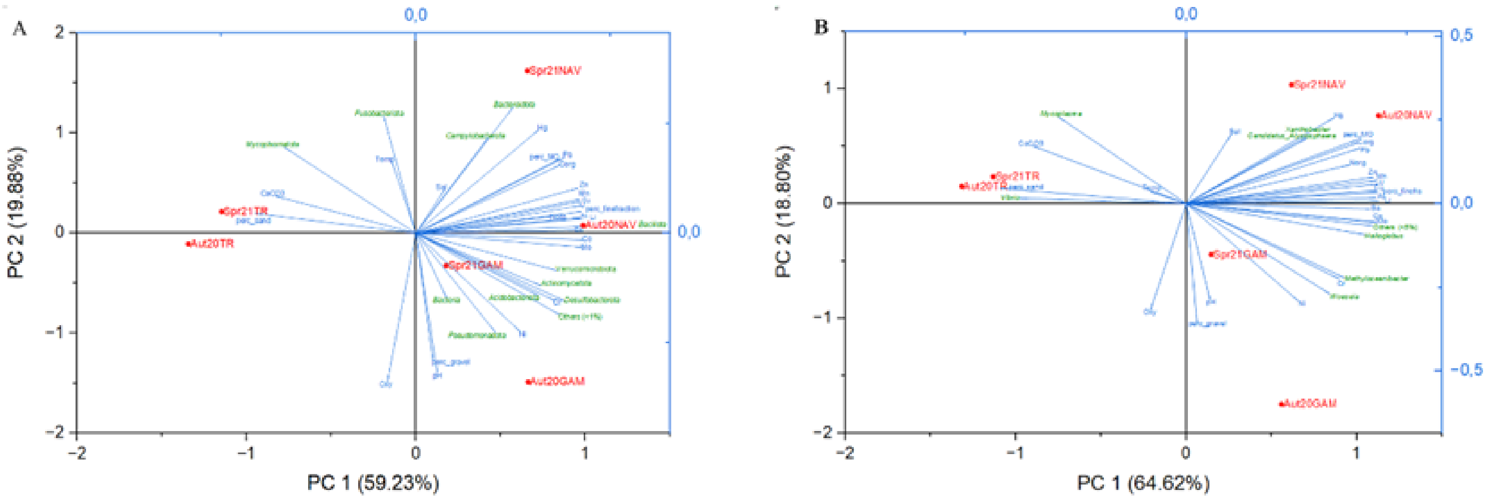

Figure 18 Principal Component Analysis (PCA) of the samples within the microorganisms and the sediment characteristics at the bacterial phylum (A) and fungal phylum(B).

The observed patterns in both the bacterial and fungal communities of the *Cerastoderma edule* gut microbiome illustrate the complex interplay between environmental factors, anthropogenic activities, and seasonal variations. These results highlight the importance of considering both natural and anthropogenic factors in understanding host-associated microbial diversity and its ecological function in estuarine environments. Furthermore, the differential responses of gut bacterial and fungal communities to environmental drivers provide complementary perspectives on host–environment interactions. While bacterial communities appear to be more directly affected by contamination levels, fungal communities show pronounced seasonal variability. This suggests that they may play different ecological roles and have different adaptive strategies within the host gut ecosystem.

3.5 Conclusions

The findings highlight the potential gut microbiome of *C. edule* as a bioindicator for the assessment of estuarine environmental status. The sensitivity of this macroinvertebrate to both natural and anthropogenic sources of contamination underline its utility as a powerful tool for assessing ecosystem disturbance and host health. However, beyond relative abundance, microbial functional traits must also be considered, as higher diversity does not necessarily indicate a balanced system.

Furthermore, investigating the functional roles of these microbial taxa may provide deeper insights into their contributions to host resilience and estuarine ecosystem services. This study highlights the potential of the gut microbiome as a sentinel of environmental and host health, but further research in metatranscriptomics is needed to confirm the microbial activity within the host and functional responses to anthropogenic pressures. In addition, it is essential that future studies include additional contamination sources, such as organic pollutants and emerging contaminants, to fully assess their influence on gut microbiome composition and function. In addition, expanding this research to other estuarine macroinvertebrates and to *C. edule* populations from different geographical locations is likely to be important for conserving biodiversity in estuaries, understanding ecosystem resilience, and supporting sustainable resource management to mitigate human pressures and the effects of climate change.

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B Appendix

Table 10 Measured sediment and water parameters from *Cerastoderma edule* sampling stations across three estuarine sites: TR (low impact), NAV (moderate to high impact), and GAM (intermediate impact) , during Spring 2021 (Spr21) and Autumn 2020 (Aut20). Variables include granulometric fractions (Fine Fraction, Sand, Gravel), organic matter (OM), trace metal concentrations (Li, Mn, Ni, Cr, U, Ba, Mo, Co, Cu, Zn, As, Pb, Hg), carbon and nitrogen content (C_{org}, N_{org}), calcium carbonate ($CaCO_3$), and in situ water (Temperature, Salinity (Sal), Dissolved Oxygen (Oxy), and pH). Values are shown for each sampling point.

Season	Location	Station	FineFraction (%)	Sand (%)	Gravel (%)	OM (%)	Li (mg/kg)	Mn (mg/kg)	Ni (mg/kg)	Cr (mg/kg)	U (mg/kg)	Ba (mg/kg)	Mo (mg/kg)
Aut20	**NAV**	**1**	28,283	66,954	4,764	2,252	27,362	99,691	15,719	26,666	1,900	259,062	3,440
		2	25,130	72,178	2,692	1,786	27,164	99,403	21,687	25,062	1,937	257,906	3,414
		3	52,110	46,360	1,530	3,187	37,845	135,201	18,805	38,064	2,844	250,198	5,899
	GAM	**1**	13,592	68,760	17,647	0,984	25,902	69,018	11,426	17,556	1,538	264,675	1,801
		2	13,556	70,925	15,518	0,828	23,431	89,725	253,115	429,682	1,157	237,014	8,964
		3	20,158	66,540	13,301	0,744	27,645	93,254	15,591	26,343	1,522	254,848	1,527
	TR	**1**	2,015	97,126	0,859	0,393	11,895	43,283	5,916	4,206	0,456	256,235	0,046
		2	0,157	96,007	3,837	0,476	11,442	50,147	9,825	4,571	0,481	223,193	0,013
		3	2,417	92,478	5,106	0,453	12,990	55,318	9,247	5,774	0,430	200,739	0,029
Spr21	**NAV**	**1**	26,487	71,901	1,612	2,311	27,174	94,733	14,097	29,319	1,588	245,579	2,051
		2	20,419	78,038	1,542	1,857	29,602	106,542	16,865	30,185	1,875	263,227	2,150
		3	30,569	66,586	2,845	2,198	30,313	110,401	16,465	29,635	2,189	244,417	4,699
	GAM	**1**	11,924	66,272	21,804	1,035	19,695	59,817	10,207	13,346	1,207	230,801	1,358
		2	15,180	68,197	16,623	0,958	22,991	69,292	11,029	18,613	1,353	241,009	0,877
		3	21,795	65,809	12,396	1,543	29,748	102,558	16,795	28,954	1,586	261,044	1,738
	TR	**1**	3,754	94,913	1,333	0,600	14,031	50,811	7,796	6,719	0,501	262,873	0,064
		2	3,049	84,301	12,650	0,449	13,627	60,949	8,270	6,640	0,599	206,240	0,023
		3	2,144	95,524	2,332	0,470	12,937	46,507	12,357	4,979	0,468	247,236	<LQ

Table 10 - Continuation

Season	*Location*	*Station*	*Co (mg/kg)*	*Cu (mg/kg)*	*Zn (mg/kg)*	*As (mg/kg)*	*Pb (mg/kg)*	*Hg (mg/kg)*	*Corg (%)*	*Norg (%)*	*CaCO3 (%)*	*Temp (°C)*	*Sal*	*Oxy (mg/L)*	*pH*
Aut20	**NAV**	**1**	4,350	33,983	86,455	11,857	25,820	0,292	0,697	0,147	2,199	13,333	15,800	10,493	7,593
		2	4,377	33,094	108,189	14,474	29,785	0,391	0,655	0,226	1,991	13,367	16,000	8,843	8,573
		3	6,114	31,536	92,282	12,647	26,277	0,190	1,116	0,516	2,175	13,967	15,933	9,467	8,847
	GAM	**1**	2,912	9,410	35,100	6,270	18,470	0,066	0,264	0,031	1,236	16,367	14,400	15,517	8,680
		2	7,304	25,585	38,755	7,793	16,277	0,058	0,294	0,040	1,336	17,633	14,933	24,000	8,615
		3	4,633	26,549	51,255	9,784	18,957	0,073	0,390	0,049	1,179	16,467	14,833	14,100	8,502
	TR	**1**	0,702	2,231	5,266	2,024	14,085	0,015	0,103	-0,026	2,890	17,300	14,767	14,567	8,107
		2	0,694	1,627	5,460	1,759	11,883	0,028	0,134	-0,089	3,731	16,933	15,800	14,033	8,167
		3	0,885	3,464	13,008	1,043	11,322	0,033	0,133	-0,125	4,051	16,200	13,567	13,167	7,974
Spr21	**NAV**	**1**	4,570	45,924	127,330	10,837	31,479	0,283	0,564	0,055	1,356	23,133	14,333	4,267	7,496
		2	4,890	43,585	141,015	14,444	35,373	0,468	0,631	0,057	1,696	22,300	16,200	7,000	8,045
		3	4,512	24,086	59,499	8,789	21,206	0,181	0,900	0,082	1,922	26,233	14,367	4,733	8,002
	GAM	**1**	2,366	7,900	28,370	3,671	15,712	0,075	0,169	0,021	1,238	23,633	16,433	8,333	8,473
		2	3,533	12,119	40,682	7,524	16,815	0,070	0,292	0,036	1,437	23,933	15,600	8,233	7,850
		3	5,089	21,450	57,109	9,365	19,728	0,117	0,409	0,044	1,268	23,300	10,100	9,733	8,054
	TR	**1**	1,008	4,416	11,312	0,948	14,635	0,051	0,125	0,014	3,550	20,933	14,867	8,100	8,333
		2	0,938	3,320	9,762	1,390	11,648	0,053	0,104	-0,044	4,571	21,033	15,067	8,700	8,266
		3	0,795	3,291	7,400	0,335	13,149	0,044	0,137	-0,018	3,071	20,267	15,967	9,067	8,368

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4

GUT MICROBIOME RESPONSES IN THE COMMON COCKLE *CERASTODERMA EDULE* TO A TRACE METAL-ENRICHED ESTUARINE SEDIMENT: AN ASSESSMENT OF THE EFFECTS OF MERCURY ENRICHMENT

This Chapter is Manuscript Preparation to Submission

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4.1 Abstract

Mercury (Hg) contamination in estuarine sediments poses a significant ecological threat to benthic organisms, yet its influence on host-associated microbiomes remains poorly understood. This study investigated the short-term effects of Hg-enriched sediments on the gut microbiome of the bivalve *Cerastoderma edule*, characterizing bacterial and fungal communities by 16S rRNA and ITS amplicon sequencing, respectively. Experimental exposure revealed distinct taxonomic and functional shifts in the gut microbiome under Hg stress. Bacterial communities showed greater sensitivity, with marked changes in the relative abundance of the *Desulfobacterota, Campylobacterota* and *Spirochaetota* phyla, groups known for sulfur metabolism and potential involvement in mercury transformation, when exposed to enriched sediments. Functional predictions indicated a decrease in fermentative and phototrophic pathways, and an increase in sulfur- and nitrogen-associated functions, stress-tolerant phenotypes, and mobile genetic elements. Fungal communities, while more taxonomically stable, showed increased alpha diversity and subtle functional guild shifts, including higher relative representation of plant pathogens and ectomycorrhizal fungi in Hg-enriched treatments. These changes suggest that fungal responses, although less pronounced, may still contribute to ecological restructuring under contaminant pressure. Redundancy analysis revealed a clear separation of microbial communities, particularly bacteria, along gradients of sediment Hg concentration, highlighting the role of mercury as a structuring factor. Overall, these results demonstrate the responsiveness of the *C. edule* gut microbiome to sediment-associated Hg contamination and support its potential as a sensitive and integrative biomarker for environmental monitoring in estuarine ecosystems.

Keywords: Functional prediction; Community resilience; Environmental biomonitoring; Estuarine ecotoxicology; Metal-microbiome interactions

4.2 Introduction

Estuarine ecosystems are among the most productive and biodiverse environments on Earth, where freshwater and marine systems converge to create dynamic transitional zones. Their ecological and economic value stems from the diverse biological communities they support and the services they provide, including nursery habitats, nutrient cycling, and fisheries productivity (Barbier et al., 2011; Crump & Bowen, 2024; Elliott & Whitfield, 2011; Fortune et al., 2023; Kennish, 2002; C. C. Vaughn & Hoellein, 2018). However, estuaries are increasingly subjected to anthropogenic pressures such as industrial discharge, urban runoff, aquaculture effluents, and wastewater treatment outflows. These activities contribute to the accumulation of organic and inorganic pollutants, with trace metals being of particular concern due to their persistence, potential toxicity, and natural occurrence in sediments (Caeiro et al., 2005; Chapman & Wang, 2001; Eggleton & Thomas, 2004; Kennish, 2002; Loring, 1991; Piló et al., 2015; Teixeira et al., 2008).

Sediments act as both sink and secondary sources of contaminants, especially in low-energy estuarine systems where hydrodynamic conditions promote the retention of fine particles and organic matter. This promotes the accumulation of trace metals, often bound in forms with high bioavailability, which can subsequently be remobilized if environmental conditions change, such as during sediment resuspension (Fernández-Cadena et al., 2020; Mil-Homens et al., 2014). Sediment composition, such as grain size and organic matter content, plays a crucial role in modulating the mobility and ecological impact of such contaminants (Ferreira et al., 2003; R. Martins et al., 2012). Benthic organisms, particularly infaunal macroinvertebrates, are thus permanently exposed to these contaminants and serve as critical sentinels of sediment quality (Carreira et al., 2013; Lobo et al., 2010; M. Martins et al., 2013).

The gut microbiome is increasingly recognized as a sensitive and integrative indicator of environmental health (Adamovsky et al., 2018; Z. Li et al., 2023; Ribas et al., 2023; Sehnal et al., 2021; Walter et al., 2019). Comprising bacteria, archaea, fungi, protozoa, and viruses, the microbiome contributes to digestion, nutrient cycling, immune regulation, and detoxification (Liu et al., 2021; Offret et al., 2020; Pierce & Ward, 2019). In marine ecosystems, microbial communities associated with host organisms, such as bivalves, respond rapidly to environmental changes, including fluctuations in temperature, salinity, and other physicochemical parameters, making them valuable bioindicators (Offret et al., 2020; Pierce & Ward, 2019). Disruptions in microbiome structure (dysbiosis) have been linked to increased disease susceptibility and reduced host fitness in marine bivalves (Paillard et al., 2022). The adaptability of these microbial communities is essential to host resilience in the face of both natural variability and anthropogenic stressors.

Bivalves, such as the common cockle *Cerastoderma edule*, are particularly well-suited for microbiome-based environmental assessments. As infaunal filter-feeding invertebrates, they are permanently exposed to both water and sediment-associated contaminants. Their widespread distribution, ecological relevance, and commercial value, especially in shellfish harvesting, make them an ideal bioindicator species (Malham et al., 2012; Offret et al., 2020; Paul-Pont et al., 2010; Pierce & Ward, 2019). Moreover, the bivalves gut microbiota reflects both internal physiological and external environmental conditions (Pierce et al., 2019; Offret et al., 2020). Changes in gut microbial communities can provide insights into both environmental quality and organism health. This is especially pertinent given that many bivalves, including oysters, are consumed raw, raising questions about potential human exposure to pathogenic or dysbiosis microbial assemblages (Akter et al., 2023; Lattos et al., 2021; Masanja et al., 2023).

Most microbiome studies in marine invertebrates have focused predominantly on bacterial communities, typically characterized through 16S rRNA gene sequencing (Glasl et al., 2016; Z. Li et al., 2023; Pagán-Jiménez et al., 2019; Suzzi et al., 2022). However, the integration of fungal profiles via Internal Transcribed Spacer (ITS) sequencing has been proposed as a means to enhance the understanding of host-associated microbiota, given that fungi play key roles in organic matter degradation, modulation of host immunity, and inter-microbial interactions (Claus et al., 2016; Hiergeist et al., 2015). Co-analysis of fungal and bacterial taxa can therefore provide a more comprehensive view of microbiome dynamics and functional responses. The simultaneous use of these molecular markers offers a robust framework for assessing the impacts of environmental stressors, including trace metal contamination, on distinct microbial domains and their functional potential within host organisms (Amend et al., 2019; S. Li et al., 2022; Pawlowska, 2024).

This study aimed to evaluate how short-term exposure to environmentally relevant trace metal contamination, including mercury enrichment, affects the bacterial and fungal gut microbiome of the bivalve *Cerastoderma edule*. Specimens were collected from a reference site in the Sado Estuary and exposed in a controlled bioassay to sediments from a known contaminated site (NAV) and to the same sediment spiked with mercury, following OECD guidelines (OECD, 2008). We hypothesized that short-term exposure to contaminated, and mercury-spiked sediments would induce significant shifts in the composition of the gut microbiome of *C. edule*, and that the magnitude and direction of these shifts would differ between bacterial and fungal communities, with specific taxa serving as potential indicators of sediment quality and host health under trace metal stress.

4.3 Materials and methods

4.3.1 Sediment Collection and Spiking Procedure

Surface sediments (0–3 cm) were collected from two locations in the Sado Estuary: (i) the Troia site, used as the uncontaminated sediment control and the source of the test organisms (CT - Control), and (ii) a site adjacent to the industrial area Navigator (NAV), where previous studies have identified significant metal enrichment, particularly mercury (Marujo Neves et al., 2025). Sediments were collected manually using plastic tools previously cleaned with 2% nitric acid (HNO_3) and then rinsed with deionized water to remove any residual contaminants. Samples were stored in opaque sealed containers and transported to the laboratory under dark, temperature-stable conditions to minimize physicochemical alteration.

Upon arrival, sediments from each site were separately homogenized. The mercury-spiking treatment (NAVSp) was prepared by adding an aqueous mercury standard solution to a portion of the NAV sediment, to achieve a final concentration equal to twice the maximum value previously detected in the area (0,288 $mg{\cdot}kg^{-1}$; Marujo Neves et al., 2025). The spiking procedure followed the guidelines of the OECD Test No. 218 (OECD, 2023). The chosen concentration (0.576 $mg{\cdot}kg^{-1}$) corresponds to twice the maximum mercury level historically recorded in the area (Marujo Neves et al., 2025). This level was selected to ensure a detectable biological response within the short-term exposure window, while remaining environmentally relevant to highly contaminated estuarine hotspots. The spiked sediment was thoroughly mixed and incubated for 72 hours without water or light to allow for metal equilibration.

All sediments (CT, NAV, and NAVSp) were then distributed in replicate test containers under identical conditions. After the equilibration period, seawater was added, and the sediments were allowed to stabilize for a further 72 hours before exposure to the organisms. Sediment samples were collected at the beginning (T0) and end (T7) of the bioassay to assess metal concentrations and to evaluate potential geochemical changes during the exposure period.

4.3.2 Experimental Design and Sample Processing

A total of 122 adult cockles (*Cerastoderma edule*) were collected from Tróia (Sado Estuary, Portugal) in September 2024 and transported to the laboratory in containers filled with ambient seawater and sediment from the collection site. Upon arrival, 10 individuals were immediately preserved at -80 °C to establish the field gut baseline condition (Field), and subsamples of soft tissue from these individuals were taken for mercury analysis. The remaining 112 individuals were placed together in a single aerated rectangular tank maintained at 18 ± 1 °C, with a 3 cm layer of sediment from the collection site and acclimated for 15 days. During acclimation, cockles were fed daily with 3 mL of a stock culture of *Tetraselmis* sp. ($\approx 1.05 \times 10^5$ cells·mL^{-1}) per 16 individuals, and partial water changes (~20% of total volume) were carried out every two days.

After acclimation, 16 individuals were randomly selected, measured for shell length (±0.1 mm) and wet weight (±0.01 g), and immediately frozen at –80 °C to serve as the time-zero control group (T0). The remaining 96 cockles were randomly assigned to six experimental units (plastic containers: 14 cm height × 39 cm length × 28 cm width) containing a 3 cm layer of sediment and 10 L of aerated seawater. Three treatments were conducted in duplicate: (i) CT, sediment from the Tróia site; (ii) NAV, sediment from a historically contaminated site near the Navigator industrial area (Marujo Neves et al., 2025); and (iii) NAVSp. Each replicate unit contained 16 cockles.

Throughout the 7-day exposure period, temperature, salinity, and pH were monitored daily, and ammonia levels were measured to ensure animal health. Dissolved oxygen was not directly measured; however, aeration was maintained in all containers to minimize hypoxic conditions. Cockles were fed daily following the same regime as during acclimation. Partial water changes (2 L, ~20% of total volume) were performed every two days. Mortality was recorded, and dead individuals were removed. At the end of exposure (T7), surviving cockles were measured, weighed, and frozen at -80 °C. Dissections were conducted under sterile conditions. Whole guts were removed for microbiome analysis, and muscle tissue from the same individuals was retained for mercury determination. For each treatment replicate, three biological pools were prepared, each consisting of three to five individuals, resulting in a total of six pools per treatment (two aquaria per treatment × three pools per aquarium). For the Field and T0 groups, only three pools were prepared in total. For mercury analysis, approximately 100 mg of muscle tissue per individual was subsampled from each pool and combined. Variations in pool size occurred due to post-exposure mortality. All gut and sediment samples for microbiome analysis were stored at -80 °C until processing.

In addition to gut samples, sediment subsamples from each treatment container were collected at T0 and T7 for microbiome analysis. Water samples for microbiome analysis were collected from the storage tank used for water changes during the bioassay. All sediment and water samples were immediately frozen at -80 °C until DNA extraction (Figure 19).

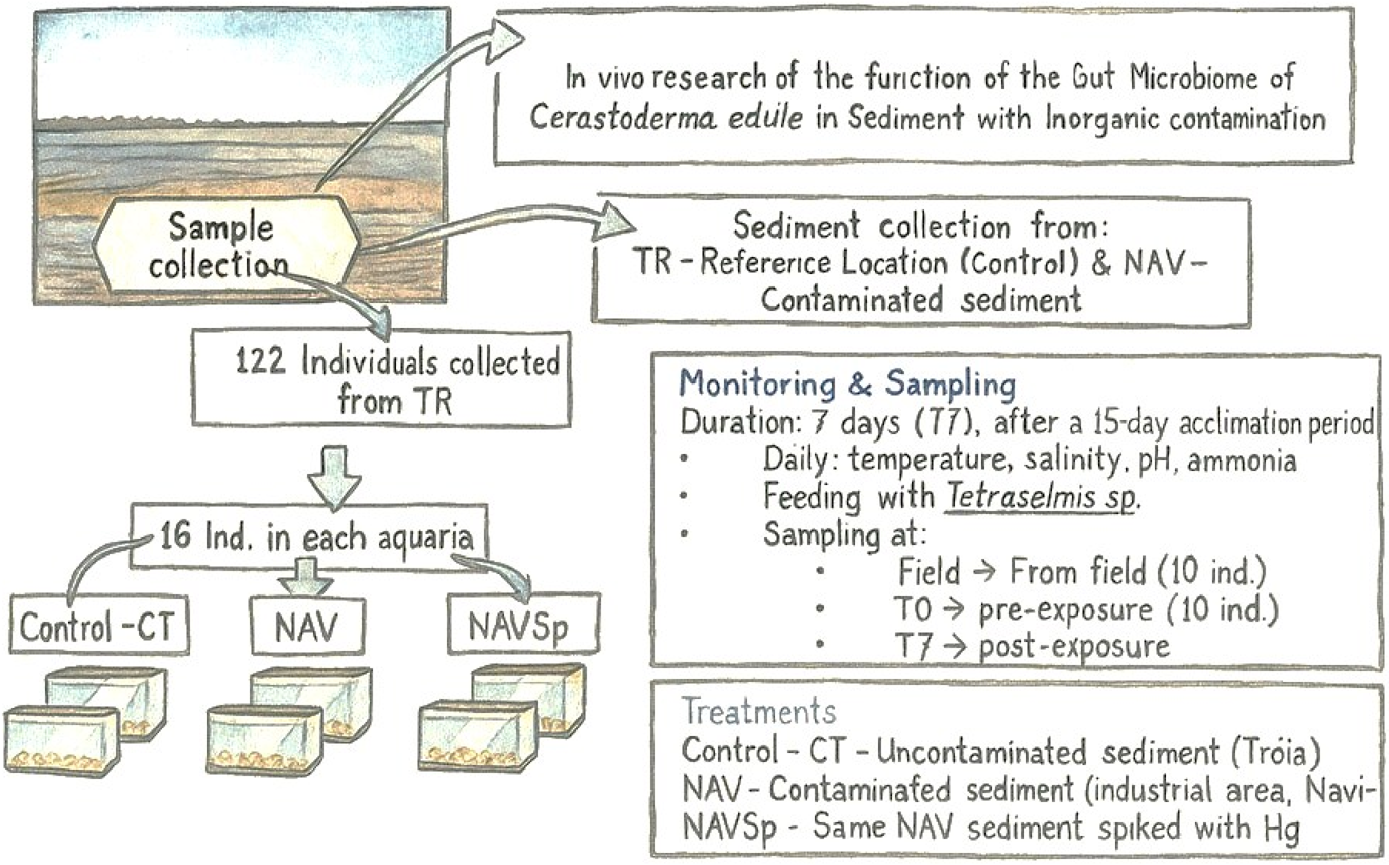


Figure 19 Schematic overview of the experimental design used to evaluate the function of the gut microbiome of *Cerastoderma edule* under exposure to contaminated sediment.

* Created by the author and lightly refined with a generative AI image tool.

4.3.3 Mercury Analysis

Total Hg was measured in dried and ground sediment samples (average weight 15 mg) and in dried and ground cockle muscle tissue (~100 mg per individual, pooled as described in Section 2.2). Analyses were performed using atomic absorption spectrophotometry with a UV detector set at 253.6 nm (LECO AMA 254), with an estimated limit of quantification of 0.07 mg·kg^{-1}. In both matrices, samples were thermally decomposed at 750 °C within a combustion tube under an oxygen flow. The resulting gases were transported over a catalyst using oxygen as carrier gas. Impurities were removed in the catalytic section, and mercury vapour was collected in a gold amalgamator. This amalgam was then heated to 900 °C, releasing the mercury vapour into the detection complex for quantification. Concentrations were calculated using an internal calibration curve (Costley et al., 2000) and expressed in mg·kg^{-1} dry mass. Quality control included procedural blanks, sample duplicates, and certified reference materials (CRM)

MESS-4 (marine sediment CRM for trace metals, certified Hg value: 0.09 ± 0.04 $mg \cdot kg^{-1}$) and ERM-BB422 (fish protein CRM for trace metals, certified Hg value: 0.601 ± 0.030 $mg \cdot kg^{-1}$). Recovery for MESS-4 (n = 4) was 93%. For ERM-BB422, two analyses were performed for each pool of muscle tissue.

4.3.4 Sediment analysis

Gran-size analysis was used to characterize the percentages of three sediment fractions gravel (> 2mm), sand (2 - 0.063 mm), and fine fraction (<0.063 mm), after hydraulic sieving following H2O2 treatment and CALGON disaggregation. Organic matter content was determined in duplicate for each sediment sample using the loss-on-ignition (LOI) method. For this, approximately 200 mg of previously dried sediment was combusted in a muffle furnace at 450 °C for 2 hours, and organic matter percentage was calculated from the weight loss after ignition.

4.3.5 DNA Extraction, Library Preparation, Bioinformatic and Statistical Analyses

Total DNA was extracted from gut, sediment, and water samples using specific commercial kits, following the manufacturers' instructions. For gut samples, the MagBeads FastDNA Kit for Feces (MP Biomedicals, USA) was used. Sediment samples were processed using the NzySoil gDNA Isolation Kit (NZYTech, Portugal), and water samples were extracted using the DNeasy PowerWater Kit (QIAGEN, Germany).

Amplification of the V3–V4 region of the bacterial 16S rRNA gene was performed using the universal primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Lu et al., 2015). Fungal ITS1 region amplification employed the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3')(Gardes & Bruns, 1993). Amplified products were verified via fragment analysis on the LabChip GX system (Perkin Elmer, USA).

Library preparation and sequencing were conducted by Biomarker Technologies Co., Ltd. (Beijing, China). Sequencing was performed on the Illumina NovaSeq 6000 platform in paired-end mode (2 × 250 bp). Bioinformatic analyses, including quality filtering, OTU clustering, and taxonomic assignment, were carried out using the BMKCloud platform (www.biocloud.net). Taxonomic classification was performed using appropriate reference databases (SILVA for 16S and UNITE for ITS).

Bacterial and fungal datasets were processed in parallel and examined independently and jointly to compare taxonomic and functional patterns across domains, including possible indirect fungal responses mediated by bacterial shifts. Alpha diversity (Shannon, Simpson, ACE, Chao1) and beta diversity (Bray–Curtis) were calculated from rarefied OTU tables (genus level), ensuring inter-sample comparability. Community structure was visualized via PCoA and tested using PERMANOVA. RDA was performed at the phylum level for bacterial (16S) and fungal (ITS) datasets, constrained by sediment Hg concentration. Functional potential of bacterial communities was inferred using FAPROTAX (Louca et al., 2016) and BugBase (Ward et al., 2017), and fungal guilds were predicted with FUNGuild (Nguyen et al., 2016).

4.4 Results

4.4.1 Sediment Characterization

At the start of the experiment (T0, immediately prior to organism exposure), control treatment sediment (CT) was composed of 92.7% sand, 5.3% gravel, and 2.0% fine fraction (<0.063 mm), with organic matter content of 0.0035%. After seven days (T7), the control sediment contained 87.1% sand, 10.8% gravel, and 2.2% fine fraction, while organic matter decreased to 0.0006%. These small differences are attributable to natural sediment heterogeneity rather than treatment effects. Sediments from the historically contaminated site adjacent to the Navigator industrial complex (NAV) at T0 consisted of 88.5% sand, no detectable gravel, and 11.5% fine fraction, with organic matter of 0.0031%. After seven days (T7), NAV sediment composition was 88.3% sand, 0.8% gravel, and 10.9% fine fraction, with organic matter slightly higher at 0.0035%. For the mercury-spiked NAV sediment (NAVSp), grain size was assessed only at the end of exposure (T7), showing 86.9% sand, 1.4% gravel, and 11.7% fine fraction, with organic matter of 0.0028%. Overall, grain size distributions across treatments remained stable during the 7-day exposure, with minor changes reflecting the heterogeneous nature of estuarine sediments.

4.4.2 Mercury in Sediments

Mercury concentrations in the sediments followed the expected contamination gradient. At the start of the experiment (T0), concentrations were 0.10 mg·kg^{-1} in the control sediment (CT) and 0.08 mg·kg^{-1} in the historically contaminated sediment from the Navigator site (NAV). The NAVSp treatment was prepared by spiking NAV sediment with mercury before exposure, as described in the Methods, and was therefore only analyzed at T7. At the end of the 7-day exposure (T7), mercury concentrations were highest in NAVSp (0.24 mg·kg^{-1}), followed by NAV (0.07 mg·kg^{-1}) and CT (0.02 mg·kg^{-1}). Differences between T0 and T7 in CT and NAV remained within the range of natural variability.

4.4.3 Mercury in bivalve

Mercury concentrations in *C. edule* spanned nearly one order of magnitude, from 0.075 to 0.79 mg·kg^{-1} dry weight (Fig. 1). Field-collected bivalves presented the lowest concentrations (median = 0.15 mg·kg^{-1}), followed by T0 (median = 0.27 mg·kg^{-1}) and control (CT) groups (median = 0.31 mg·kg^{-1}). The highest values were recorded in NAV (median = 0.35 mg·kg^{-1}) and NAVSp (median = 0.49 mg·kg^{-1}) treatments. Statistical analysis ($p < 0.05$) indicated significant differences among most groups, except between T0 and CT. Significant differences are marked in Figure 20. The elevated mercury levels in NAV and NAVSp specimens reflect the higher mercury content of the sediments from which these treatments were derived, with NAVSp showing the greatest bioaccumulation due to the deliberate spiking of NAV sediment.

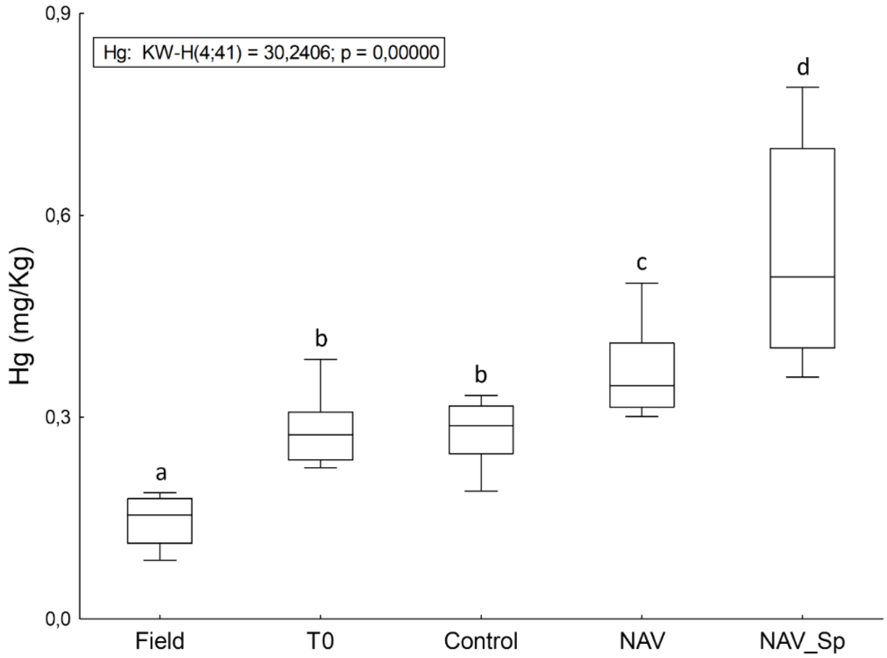


Figure 20 - Mercury concentrations (mg/kg dry weight) in *Cerastoderma edule* individuals exposed to different sediment treatments. Treatments include field-collected specimens (Field), baseline group prior to exposure (T0), control sediment at day 7 (Control), Navigator sediment at day 7 (NAV), and spiked Navigator sediment at day 7 (NAVSp). Boxes represent interquartile ranges, horizontal lines indicate medians, and whiskers show the full data range. Statistical differences among treatments were assessed using the Kruskal–Wallis test ($H = 30.24$, $df = 4$, $p < 0.001$).

4.4.4 Gut and Environmental Microbiome Composition

4.4.4.1 Alpha diversity

4.4.4.1.1 16S ribosomal RNA (rRNA) gene sequencing – Bacterial communities

Environmental samples from seawater (T0W) and sediments (T0SCT, T7SCT, T7SNAV) exhibited high bacterial richness, with OTU counts at the kingdom level ranging from approximately 50.000 to 68.000. Sediments consistently displayed higher diversity than seawater, highlighting the complexity of benthic microbial communities. Full alpha diversity results for environmental samples are provided in Appendix C in Figure 29.

In bivalve gut samples (Field, T0, Control, NAV, NAVSp), OTU counts were generally lower than in sediments but showed substantial variability among treatments. Field samples exhibited ACE values between 200 and 300 and Shannon indices between 3 and . T0 samples ranged from 53.746 to 67.255 OTUs, with ACE values between 250 and 340 and Shannon indices from 4.3 to 6.2. Control samples displayed ACE values from 198 to 442 and Shannon indices between 3.6 and 5.7. NAV and NAVSp samples showed comparable diversity across all metrics, with Simpson indices from 0.7 to 0.9, Shannon indices between 3.0 and 5.0, and Chao1 and ACE values between 200 and 350 (Figure 21).

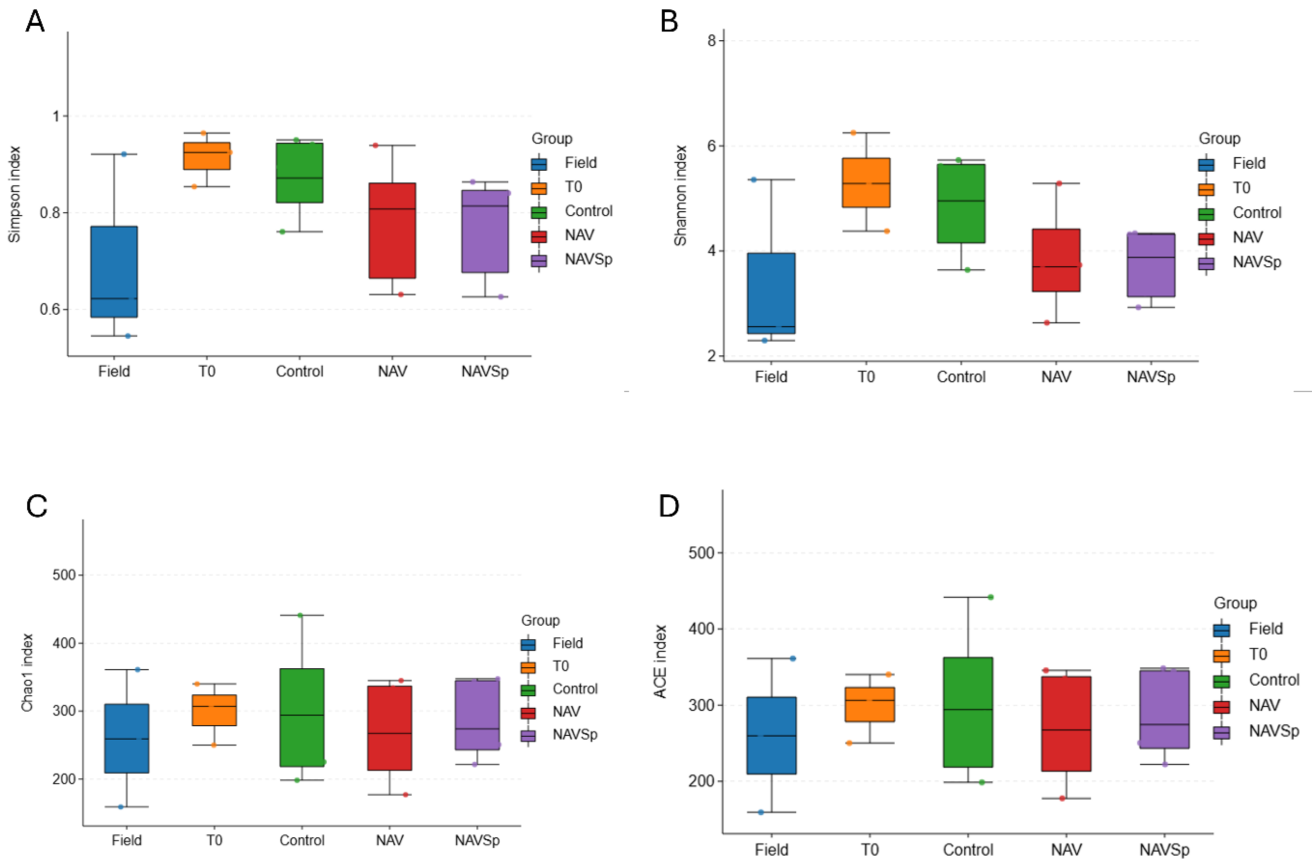


Figure 21 - Alpha diversity indices of bacterial communities associated with *Cerastoderma edule* gut samples across experimental groups. Box plots show Simpson, Shannon, Chao1, and ACE indices (A–D), calculated at the genus level from 16S rRNA gene sequencing data. Indices were derived from rarefied OTU tables normalized by sequencing depth. Comparisons between groups were performed using Student's T-tests.

4.4.4.1.2 Internal Transcribed Spacer (ITS) sequencing – Fungal communities

Internal Transcribed Spacer (ITS) sequencing was used to characterize fungal communities. Environmental samples, particularly sediments, exhibited higher OTU counts compared to gut samples. Sediment samples from the control treatment at T0 and T7 (T0SCT, T7SCT) and from the Navigator site at T7 (T7SNAV) showed OTU counts ranging from approximately 27.000 to 67.000 at the kingdom level. Seawater samples (T0W) displayed slightly lower richness, with counts between 41.000 and 45.000 OTUs. Alpha diversity indices (ACE, Chao1, Simpson, Shannon) varied across environmental sample types. Sediment samples exhibited ACE and Chao1 values exceeding 2.800 and Shannon indices above 5.4 (Appendix C Figure 30). Seawater samples showed a similar pattern, with ACE and Chao1 values approaching 2.900 and Shannon indices near 5.4 (Appendix C Figure 30)

Gut samples (Field, T0, Control, NAV, NAVSp) exhibited lower fungal richness, with OTU counts ranging from approximately 38.000 to 51.000. Diversity indices for gut samples ranged from ACE and Chao1 values of 2,600–3,000 to Shannon indices between 5.3 and 5.6. Among gut samples, NAV and NAVSp showed higher median richness estimates and greater variability than Field, T0, and Control groups (Figure 22). Specifically, NAV and NAVSp Chao1 and ACE values ranged from approximately 200 to 350, with elevated outliers, whereas the remaining groups had restricted ranges, with medians tending towards 280–300.

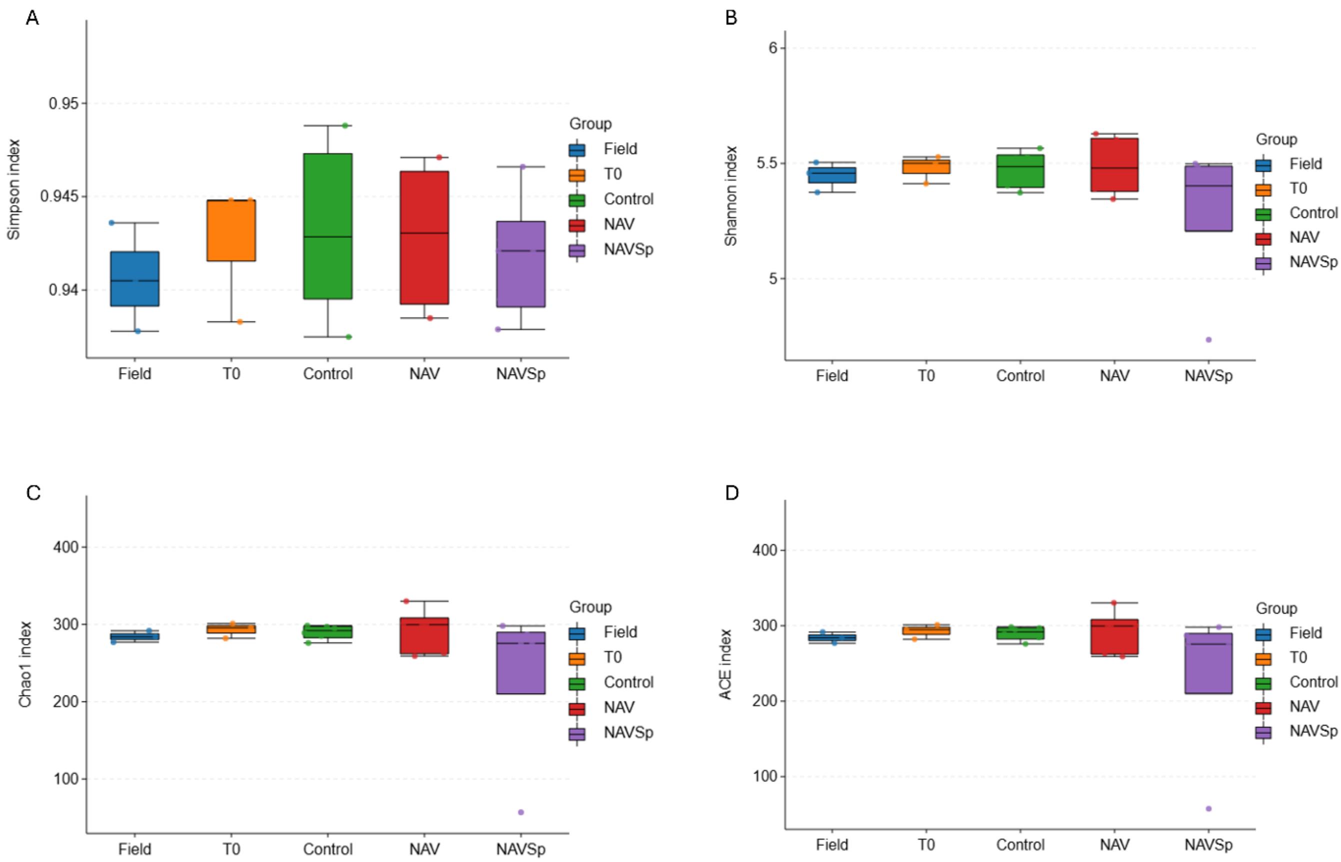


Figure 22 - Alpha diversity of fungal communities in the gut microbiota of *Cerastoderma edule* across experimental treatments. Diversity was assessed at the genus level using four indices: (A) Simpson, (B) Shannon, (C) Chao1, and (D) abundance-based coverage estimator (ACE), based on internal transcribed spacer (ITS) region sequencing. Values were derived from rarefied datasets to ensure consistent sequencing depth across samples. Comparisons between groups were performed using Student's T-test

4.4.4.2 Beta diversity

Principal Coordinates Analysis (PCoA) was used to assess differences in microbial community composition across all sample types (gut, sediment, and seawater), separately for bacterial (16S) and fungal (ITS) datasets. The PCoA based on 16S rRNA gene data (Appendix C Figure 31) showed a clear separation among gut, sediment, and seawater samples (PERMANOVA, R^2 = 0.76, p = 0.001), indicating strong differentiation of bacterial communities between environmental compartments. In contrast, the ITS-based PCoA (Appendix C Figure 31) indicated considerable overlap between sample types, with no statistically significant differences (PERMANOVA, R^2 = 0.233, p = 0.598).

In gut samples only (Fig. 4), bacterial communities (Figure 23) showed moderate clustering by treatment, with partial separation between the mercury-spiked sediment treatment (NAVSp) and the control sediment group (Control), and one NAV sample appearing as an outlier. Fungal communities (Figure 23) exhibited substantial overlap between treatments, with one NAVSp sample located outside the main cluster. PERMANOVA results indicated significant treatment effects for bacteria (R^2 = 0.349, p = 0.001) but not for fungi (R^2 = 0.138, p = 0.848).

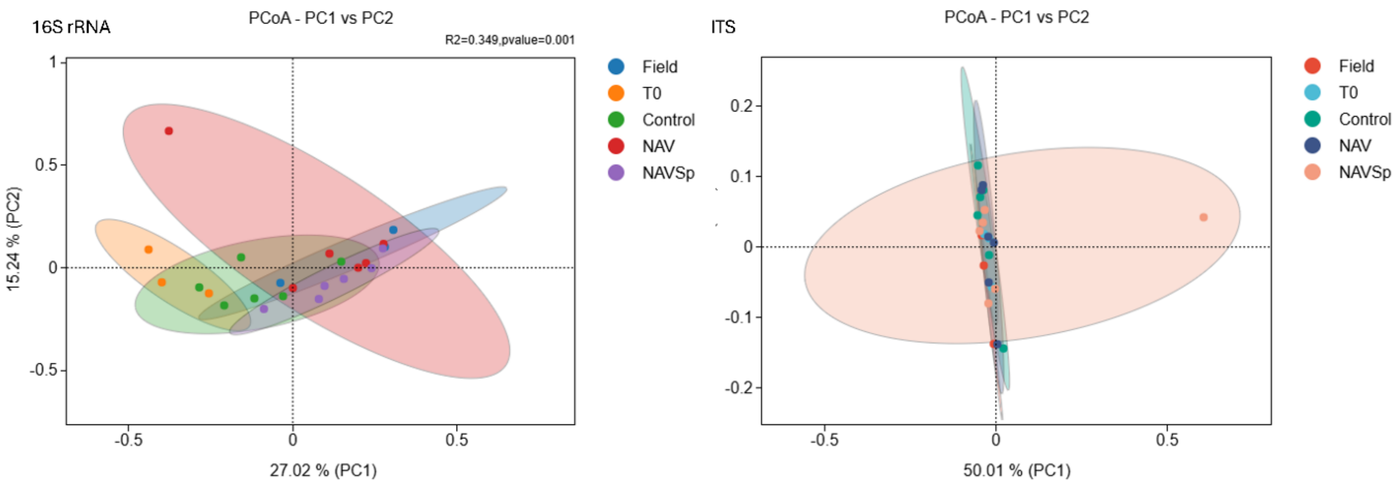


Figure 23 - Principal Coordinates Analysis (PCoA) of gut microbiome communities (16S and ITS) in *Cerastoderma edule* across experimental treatments. PCoA was performed using Bray–Curtis dissimilarity matrices at the genus level. Values indicate PERMANOVA results for each dataset. “Field” refers to bivalves collected directly from the field prior to acclimation, “T0” to bivalves sampled after acclimation but before exposure, “Control” to bivalves exposed for 7 days to clean sediment, and “NAV” and “NAVSp” to bivalves exposed for 7 days to contaminated or mercury-spiked sediment, respectively.

4.4.4.3 Taxonomic profiles

Taxonomic composition profiles of bacterial (16S) and fungal (ITS) communities across all sample types (water, sediment, and gut) are presented in Appendix C, Figure 33. Bacterial communities were dominated by *Proteobacteria, Bacteroidota*, and *Firmicutes*, with clear differences in relative abundance between environmental (sediment and water) and gut samples. Fungal communities were largely composed of *Ascomycota* and *Basidiomycota*, with relatively similar phylum-level profiles across sample types.

The gut microbiome of *C. edule* was consistently dominated by *Proteobacteria* and *Firmicutes* across all treatments. Relative abundance of *Bacteroidota*, *Cyanobacteria*, and *Actinobacteriota* varied between treatments (e.g., *Bacteroidota*: Field 19.6%, NAVSp 4.6%; *Cyanobacteria*: Field 5.2%, NAVSp 13.8%; *Actinobacteriota*: Field 1.9%, NAVSp 1.6%) (Fig. 5A). *Spirochaetota, Campylobacterota, Desulfobacterota*, and *Fusobacteriota* were detected in all gut samples. *Campylobacterota* was more abundant in NAV (0.96%) and NAVSp (1.01%) compared to T0 (0.03%), while *Desulfobacterota* increased from 0.28% (Field) to 1.27% (NAVSp). *Fusobacteriota* ranged from 0.29% (Field) to 0.57% (NAV) and 0.45% (NAVSp). At the genus level, Mycoplasma, *Shewanella*, and *Endozoicomonas* were the most abundant taxa in gut samples, with shifts in prevalence between treatments (Figure 24B). *Roseimarinus* was more abundant in T0, while *Vibrio* occurred at higher relative abundance in NAV and NAVSp. *Unclassified_Nostocaceae* was elevated in T0, and *Unclassified_Cyanobacteriales* and *Pseudophaeobacter* were enriched in NAVSp.

Fungal gut communities across treatments were dominated by *Ascomycota, Basidiomycota,* and *Rozellomycota* (Figure 24C). *Ascomycota* was the most prevalent phylum, with similar relative abundance across treatments: Field (68.4%), T0 (67.5%), CT (67.4%), NAV (67.9%), and NAVSp (66.8%). *Basidiomycota* ranged from 10.1% (Field) to 11.9% (NAVSp), while *Rozellomycota* decreased slightly in NAVSp (4.9%) compared to Field (7.2%) and T0 (6.4%). At the genus level, NAVSp was characterised by higher relative abundance of unclassified *Fungi* (13.3%), *Fusarium* (11.9%), and *Penicillium* (5.5%) compared to Field and T0 (Figure 24D). *Clonostachys* (1.1%) and *Plectosphaerella* (1.3%) were also more abundant in NAVSp. In NAV, *Trichoderma* 2.4%) and *Talaromyces* (2.6%) showed values comparable to T0 and higher than Field.

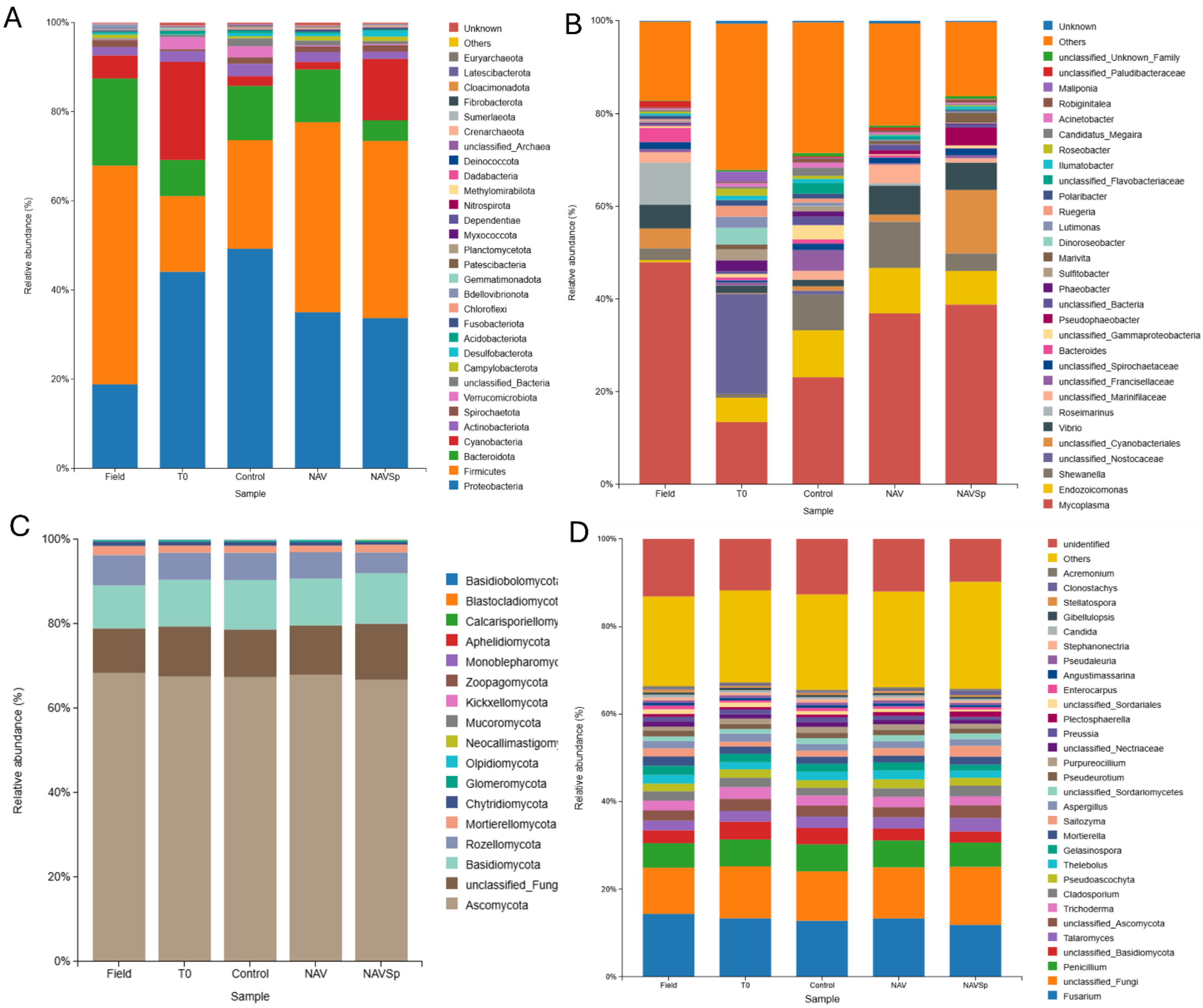


Figure 24 - Taxonomic composition of the bacterial (16S rRNA) and fungal (ITS) gut microbiomes of *Cerastoderma edule* across treatments. Bacterial (A) and fungal (C) community composition at the phylum level. Bacterial (B) and fungal (D) community composition at the genus level, only taxa with >1% relative abundance is shown.

4.4.5 RDA constrained by sediment mercury concentration

Redundancy Analysis (RDA) constrained by sediment mercury concentration at the phylum level revealed distinct ordination patterns among the gut microbial communities of *C. edule* across treatments (Figure 25). RDA1, representing the primary axis of variance constrained by experimental treatments, differentiated the groups clearly. Samples from the mercury-spiked treatment (NAVSp) had the highest positive RDA1 values, corresponding to higher relative abundance of *Desulfobacterota, Campylobacterota*, and *Spirochaetota* compared with other treatments. In contrast, negative RDA1 values were associated with *Bacteroidota* and *Actinobacteriota* in both Control and Field samples. T0 samples also displayed negative RDA1 scores, grouping more closely with Field and Control communities.

Analysis of ITS phylum-level data revealed treatment-related variation with more subtle clustering patterns (Figure 25). Field and T0 samples were generally positioned towards positive RDA1 values, correlating with *Mortierellomycota*, whereas NAVSp samples displayed a broader range of scores, including strongly negative values associated with *Glomeromycota* and *Mucoromycota.* Control samples occupied an intermediate position, with no consistent trend along the RDA1 axis

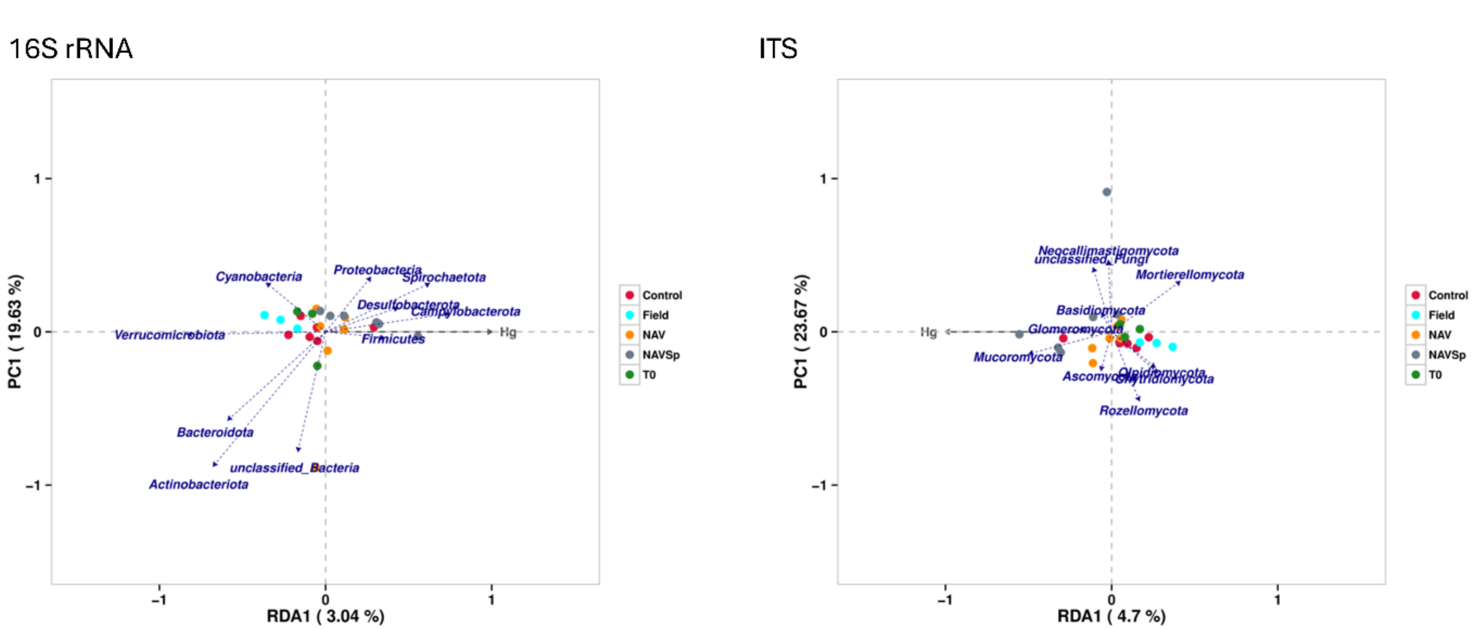


Figure 25 - Redundancy Analysis (RDA) of gut microbiome communities in *Cerastoderma edule* across treatments, constrained by sediment mercury concentration. Analyses were performed separately for bacterial (16S rRNA gene) and fungal (ITS rDNA) datasets at the phylum level. Field (blue), T0 (green), Control (red), NAV (orange), and NAVSp (grey). Arrows indicate the direction and strength of correlation of each phylum with the ordination axes.

4.4.6 Functional Inference from Microbiome Community Profiles

Functional prediction analysis using FAPROTAX was performed to compare the mercury-spiked treatment (NAVSp) with the other experimental groups, in order to identify functions most affected by Hg enrichment (Figure 26). Chemoheterotrophy and aerobic chemoheterotrophy were the most abundant predicted functions in all groups, with NAVSp values (29.2%

and 28.9%, respectively) comparable to NAV (35.0% and 29.9%) and Control (30.6% and 29.0%). In contrast, NAVSp showed reduced proportions of functions related to fermentation (0.3% vs. 5.1% in NAV), animal parasitism or symbiosis (0.9% vs. 4.5% in Control), and phototrophic activity, including photoautotrophy and oxygenic photoautotrophy (7.0% vs. 8.2% in T0). Cyanobacterial metabolism was also lower in NAVSp (6.9%) compared to T0 (8.2%). Functions associated with nitrate respiration (2.0% vs. 3.9% in Control), and dark sulfur oxidation (0.1% vs. 0.9% in Control) were reduced in NAVSp. Conversely, nitrogen fixation (0.3%) and fumarate respiration (0.2%) were slightly higher in NAVSp compared to Field (both 0%) and T0 (0.1% and 0%, respectively).

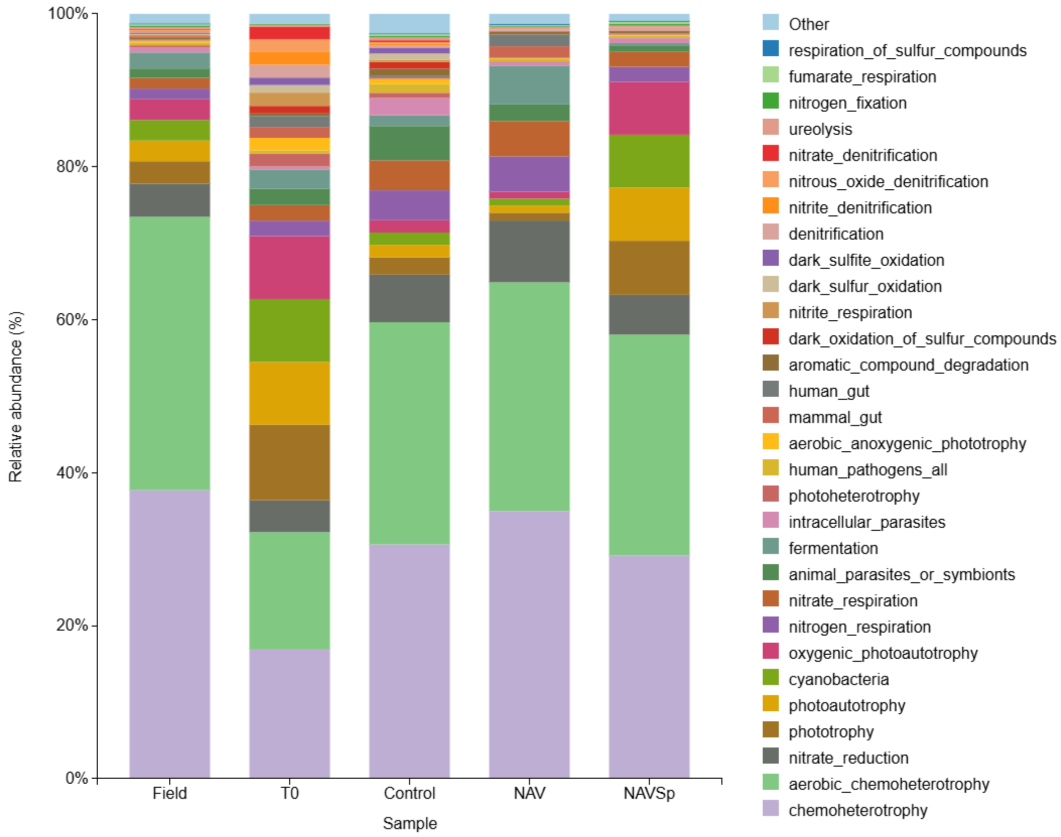


Figure 26 -Relative abundance of microbial functional groups predicted by FAPROTAX analysis in different gut microbiome samples. Functional categories are presented as stacked bar plots showing the proportional contribution of each function in the gut of organisms.

The analysis of bacterial phenotypic traits, conducted using BugBase, revealed distinct profiles across treatments (Figure 27). In NAVSp samples, Aerobic taxa constituted 7.3% of the community, a value similar to NAV (6.5%) but lower than Control (12.1%) and T0 (12.0%).

Anaerobic taxa represented 4.3% in NAVSp, similar to Control (4.4%) but lower than NAV (8.8%) and Field (11.7%). Taxa containing Mobile Genetic Elements accounted for 21.2% in NAVSp, aligning with T0 (22.1%) and higher than Field (14.4%) and Control (17.8%). Facultatively Anaerobic taxa were less prevalent in NAVSp (2.6%) compared with NAV (4.2%) and Field (3.1%). Potential Biofilm-Forming taxa in NAVSp were 12.0%, identical to NAV (12.0%) but lower than Control (15.9%). Gram-Negative bacteria predominated across all treatment groups, while Gram-Positive taxa remained at minimal levels, averaging 1.3%. Potentially Pathogenic taxa represented 9.0% in NAVSp, falling between Control (8.2%) and NAV (13.6%). Stress-Tolerant taxa reached 20.1% in NAVSp, comparable to T0 (21.6%) and higher than Field (13.2%) and Control (17.1%).

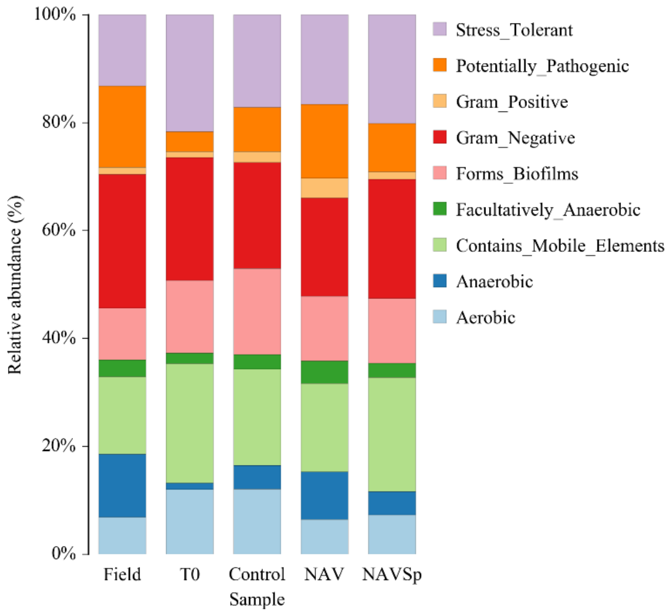


Figure 27 - Relative abundance (%) of predicted microbial phenotypes from BugBase analysis of *Cerastoderma edule* gut communities across treatments: Field, T0 (baseline), Control, NAV (natural sediment), and NAVSp (Hg-spiked sediment).

Using FUNGuild, a comprehensive evaluation of fungal functional guilds was performed on the gut communities of *Cerastoderma edule* across treatments. Overall, all treatments were dominated by Undefined Saprotrophs (35–38%), Wood Saprotrophs (16–19%), and Plant Pathogens (10–14%) (Figure 28). A slight increase in the occurrence of Plant Pathogens was observed in NAVSp (14.7%) compared to Field (10.4%) and Control (10.3%). Dung Saprotrophs decreased moderately in NAVSp (8.5%) relative to Field (10.2%) and Control (9.9%). Notably, Clavicipitaceous Endophytes were detected exclusively in NAVSp, reaching 5.85% relative abundance, and were absent from all other treatments. Leaf Saprotrophs were more abundant in NAVSp (0.59%) compared to lower levels in Field (0.15%) and Control (0.11%). Similarly, Ectomycorrhizal fungi showed a higher prevalence in NAVSp (1.41%) than in other treatments, which ranged from approximately 1.0% to 1.1%.

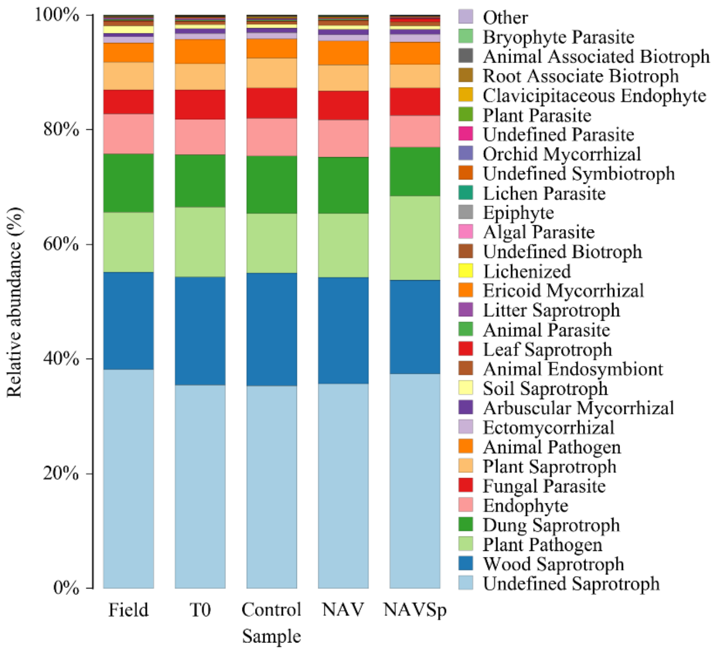


Figure 28 - Relative distribution (%) of trophic modes and functional guilds of fungi associated with the intestine of *Cerastoderma edule*, obtained based on FUNGuild prediction.

4.5 Discussion

Overall, the experimental addition of mercury (Hg) was successful, as confirmed by the elevated concentrations detected in both sediments and organism tissues, validating the effectiveness of the spiking procedure. The use of both bacterial (16S rRNA gene) and fungal (ITS region) markers proved valuable in detecting the gut microbiota response to contaminant exposure, underscoring the utility of dual-marker approaches for ecotoxicological assessments.

Bacterial communities showed greater sensitivity and variability in response to environmental changes, whereas fungal profiles remained comparatively stable. This is consistent with Li et al. (2022), who reported that while both bacterial and fungal assemblages coexisted in the gut and tissues of Perna canaliculus (Gmelin, 1791), bacterial communities displayed more pronounced shifts across tissue types and environmental gradients. Similarly, Pawlowska (2024) highlighted the ecological resilience of fungal symbionts under stress, attributing this stability to the evolutionary conservatism of host-associated fungi and their metabolic versatility.

Despite this apparent structural stability, fungal alpha diversity increased in NAV and NAVSp treatments, indicating that even resilient fungal communities may undergo compositional reorganization in response to contaminant-induced pressure. An alternative, non-exclusive explanation is that such reorganization could be secondary to bacterial community changes, for example, following the decline of dominant bacterial groups, fungi may expand into newly available niches, as documented in human microbiomes under dysbiosis conditions (Pérez, 2021). This aligns with the emerging understanding that marine fungal communities, though less dynamic than bacterial ones, can shift under persistent stressors (Richards et al., 2012).

Clear distinctions emerged between water, sediment, and gut microbiomes. The gut microbiome exhibited unique taxonomic and functional signatures compared to environmental samples. While bacterial communities varied substantially between sample types, fungal profiles were more consistent, supporting the hypothesis of functional stability in fungi. However, as noted by Djemiel et al. (2022), most functional prediction tools are adapted to bacterial taxa, limiting the depth of fungal ecological interpretation. This reinforces the need to integrate ITS-based fungal profiling and metatranscriptomics into environmental microbiome studies to better understand their roles in host health and ecosystem processes (Amend et al., 2019).

The increase in *Cyanobacteria* at T0 was linked to *unclassified_Nostocaceae*, whereas in NAVSp it was linked to *unclassified_Cyanobacteriales*. Pierce and Ward (2019) also reported the predominance of *Cyanobacteria* in the gastrointestinal tract of bivalves. These shifts may be associated with environmental factors and dietary patterns, consistent with Vaughn et al. (2024), who found strong microbiome restructuring in mussels introduced into novel habitats. However, the high proportion of *unclassified Cyanobacteria* limits functional inference. Expanding functional annotation databases and applying metatranscriptomics could elucidate the ecological roles of these groups in bivalve gut physiology and their responses to environmental change.

In NAVSp, there was a higher relative abundance of *Campylobacterota,* yet no corresponding increase in predicted sulfur compound oxidation pathways. This apparent discrepancy likely reflects the limitation of functional inference at high taxonomic levels, while many *Campylobacterota* genera can oxidize sulfur compounds in anoxic or contaminated settings (Costa & Iraola, 2019; Alamoudi et al., 2025), the absence of genus or species-level resolution makes confirmation difficult. Jurinović et al. (2023) reported *Campylobacter* spp. in Croatian coastal bivalves, suggesting a wider distribution of these bacteria in marine systems than previously recognized. Further targeted functional studies are needed to clarify their ecological roles in mercury-impacted sediments.

Functional predictive analysis revealed clear differences among treatments, especially in NAVSp. Chemoheterotrophy and aerobic chemoheterotrophy remained dominant functions, but fermentation and oxygenic photoautotrophy pathways declined in NAVSp, possibly due to reduced oxygen availability and changes in organic matter load in sediments, which directly affect anaerobic and photosynthetic taxa (Franco et al., 2018). These declines may reflect the sensitivity of fermentative and photosynthetic groups to mercury toxicity, potentially leading to the loss of specialized strains.

These predictions were supported by FAPROTAX and BugBase outputs, which showed increased stress-tolerant phenotypes, mobile genetic elements, and potential pathogenicity in NAVSp. Such traits are characteristic of microbial adaptation to toxic environments, where horizontal gene transfer enhances resilience (Louca et al., 2016; Douglas et al., 2020).

In contrast, small increases in nitrogen fixation and fumarate respiration in NAVSp suggest the activation of alternative metabolic routes, possibly as an adaptive mechanism to Hg-induced stress. This functional substitution aligns with the concept of microbial functional resilience, whereby different taxa compensate for function loss in sensitive lineages to maintain essential metabolic processes. Vigneron et al. (2021) showed that *Desulfobacterota* spp. can perform multiple biogeochemical cycles, sulfur, iron, and nitrogen, particularly in mercury-contaminated settings. Likewise, Figueiredo et al. (2018) demonstrated that estuarine microbial communities can perform both mercury methylation and demethylation, indicating complex functional restructuring under chemical stress.

A slight reduction in strict anaerobes in NAVSp further supports this scenario, likely reflecting the inhibitory effects of Hg on sensitive anaerobic taxa. This mirrors observations from heavy metal–impacted estuarine sediments, where contamination reduces anaerobic guild abundance and alters community structure (Covelli et al., 2021). Together, these findings indicate a functionally plastic gut microbiome capable of reorganizing its metabolic potential in response to sediment-bound inorganic contaminants.

ITS-based fungal analysis revealed more subtle, yet ecologically relevant, shifts in NAVSp. Although dominant phyla (*Ascomycota, Basidiomycota, Rozellomycota*) remained stable, NAV and NAVSp showed higher fungal richness and diversity (Chao1, ACE indices), suggesting secondary colonization or compositional restructuring under environmental stress. This stability in functional guild composition despite taxonomic changes supports the concept of functional redundancy. Increased representation of plant pathogens and ectomycorrhizal fungi in NAVSp may reflect ecological reorganization under mercury influence, though in bivalve gut systems such guilds might arise from ingestion of sediment or detritus-associated fungi rather than direct host association. FUNGuild predictions confirmed rises in Plant Pathogens and Leaf Saprophytes, alongside decreases in Wood Saprophytes and Endophytes. Such changes likely favor niche-specialized or opportunistic taxa adapted to altered substrates or stress (Zhang et al., 2024; Gao et al., 2024). Similar guild-level reorganizations have been reported in other estuarine fungal communities exposed to contamination (Li et al., 2023).

RDA analysis (performed using only Hg as the explanatory variable) revealed distinct responses of bacterial and fungal communities to contamination. NAVSp bacteria clustered along RDA1, aligned with the Hg gradient, and were mainly affiliated with *Campylobacterota, Desulfobacterota,* and *Spirochaetota*, phyla often linked to sulfur metabolism and polluted environments (Humphrey et al., 2007; Rincón-Tomás et al., 2024).

Overall, the results align with prior work demonstrating the utility of microbiome analyses in ecotoxicology and environmental monitoring (Adamovsky et al., 2018; Duperron et al., 2020). The gut microbiome of *Cerastoderma edule* displayed rapid, multifaceted responses to Hg-enriched sediments, including taxonomic restructuring, functional shifts, and phenotypic adaptations. These findings strengthen the case for using host-associated microbiomes as sensitive sentinels of environmental quality in estuarine ecosystems.

4.6 Conclusions

This study underscores the responsiveness of *Cerastoderma edule* gut microbiome to short-term exposure to mercury-enriched sediments, revealing taxonomic, functional, and phenotypic shifts that underscore its potential as a sensitive bioindicator of environmental contamination. Bacterial communities exhibited significant plasticity, while fungal communities demonstrated comparatively greater stability, though they were not entirely unresponsive, particularly under chemically stressed conditions.

In order to further substantiate these findings, future research should prioritize metatranscriptomics validation to confirm predicted functional shifts and elucidate how microbial pathways are modulated under contaminant pressure. Furthermore, to strengthen the interpretation of mercury-specific effects, the study design included a reference sediment with known background contamination levels. This allowed us to disentangle general responses to sediment disturbance from those specifically induced by mercury enrichment. The consistent microbiome shifts observed only under the mercury-spiked treatment reinforce the conclusion that Hg was the primary driver of the observed microbial alterations.

It is also essential to conduct long-term exposure studies to accurately assess the temporal dynamics of fungal communities and to differentiate between resilience and delayed shifts. In conclusion, the integration of host physiological metrics, such as oxidative stress and immune response, with microbiome data would facilitate a more comprehensive understanding of contaminant effects. This, in turn, would serve to reinforce the case for the gut microbiome as a reliable ecological health sentinel within the context of estuarine monitoring frameworks.

C Appendix

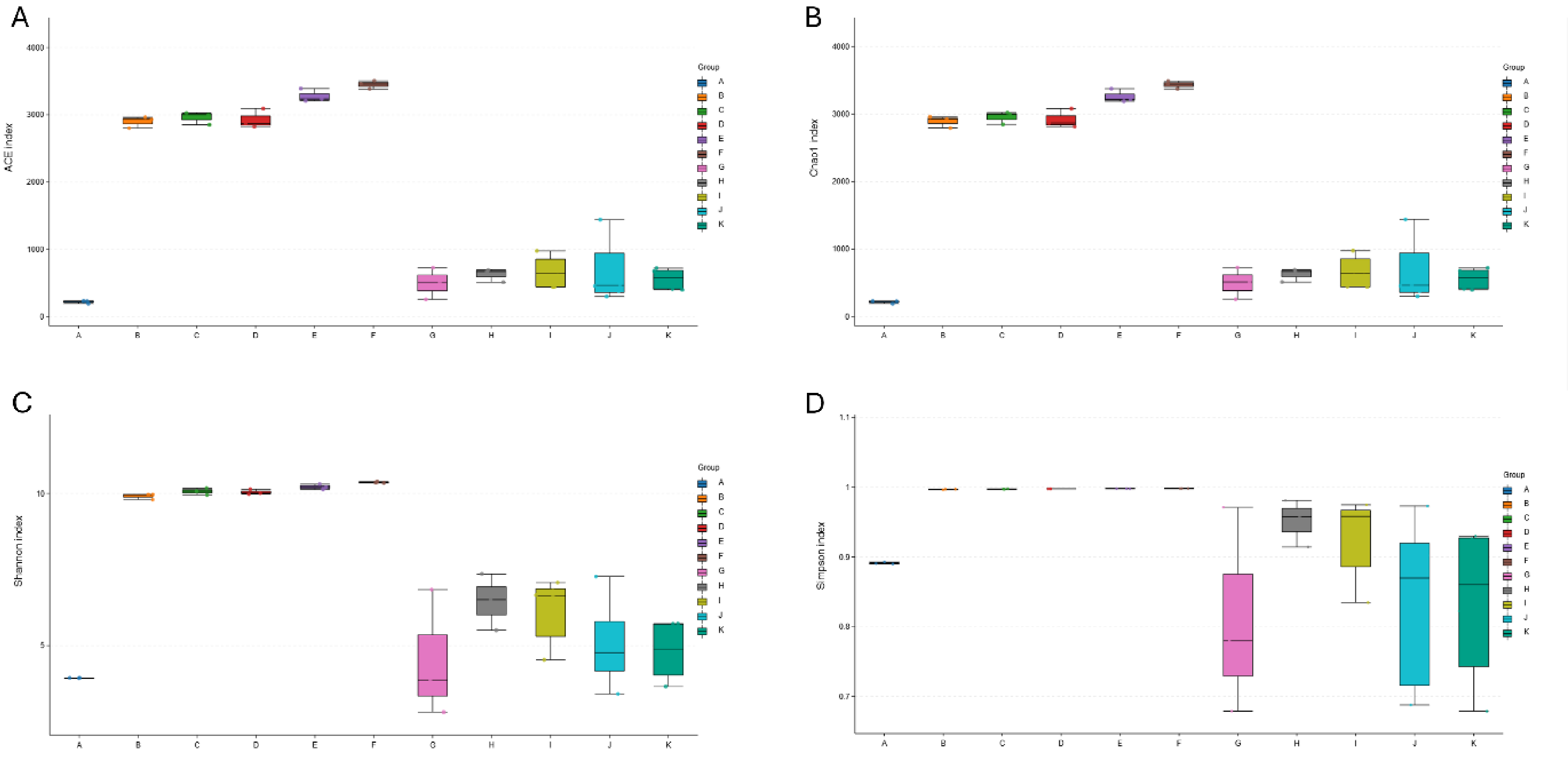


Figure 29 - Alpha diversity metrics of the bacterial microbiome across experimental treatments. (A) ACE index. (B) Chao1 index. (C) Shannon diversity index. (D) Simpson diversity index. Boxplots represent median, interquartile range, and outliers of diversity values for each treatment group. Abbreviations: W = water; S = sediment; G = gut; A = T0W; B = T0S Control; C = T7S Control; D = T0S NAV; E = T7S NAV; F = T7S NAVSp; G = TFieldG; H = T0G; I = T7G Control; J = T7G NAV; K = T7G NAVSp.

ITS

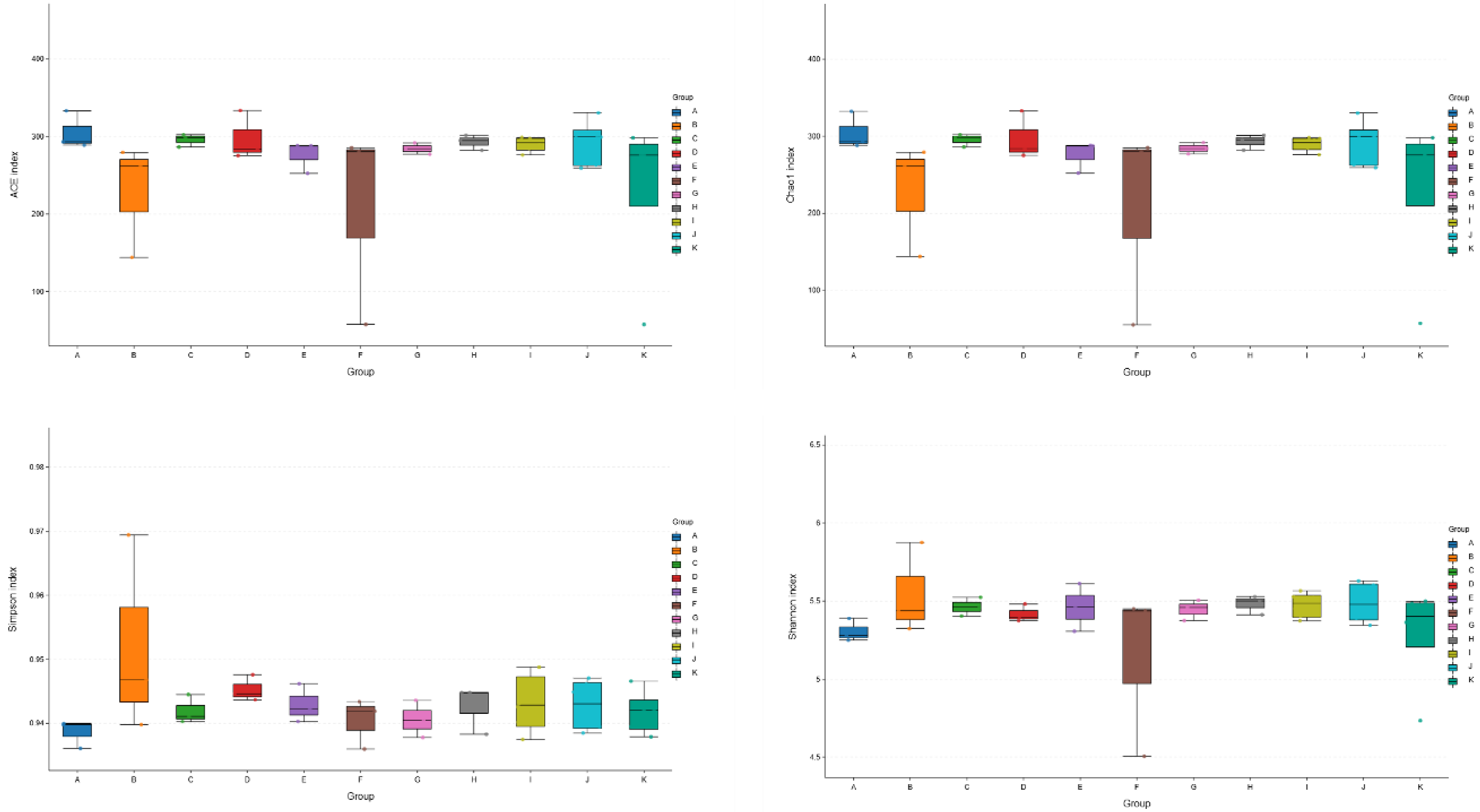
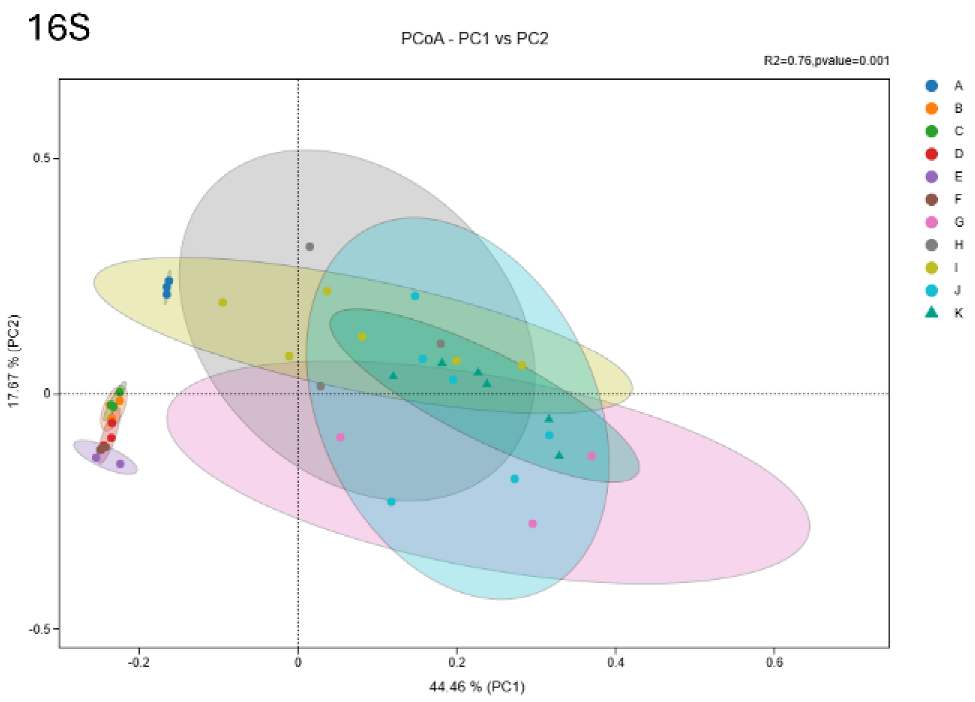


Figure 30 Alpha diversity metrics of the fungal microbiome across experimental treatments. (A) ACE index. (B) Chao1 index. (C) Shannon diversity index. (D) Simpson diversity index. Boxplots represent median, interquartile range, and outliers for each treatment group. Abbreviations: W = water; S = sediment; G = gut; A = T0W; B = T0S Control; C = T7S Control; D = T0S NAV; E = T7S NAV; F = T7S NAVSp; G = TFieldG; H = T0G; I = T7G Control; J = T7G NAV; K = T7G NAVSp



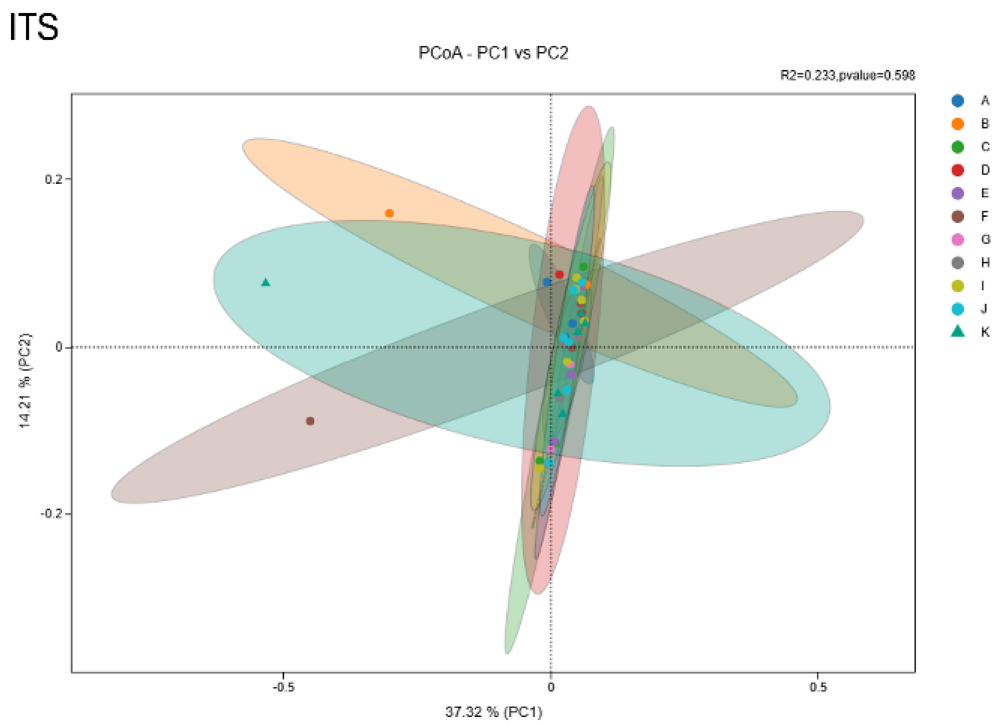


Figure 31 - Principal Coordinates Analysis (PCoA) of microbiome communities across treatments. Left graphic: Bacterial communities (16S rRNA); right graphic: Fungal communities (ITS). The plots display Bray–Curtis dissimilarity-based ordination of samples along the first two principal coordinate axes (PC1 and PC2). The ellipses represent 95% confidence intervals for each treatment group. R^2 and p-values from PERMANOVA indicate the amount of variance explained by treatment effects and their statistical significance. Abbreviations: W = water; S = sediment; G = gut; A = T0W; B = T0S Control; C = T7S Control; D = T0S NAV; E = T7S NAV; F = T7S NAVSp; G = TFieldG; H = T0G; I = T7G Control; J = T7G NAV; K = T7G NAVSp

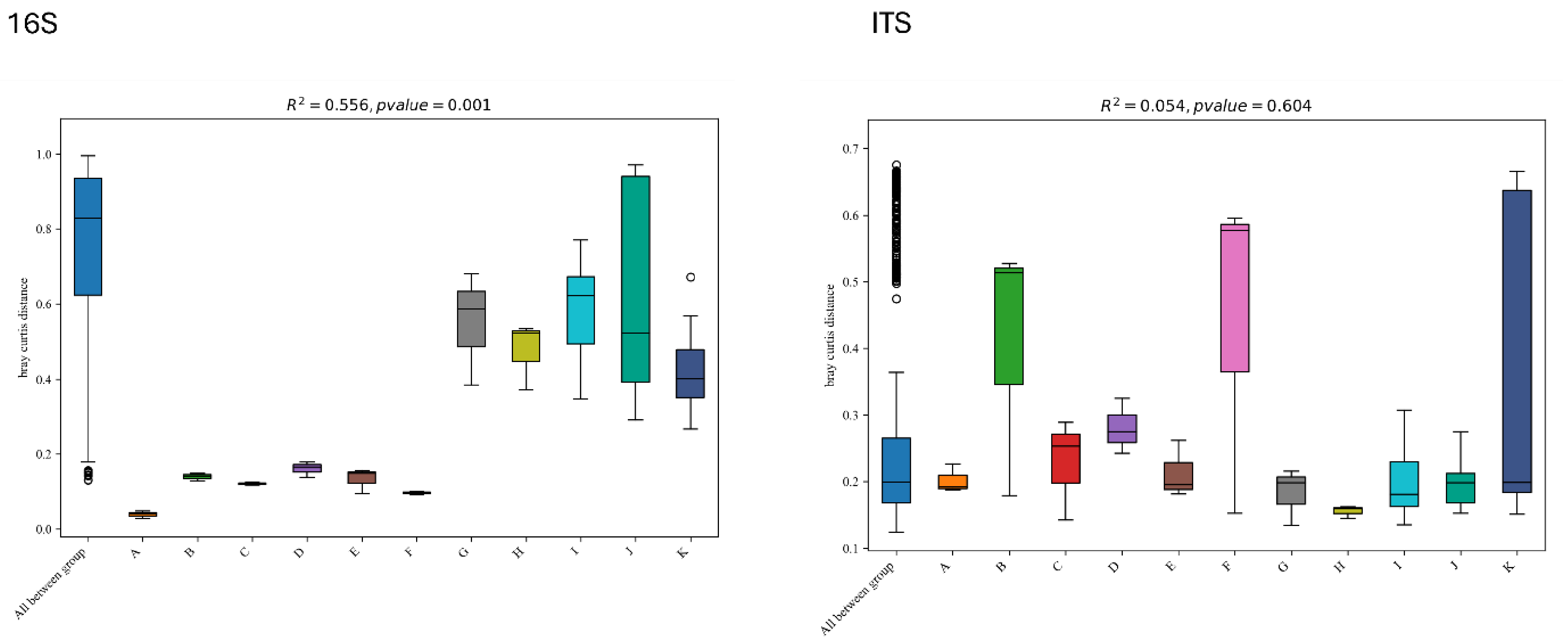


Figure 32 -Beta diversity of microbiome communities across treatments. Boxplots represent Bray–Curtis dissimilarity distances within and between groups. Left graphic: Bacterial communities (16S rRNA); right graphic: Fungal communities (ITS). Higher values indicate greater community dissimilarity. R^2 and p-values from PERMANOVA tests indicate the proportion of variance explained by treatment and the statistical significance of group differences. Abbreviations: W = water; S = sediment; G = gut; A = T0W; B = T0S Control; C = T7S Control; D = T0S NAV; E = T7S NAV; F = T7S NAVSp; G = TFieldG; H = T0G; I = T7G Control; J = T7G NAV; K = T7G NAVSp

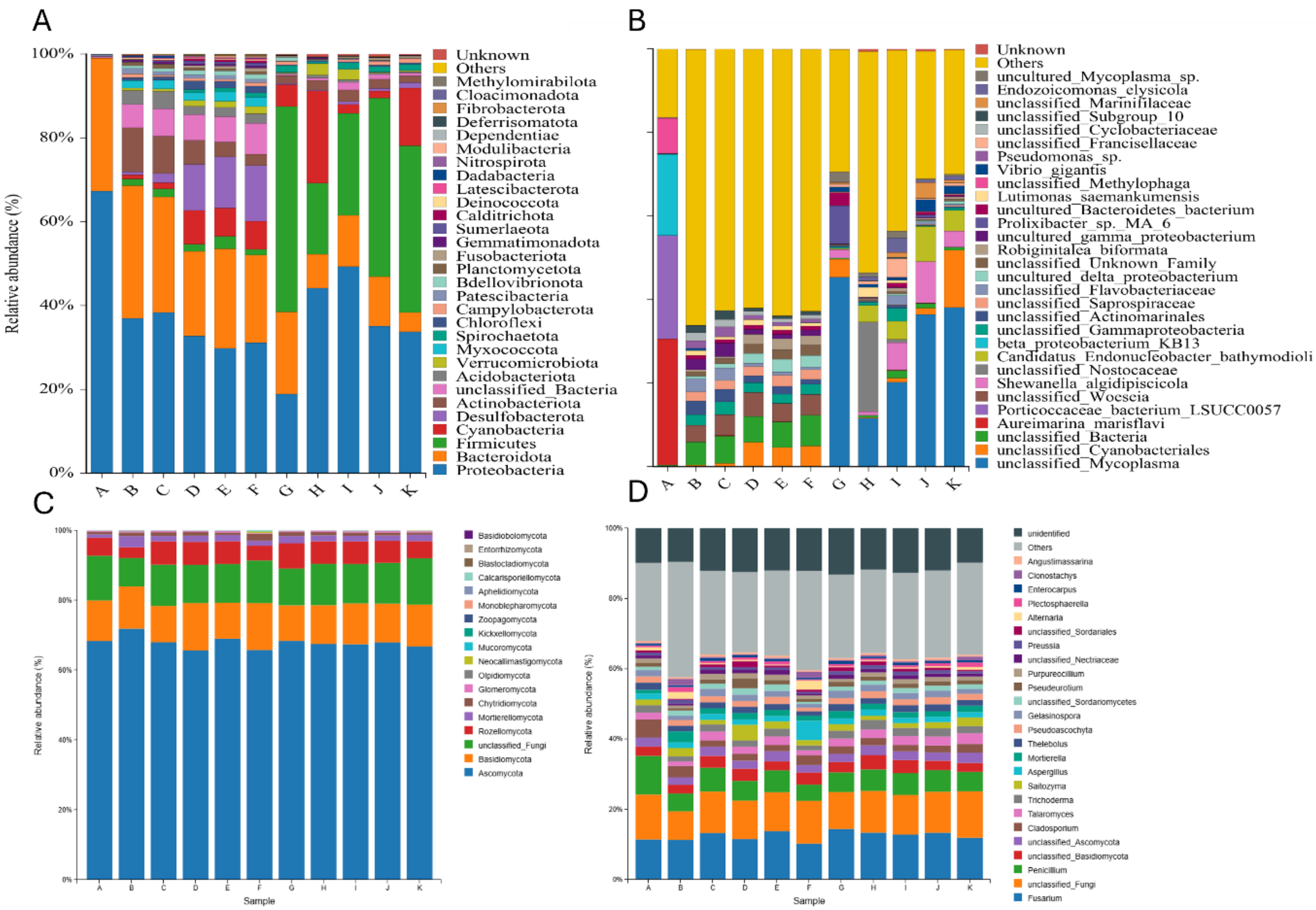


Figure 33 - Taxonomic profiles of the microbiome across experimental treatments. (A) Bacterial community composition at the phylum level. (B) Bacterial community composition at the genus level. (C) Fungal community composition at the phylum level. (D) Fungal community composition at the genus level. Relative abundances are presented as mean proportions across replicates within each treatment group. Abbreviations: W = water; S = sediment; G = gut; A = T0W; B = T0S Control; C = T7S Control; D = T0S NAV; E = T7S NAV; F = T7S NAVSp; G = TFieldG; H = T0G; I = T7G Control; J = T7G NAV; K = T7G NAVSp.

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5
General Discussion

The main objective of this thesis was to elucidate the function of the gut microbiome of macrobenthic organisms in estuarine sediments exposed to different levels of inorganic contamination. The objective was clearly delineated into three primary research areas:

1. To characterize the sediments and macrobenthic communities of the selected sites in the Sado estuary, allowing an ecological and environmental baseline to be established, and to support the selection of a representative macrobenthic species to be used as a model organism for subsequent microbiome analyses.
2. To evaluate the diversity, composition and predictive function of the gut microbiome of the bivalve *Cerastoderma edule* under real environmental conditions (*in situ*).
3. To investigate the response of the microbiome to a controlled exposure to a specific contaminant (Hg), through a laboratory bioassay (*in vivo*).

An integrated analysis of the results obtained across the three main chapters of this thesis highlights how the gut microbiome of *Cerastoderma edule* reflects and responds to environmental stressors in estuarine ecosystems, particularly sediment-associated inorganic contamination.

In Chapter 2, the ecological characterization of the Sado estuary demonstrated marked spatial heterogeneity in sediment properties, including grain size, organic matter, and trace metal content, across the sites of Tróia (TR), Gambia (GAM), and NAV. These locations represent a gradient of anthropogenic impact, ranging from low (TR) to moderate (GAM) and high (NAV), with industrial and port activities concentrated near NAV (Caeiro et al., 2005; Monteiro et al., 1995). The three selected sites exhibited varying levels of anthropogenic influence, which were reflected in the AMBI ecological quality assessments. The application of the AMBI index classified all sites as "slightly disturbed", their macrobenthic community structure was different in each location, reinforcing the sensitivity of benthic assemblages to localized environmental pressures (Borja et al., 2000). These findings established the baseline context for the study and confirmed *C. edule* as a suitable sentinel species, present in all sampled areas and exposed to both sediment and water column influences. A methodological highlight of this chapter was the integration of morphological and molecular approaches (18S rRNA and COI metabarcoding) for species identification, combined with the use of the Enrichment Factor (EF) to evaluate the origin of sedimentary metals. This combined application not only enhanced biodiversity assessments by detecting cryptic, non-indigenous, or morphologically indistinct taxa, but also identified which trace metals were enriched. However, molecular detection alone does not guarantee the physical presence of organisms, since environmental DNA may reflect traces rather than viable populations. Morphological identification therefore remains essential to validate molecular findings, particularly in the case of non-indigenous species, where physical confirmation is critical for ecological monitoring and management (Brown et al., 2016; Duarte et al., 2023; Lobo et al., 2017).

Building on this baseline, Chapter 3 revealed that the gut microbiome of *C. edule* varied significantly between sites. Despite the persistence of core microbial taxa across all locations, their relative abundances and predicted functions varied, indicative of functional redundancy (Puente-Sánchez et al,. 2024; Pierce and Ward, 2019; Fan et al,. 2013; Wemheuer et al,. 2020) and local environmental adaptation. TR, the least impacted site, hosted a more stable microbial community with predominance of *Vibrio* spp. and consistent fungal guilds. GAM, subject to aquaculture inputs, exhibited adaptations likely driven by higher organic matter and nutrient availability. The Akter et al,. (2024) study provided confirmation that dietary influences can impact the composition of the gut bacterial community. Furthermore, research has demonstrated the capacity of aquaculture activities to influence the gut microbiome of fish, shellfish and bivalves (Desriac et al., 2014; Diwa et al., 2022). The NAV site, rich in heavy metals, was associated with increased bacterial diversity, potentially reflecting the need for functional recruitment under chronic stress (Shade et al., 2012; Allison & Martiny, 2008).

The *in situ* findings directly informed the design of the bioassay experiment in Chapter 4. Organisms and sediments from TR (the least contaminated site) served as references, while NAV sediments, with the highest enrichment factor for mercury, were tested both in their natural state and spiked with Hg. This setup allowed for a focused assessment of mercury-induced microbiome disruption.

Under controlled exposure, Chapter 4, bacterial communities in *C. edule* responded markedly to mercury, displaying an increase in taxa such as *Desulfobacterota* and *Campylobacterot*a, associated with sulfur metabolism and metal transformation (Alamoudi et al,. 2025; Robador et al,. 2016;). Functional predictions using FAPROTAX and BugBase indicated a shift toward stress-tolerant and sulfur-reducing phenotypes, with reduced fermentative and phototrophic activity. Fungal communities were less affected in composition but showed increased diversity and shifts in ecological guilds, including a rise in plant-pathogenic and ectomycorrhizal groups, suggesting subtle stress responses . The addition of functional inference (FAPROTAX for bacteria; FUNGuild for fungi) enhanced the ecological interpretation of taxonomic data. Although such tools rely on taxonomic proxies and may underrepresent functions of poorly described taxa (Djemiel et al,. 2022; Nguyen et al,. 2016), they offer valuable insight when supported by metagenomics or transcriptomics (Rey-Campos et al,. 2022; Shaffer et al,. 2022). The integration of multiomic approaches will be key for the accurate detection of genes linked to resistance, detoxification, and metabolic adaptation. These results affirm the gut microbiome's responsiveness to inorganic contaminant exposure, validating its role as a sensitive and integrative sentinel bioindicator. Importantly, the combination of in situ and *in vivo* approaches enabled the separation of ambient environmental influences from contaminant-specific effects, a critical step in robust ecological risk assessment (Evariste et al., 2019; Jin et al., 2017).

The Sado estuary itself represents a dynamic and multifactorial system. Its northern areas are influenced by chemical industries and shipyards, the north central zone by aquaculture and shellfish farming, and the southern zone by agriculture and recreational use. This complexity promotes fine particle retention and contaminant accumulation, especially under low hydrodynamics (Zhang et al,. 2019; Cabrita et al,. 2020; Caeiro et al,. 2005, 2009; Cortesão et al,. 1995; Lillebø et al,. 2011), underscoring the need for spatially explicit ecological management. Beyond its role as an environmental sensor, the gut microbiome of *C. edule* can function as an ecological vector for microorganisms. The continuous microbial exchange between the digestive tract and the sediment, mediated by processes such as bioturbation and bioirrigation (Vasquez-Cardenas et al,. 2016; Widdicombe et al,. 2000; Foshtomi et al,. 2015; Kristensen & Kostka, 2005), suggests active connectivity between host and environment.

The results of this work indicate that exposure to mercury-enriched sediments led to the proliferation of pathogenic or potentially pathogenic taxa, such as *Vibrio* spp. And *Campylobaterota*, in the gut microbiome of cockles. This phenomenon has already been described in other studies involving oysters and mussels, where metal-induced dysbiosis favored pathogenic outbreaks (Lokmer & Wegner, 2015; Destoumieux-Garzón et al., 2020; Montúfar-Romero et al,. 2025; Jurinović et al,. 2022; González et al,. 2022; Yi-Feng et al,. 2019; Rincón-Tomás et al,. 2024;). In the case of *C. edule*, we observed that more contaminated environments like NAV were associated with a higher relative abundance of *Vibrio, Campybacterota and Desulfobacterota*, suggesting microbial imbalance with potential implications for host health and food chain contamination risks. This ecological link was reinforced by Albuixech-Martí et al. (2021), who demonstrated the transmission of *Vibrio* spp. from *C. edule* to shorebirds through the food chain. Such dynamics reveal a potential for interspecies and intercompartmental microbial connectivity, relevant to public health, conservation, and bio surveillance efforts.

Additionally, microbiomes participate in essential holobiont processes, including digestion, nutrient assimilation, and detoxification of compounds (See et al,. 2025; Pogoreutz et al,. 2022; Glasl et al,. 2016; Gastaldi et al,. 2024). The loss of functional microbial groups, such as fermenters and nitrogen fixers, can compromise nutrient recycling and negatively affect benthic primary productivity. For instance, the microbial communities comprising *Vibrio* sp., *Bacteroides and Pseudomonas* within oysters play a pivotal role in the process of digestion, including the breakdown of complex carbohydrates and the production of indispensable amino acids (Pierce and Ward, 2019). This perspective reinforces the microbiome's role not only as a biomarker for environmental stressors, but also as a critical functional component of estuarine ecosystem resilience.

For this reason, integrating taxonomic analysis, functional inference and ecological context is essential for achieving a holistic understanding of the gut microbiome's role as both an ecological sensor and modulator. This work underscores the importance of integrative approaches that combine in situ and in vivo data, molecular tools, and classical ecological indicators. Such approaches facilitate a more comprehensive understanding of the impact of multiple stressors on sentinel organisms and their microbiomes. The perspective of bivalves as dynamic mediators of microbial connectivity has the potential to expand the scope of applications in the domain of microbiome studies, particularly in the fields of environmental biomonitoring, food safety, public health, and remediation strategies within estuarine ecosystems.

6 Conclusions

This thesis presents a significant contribution to our understanding of how sediment-associated inorganic contamination influences the structure and function of the gut microbiome in estuarine macrobenthic organisms, with the common cockle *Cerastoderma edule* selected as a focal species. Using an integrated approach, comprising ecological site characterization, in situ microbiome profiling, and a controlled bioassay, it was possible to:

- Confirm the presence of distinct levels of ecological disturbance at the selected sites in the Sado estuary. The NAV site, which is located near shipyards and industrial activities, exhibited the highest enrichment of trace metals and the most severely impacted macrobenthic community. Troia (TR) exhibited the lowest degree of disturbance, while the ecological condition of GAM was intermediate, possibly due to aquaculture inputs.
- Demonstrate that the gut microbiome of *C. edule* is sensitive to environmental gradients. While bacterial communities exhibited high responsiveness to contamination and local pressures, fungal communities showed greater structural resilience, though subtle functional shifts were still evident.
- Through a bioassay experiment, to validate that exposure to mercury-spiked sediments induces compositional and functional changes in the intestinal microbiome, particularly in bacterial taxa involved in sulphur and nitrogen cycling, oxidative stress responses and resistance traits. Fungal communities exhibited increased diversity and slight shifts in ecological guilds.

These findings highlight the importance of the gut microbiome as a functional and sensitive bioindicator that provides complementary information to traditional benthic assessments, particularly in estuarine systems under multiple stressors.

The combined use of morphological and molecular tools (18S, COI, 16S rRNA and ITS) enabled more accurate biodiversity assessments and facilitated the detection of cryptic, non-indigenous or developmentally distinct taxa. This integrative approach strengthens ecological monitoring efforts and highlights the value of classic taxonomy and advanced molecular biology.

However, there are still several methodological challenges. Methods for functional inference (e.g., FAPROTAX, BugBase and FUNGuild) rely heavily on taxonomic resolution and the robustness of databases, which can limit their accuracy, particularly for underrepresented taxa and marine fungi. Improvements in genetic reference libraries and the adoption of metagenomics and metatranscriptomics tools would be essential for overcoming these constraints and enabling the direct detection of functional genes, including those associated with metal and antibiotic resistance.

Furthermore, this study highlights the importance of understanding the connection between sediments and organisms in estuarine systems. As a reflection of environmental conditions, the gut microbiome also plays an active ecological role. It participates in nutrient cycling and contributes to host digestion and immunity. It may also act as a vector for microbial dispersion, including potential pathogens such as *Vibrio* spp., which proliferate in disturbed and Hg-enriched sediments. This is consistent with literature demonstrating the dual role of *Vibrio* as both commensal and pathogen under environmental stress, with potential implications for ecosystem and public health.

This thesis provides significant evidence that the gut microbiome functions as an integrator of multiple environmental stress factors and represents a valuable and sensitive tool for assessing ecological quality in estuarine environments. The application of molecular approaches to gut microbiome analysis, specifically the use of 16S rRNA and ITS markers, enabled the characterization of the microbial diversity and functional profiles associated with inorganic contamination. These methodologies complement traditional monitoring tools and are essential for reveling of patterns that would otherwise remain undetectable. The data generated contributes significantly to the understanding of organism-environment interactions and provides a valuable basis for the future integration of microbiome-based indicators into estuarine conservation strategies, biomonitoring programs, and environmental health policies.

6.1 Future Perspectives

The findings of this thesis highlight several promising avenues for future research and practical applications in estuarine monitoring and management. Based on the results obtained and the identified knowledge gaps, the following are proposed:

1. Broaden species coverage to include other macrobenthic taxa with different feeding strategies, sediment interaction, and ecological niches. This would help to assess the generalizability and robustness of the gut microbiome as a bioindicator across taxonomic and functional diversity.

2. Conduct longitudinal and seasonal studies to capture the temporal variability in the gut microbiome and assess whether microbial communities exhibit resilience or 'ecological memory' following exposure to stressors such as heavy metals or salinity fluctuations. This is crucial for predicting ecosystem stability and the potential for microbial recovery over time.

3. Apply multi-omics approaches, including metagenomics, metatranscriptomics, metaproteomics and metabolomics, to overcome the limitations of inference-based tools such as FAPROTAX, BugBase and FUNGuild, and enable the direct detection of functional genes and microbial pathways linked to metal resistance, antibiotic degradation or stress responses.

4. Conceive bioassays that are oriented towards the manipulation of the microbiome, for example through the utilization of antibiotics or probiotics, with a view to furthering our understanding of host–microbiome–contaminant interactions. These experimental designs could help to assess the physiological consequences and adaptive potential under realistic contamination scenarios.

5. Investigate microbiome-based early-warning tools for detecting pathogenic taxa (e.g., *Vibrio* spp.) and shifts in host health before community-level changes occur. These early signals could enhance the responsiveness of ecological management.

6. Support environmental remediation and restoration strategies by using microbial indicators to reflect functional connectivity between sediments and benthic organisms. This could guide intervention prioritization and evaluate the effectiveness of ecological management.

7. Promote integration of microbiome-based metrics into regulatory frameworks by advocating for their inclusion as complementary indicators within national and European monitoring programs, such as the Water Framework Directive (WFD) and the Marine Strategy Framework Directive (MSFD).

This thesis establishes a robust scientific basis for developing microbiome-informed indicators to enhance ecological monitoring, improve risk assessment and inform the sustainable management of estuarine and coastal systems under multiple stressors.

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Joana F. Marujo Neves

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