# Effects of historical contamination on invertebrates' communities from vegetated and non-vegetated areas


Rita Pereira Faião

Master's Degree in Ecology and Environment

Biology Department


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**Supervisor**

Ph.D. Marina Dolbeth, Assistant researcher, CIIMAR-UP

**Co-supervisor**

Ph.D. Daniel Crespo, Assistant researcher, CIIMAR-UP

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

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By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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Rita Pereira Faião

30 September 2022

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# Resumo

Os estuários possuem uma longa carga histórica enquanto destino de contaminantes de águas residuais urbanas e industriais, sendo a grande maioria sem tratamento. A elevada concentração de contaminantes torna-se tóxica para a fauna e flora. Afeta a diversidade e abundância das comunidades de macrofauna bentónica, algumas das quais são dominadas por espécies que não são tolerantes a alterações físicas e químicas. É essencial compreender os efeitos da concentração de contaminantes nas comunidades bentónicas em estuários e tomar medidas para mitigar esses efeitos. É neste contexto que se realiza o projeto RemediGrass, com o objetivo de recolonizar os fundos de ervas marinhas (*Zostera noltei*) como Infraestrutura Verde e Azul para biorremediar a contaminação histórica com mercúrio (Hg) do Largo do Laranjo, na Ria de Aveiro (Portugal). Para avaliar o papel da recolonização das ervas marinhas como uma Solução Baseada na Natureza (em inglês NBS) para o restauro do ecossistema, o projeto aproveita as condições privilegiadas do campo e o gradiente de contaminação. O objetivo será promover a biodiversidade local, minimizando os impactos da contaminação histórica. Neste âmbito, foram comparadas as comunidades bentónicas ao longo de um continuum de presença-ausência da erva marinha em duas áreas, uma localizada em pradarias bem estabelecidas de erva marinha e outra na área contaminada historicamente, onde foi realizado um transplante de erva marinha. Os resultados demonstram uma forte influência da pradaria de ervas marinhas na composição e abundância da comunidade bênticas ao longo do gradiente espacial. A maior diversidade foi observada nas pradarias bem estabelecidas de *Zostera noltei*, em comparação com as outras áreas. Na zona de contaminação histórica, é também evidente a influência da presença de *Z. noltei*, embora em menor escala. Apesar das diferenças nos indicadores de comunidade não serem tão expressivas, algumas espécies-chave como *Peringia ulvae*, *Hediste diversicolor* e *Scrobicularia plana* seguiram o gradiente, apresentando um aumento de abundância desde os lamaçais até aos locais de *Zostera*, em particular na área de recolonização.

Palavras-chave: comunidades bentónicas, estuários, restauro ecológico, contaminação histórica, *Zostera noltei*

# Abstract

Estuaries have a long historical burden as a destination for contaminants from urban and industrial wastewater, with the vast majority untreated**.** The high concentration of contaminants becomes toxic to the fauna and flora, affecting the diversity and abundance of benthic macrofauna communities, some of which are dominated by species that are not tolerant to physical and chemical alterations. It is essential to understand the effects of this historical contamination on benthic estuarine communities and take actions to minimize such effects. It is in this context that the RemediGrass pilot project was carried out, with the aim to recolonize seagrass beds (*Zostera noltei*) as a Green and Blue Infrastructure to bioremediate the historical mercury (Hg) contamination of Laranjo Bay, at Ria de Aveiro coastal lagoon (Portugal). To evaluate the role of seagrass recolonization as a Nature Based Solution (NBS) for ecosystem restoration, the project takes advantage of the privileged field conditions and contamination gradient. The goal is to promote local biodiversity, while minimizing the impacts of the historical contamination. Within this framework, we compared the benthic communities along the presence-absence continuum of the seagrass in two areas, one located at a well-established seagrass meadow and another at the historical contaminated area, where a seagrass transplant was held. The results demonstrated a strong influence of the seagrass meadow on the benthic communities' diversity and abundance along the spatial gradient. The highest diversity was observed in the well-established *Zostera noltei* meadows, compared to the other areas. In the historical contamination area, it is also evident the influence of the *Z. noltei* presence, yet on a smaller level. Despite the differences on community indicators not being as expressive as expected, some key species such as *Peringia ulvae*, *Hediste diversicolor* and *Scrobicularia plana* followed that continuum, with an increase in abundance from Bare mudflats to *Zostera* sites, particularly in the recolonization area.

Keywords: benthic communities, estuaries, ecological restoration, historical contamination, *Zostera noltei*

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# List of Abbreviations

CESAM - Centre for Environmental and Marine Studies

CIIMAR – Interdisciplinary Centre of Marine and Environmental Research of the University of Porto

GBI – Green and Blue Infrastructure

GLS – Generalized Least Squares

H' – Shannon Wiener index

IPCC - Intergovernmental Panel on Climate Change

NBS – Nature-Based Solutions

OSPAR - Convention for the Protection of the Marine Environment of the North-East Atlantic

UNEP – United Nations Environment Program

WFD - Water Framework Directive

# 1. Introduction

The last decade has been marked by the word "climate crisis", being one of the defining issues of our time. The evidence is clear. The impacts caused by climate change, aggravated by anthropogenic greenhouse gas emissions, are clearly apparent, increasing the threat to the viability and resilience of ecosystems and the human societies that depend on them (The Royal Society, 2020). The IPCC report of 2021 (IPCC Climate Change, 2021) indicates that if greenhouse gases continue to have high emission levels at the current rate, the negative consequences on the planet will be increasingly aggravated (Malhi et al., 2020). Examples of such consequences are unprecedented sea level rise, shifts in growing seasons, loss of biodiversity, and increased frequency of extreme weather phenomena (Malhi et al., 2020; The Royal Society, 2021).

Around 40 percent of the world´s human population lives within 100 kilometres of the coast, and more are moving there. By 2025, 35 percent of people living at a safe distance from the coast will move into a danger zone, increasing the pressure on coastlines and exposing people's homes and businesses to flooding, storm damage and sea level rise (Honeyborne et al., 2017). As the tendency of sea level rise grows, coastal habitats are being squeezed and pressured also due to other anthropogenic pressures. The construction of fortifications to protect low-lying areas are increasing. Since coastal habitats are merely seen as development places and not as locations for Nature, this is causing a forced and rapid change in the coastline, with consequent loss of wildlife habitats. This may result in the disappearance of critical ecological habitats, decreasing nursery areas for marine life, poor water quality, and loss of feeding areas for migratory birds (Honeyborne et al., 2017).

Is necessary to understand the ecological dynamics of environmental impacts to identify hotspots of vulnerability and resilience, and to intervene by enhancing the resilience of the biosphere to climate change (Malhi et al., 2020). Indeed, ecosystems and their habitats may play a key role in mitigating and adapting to climate change and rehabilitating from human impacts through different mechanisms while promoting biodiversity  Nature Based Solutions (NBS) - (Malhi et al., 2020). Yet, the resilience of ecological communities requires long-term perspectives to increase our understanding of communities' responses to change (The Royal Society, 2020), and how we can rely on them to achieve environmental sustainability goals. It is in this aspect that the seagrasses appear as heroines of the Ocean. Located in coastal areas, estuaries and lagoons, there is evidence that seagrass meadows provide NBS that help fight climate change due to their potential to sequester and store carbon (United Nations Environment Programme, 2020). They form some of the most productive ecosystems, representing a very important role in providing high-value ecosystem services (Table 1), such as acting as nurseries for juvenile fish species, including commercial explored species. They can also act as hotspots for marine biodiversity, including charismatic species such as seahorses and sea turtles. Thus, seagrass meadows can be essential tools to fulfill international environmental commitments, from the Sustainable Development Goals to the Paris Agreement (Arendal, 2019).

Table 1 - List of ecosystem services associated with seagrass and their classification (Source: GRID Arendal, 2019)

| Seagrass Ecosystem Services | | |
|---|---|---|
| Section | Class | Group |
| Provisioning | Fisheries | Support global fisheries and provide nursery habitats for commercially targeted Fish, Bivalve and Crustacean Species. |
| | Biodiversity | Can be hotspots of marine biodiversity, including protected and charismatic species such as Dugongs, Sea Turtles, Sharks, and Seahorses. |
| Regulation & Maintenance | Water Filtration | Natural filters, trapping sediments and excessive nutrients out of the water. |
| | Phytoremediation | Remediates contaminated environments by accumulating heavy metals |
| | Disease Control | Control human, fish, and coral diseases by reducing exposure to pathogens. |
| | Climate Regulation | Store large amounts of carbon in the biomass and sediment below, helping to mitigate climate change. |
| | Ocean Acidification Buffer | Regulate the chemical composition of seawater by releasing oxygen and removing carbon dioxide during daylight, oxygenation water and buffering ocean acidification. |
| | Coastal Protection | Prevent coastal erosion and protection from flooding and storm surges. |
| Cultural | Tourism | Provide cultural services such as a sense of identity for local communities and opportunities for recreational activities (birdwatching, diving, fishing) |

However, despite the growing prominence they receive, seagrass meadows are considered a forgotten habitat. They are under surface water, hidden from the public eye, staying out of sight and out of mind, overshadowed by the lack of "charisma" and the bright colors of coral reefs (UNEP, 2020), despite being present on coastlines worldwide, in 159 countries on six continents, and covering an area of over 300,000 $km^2$ (UNEP, 2020).

From the worldwide distribution of seagrass meadows identified so far, only 26% are found in Marine Protected Areas (MPA), while warm-water corals have a percentage of 40%, mangroves of 43%, saltmarshes of 42% and cold-water reefs 32%, being the seagrasses one of the least protected marine ecosystems (Potouroglou et al., 2020; UNEP, 2020).

The pressure and increase of human activities in coastal ecosystems, combined with the impacts of the past, present, and future, represent big threats to seagrass meadows (Griffiths et al., 2019). A study by Waycott et al. (2009) shows that since 1879, seagrass meadows have declined in all areas of the globe, most of them declined more than predicted where: 58% of sites declined, 25% increased, and 17% exhibited no detectable change.

The loss of seagrass meadows on a global scale represents profound consequences for coastal biodiversity, and status of the environment and economy (Cunha et al., 2014). The overall scale of habitat loss is uncertain, due to the reduced research from regions such as Southeast Asia, the Caribbean, and the western Indian Ocean (Tan et al., 2020). It is essential to integrate the management of coastal areas in the environmental policies of the countries, to protect coastal habitats from the multiple pressures they suffer. In the lack of adequate legislation, seagrass meadows remain at risk of decline, by the absence of instruments that recognize this habitat status or minimize impacts through systems and processes of regulation and conservation (Griffiths et al., 2019).

However, there are several national and legislative directives that protect the seagrasses. The protection of seagrass and their species in Europe varies between countries, with different levels of protection, from international directives and conventions to national and regional level. On an international level, seagrasses are covered by the Habitat Directive (192/42/EEC), OSPAR and Bern Convention protecting the seagrasses species (OSPAR protects 3 out of the 4 European in Atlantic coasts), and the Helsinki Convention on the Protection of the Marine Environment of the Baltic Sea Area (HELCOM). The IUCN International Red List considers all seagrasses species from Europe as "Least Concern". There are also conventions that indirectly protects the seagrasses habitats, such as the Ramsar Convention and Barcelona Convention (protection of the seagrass habitats in the Mediterranean Sea). Most of the seagrass sites are protected as Marine Protect Areas, with Ramsar sites included (Sousa et al., 2019; De Los Santos et al., 2014).

Despite of the growing relevance and focus that seagrass meadows receive from the scientific community, and their undeniable importance, these habitats still remain unknown to the public eyes. Outreach actions to local communities, to stakeholders, and environmental education of those that use and benefit the most from the seagrasses, is an important key for behaviour change and protection of the seagrass meadows. In this sense, within the scope of this project, an outreach activity was performed to high school students (Attachment 1).

## 1.1 Estuaries and benthic communities

Several seagrass meadows occur in estuarine systems. Still, estuaries can be subjected to significant anthropogenic pressures due to the location of industrial and metropolitan activities near these areas (Chapman & Wang, 2001; Rodgers et al., 2020). Indeed, several estuaries have received contaminant discharges in the past from industrial sources (Pereira et al., 2009). This practice has declined with the implementation of legal frameworks for water use (Dolbeth et al., 2016). Nevertheless, sediments may still present historical contamination in restricted areas, as in Ria de Aveiro's Laranjo Bay, with associated effects on the benthic invertebrate species ( Nunes et al., 2008).

Benthic invertebrate organisms, or benthos, live within or on the surface of the seafloor (Cusson & Bourget, 2005; Crespo & Pardal, 2020). Due to their limited mobility that unables them from escaping the stressors and to species' different tolerances to stress, benthic communities are considered good indicators of ecological and environmental quality status, serving as a tool to assess the environmental impacts of human pressures (Borja et al,. 2011; Crespo & Pardal, 2020).

Benthos play important roles in ecological functions with fundamental importance in the overall balance of aquatic systems: the occurring processes in the benthic compartment and interplay between sediments and water column influence some important ecosystem functions and services supplied by aquatic systems (Crespo &

Pardal, 2020). For instance, these individuals can affect nutrient cycling and the overall water quality by their own filtration activity. In this scope, alterations and stressors affecting these invertebrates through the trophic web, may affect the overall aquatic ecosystem. Besides their ecological relevance, direct economic values are also associated with benthic communities, as food sources for commercially explored species such as shrimps, bivalves or lobsters, destined for human consumption (Chapman & Wang, 2001, Crespo & Pardal, 2020).

Benthic communities also face threats to their diversity and integrity. These invertebrates can be very sensitive to physical and chemical alterations, suffering mainly from fishing, contamination pollutants or toxins, eutrophication, ocean acidification and temperature increases (Crespo & Pardal, 2020; Dolbeth, et al., 2021).

## 1.2. Project scope

The present dissertation is integrated within the RemediGrass project, focusing on the effects of historical contamination and on the tools to minimize the impacts of contamination on benthic communities.

Remedigrass project main aim is to improve the scientific knowledge on the ecological recovery process of historically contaminated estuaries, using seagrass beds, *Zostera noltei* Hornemann (1832) recolonization as a green and blue infrastructure (GBI), to bioremediate the historical contamination by Hg (mercury) on Ria de Aveiro. Focusing to evaluate the role of seagrass re-colonization as a NBS for ecosystem restoration, the project takes advantage of the privileged field conditions and contamination gradient, to promote local biodiversity, and minimizing the impacts of the historical contamination.

This project is in line with Target 2 of the EU Biodiversity Strategy to 2020, which states that "By 2020, ecosystems and their services are maintained and enhanced by establishing green infrastructure and restoring at least 15% of degraded ecosystems".

To evaluate this, we compared the benthic communities along the spatial gradient of the seagrass cover at three level habitats (*Zostera*, Adjacent and Bare sediments), located at two areas, a well-established seagrass meadow and another at the historical contaminated area, where a seagrass transplant was held.

## 1.3. Objectives and Hypothesis

The present work was carried out within the scope of the RemediGrass project, with the main objective of evaluating the role of seagrass (*Zostera noltei*) in the rehabilitation of degraded ecosystems, focusing on the evolution of the benthic communities. This work has the underlying hypothesis that the benthic communities adapted well to the transplant, and ultimately the communities in the transplanted area will resemble those from the source meadows area, considering diversity and abundance/biomass levels. We tested this along a seagrass spatial continuum and expected to have an evolution of the values along the continuum, where the highest values would be represented in the seagrass meadows and decrease gradually to the areas without.

# 2. Materials and Methods

## 2.1. Study Area

### 2.1.1 Ria de Aveiro

Ria de Aveiro is a shallow mesotidal coastal lagoon, located along the northwest coast of Portugal, with an extension of 45 kilometers from Ovar to Mira (Fig. 1). The lagoon has 10 kilometers wide (Lillebø et al., 2015), and a total area of 82 km$^2$ during the high tide, and 66 km$^2$ at low tide. It goes through the localities of Aveiro, Estarreja, Ílhavo, Mira, Murtosa, Ovar and Vagos. The Ria contacts with the Atlantic Ocean by a single connection – Barra – and has 4 main channels, the Ovar channel, Ílhavo channel, Mira channel, and Espinheiro channel, forming islands, inner basins, and mudflats (Libellø et al., 2015). Ria is rich in habitats, due to the complexity of the channels and islands, large intertidal mudflat areas and seagrass meadows of *Zostera noltei* and one of the largest continuous salt marshes in Europe (Lillebø et al., 2018).

Ria de Aveiro provides ecological, economic, and social services essential to the local community (Dolbeth et al., 2016). From an ecological point of view, it is an important area in the national context, because it is the habitat of several species of flora and fauna that are supported by the dynamics of the lagoon (Sousa et al., 2013). The Ria ecosystem has a complex system of natural values and functions, consisting of a wide variety of habitats, such as seagrass meadows, and salt pans, intertidal mudflats, coastal dunes, and agricultural areas (ICNB, 2006; PLRA/AMBIECO, 2011), which serve as nurseries, food sources and protection for marine life, such as bivalves, crustaceans, fish, and birds (Sousa et al., 2013).

Due to its environmental and landscape qualities of high ecological value, the Ria de Aveiro is considered a Special Protection Zone (SPZ) for birds (Decree-Law 384-B/99 of 23 September) and a Site of Community Importance (SCI) under the Habitats Directive.

### 2.1.2.Laranjo Bay

The Laranjo Bay was selected as a Focus area for the present study (fig. 1). It is a shallow area, which used to have continuous discharges of mercury (Hg) from the effluents of the Estarreja Chemical Complex in the past, from 1950 to 1994 (Oliveira et al., 2022). Mercury is considered one of the highest priority environmental pollutants by the European Water Framework Directive (WFD), and on a global scale. Anthropogenic mercury can enter the coastal ecosystems in its organic form through diffuse sources or via discharge points (Pereira et al., 2008).

Laranjo Bay used to be one of the most contaminated systems in Europe by mercury (Válega et al., 2008), yet this contamination is nowadays considered as historical and restricted to a 2km$^2$ area (Lillebø et al., 2015). With the restrictions imposed at EU-level, the concentration levels of mercury in this bay have diminished considerably. Nevertheless, the levels of mercury in the sediments in this restricted Laranjo area are still relevant with concentrations around 5 mg.kg$^{-1}$ assessed in a recent study (Oliveira et al., 2022). Thus, sediments in this area are still considered highly contaminated, according to the OSPAR convention that defines a maximum value of 0.15 mg.kg$^{-1}$ (OSPAR Commission, 2004).

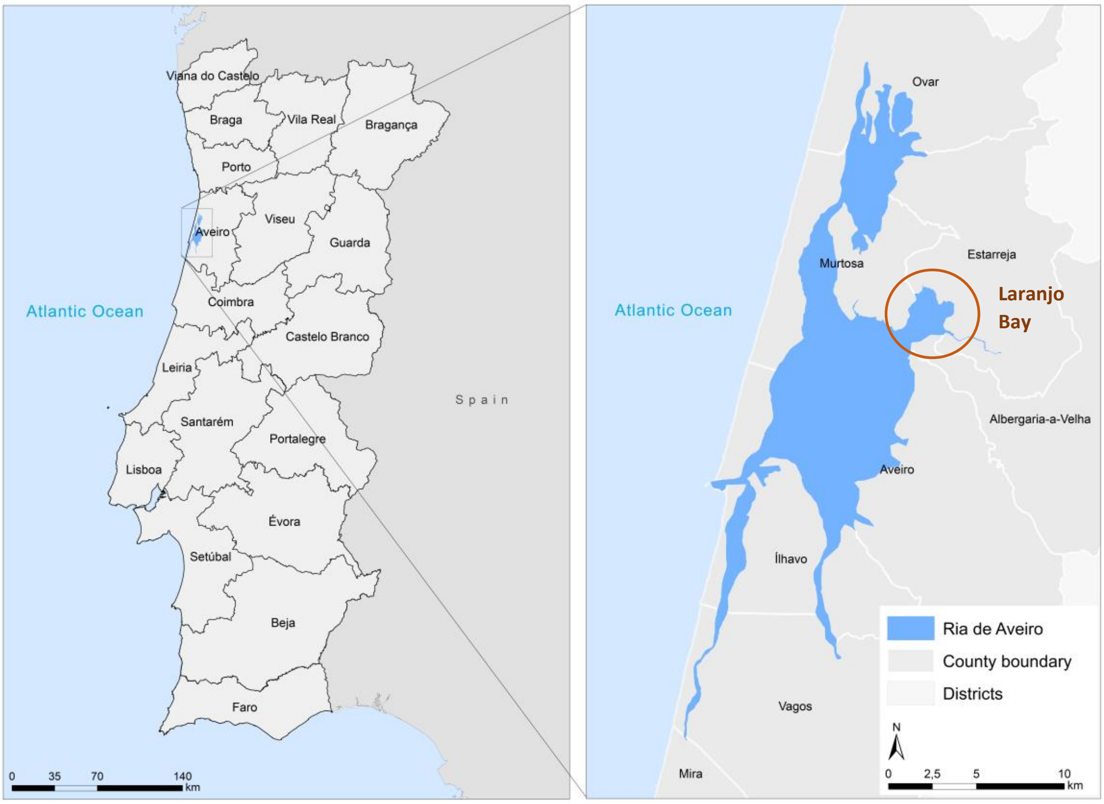


Fig. 1 - Frame map of the Ria de Aveiro and Laranjo Bay in mainland Portugal. Source: CAOP 2021. Map created with ArcGIS 10.8.1.

## 2.2. Pilot restoration program

Within Remedigrass project, a seagrass pilot restoration program has been implemented in the historical contaminated area, as a rehabilitant mechanism to recover the degraded area. The presence of the seagrass stabilizes the sediments, by reducing local water currents and sediment resuspension, while coping with moderate anthropogenic contamination (Waycott et al., 2009). Therefore, the seagrass could be used as an efficient green infrastructure to recover and avoid contamination of the resident biota.

The methodologies of the project include having one area of the Ria with healthy seagrass meadow (Source Area), from which a transplant was taken to another area (Laranjo Bay) with a historical contamination by mercury (Focus Area). *Z. noltei* small patches, named as SOD's (Costa et al., 2022) were collected in the Source Area and transplanted into the Focus Area, in a mosaic structure (Fig. 2) to allow its expansion through time and without removing a significant portion of the seagrass from the source area. Due to several constrains, including a severe winter and logistic difficulties created by COVID-19 confinements, a first transplant was lost in the spring of 2020. However, a successful transplant was held in July of 2020.

Fig. 2 - Transplantation of *Zostera noltei* in the Focus area in a mosaic structure.

Within each area, the Source and the Focus one, three sites were selected (Fig. 3), following the presence-absence continuum of the seagrass in two areas:

Table 2 - Coordinates of the Source and Focus Areas' sites.

| Area | Site | Coordinates |
|---|---|---|
| Source | *Zostera* Source | 40°43'22.22"N / 8°39'27.70"W |
| | Adjacent Source | 40°43'21.87"N / 8°39'26.46"W |
| | Bare Source | 40°43'19.99"N / 8°39'19.99"W |
| Focus | *Zostera* Focus | 40°43'49.81"N / 8°37'2.04"W |
| | Adjacent Focus | 40°43'49.80"N / 8°37'2.24"W |
| | Bare Focus | 40°43'47.16"N / 8°37'1.70"W |

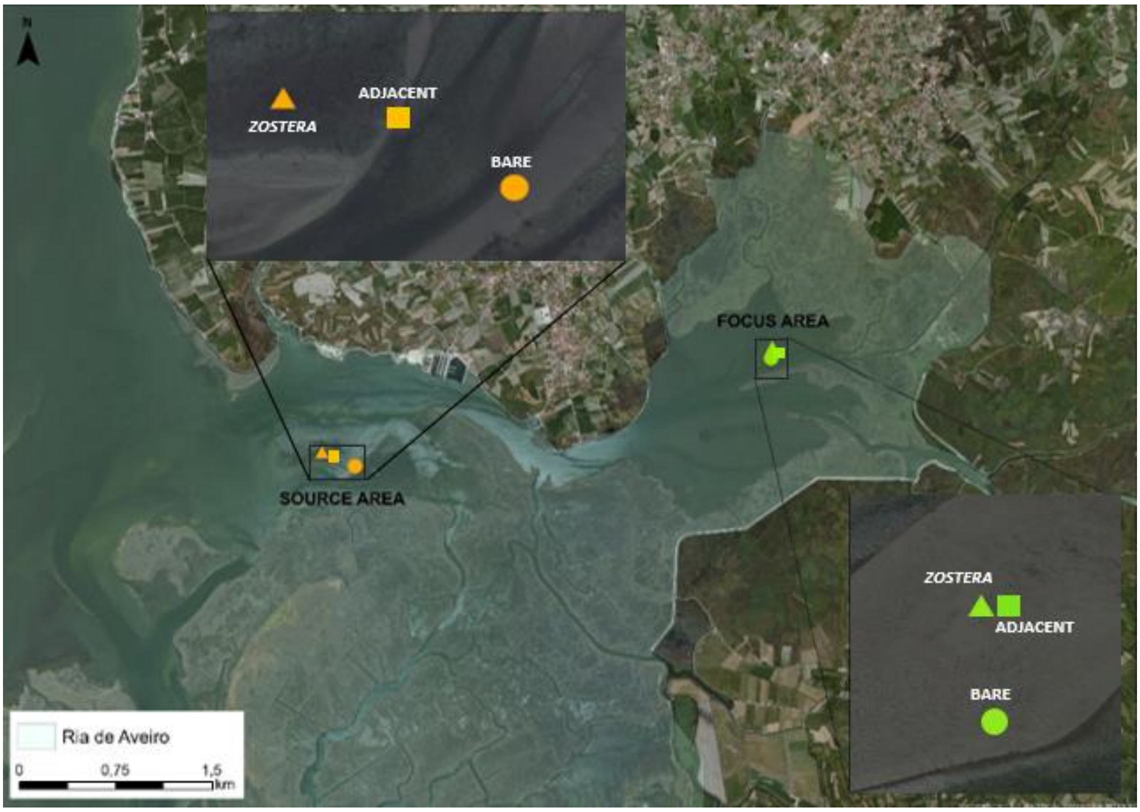


Fig. 3 - Source Area and Focus Area sampling areas of the Remedigrass Project, in the Ria de Aveiro. Map generated with ArcGIS 10.8.1.

## 2.3. Biological data

### 2.3.1. Collection and Processing

For the execution of this work, two field campaigns were carried out at the study site on 23rd June and 21st September of 2021, 1 year after the successful transplant of *Z. noltei* in the Laranjo area. At each Source and Focus area, 5 replicates were taken at each sampling site (Bare, Adjacent and *Zostera* sites), resulting in a total of 15 replicates per location, and 30 replicates on each field campaign. Each sample was collected with a polyethylene corer (Fig. 4A and Fig. 4B) with a circumference area of 0.0117 $m^2$, then placed in a net bag with 0.5mm mesh (Fig. 4C). These were washed *in situ* (Fig. 4D) and stored in a plastic bag. On that same day, the samples were transported to the laboratory and frozen (-20ºC) until further processing.

At the field and at each sampling campaign, physical-chemical parameters (temperature, salinity, pH and $O_2$) of the water were measured *in situ* from the interstitial water pools. Additional sediment samples were collected for grain size analyses and Hg concentration quantification. These sediment samples were further processed by the CESAM team for grain size analysis through sequential sieve sizes analysis, sediment organic matter and Hg concentration following the procedures by Oliveira et al. (2018).


Fig. 4 - (A) The sampling polyethylene core used to survey the benthic communities, (B) sample being removed with core in Bare site, (C) sample being placed in net 0.5mm bag, (D) sample washing in the estuary.

The laboratory work was divided into two phases: processing and identification of the invertebrates (sorting, taxonomic identification, abundance, and evaluation of biomass). First, the sample is washed in a sieve with a spacing of 0.5mm, and then screened. *Zostera noltei* sorting and quantification was also carried out, separating the leaves and rhizomes.

All organisms were sorted and identified under the stereoscopic microscope or the compound microscope, to the lowest possible taxonomic resolution. After identification, the next step was to dry the invertebrates to evaluate their biomass as ash free dry mass (AFDM). All individuals were placed in crucibles, all previously weighed, placed on a tray by group and sample (Fig. 5A), using a stove at 60ºC during the minimum period of 48h. After drying, the individuals were weighed again, and placed in a laboratory muffle oven, with the crucibles, at 450ºC for 8h. The case of individuals of very small sizes, were placed on the muffle on an appropriate metal plate, with small holes suitable for their dimensions (Fig. 5B).

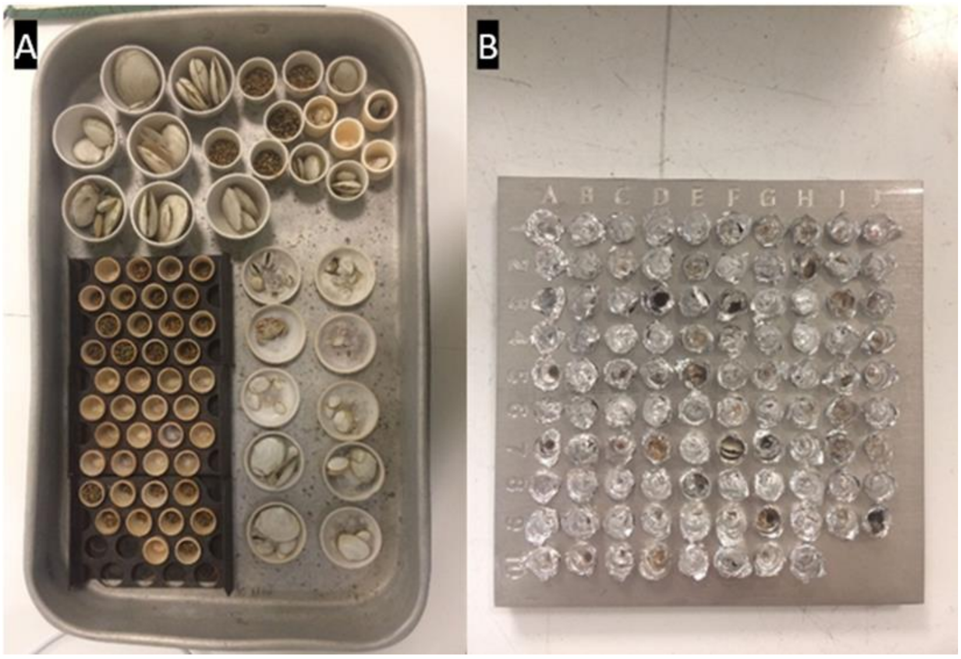


Fig. 5 - (A) Tray with the invertebrates in the crucibles. (B) invertebrates in aluminum crucibles on metal plate for biomass quantification.

## 2.4. Statistical Analysis

We computed diversity indices for benthic communities, namely the Species richness (as the species number) and the Shannon Wiener (H'), as well as total density and biomass. A statistical analysis was carried to evaluate the differences between these indicators and for mercury concentration, key species (density and biomass), and *Zostera* biomass (total, leaves, and rhizomes). Differences were sought in these measures using two factors, contamination (Contaminated or Non-contaminated) and habitat (*Zostera*, Adjacent and Bare). Linear regressions were performed, however, since the requirements of normality and homogeneity were not achieved, the Generalized Least Square (GLS) extension was applied to the regression model. The most adequate GLS extension was established, using a restricted maximum-likelihood (REML) estimation (Zuur et al., 2009) and with the lowest Akaike Information Criteria

(AIC). Afterwards, a manual backward step regression was made with the maximum-likelihood (ML) ratio test. The best model was chosen based on the p-value. All the p values lower than 0.05 were evaluated as statistically significant. The analysis was performed within "R" software, a statistical and programming environment (R Development Core Team, 2020), using the *vegan* package (Oksanen et al., 2022) for H', with the *nlme* package (Pinheiro et al., 2014; Pinheiro & Bates, 2000) for GLS, and all the plots were made with the *ggplot* package (Wickham, 2016).

# 3. Results

## 3.1. Environmental data

For the environmental data, we do not present values for the Adjacent site, as this was too close the *Zostera* one, so, their values were assumed to be the similar. The *Zostera* Source presented higher values for pH and $O_2$ than the *Zostera* Focus (Table 3). Within the Bare sites, the Bare Focus had higher values of Hg concentrations and organic matter than Bare Source. For temperature, the Bare Source and Zostera Focus sites presented higher values than the others. Overall, the variation within these environmental data was similar between sampling dates, except for the $O_2$% (Table 3).

Table 3 - Mean and standard deviation values of the physical-chemical variables at each sampling site on the two dates.

| | Dates | *Zostera* Source | Bare Source | *Zostera* Focus | Bare Focus |
|---|---|---|---|---|---|
| Hg ($mg.kg^{-1}$, mean ± s.d) | 23.06.21 | 0.70 ± 0.03 | 0.41 ± 0.05 | 2.39 ± 0.36 | 13.14 ± 3.94 |
| | 21.09.21 | 0.55 ± 0.25 | 0.57 ± 0.01 | 1.88 ± 0.48 | 9.11 ± 2.19 |
| organic matter (%, mean ± s.d) | 23.06.21 | 8.73 ± 0.52 | 4.89 ± 0.64 | 3.78 ± 0.63 | 7.61 ± 0.61 |
| | 21.09.21 | 10.17 ± 0.43 | 4.59 ± 1.15 | 4.94 ± 0.41 | 8.88 ± 0.68 |
| pH (mean ± s.d) | 23.06.21 | 8.58 ± 0.08 | 7.77 ± 0.10 | 7.68 ± 0.26 | 8.15 ± 0.08 |
| | 21.09.21 | 8.43 ± 0.07 | 7.89 ± 0.00 | 7.82 ± 0.02 | 7.63 ± 0.04 |
| temperature (ºC, mean ± s.d) | 23.06.21 | 21.93 ± 0.06 | 26.63 ± 1.48 | 25.83 ± 1.97 | 24.77 ± 0.65 |
| | 21.09.21 | 19.67 ± 0.06 | 29.97 ± 0.55 | 28.3 ± 0.17 | 16.4 ± 0.10 |
| $O^2$ (%, mean ± s.d) | 23.06.21 | 166 ± 3.61 | 92.33 ± 2.89 | 79.33 ± 18.34 | 101 ± 15.59 |
| | 21.09.21 | 97.37 ± 2.76 | 82.53 ± 7.35 | 79.83 ± 5.45 | 44.57 ± 5.24 |

Regarding Hg concentration in the sediments, considering the 4cm depth, the Bare Focus site presented a much higher value (the average of the two sampling moments) than all the other sites, 11.13 $mg.kg^{-1}$, while the second highest was observed for the

*Zostera* Focus, but still a connsiderably lower value than in the Bare mudflats, 2.14 mg.kg$^{-1}$ (F-test= -6.05, p-value= 0, Fig. 6). In the Source area, the difference between the values of the two sites was very small, with the *Zostera* site having a slight increase from the Bare site (F-test= 1.59, p-value= 0.12, Fig. 6).

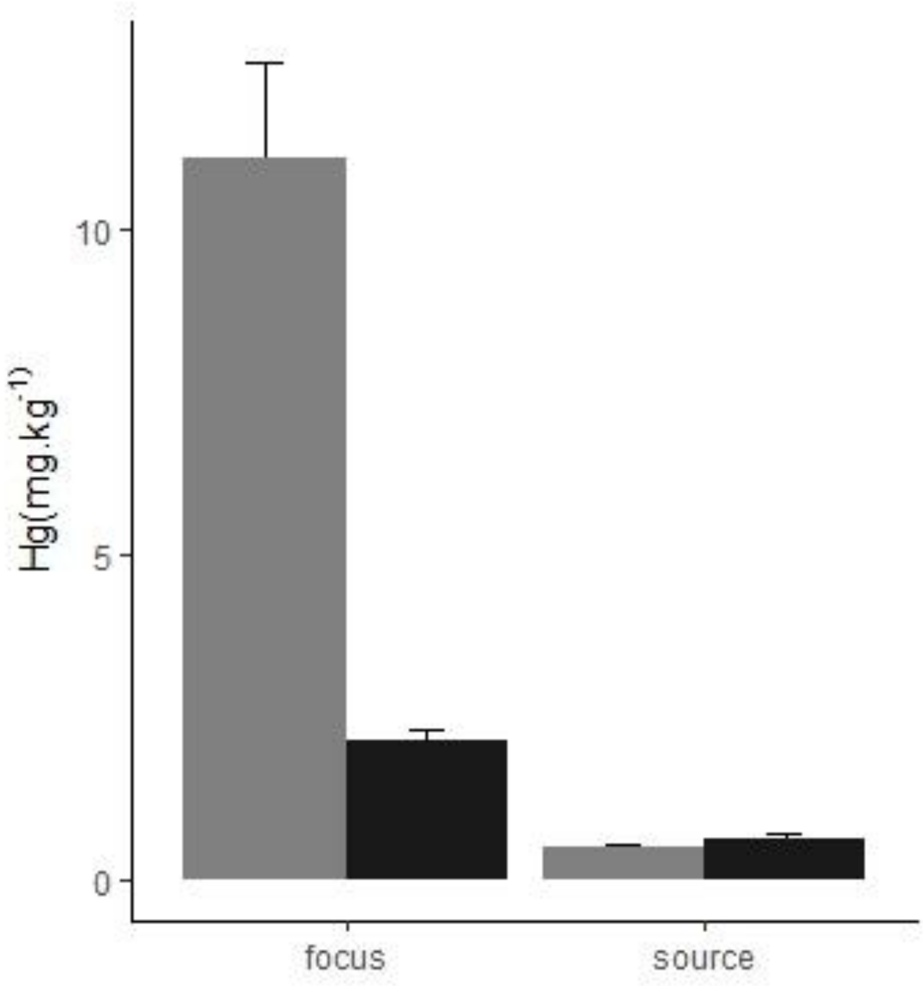


Fig. 6 - Mercury concentrations in the sediments (mean ± s.e., n = 6) considering the 4cm into the sediments considering two sites of each area, averaged for the two sampling dates. For comparison, values of each site are presented: Bare ; *Zostera* .

The sediment in *Zostera* Focus site was classified as sand in both dates, dominated by medium sand (Table 4). The sites Bare Source and *Zostera* Source were classified as muddy sand. In the median values, the Focus area has the highest values in both sites, except between the Bare sites of the September date. Both Focus sites present a dominance of medium sand, while in the Source sites, *Zostera* has a dominance of muddy sand, and in the Bare fine sand is the dominant textural group.

Table 4 - Median grain size analysis for the sampling site on the two dates.

| | Dates | *Zostera* Focus | Bare Focus | *Zostera* Source | Bare Source |
|---|---|---|---|---|---|
| Textural group | 23.06.21 | Sand | Muddy sand | Muddy sand | Muddy sand |
| | 21.09.21 | Sand | Muddy sand | Muddy sand | Sand |
| Median (µm) | 23.06.21 | 272.9 | 172.7 | 82.31 | 131.6 |
| | 21.09.21 | 285 | 134.7 | 125.2 | 157.4 |
| Mud (%) | 23.06.21 | 8.5 | 14.5 | 29.1 | 19 |
| | 21.09.21 | 7.7 | 18.7 | 29.8 | 8.2 |
| Medium sand (%) | 23.06.21 | 43.5 | 34.9 | 13.7 | 15.2 |
| | 21.09.21 | 41.8 | 21.4 | 19.2 | 29.5 |
| Fine sand (%) | 23.06.21 | 18.7 | 21.9 | 19.3 | 32.4 |
| | 21.09.21 | 17 | 24.8 | 18.1 | 37.7 |

The biomass of *Zostera noltei* is consistently higher in the Source area (Fig. 7), independently of considering only leaves, rhizomes, or the total values. In the Focus area, the values between *Zostera* rhizomes and *Zostera* leaves were similar, with *Zostera* rhizomes having 65 g.m$^{-2}$ and *Zostera* leaves 75 g.m$^{-2}$. In the source area, differences were more noticeable, where *Zostera* leaves reached the highest value (138.91 g.m$^{-2}$). Regarding *Zostera* total biomass, it presents a larger difference between the Focus and Source area, however with no statistical differences (F-value= 1.93, p-value= 0.07, Fig. 7).

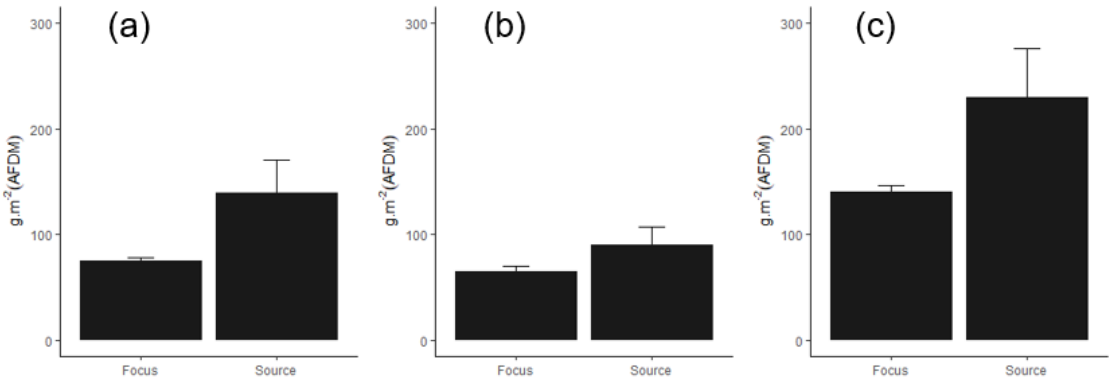


Fig. 7 - Biomass of *Zostera* leaves (a), *Zostera* rhizomes (b) and *Zostera* total (c) in the two areas, considering the *Zostera* sites (mean ± s.e., n = 10).

# 3.2. Community Responses

## 3.2.1. Benthic community descriptors

In total, 26 species were identified (Table 5) and 5,292 invertebrates were counted. The most abundant group was the Gastropoda with a total of 3772 individuals, followed by Polychaeta with 1181 individuals.

Table 5 - List of identified species considering all selected sites in Ria de Aveiro.

| Phylum | Class | Species |
|---|---|---|
| Annelida | Polychaeta | *Alkamaria romiji (*Horst, 1919) |
| | | *Capitella capitata* (Fabricius, 1780) |
| | | *Glycera alba* (Müller, 1776) |
| | | *Hediste diversicolor* (O.F. Müller, 1776) |
| | | *Mysta picta* (Quatrefages, 1866) |
| | | *Sabellaria* sp. |
| | | *Spio filicornis* (Müller, 1776) |
| Arthropoda | Hexanauplia | *Copepoda* sp. |
| | Insecta | *Dolichopus* sp. |
| | Malacostraca (Order Amphipoda) | *Amphitoe rubricata* (Montagu, 1808) |
| | | *Amphitoe valida* (S.I. Smith, 1873) |
| | | *Corophium volutator* (Pallas, 1766) |
| | | *Ericthonius difformis* (H. Milne Edwards, 1830) |
| | | *Gammaropsis sp.* (Lilljeborg, 1855) |
| | | *Mysidacea* (Haworth, 1825) |
| | Malacostraca (Order Decapoda) | *Carcinus maenas* (Linnaeus, 1758) |
| | Malacostraca (Order Isopoda) | *Cyathura carinata* (Krøyer, 1847) |
| | | *Sphaeroma serratum* (Fabricius, 1787) |
| | | *Crangon crangon* (Linnaeus, 1758) |
| Molusca | Bivalvia | *Ensis siliqua* (Linnaeus, 1758) |
| | | *Cerastoderma edule* (Linnaeus, 1758) |
| | | *Scrobicularia plana* (da Costa, 1778) |
| | Gastropoda | *Haminoea hydatis* (Linnaeus, 1758) |
| | | *Littorina* sp. |
| | | *Omalogyra atomus* (Philippi, 1841) |
| | | *Peringia ulvae* (Pennant, 1777) |

The analysis on the total community average density presented the highest values for the *Zostera* sites. For the biomass, the *Zostera* site had the highest value within the Source area, while the Bare site attained highest biomass community levels within the Focus area. Comparing both areas, the Focus area had higher biomass values than the Source area. It is also noticeable that the Bare sites presented, in general, higher biomass values than the Adjacent sites (Bare Focus*Adjacent Source: F-value= -0.69, p-value= 0.49, Fig. 8).

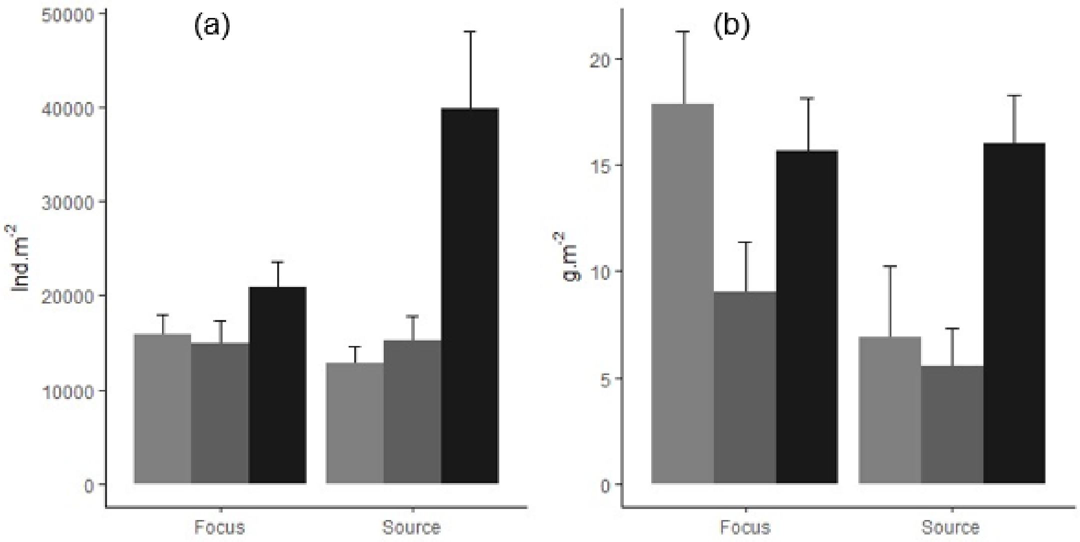


Fig. 8 - Total community average (mean ± s.e., n = 5) density (a) and biomass (b) in the two areas, considering the three sites. For comparison, values of each site are presented: Bare ; Adjacent ; *Zostera* .

Regarding community diversity indicators, we found the highest species richness values for the Bare mudflats and lowest for the Adjacent ones (on average 9.6 s.d and 7.2 s.d respectively, Fig. 9). Still, we only found statistically significant differences for the Source area, when comparing the Adjacent with the Bare site (F-test= 3.017, p-value= 0.003, Fig 9). When weighing diversity through the Shannon Wiener index, we found similar results for those measured with density, where the Bare mudflats have the highest values. Indeed, for the Focus area, the trends were the same, despite not statistically significant (*Zostera* Focus*Adjacent Source: F-value= -1.61, p-value= 0.11, Fig. 9): decreasing from the Bare mudflat, seagrass meadow to the Adjacent for the density, and the increasing trend for the biomass, with the highest value for the seagrass, in the Focus area (Fig. 9).

When comparing sites in the Source area, the Adjacent site had always the lowest diversity, followed by the *Zostera* meadows and, finally, the Bare mudflats, with the highest values. Still, results were not statistically different for the *Zostera* and Bare mudflats (*Zostera* Focus*Bare Source: F-value= -1.24, p-value= 0.22, Fig 9). Overall, diversity was higher for the Source area compared to the Focus one, except when weighted by density (F-value= 3.83, p-value= 0.06, Fig. 9).

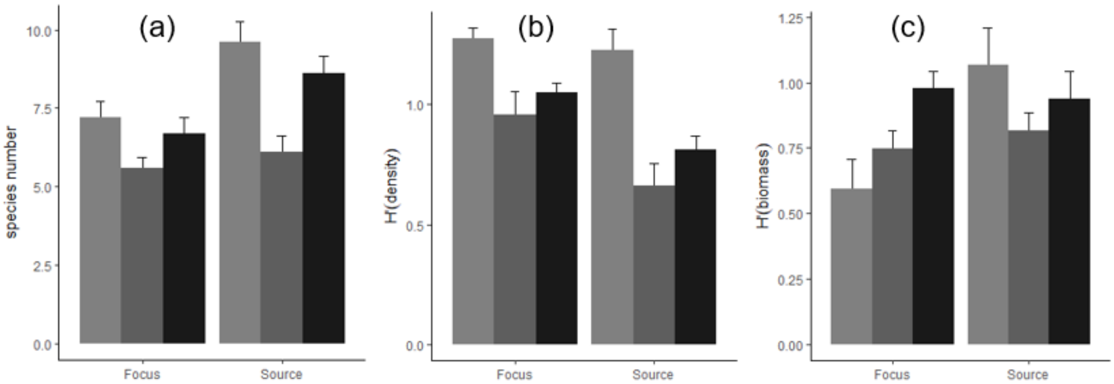


Fig. 9 - Community diversity indicators for the communities (mean ± s.e., n = 5) as (a) species number, (b) Shannon-Wiener measured taking into account density, and (c) Shannon-Wiener measured taking into account biomass. For comparison, values of each site are presented: Bare ; Adjacent *; Zostera* .

## 3.2.2.Species-specific responses

Three key species were selected for more detailed analysis: *Peringia ulvae, Hediste diversicolor, Scrobicularia plana*. This selection was based on each species contribution to the total biomass, as dominant in the community.

For these three key species, their density was always the highest in the *Zostera* site, in both areas (Fig. 10). However, the same result did not happen for the biomass, as *H. diversicolor* and *S. plana* had higher biomass in the Bare mudflats of the Focus area, compared to other areas (Fig. 10). The species *P. ulvae* is the only with an equal trend for density and biomass variation for both areas, increasing from Bare mudflats, Adjacent to *Zostera* (Biomass: *Zostera* Focus*Adjacent Source: F-value= -5.08, p-value<0.001; Density: Adjacent Focus**Zostera* Source: F-value= 1.79, p-value= 0.07, Fig. 11Pd and Fig. 10Pb). *Hediste diversicolor* had the same results regarding density (Adjacent Focus**Zostera* Source: F-value= 5.00, p-value<0.001, Fig. 10Hd). As for *S. plana* species, a different density trend was observed for both areas: the Adjacent site has the lowest value, increasing to Bare and *Zostera* (Adjacent Focus**Zostera* Source F-value= 2.29, p-value= 0.02, Fig. 10Sd).

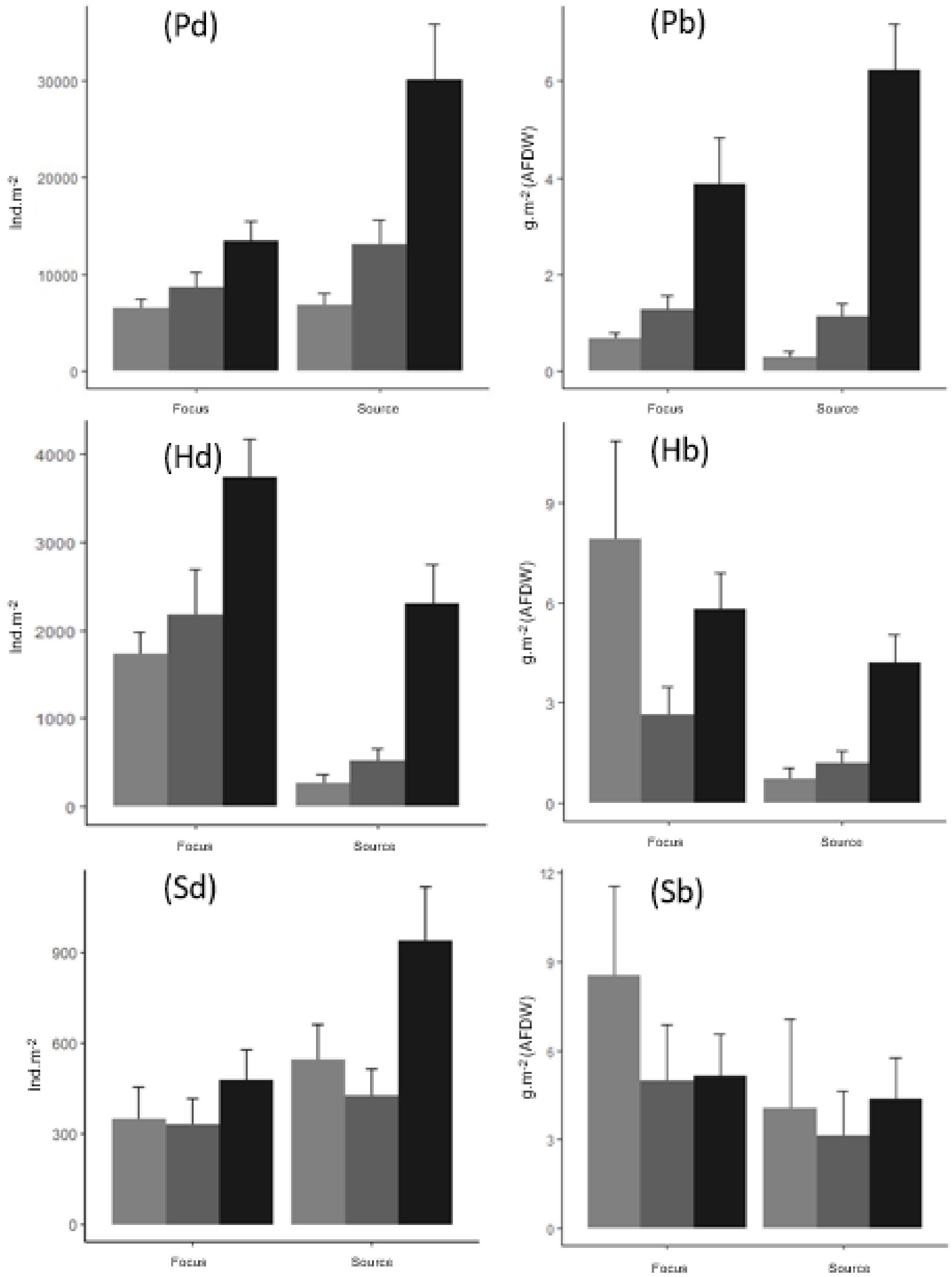


Fig. 10 - Species-specific responses (mean ± s.e., n = 10) for *Peringia ulvae* (P), *Hediste diversicolor* (H), *Scrobicularia plana* (S), regarding density (d) and biomass (b) in the two areas, considering the three sites. For comparison, values of each site are presented: Bare ; Adjacent ; *Zostera* .

# 4. Discussion

Seagrasses form some of the most productive ecosystems in the world, supporting highly diverse communities (Waycott et al., 2009; Duarte et al., 2008). Our results partially support that notion, with higher density and biomass of key-species found within the seagrass meadows. However, for the community biomass and diversity and one of the key estuarine species values were generally higher in the Bare mudflats, without the presence of the seagrass. Regarding biomass, we can verify that in the Focus area, the Bare site presents the highest value. This result can be related with the influence of the species *Scrobicularia plana,* as a species that presents the highest biomass and has a typical preference for muddy habitats (Pizzolla, 2002), which is characteristic with the Bare Focus site (predominance of the muddy sand textural group). It is also noteworthy that, despite the highest Bare mudflat values, the *Zostera* sites presented higher values for all the community levels and species indicators than the Adjacent areas, indicating an increase in complexity associated to the meadows compared to their neighboring Bare areas.

Our analysis held somewhat surprising results considering our initial hypothesis. We expected a gradual increase of the community diversity indicators values from the Bare, Adjacent, to the *Zostera* sites in all areas, in which the Bare would have the lowest and the *Zostera* the highest value, independently of belonging to the Focus or Source area. The presence of the seagrass, as a habitat forming species, usually reflects into higher benthic diversity and productivity (Dolbeth et al., 2007; Grilo et al., 2008; UNEP, 2020). The Bare sites showed higher H' and species richness values than expected when comparing with the *Zostera* sites. These larger values found in the Bare areas could be partially justified by the influence of *Peringia ulvae* on the Shannon-Wiener index. *Peringia ulvae* was among the most dominant in both *Zostera noltei* sites, as also observed in other estuarine systems, such as Mondego Estuary, together with the species *H. diversicolor* (Crespo et al., 2017). The species is also a pioneer one in systems recovering from disturbance (Cardoso et al., 2013). This dominance in the *Zostera* sites may be masking the effects of the presence of the vegetation and contributing to the decrease of H' since the index accounts for both the species richness and its evenness (Strong, 2016). As tested by Strong (2016), the Shannon-Wiener index is an imperfect and biased measure of diversity due to the unequal roles of evenness. Another study made in Ria de Aveiro by Nunes et al.

(2008), also detected low values for this indicator due to the abundance of the species *Peringia ulvae*, not considering the H' as good indicator.

In a more general analysis, within the perspective of the seagrass restoration project, the present results show some positive signs of a recovery from the benthic communities. From a perspective of biodiversity and abundance/biomass restoration, we have explored the cumulative effects from the seagrass recolonization with one year of success, and with some signs of Hg attenuation of the sediments and interstitial waters (Oliveira et al., 2022). Considering that benthic communities may take 2 to 7 years to recover from disturbance (Borja et al., 2010), we are already beginning to see some positive trends in the community indicators. Indeed, diversity values were not that different between Focus and Source areas, also considering that diversity in temperate estuarine areas is generally low due to their naturally challenging conditions (McLusky & Elliott, 2004). The transplant technique based on SODs may partially influence this result, as a sediment portion and the epifauna on the SODs are also transplanted (Costa et al., 2022). Still, these initial seagrass patches are a small cover compared to their expansion after one year.

The historical contamination in the Focus area may also influence the benthic recovery, since generally lower diversity and abundance have been found in these contaminated areas in the past (Nunes et al., 2008). Indeed, the Hg concentrations found in the Bare site of the focus area were still within the toxic levels for benthic invertebrates (e.g., >3mg/kg, Conder et al. 2015), despite being in lower concentration at the surface (Oliveira et al. 2022). Although with the current data and temporal scale it is difficult to ascertain that effect, the recovery trends along the *Zostera* continuum at the Focus area are a sign that the restoration may be starting to promote their benthic communities. However, we cannot disclose all the complexity generated by putting a new habitat, because, regarding biodiversity and community density, this new habitat has not yet reached the values of the original source.

In the case of the specific responses of the key species - *Peringia ulvae, Hediste diversicolor and Scrobicularia plana*, we found increasing density patterns along the *Zostera* continuum at the Focus area, consistent with a gradual recovery scenario and considering that the transplant site used to be a Bare mudflat. On the one hand, the relative tolerance of the species to Hg contamination may be beneficial for this recovery (Cardoso et al., 2013; Coelho et al., 2008); on the other, the new habitat may be promoting this increase, as discussed above for *P. ulvae.*

Nevertheless, we found different trends for the biomass of *Hediste diversicolor* and *Scrobicularia plana* in the Focus area, where values were highest for the Bare mudflat. The grain size, with muddier sediments and higher organic matter at the Bare mudflats, may be contributing to these results, which might be linked to the habitat preferences of the species (Pizzolla, 2002;  Budd, 2008). Regarding *H. diversicolor* population, the high abundance can also be related to its rapid recovery time, enhanced by the adult migration from adjacent areas (Ashley, 2016) and high physiological tolerance to extreme environmental factors, thus, highly opportunistic (Budd, 2008).

This pilot restoration project was developed to solve a very concrete problem with a very defined purpose. Still, efforts to reduce the loss of seagrass habitats have been occurring all over the world. Ecological restoration practices have become more relevant to conservation and natural resource management, as well as providing strategies that present realistic and concrete responses to sustainability. With the acceleration of environmental degradation, traditional recolonization practices are no longer sufficient (Pazzaglia et al., 2021). The recolonization of seagrasses is yet somewhat immature, since there are still major gaps. The development of these practices is necessary to foster the success of future programs (Tan et al., 2020).

When a restoration project is performed, there are different possible scenarios (Borja et al., 2010), dependent on the intensity, space, and time of disturbances. In our project, redirection through ecological restoration, where anthropogenic intervention assists secondary succession, is the one related to our case. However, as stated by Borja et al. (2010), some systems may not reach the state of the historical ecosystem and the historic environmental homeostasis, but may achieve an alternative state, with ecosystem structure, fostered by the presence of appropriate organisms. In our case, our three species (*H. diversicolor*, *S. plana* and *P. ulvae*) represent an important role in this matter, fostering the alternative state of the system.

# 5. Conclusion

This study provides evidence that a seagrass transplant action can help to enhance the stabilisation and evolution of the benthic communities. The continuous monitoring and maintenance of the transplanted areas is essential to guarantee good results, and to analyse the invertebrates' responses and adaptability to the recolonized area. Given the importance of benthic communities, it was already possible to verify some recovery in the ecosystem functions just one year after a successful transplant. So, it becomes increasingly evident the relevance of the efforts to recover and protect these habitats. Effective adoption of protective actions will benefit the benthic communities living in the seagrass meadows, enhancing their ecosystem services, contributing to environmental regulation and maintenance, and creating mutual benefits for the benthic communities and those that depend on productivity and on the nurseries of commercial species that this habitat provides.

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# Attachments


Attachment 1 - Outreach to high school students, at José Macedo Fragateiro High School, in Ovar. Date: 20 January 2022.