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Assessing macrobenthic community functional response to natural and human pressures in estuarine ecosystems using biological trait analysis (BTA)

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Abstract

Understanding an ecosystem's resilience, and potential for recovery in the face of anthropogenic and natural disturbance is crucial. Due to the influx of fresh and sea water, estuaries are naturally stressed ecosystems, but they are also vulnerable to high levels of human pressure. Benthic macroinvertebrates are a well-known component of estuaries, playing an important role in regulating their functioning. These communities respond predictably to disturbance due to their varying traits. Trait-based approaches can be useful to detect anthropogenic and natural pressures, since these will act as filters, selecting species with more suitable traits. The present dissertation investigates the macrozoobenthic community functional response to natural and human disturbances using Biological Trait Analysis (BTA) and Functional Diversity Indices (FDI). The study was carried out in two temperate estuaries using different case studies: i) the recipient area of a WWTP located in the Tagus estuary, after the implementation of secondary and tertiary treatments, to understand the effects of water quality improvement (WQI) and ii) the estuarine gradient of the Mira estuary to investigate the effects of climate change. Nine traits were selected, covering different aspects of morphology, behavior, and life history of each taxa, which might respond to disturbance. BTA combined with FDI, were able to detect spatial and temporal changes along the different disturbance gradients. Functional Richness index (FRic) proved to be the more effective FDI to detect different types of disturbance. The Mira estuary remained mostly stable at a functional level, despite the increase in species resulting from the augmented saltwater intrusion, showing a highly resilient community in an estuary strongly influenced by natural variations. The area near the Tagus WWTP continues to be under pressure, however there were improvements in functional diversity associated to WQI. This functional-based approach demonstrated the potential to be incorporated in estuarine monitoring/quality assessment programs.

Keywords: Estuaries; Climate change; Macroinvertebrates; Organic pollution; Functional traits

Resumo alargado

Compreender como os ecossistemas podem lidar com as pressões antropogénicas e perturbações naturais é fundamental para a determinação da sua resiliência e capacidade de recuperação após perturbações. Os estuários estão entre os ambientes mais produtivos, dinâmicos e complexos do mundo; no entanto, estão também sobre a influência de múltiplas pressões, naturais ou antropogénicas. As comunidades de macroinvertebrados bentónicos são uma componente fundamental dos estuários, tendo sido amplamente utilizadas como indicadores para avaliar e monitorizar os impactos humanos em sistemas aquáticos, uma vez que desempenham papéis importantes no funcionamento dos ecossistemas e respondem de forma previsível a muitos tipos de pressão. As abordagens tradicionais para avaliar as mudanças no funcionamento dos ecossistemas relacionadas com perturbações, têm-se centrado frequentemente em componentes estruturais, tais como a composição taxonómica, aplicando índices taxonómicos e de qualidade ambiental baseados na sensibilidade/tolerância das espécies à pressão. Estas métricas, embora tenham a capacidade de descrever padrões espaciais e temporais na composição e estrutura das comunidades de macroinvertebrados, não conseguem captar os mecanismos subjacentes às relações espécie-ambiente-pressão. Abordagens baseadas em atributos funcionais fornecem associações mais claras aos serviços ecossistémicos, uma vez que as características funcionais dos organismos refletem a forma como os mesmos influenciam os processos ecossistémicos e por serem úteis na deteção de pressões, uma vez que estas atuam como crivos, selecionando espécies com características mais adequadas. Em ecologia, as abordagens baseadas em características funcionais são frequentemente conhecidas coletivamente como "*Biological Trait Analysis*" (BTA). As tendências de perda de biodiversidade associadas a pressões antropogénicas à escala local também devem ser contempladas no contexto do stress natural e das alterações climáticas, uma vez que ambas condicionam o funcionamento dos ecossistemas. Nas últimas décadas tem havido diversas intervenções em Portugal com o objetivo de melhorar a qualidade da água, nomeadamente: a construção de estações de tratamento de águas residuais (ETAR) e a eliminação, ou a reconversão de áreas industriais. O presente estudo teve como principal objetivo investigar a resposta funcional de comunidades de macroinvertebrados bentónicos a pressões naturais e antropogénicas utilizando BTA e Índices de Diversidade Funcional (FDI). O estudo foi realizado em dois estuários portugueses utilizando diferentes casos de estudo: i) a área limítrofe ao emissário de uma ETAR localizada no estuário do Tejo, após melhorias infraestruturais, para compreender os efeitos na qualidade da água e ii) e o gradiente estuarino do Mira para avaliar os efeitos das alterações climáticas.

Os dados biológicos e ambientais utilizados neste estudo foram recolhidos: ao longo de um período de seis anos (2009-2013; 2019), durante o verão, em três radiais (IA, IB, IC) da zona euhalina do estuário do Tejo, sob a influência direta da ETAR de Alcântara, e dois transeptos de controlo localizados a jusante (C1 e C2); e em 1984 e 2019 em oito locais situados ao longo do gradiente estuarino do Mira, para abranger comunidades bentónicas presentes nas diferentes massas de água: três no euhalino; três no polihalino e dois no mesohalino. Nove atributos foram selecionados para investigar a resposta funcional bentónica às perturbações ambientais. Cada característica foi subdividida em diversas modalidades totalizando 40 categorias. As informações sobre os atributos funcionais foram compiladas principalmente através de bases dados na rede, bem como de publicações científicas e guias de identificação de espécies. Para proceder à BTA a nível das comunidades foram calculadas matrizes de amostras x atributos (LQ) multiplicando a tabela de amostras x espécies (L) pela tabela de espécies x atributos (Q). Foi aplicada a "*Fuzzy Coding approach*" (FC) permitindo atribuir valores a múltiplas categorias de um determinado atributo funcional. Foram calculados seis FDI: riqueza funcional (FRic); equitabilidade funcional (FEve); divergência funcional (FDiv); dispersão funcional (FDis); Entropia

quadrática de Rao (RaoQ) e redundância funcional (FRed) com base em matrizes de distâncias de Gower para ambos os estuários. Adicionalmente, testes de Kruskal-Wallis e Dunn foram realizados, para identificar diferenças nos FDI entre os anos e zonas de ambos os estuários. Os resultados obtidos nas tabelas LQ foram analisados com recurso a análises multivariadas, nomeadamente análise de variância multivariada permutacional (PERMANOVA) e análise de coordenadas principais (PCoA), para detetar padrões espaciais e temporais nas características funcionais ponderadas das comunidades bentónicas. Adicionalmente as características funcionais e variáveis ambientais foram testados quanto a sua correlação com os dois primeiros eixos da PCoA.

Foram recolhidos 164 taxa no estuário do Tejo, ao longo dos 6 anos. Os taxa que apresentaram maior abundância média foram os poliquetas *Tharyx* sp., *Aphelochaeta* sp., *Streblospio shrubsolii* e *Nephtys hombergii*. Não foram encontradas diferenças significativas através da BTA entre as comunidades de antes e depois das melhorias da ETAR no estuário do Tejo. As únicas diferenças detetadas foram entre 2019 e os anos de 2011 e 2013, provavelmente devido à afetação das comunidades pela ocorrência de uma seca prolongada em 2019. Porém, a análise estatística mostrou diferenças nos atributos relacionadas com a distância à fonte emissora, principalmente entre a radial de impacto mais próxima e a mais distante. As amostras da IC surgiram associadas principalmente a atributos funcionais relacionados com níveis mais baixos de perturbação, como espécies suspensívoras (F_S) de tamanho médio a grande (MS20_50) fixas (LP_A) e com preferência por substratos mais grossos (S_H). No entanto, esperava-se que as restantes características associadas a estas estações, vida curta e tolerância à poluição, estivessem mais representadas na radial IA, uma vez que espécies com estas características são mais propícias a suportar pressões, sugerindo que esta área ainda está sob stress. A maioria das amostras da IA são caracterizadas por espécies oportunistas de 1ª ordem, mostrando que este atributo funcional responde bem à poluição orgânica. Após as melhorias, não houve diferença significativa entre as características das comunidades bentónicas de 2009 para os anos seguintes.

Os resultados dos FDI corroboraram os da BTA, uma vez que nenhum índice foi capaz de detetar diferenças entre anos no Tejo. Embora as variações temporais não tenham sido significativas, houve um aumento na diversidade funcional e do FRic após as melhorias. O FRic foi o único índice que apresentou diferenças espaciais significativas (entre IA e IC), aumentando com a distância do emissário. Mesmo com flutuações ao longo dos anos, o FRic nunca voltou aos valores anteriores às obras. No entanto, esse aumento está muito correlacionado com um incremento da riqueza especifica. Ainda existe um impacto do emissário da ETAR nas comunidades bentónicas, no entanto, é fortemente sobreposto por outros fatores, como variações naturais, o que dificulta a identificação de tendências funcionais a nível temporal associadas às melhorias na qualidade da água.

Foram recolhidos 143 taxa no estuário do Mira. A comunidade de 1984 tinha espécies dominantes diferentes da comunidade de 2019, para as mesmas zonas. A BTA do estuário do Mira revelou diferenças significativas nos atributos funcionais entre os anos. A comunidade de 2019 caracterizou-se principalmente pela presença de predadores tubícolas de pequenas dimensões com preferência por sedimentos mais finos (areia e vasa). O aumento na proporção de predadores está provavelmente ligado a um incremento na disponibilidade de presas, uma vez que a comunidade de 2019 apresentou maior riqueza especifica. Não houve evidência de um aumento de espécies com maior afinidade por temperaturas mais elevadas, no entanto, como os estuários são ambientes altamente complexos com espécies adaptadas a instabilidade na temperatura e com elevada tolerância térmica, pode ser complicado detetar estas alterações. Foi identificado um gradiente espacial entre as zonas euhalina e mesohalina: a primeira correlacionada com um maior número de espécies de vida longa e de vida muito curta, espécies sensíveis à poluição com preferência por maior salinidade e águas mais quentes: contrariamente a segunda correlacionada com espécies suspensívoras, tolerantes à poluição com preferência por águas frias e de baixa salinidade. A representatividade de espécies com elevada longevidade e sensíveis à

poluição, sugere que esta área não se encontra muito perturbada. Ao contrário da BTA, nenhum dos FDI foi capaz de detetar diferenças entre zonas no Mira. FRic e FRed foram os únicos índices capazes de evidenciar diferenças significativas entre os anos. A "migração" de espécies "marinhas" para o interior do estuário poderá ter sido uma das razões a causar este aumento da riqueza específica e consequentemente da FRic. Embora existam evidências de uma FRic mais elevada em 2019, tal aumento foi influenciado por um incremento na riqueza especifica. Estes resultados implicam que, apesar de algumas disparidades funcionais entre comunidades, estas permaneceram praticamente inalteradas entre os anos, em termos da maioria dos componentes da diversidade funcional.

A combinação da BTA e do índice FRic provou ser a abordagem mais eficaz para explicar as alterações nas comunidades macrozoobênticas causadas por diferentes níveis de pressão. Além disso, as características funcionais das comunidades foram fortemente influenciadas por variações naturais, especialmente no estuário do Mira. Este ecossistema manteve-se maioritariamente estável a nível funcional, apesar do aumento de espécies e de FRic, evidenciando uma elevada resiliência das comunidades bentónicas num estuário fortemente influenciado por variações naturais. A zona próxima da ETAR de Alcântara continua sob pressão, mas registaram-se melhorias a nível da diversidade funcional. O próximo passo crítico deverá ser desenvolver uma base de dados abrangente, fiável e de acesso aberto de atributos funcionais das espécies macrobentónicas da Península Ibérica Atlântica.

Palavras-chave: Estuários; Alterações climáticas; Macroinvertebrados; Poluição orgânica; Características funcionais

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1. Introduction

Understanding how an ecosystem can cope with anthropogenic pressures and natural disturbance is fundamental for the determination of its resilience, resistance, and recovery capacity after disturbance (Vinagre et al., 2017). Coastal areas, in particular, estuarine ecosystems are among the most productive, dynamic, and complex environments in the world (Bebianno et al., 2017; Liu et al., 2003), however, they are under the influence of multiple stressors and disturbances (Veríssimo et al., 2012), naturally or anthropogenically driven. Estuaries constitute naturally stressed, highly variable ecosystems, due to fresh and marine water input, but are also particularly exposed to high degrees of anthropogenic stress (Dauvin, 2007; Elliott and Quintino, 2007). These anthropogenic pressures often stem from inputs coming from metropolitan areas located at the estuaries margins or originating from upstream areas (Mucha et al., 2005). Both anthropogenically and naturally driven stresses, may affect these ecosystems, causing degradation in ecological quality, impacting biodiversity and functioning (Micheli et al., 2016), and thus, compromising their ability to sustain ecosystem services (Halpern et al., 2008; Thrush et al., 2008).

Macrobenthic communities are a well-known component of estuarine ecosystems, having a fundamental role in regulating the functioning of aquatic systems and being an essential part of the food web (Villnäs et al., 2012). This group of organisms have been widely used as indicators for assessing and monitoring the extent and magnitude of human impacts over aquatic systems (Chainho, 2008; Sousa, 2016) as they usually show specific responses to environmental changes (Quintino et al., 2006) and have important roles in ecosystem functioning (Hu et al., 2019; van der Linden et al., 2017). Divergent characteristics of these communities, such as, very diverse physiological tolerances, feeding modes, adaptability, and resilience to different levels of disturbance, make them respond predictably to many kinds of natural and anthropogenic pressures (for further details, see Chainho, 2008 and Rosenberg, 1978). Because of that, macrobenthic composition and abundance has been used as one of the biological elements to assess the ecological quality status (EcoQS) of European transitional waters in the aim of the implementation of the Water Framework Directive (WFD) (2000/60/EC; Annex V, 1.1.4).

In transitional environments, natural disturbance (e.g., highly variable hydrodynamic conditions, temperature, salinity, and oxygen concentration) constrains the benthic fauna, therefore, macroinvertebrate communities are usually composed by a low number of species, low taxonomic diversity (Dauvin, 2007) and by species capable of coping with stress (natural and human-induced disturbed conditions) (Elliott and Quintino, 2007). Consequently, the dominance of tolerant species does not always guarantee a correct assessment of EcoQS, as it might be undervalued due to high levels of stress caused by natural variations. Due to this, the number of species and taxonomic diversity cannot be considered reliable measures of ecosystem functioning if no appropriate references of good status are defined (Marchini et al., 2008).

Urban sewage, industrial effluents, the diffuse contamination originated by runoff of nutrients and pesticides from agriculture activities, fishing, dredging activities as well as pollution caused by the transport sector, constitute the main anthropogenic perturbations that cause significant impacts on water quality; also conditioning the structure and dynamics of biotic communities in estuarine systems (Chainho et al., 2013). In the last decades there have been several interventions with the purpose of

improving water quality, specifically the construction of wastewater treatment plants (WWTPs) the elimination, or the reconversion of industrial areas and the implementation of European directives, such as: the European Urban Wastewater Directive (Council Directive 91/271/EEC amendment by the Directive 98/15/EC) that framed the requirements for water quality and discharges from urban wastewater treatment plants to sensitive areas; and the WFD that requires the application of measures to achieve the Good status and prevent further degradation of the hydromorphological, chemical and ecological status of water bodies through the implementation of the River Basin Management Plans.

Comprehending the processes shaping biological communities under multiple disturbances is a core challenge in ecology and conservation science (Mouillot et al., 2013). Classical approaches to assess changes in species-environment relationships (ecosystem functioning), regarding disturbance in marine and transitional ecosystems have often been focused on structural components such as taxonomic composition, applying taxonomic-based indices (e.g. richness, diversity, and abundance of species) and environmental quality indices based on species sensitivity/tolerance do disturbance (Aarnio et al., 2011; Borja et al., 2004; Muniz et al., 2013; Muxika et al., 2007; Vandewalle et al., 2010) such as the Azti-Marine Biotic Index (AMBI) (Borja et al., 2000; Borja et al., 2007). These metrics may describe spatial and temporal patterns in the communities composition and structure (van Der Linden et al., 2016a). However, they do not capture the causal mechanisms underlying species–environment relationships (Mouillot et al., 2013; Statzner and Bêche, 2010; Stuart-Smith et al., 2013; Verberk et al., 2013), frequently being deficient when investigating processes that sustain an ecological system (Munari, 2013). Moreover, the strength of traditional taxonomic-based metrics to assess human-induced effects is still not clear and their use raises several concerns (for further details, see Veríssimo et al., 2012). Growing awareness that changes in biodiversity may potentially modify ecosystem functioning led to the perception of the importance of functional structure to understand the effects of human impacts on community functioning (Darr et al., 2014) and, for this reason, recent research has been moving toward incorporation of functional attributes to evaluate the effects of disturbance (Veríssimo et al., 2012). From a biological viewpoint, the functional structure of a community can be represented by a set of traits, which are morphological, behavioral, or ecological characteristics of an organism, displayed by the observed species and usually measured at the individual level (Paganelli et al., 2012). However, these traits are also often applied to characterize the ecology of a given species serving as a proxy on their potential resilience and resistance to environmental disturbances (van den Brink et al., 2011).

Trait-based approaches provide clearer associations to ecosystem services since the functional characteristics of the organisms involved are the main properties by which organisms influence ecosystem processes, rather than by their taxonomy (Grime, 1997; Petchey and Gaston, 2006). Additionally, trait composition can be useful to detect anthropogenic and natural pressures, since these will act as filters, selecting species with more suitable traits (Mathers et al., 2017). In marine and coastal ecology, trait-based approaches are often collectively known under the name "biological trait analysis" (BTA) (van Der Linden et al., 2016a), which is a useful analytical method for addressing ecological functioning and to improve the understanding on how disturbance and/or environmental gradients impact communities (Bremner et al., 2003; Veríssimo et al., 2012).

Trends of biodiversity loss linked to local and regional scale human-mediated pressures must also be contemplated in the context of natural stress fluctuations and anthropogenically driven climate change (Birchenough et al., 2015; Firth and Hawkins, 2011) as these are compromising ecosystem function (Covich et al., 2004). Cascading effects of climate change, such as sea level rise, more common extreme weather events and, above all environmental warming will impact all functional levels, from structural traits to population dynamics and ecosystem structure and functioning, and consequently the

services they provide (Bosch-Belmar et al., 2021). In Portugal, the application of BTA to estuarine systems is scarce, having mostly been applied in the Mondego estuary to investigate: the effect of hydromorphological disturbance on benthic communities (Alves et al., 2014; van der Linden et al., 2016b); changes in distribution after seagrass bed restoration (Dolbeth et al., 2013); response of the subtidal benthic invertebrate communities to high variability of environmental conditions (van der Linden et al., 2012) and the effects of management measures following environmental restoration (Veríssimo et al., 2012). It has been also applied in the Tagus estuary, only addressing the effects of metal contamination (Piló et al., 2016), in the Mira estuary, regarding functional changes of benthic nematode assemblages during natural recovery of seagrass meadows (Materatski et al., 2016), and more recently in the Sado estuary to study nematode taxonomic and functional responses to estuarine gradients (Sroczyńska et al., 2021). To the best of my knowledge there is no research concerning the effects of wastewater treatment plants improvements and upgrades on macrobenthic communities functions, neither to investigate the effects of climate change, using BTA in Portuguese estuaries.

To fill these knowledge gaps, the present work aims at investigating the functional response of the macrobenthic communities to natural and anthropogenic pressures using two distinct case studies:

- the Tagus estuary (Lisbon, Portugal), in the recipient area of the Alcântara WWTP, after the improvements expected with the enhancement of secondary treatment and implementation of tertiary treatment;
- the Mira estuary (Odemira, Portugal), to investigate the effects of climate change on the benthic communities along the estuarine gradient.

The assessment of macrobenthic community functional response in these case studies aimed at:
- ascertain potential differences in the capacity of BTA and FDI to detect local anthropogenic disturbance and to detect natural variations due to climate change;
- provide a framework capable of detecting different types of disturbance;
- extend the current knowledge on how anthropogenic disturbance and climate change may impact macrobenthic communities in estuarine ecosystems, contributing to a better understanding of the ecological functioning of these communities within transitional systems.

2. Materials and methods

2.1. Study area

Two temperate estuarine systems located on the Portuguese coast (Tagus and Mira) were included in this study.

The Tagus estuary is one of the largest European estuaries, located in the most populated area of Portugal. With an area of 340 km^2, this mesotidal estuary with semi-diurnal tides receives most freshwater flow from the Tagus River, which has a high annual average riverine flow (400 m^3 s^{-1}) (Piló et al., 2016). This system receives effluents from industrial, agricultural, and urban sources, being highly disturbed mainly by the inflow of effluents from about 2.5 million Great Lisbon Area inhabitants (Chainho et al., 2010). The present study focused on the intermediate area of the estuary, more exactly the area adjacent to the discharge of the Alcântara WWTP effluents (Fig. 1), which has the capacity to treat the wastewater of at least 756,000 inhab.eq (Azeda et al., 2013). The northern areas of this wetland were classified as a natural Reserve in 1976 (Tagus Estuary Nature Reserve). Furthermore, a larger

extent, which includes intertidal areas, was also assigned as a Special Protection Area (SPA) in 1994, under European Union legislation (Birds Directive 79/409/EEC), in order to preserve the national value of this estuary (Moreira, 1999).

Macrobenthic and environmental data were collected in the Tagus estuary under the monitoring program of the benthic macroinvertebrates developed by the Municipality of Lisbon and MARE - Marine and Environmental Sciences Centre of ULisboa. The main objective of this program was to test if the improvement of the secondary treatment and the introduction of tertiary treatment of effluents in 2009 in the WWTP of Alcantra, led to an improvement on the ecological status of adjacent benthic macroinvertebrates assemblages (Azeda et al., 2013). Data used for this study was gathered over a period of six years (2009-2013; 2019), during summer. This season was selected in all of the datasets to avoid the natural stress induced by high freshwater inputs in estuarine environments during winter and high levels of productivity in spring (intense reproduction period of benthic macroinvertebrates occurs in spring in Portugal) (Silva et al., 2006).

The Mira estuary is a transitional water body with a narrow channel shape (Fig. 2), located in the protected area of Parque Natural do Sudoeste Alentejano e Costa Vicentina (PNSACV). This system is naturally highly dynamic but nearly undisturbed when compared to the other Portuguese estuaries, that are exposed to higher human pressure (Medeiros et al., 2012), and hence, may be less exposed to nutrient and chemical pollution (Castro and Freitas, 2006). Even though there are some potentially polluting human activities (for further details, see Costa, 2004), it is still a system with reduced exposition to anthropogenic pressures (Cardoso et al., 2011, Vasconcelos et al., 2007).

2.2 Sampling Design and Datasets Description

2.2.1 Tagus estuary

The experimental design included a set of sampling stations in the euhaline zone of the estuary, arranged in three radials (IA, IB, IC), under the expected direct influence of the Alcântara's WWTP outfalls, and two control transects located downstream (C1 & C2), predicted to be out of the area where the effects of the effluents were significant (Fig. 1). Control stations were not established upstream, due to the high influence of port facilities in this area (highly impacted area). Impact radials were separated by approximately 50 m from each other, with the first one located at about 30 m off the shore. The control transects were established at 350 m and 500 m off the outfalls, respectively.
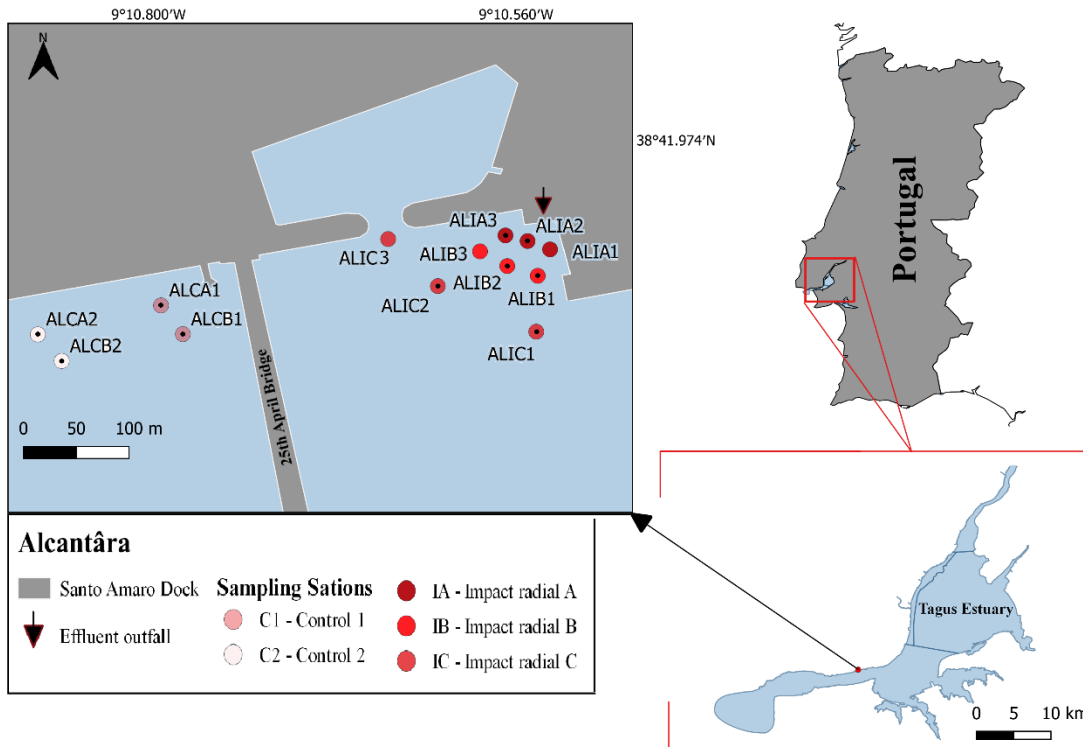
Figure 1: *Location of the sampling stations of the Alcântara WWTP monitoring program in the Tagus estuary. Stations with a black dot were used in this study.*

Environmental data collected included the sediment grain size (SGZ) and sediment total organic matter (TOM). For the determination of SGZ, samples were first dried for 48h at 60 °C and sieved through several mesh sizes and subsequently each resultant fraction was weighted. Regarding mud fraction, this was obtained following Gaudêncio et al. (1991) by calculating the difference to initial dried sample weight. TOM was obtained from difference between the weight of samples dried for 48h at 60 °C and weight obtained by loss on ignition (550 °C during, at least, 4h in a muffle).

Samples were collected using a Day grab 0.1 m^2 in a total of nine impacted stations and four control stations per year. Only six of the nine impacted stations, two from each impacted radials, were used in this study analysis. These sampling stations were chosen since they were the only with comparable data for all the years allowing to analyze changes on the macrobenthic communities after improvements made in the Alcântara's WWTP. Samples were later fixed and preserved in a 4% buffered formalin solution and stained with Bengal Rose. Fixed samples were posteriorly washed to remove formalin and reserved in alcohol 70%. Specimens were then sorted, counted, and identified to the lowest possible taxonomic level.

2.2.1 Mira estuary

Benthic community samples were collected at 8 sites located along the Mira estuarine gradient, to encompass benthic communities occurring at the different water bodies identified in that transitional system in the aim of the WFD: WB1, covering the euhaline area according to the Venice System (1958) (stations MR11, MR12, MR13), WB2, corresponding to the polyhaline area (stations MR24, MR25, MR26), and WB3, covering the mesohaline area (Stations MR37, MR38) (Fig. 2). No oligohaline stations were used in this study since there was no corresponding data between the projects. Three

random replicates were collected at each sampling station using a van Veen grab (0.1 m^2) and sieved in situ through a 1 mm mesh sieve. Fixed samples were posteriorly washed to remove formalin and reserved in alcohol 70%. Specimens were then sorted, counted, and identified to the lowest possible taxonomic level. Since the available environmental data weren't comparable between the two years, they were not used in the analysis of the Mira estuary.
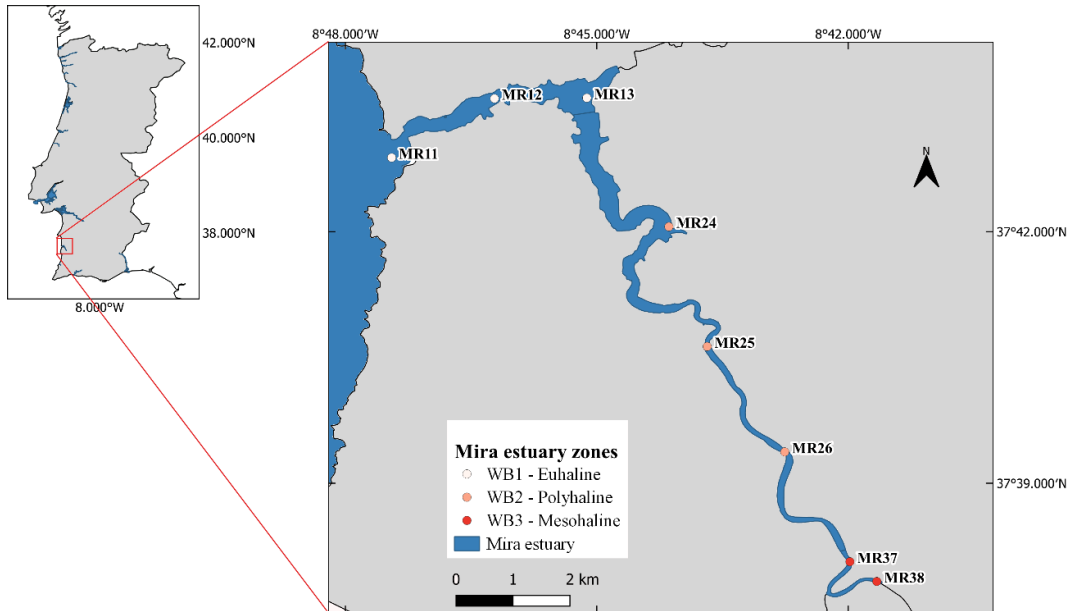
Figure 2: *Location of the sampling stations in the Mira estuary.*

A macrobenthic community dataset was created by including data collected in the Mira estuary within 2 projects:

- Andrade (1986) Ph.D. dissertation, whose main objective was to present a general and quantitative characterization of the macrobenthic communities of the Mira estuary from 1984 ;
- MESCLA project (Improve and complement the status classification criteria for transitional and coastal water bodies), using data from 2019 of the task of the project aiming to evaluate the biological quality element: "benthic macroinvertebrates for the coastal transition waters of Portugal" (Neto et al., 2020).

Data included was collected at the same locations along the estuarine gradient in 1984 and 2019 resulting in a 35-year long term change dataset. To ensure consistency only data pooled from sampling campaigns carried out during the summer were used.

2.3. Data analysis

2.3.1 Biological trait analysis

Nine traits were selected to investigate the benthic functional response, covering different aspects of morphology, behavior, and life history of each taxa, which might respond to environmental disturbance. Each trait was subdivided into several modalities adding up to a total of 40 functional categories.

An overview of the traits and categories used to characterize the functional features of the benthic macroinvertebrates is presented in Table 1. The trait modalities were assigned based on the diversity of the life functions that each trait represents and the status of knowledge on the traits. Biological traits are often able to demonstrate the link between environmental disturbances and the sensitivity or recovery potential of benthic macroinvertebrates populations, and are less influenced by natural spatial gradients, contributing to a more accurate assessment of anthropogenic impacts as compared to taxonomical approaches (Dolédec et al., 1999). Moreover, these can be a useful tool when trying to shed light on changes in benthic assemblages associated to climate change (Pacifici et al., 2017).

Table 1: Benthic macroinvertebrates biological traits and respective categories selected and analyzed in the present study, their labels, and the rationale/importance for choosing them.

Biological Trait	Trait Categories	Labels	Rationale and traits importance to indicate disturbance
Maximum size	Very small (<1 cm)	MS_1	The organisms body size is a key trait for the trophic structure of a community (Jennings et al., 2001), with crucial importance to various ecosystem functions (e.g. nutrient cycling) and dynamics of marine habitats (Blanchard et al., 2009), capable of indicating disturbance. The proportion of small-bodied invertebrates (i.e. better resilience capacity) in a community is expected to increase as a consequence of environmental/anthropogenic pressure imposed on the organisms (Norkko et al., 2013; Statzner and Bêche, 2010), therefore characterizing environments with high instability (Mouillot et al., 2013).
	Small (1-3 cm)	MS1_3	
	Small-medium (3-10 cm)	MS3_10	
	Medium (10-20 cm)	MS10_20	
	Medium-large (20-50 cm)	MS20_50	
	Large (>50 cm)	MS_50	
Adult longevity	Very short (< 1 year)	L_1	The longevity of an organism is of critical relevance for understanding temporal redistribution of nutrient processing which may shed light about the resilience of an organism in the presence of a disturbance (De Juan et al., 2007). When facing disturbances, the proportion of large slow growing taxa (longer life spans) is expected to decrease (Statzner and Bêche, 2010), with shifts from longer-lived species to less vulnerable faster growing species.
	Short (1-3 years)	L1_3	
	Medium (3-10 years)	L3_10	
	Long (> 10 years)	l_10	
Feeding guilds	Deposit feeders	F_DE	Indicates feeding interactions and food source availability, which have long been contemplated as an essential factor structuring invertebrate communities (Pearson and Rosenberg, 1987). It reflects the trophic structure, influencing energy flow and nutrient cycling and how organisms adapt to habitat changes and/or disturbance (Bremner, 2008; Webb et al., 2009). The proportion of suspension feeders in a community is expected to decrease after disturbance caused by organic pollution, while the proportion of deposit-feeders, grazers and omnivores are expected to increase (i.e. better resilience capacity) (van der Linden et al., 2016a).
	Grazers	F_GRA	
	Suspension	F_S	
	Herbivores	F_HER	
	Predator	F_PRE	
	Scavenger	F_SCA	
	Omnivores	F_OMNIP	
	Parasites	F_PARA	
AMBI ecological (sensitivity) groups (EG's)	(I) very sensitive species	EG_I	The AMBI index allow to detect anthropogenic impacts induced by different types of pressures and across different biogeographical regions (Borja et al., 2019). The proportion of taxa characterized as tolerant or opportunists (1st or 2nd orders) in a community is expected to increase after disturbance, while very sensitive species are expected to decrease (Borja et al., 2000).
	(II) indifferent	EG_II	
	(III) tolerant	EG_III	
	(IV) 2nd order opportunists	EG_IV	
	(V) 1st order opportunists	EG_V	

Developmental mechanism	Planktotrophic	DM_P	Indicates the macrozoobenthic larvae dispersal type, which is related to the dispersal potential of species and may indicate disturbance. The proportion of taxa with high dispersal potential (planktotrophic) is expected to increase after disturbance, because the extinction risk of taxa with a lecithotrophic (medium dispersal potential) and direct larval development is higher (McHugh and Fong, 2002).
	Lecithotrophic	DM_L	
	Direct	DM_D	
Living position	Burrow-dweller	LP_BD	The type of the small-scale motility and living position is a decisive survival factor for benthic communities, not only to avoid physical disturbance (Hinchey et al., 2006), but also regarding the predator–prey interactions or even the construction of biological structures. Dwellers (tube or burrow) are theoretically less vulnerable to strong water pollution and natural disturbance as opposed to free-living species (Reise, 2002).
	Tube-dweller	LP_TD	
	Free-living	LP_FREE	
	Attached	LP_A	
Salinity preference	Mesohaline (5-18)	SP5_18	Salinity and its fluctuation define the organisms' longitudinal distribution in an estuary (Medeiros et al., 2012) and may become a prime stressor factor for benthic communities (Cortelezzi et al., 2007; van Diggelen and Montagna, 2016), especially in marine-dominated estuarine communities. Also, it can work as proxy of other stressors (e.g. sediment grain size, fine material in suspension).
	Polyhaline (18-30)	SP18_30	
	Euhaline (30-40)	SP30_40	
Thermal preference	Cold waters (0-10 °C)	T_C	Indicates the macroinvertebrate thermal range preference which may be affected by thermal pollution, distance from sewage inputs or climate change. Differences in the species' responses to shifts in temperature and climate impacts depend up to a certain point on species' biological traits (Dawson et al., 2011). Ongoing climate change affects benthic communities, which tend to adjust their trait composition, to increase resilience and resistance to adapt to the new conditions (Filipe et al., 2013).
	Warm/ temperate/ subtropical waters (10-25°C)	T_W	
	Tropical waters (higher than 25 °C)	T_T	
Substrate preference	Hard substratum (from bedrock to cobbles-diameter between 64 and 256 mm).	S_H	Indicates substrate affinity of each species. Substratum provides structural support and nutrient provision. Effluent discharges and climatic pressures can influence shifts in communities' composition by affecting substrate composition.
	Gravel	S_G	
	Sand	S_S	
	Mud	S_M	

BTA at a community level (community-weighted mean - CWM approach) requires computing a samples x traits table (LQ), where trait modalities are weighted by species abundances (Fig. 3). LQ table is created by multiplying the samples x species table (L) by the species x traits (Q) table.
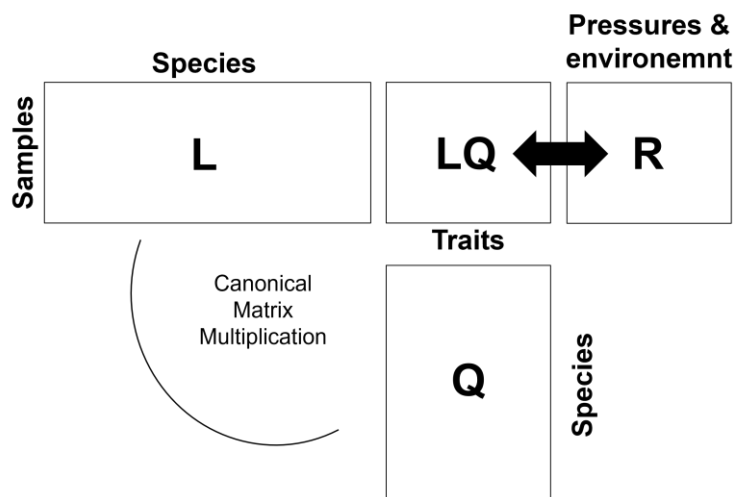
Figure 3: *Matrices and rational behind BTA: L – samples x species table, Q – species x traits table, R – samples x environmental/pressure variables table, LQ – samples x traits table.*

The Fuzzy Coding approach (FC) was applied after matrix multiplication of raw data tables as suggested by Beauchard et al. (2017). FC associates each taxon and trait category based on an affinity score (Chevenet et al., 1994). This procedure implies that to a species/genus may be given values in more than one category for a trait. For this method the coding scheme scale used ranged from 0 (no affinity) to 3 (total affinity) for a given trait (Table 2) as it is the most widely used in the literature (Degen et al., 2018) and therefore more reproducible.

FC is a reliable method as it is capable of addressing temporal and spatial variation in the traits of a given taxa (Statzner and Bêche, 2010; Tillin et al., 2006) by allowing the assessment of affinity of a taxa to multiple categories of a given trait. In addition, the FC avoids the loss of information, as it has the ability to account for ambiguous or conflicting literature information (Degen et al., 2018; Paganelli et al., 2012). To avoid bias among different traits, the affinity scores for each trait were standardized (sum for a given taxon and a given trait equaled 1) to give the same weight to each trait in posterior statistical analyses (Baptista et al., 2021; Darr et al., 2014; Péru and Dolédec, 2010).

Table 2: *Fuzzy coding approach scheme scale explanation.*

Affinity scores	Taxon affinity for a certain trait modality:
0	No affinity.
1	Low affinity.
2	High affinity.
3	Total and exclusive affinity.

Trait information was mainly compiled from online databases such as MarLIN BIOTIC – Biological Traits Information Catalogue: http://www.marlin.ac.uk/biotic/, WORMS – World Register of Marine Species: http://www.marinespecies.org, OBIS – Ocean Biodiversity Information System: https://obis.org/, Polytraits: http://polytraits.lifewatchgreece.eu, Marine Species Identification Portal: http://species-identification.org/ as well as in scientific publications and species identification guides. Due to lack of trait information for some species, in those cases modalities were assigned based on genus level and/or by expert knowledge. In cases where none of the above options were possible to

fulfill taxa trait information gaps an affinity score of 0 was attributed for all categories within a trait, which was later replaced by the mean profile of all other taxa in the function for subsequent trait analysis (Dray and Dufour, 2007).

Q matrices of both estuaries were standardized prior to the calculation of the functional indices using a "prep.fuzzy.var()" function in the package ade4, a function capable of solving the problem of missing data by replacing these values by the mean profile of the other species scores in the function.

2.3.2 Functional indices

Two Gower dissimilarity matrixes were calculated, one for each estuary, using the R package gawdis (de Bello et al., 2021) to compute the pairwise dissimilarities between species based on their normalized traits. The Gower's distance can be used to measure the difference between samples, which can contain a combination of logical, numeric, categorical, or text data (Gower, 1971). Gawdis function allows for fuzzy coded data to be used, using the fuzzy argument, to create a more balanced analysis. This is made by defining the columns (categories) that belong to each trait normalizing the contribution of each trait to the analysis. Balancing the multiple traits contribution into multi-trait dissimilarity is key for accurately interpreting the ecological effects of complex species or communities' differences (for further explanations, see de Bello et al., 2021). Subsequentially, the resulting dissimilarity matrices were used to calculate the PCoA (principal coordinate analysis) axes needed to compute the six functional indices explained bellow (Table 3) using the package FD (Laliberté et al., 2014) (Fig.4).
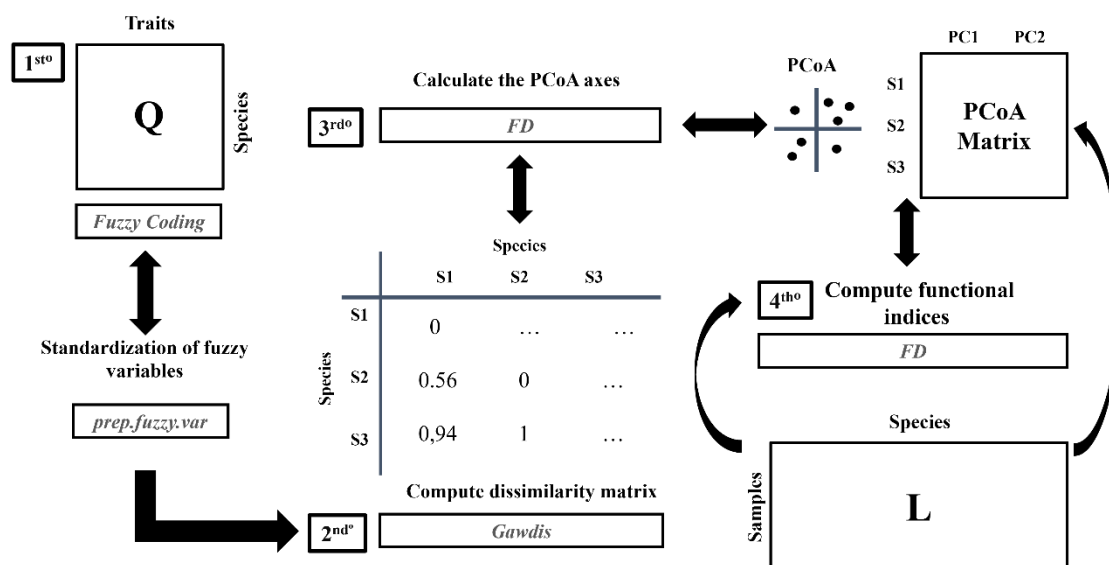
Figure 4*: Flowchart of the analysis carried out for the calculation of functional diversity indices for both estuary datasets: **1****sto*** *data standardization of the fuzzy coded Q – species x traits table; **2****ndo*** *Computation of a Gower dissimilarity matrix for both datasets using the standardized Q tables; **3****rdo*** *Calculation of the PCoA axes; **4****tho*** *PCoA axes used as traits to compute several multidimensional functional diversity indices, can be weighted by species abundance using L – samples x species table.* ***Functions used in RStudio:*** *prep.fuzzy.var () from package ade4; function gawdis() from package gawdis: Multi-Trait Dissimilarity with more Uniform Contributions and function dbFD () from package FD.*

Functional richness (FRic) was calculated as a proxy of the range of traits represented in the different benthic macroinvertebrate assemblages (years and zones) to search for changes in the volume occupied by these assemblages in the trait space. FRic does not consider species abundance (Mason et al., 2005) and it is expected to increase with lower disturbance as in the case of the Tagus estuary design.

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When significant differences were detected between groups, Kendall's (1955) correlation coefficient was performed between this FRic and species richness, since environmental filtering theory predicts that FRic may increase with species richness in more stressful environments. Functional evenness (FEve) is similar to evenness in taxonomic diversity and was used to measure the uniformity of the distribution of both species and abundance in the functional space (Villéger et al., 2008). Often when an ecosystem is disturbed, species with particular trait modalities are more abundant making the assemblages less functional even (van der Linden et al., 2016a). Functional divergence (FDiv) in the same way as above indices is expected to decrease with pressure (Mouillot et al., 2013). This index was applied to identify how abundance is distributed within the functional space, in other words, whether the most abundant species have traits similar to or distant from the centroid of the functional space (most common trait categories combination).

Functional dispersion (FDis) considers both FRic and FDiv (Mason et al., 2013) and has the capacity of measuring the average distance of a species to the abundance weighted centroid in the community trait space (Laliberté and Legendre, 2010). Communities with higher functional dispersion in theory have a more efficient use of resources. Rao's quadratic entropy (RaoQ) measure the degree of trait dissimilarity between two randomly selected individuals in the community and is expected to decrease after disturbance (van der Linden et al., 2016a).

Functional redundancy (FRed) was also calculated, as the inverted function of the ratio between Shannon diversity index (H′) and FDis; whenever this ratio increases, the FRed decreases. To compute FRic, FEve, and FDiv, a minimum of three species are required, although FDis and RaoQ only require two.

Table 3: Functional indices computed

Label	Functional Indices	Rationale	Range limits	Response to disturbance	Reference:
FRic	functional richness	Amount of trait space filled by the species in the community.	0 - ∞	-	
FEve	functional evenness	Evenness in the distribution of abundance in the trait space.	0 - 1	-	Villéger et al., 2008
FDiv	functional divergence	Degree to which abundance distribution in the trait space maximizes the divergence of trait-categories within the community.	0 - 1	-	
FDis	functional dispersion	Mean distance of individual species to the center of the trait space occupied by species.	0 - ∞	-	Laliberté and Legendre, 2010
RaoQ	Rao's quadratic entropy	Amount of trait dissimilarity between two random entities (individuals) in the community.	0 - 1	-	Botta-Dukát, 2005
FRed	Functional redundancy	Ratio between H′ and FDis, measuring the amount of species with similar trait combinations	0 - ∞	+	Adapted from van der Linden et al, 2016a

All functional indices were tested for significant differences between years and the estuary zones/distance using the Kruskal–Wallis test (Kruskal and Wallis, 1952). Two independent Kruskal–Wallis tests were performed, using years and estuary zones as factors. A p-value ≤ 0.05 was used as an indication of significant differences between assemblages. When significant differences were detected between groups, a Dunn's post hoc Test (1964) with Bonferroni corrections was performed between each independent group using the R package dunn.test ()

To explore the CWM approach, a Gower dissimilarity matrix was calculated for each estuary LQ tables. Following that, two Principal Coordinate Analysis (PCoA) were performed using the cmdscale () function from R on the resultant dissimilarity matrixes for both the Tagus and Mira estuaries to examine the spatial and temporal patterns of benthic community weighted traits. Additionally, to understand patterns of association between traits and environmental conditions in the Tagus dataset, environmental variables were represented as vectors, after being tested for association with the community-weighted trait composition (PCoA), using the "envfit" function in Vegan R packages. When correlation was > 0.2 and had an associated p value <0.05, vectors were overlapped in the PCoA graph for better visualization (Fig. 5).
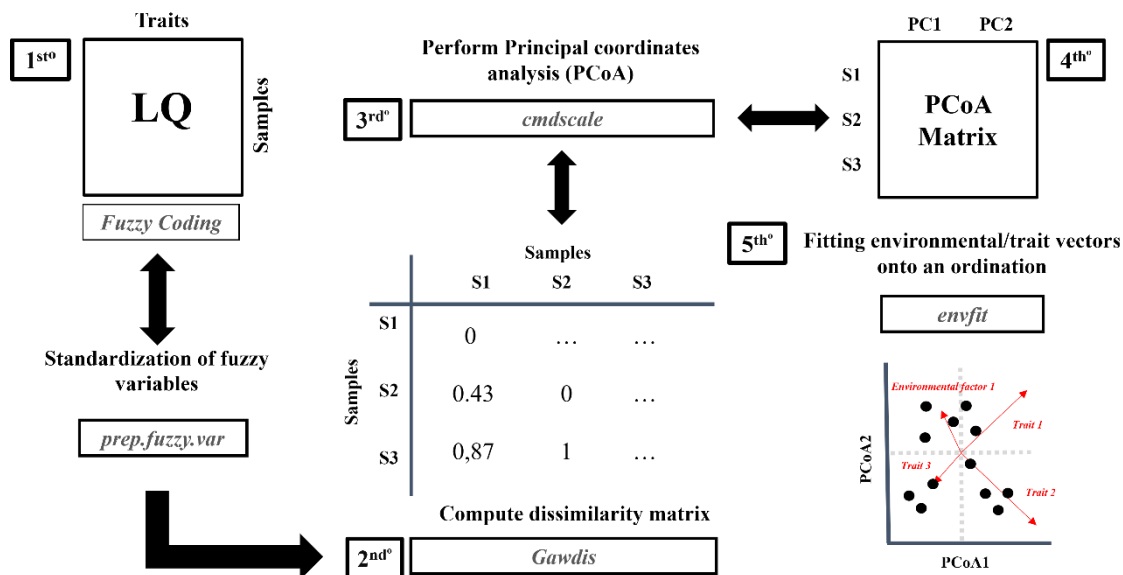
Figure 5: *Flowchart of the analysis carried out on the two LQ tables (samples x traits). Analysis carried out for the PCoA and vector analysis indices for both estuary datasets: 1^{sto} data standardization of the fuzzy coded LQ – traits x samples table; 2^{ndo} Computation of a Gower dissimilarity matrix for both datasets using the standardized LQ tables; 3^{rdo} Calculation of the PCoA axes; 4^{tho} PCoA axes used as Multi-traits to explore and to visualize similarities or dissimilarities of the samples based on trait composition; 5^{tho} Fitting environmental factors and trait vectors in to the PCoA ordinations.* **Functions used in RStudio:** *prep.fuzzy.var () from package ade4; function gawdis() from package gawdis: Multi-Trait Dissimilarity with more Uniform Contributions and function cmdscale: Classical (Metric) Multidimensional Scaling and function envfit: Fits an Environmental Vector or Factor onto an Ordination from the vegan package.*

Subsequentially, a permutational analysis of variance (PERMANOVA) using a two-way factorial design was performed, using the function "adonis" of the Vegan R package, to test for significant differences in macrozoobenthic trait assemblages between years and zones, for both estuaries:

- Tagus estuary – Year as a fixed factor (6 levels: 2009; 2010; 2011; 2012; 2013; 2019) and outfall distance/zones also as fixed factor (5 levels: 500-C2; 350-C1; 130-IC; 80-IB; 30-IA);
- Mira estuary – Year as fixed factor (2 levels: 1984 and 2019) and estuary zone also as fixed factor (3 levels: WB1; WB2; WB3).

In addition, when the PERMANOVA returned significant p-values, a pairwise PERMANOVA was performed in R using the "pairwise.adonis" function and the Holm p-value correction method for multiple comparisons (Arbizu and PairwiseAdonis, 2019). All analyses were carried out using 9999 permutations.

All statistical analyses were performed in the open-source R software (R version 4.0.3; R Core Team, 2020) and "ggplot2" package (Wickham, 2016) was used for graphical outputs.

3. Results

3.1 Tagus dataset

3.1.1 Tagus benthic macroinvertebrates community distribution

A total of 164 taxa (Table 1, Supplementary data) were collected in the Tagus estuary study area, along the 6 years. Taxa presenting higher mean abundance were mainly the polychaetes *Tharyx* sp., *Aphelochaeta* sp., *Streblospio shrubsolii* and *Nephtys hombergii,* in both impact and control stations. *Monocorophium acherusicum* and *Capitella capitata* were also very abundant in impacted stations and *Mediomastus fragilis* and *Cossura soyeri* on control stations (Table 4).

Table 4: *Alcântara macrozoobenthic most common species mean abundance by impact, control, and total stations*

	Taxa	Mean Abundance
Impact	*Aphelochaeta* sp.	12
	Streblospio shrubsolii	6
	Monocorophium acherusicum	5
	Tharyx sp.	4
	Nephtys hombergii	4
	Capitella capitata	4
Control	*Tharyx sp.*	51
	Aphelochaeta sp.	17
	Streblospio shrubsolii	15
	Nephtys hombergii	8
	Mediomastus fragilis	5
	Cossura soyeri	4
Total	*Tharyx* sp.	23
	Aphelochaeta sp.	14
	Streblospio shrubsolii	9
	Nephtys hombergii	5
	Mediomastus fragilis	4
	Monocorophium acherusicum	4

3.1.2 Tagus community-weighted mean traits temporal and spatial patterns

Control stations were removed from this analysis, as no significant differences were detected between the control and impacted stations, concerning macrozoobenthic assemblage trait composition (Table 5). Furthermore, there were replicates in the control stations with only one to three species, which could mask the functional response of the macrozoobenthos caused by outfall. The first two axes of the PCoA accounted for 31.6% and 22.3% of the spatial and temporal variance in the Tagus estuary macrozoobenthic communities. Despite the lack of a conspicuous segmentation among years in the ordination (Fig. 6A), it is possible to observe a distribution of the different years along PCo1, with older samples located in the positive axis and more recent samples in the negative axes (Fig. 6C). The allocation of samples along PCo2 seem to be associated with a significant correlation to higher planktotrophic species (DM_P) and environmental conditions characterized by muddy sediments in the positive axis (Fig. 6C and Fig. 6D). Additionally, in terms of the station's distance to the WWTP outfall there is a clearer separation between impact radials along the second axis (Fig. 6B), with the farther stations being positioned in both negative axis and associated with higher abundance of euhaline species. There is separation between the traits of benthic macroinvertebrates communities along the pollution gradient (outfall distance), which is more evident between the sampling points of 30 m to 130 m (Fig. 6 D). The vectors representing the traits showed an association of attached (LP_A) medium to large size (MS20_50) suspension feeders species (F_S) with very short live span (L1), tolerant to pollution (EG_III) and with preference for coarser substrates (S_H) to samples collected on the farthest impact radial. Most of the IA samples for all years excepting 2010 and 2019 were correlated to 1° order opportunists, showing that this vector responded to the spatial variation of organic-matter pollution, the remaining samples from IA, mainly the ones from 2019, had an association with lower salinity (SP5_18), and medium longevity species (L3_10). Prior to the Alcântara's WWTP improvements (2009) samples are scattered throughout the plot, showing no discernible pattern.

Table 5: *PERMANOVA analysis results on community-weighted traits (Gower similarity). Type of station (2 levels: Control vs Impact radial) as a fixed factor (df: degrees of freedom; MS: mean square). Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

Factor	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Type of station:						
Control (C1 and C2) vs Impact radials (IA, IB, IC)	1	0.01068	0.4964	1.97645	0.00849	**0.862**
Residuals	58	1.24835	0.021523	-	0.99151	-
Total	59	1.25904	-	-	1.00000	-
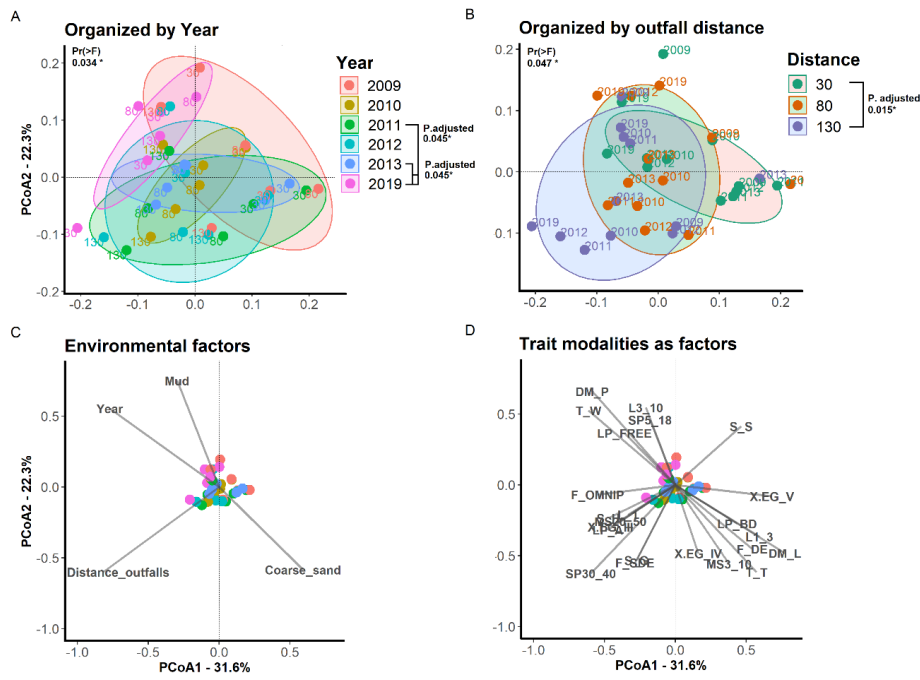
Figure 6: *Principal Coordinate Ordination (PCoA) of macrozoobenthic communities' weighted trait means at the Tagus estuary Alcântara's WWTP based on Gower's similarity coefficient colored by year (**A**) and by distance to outfall of IR (**B**) Correlation coefficients of each environmental factor (**C**) (r > 0.2) and trait modality (**D**) with the two initial PCoA axes are indicated by the lengths of the overlaid vectors: planktotrophic larvae (DM_P); temperate waters (T_W); free-living (LP_FREE); mesohaline (SP5_18); medium longevity (L3_10); omnivores (F_OMNIP); medium size (MS20_50); very short longevity (L_1); attached (LP_A); tolerant (X.EG _III); euhaline (SP30-40); suspension feeder (F_S); gravel (S_G); 2ndº order opportunists (X.EG_IV); small-medium size (MS3_10); tropical temperatures(T_T); burrow-dweller (LP_BD); short longevity (L1_3); deposit feeder (F_DE); lecithotrophic larvae (DM_L); 1stº order opportunists (X.EG_V); sand (S_S). **For a better reading of figure 6D go to Fig. A.1.** PERMANOVA analysis statistically significant results (p<0.05) are indicated for factors year (A) and outfall distance (B).*

The PERMANOVA analysis showed significant differences between years (p=0.034) and between impact radials/distance (p=0.047). Pairwise comparisons (pairwise PERMANOVA) revealed that 2011 and 2013 were significantly different from 2019, as were the radials IA (30 meters) and IC (130 m) (Table 6) this supports the hypothesis discussed above of differences on traits along the organic pollution gradient. After the improvement in the WWTP there was an increase in FRic in the impacted area (Fig. 7), however there was no clear difference between 2009 community-weighted traits and the following years.

Table 6: *Tagus PERMANOVA analysis results on community-weighted traits (Gower similarity). Year (6 levels: 2009; 2010; 2011; 2012; 2013; 2019) and Outfall Distance (3 levels: 130 m; 80 m; 30 m) as fixed factors (df: degrees of freedom; MS: mean square). Pairwise PERMANOVA results with p-value adjusted by the Holm p-value correction method. Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05.*

Factor	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	P.adjusted
Year	5	0.10190	0.050949	1.97645	0.09962	**0.034***	**-**
Pairs							
2009 vs 2010	1	0.04600376	**-**	1.6242076	0.13972631	0.148	1.000
2009 vs 2011	1	0.04732072	**-**	1.9048987	0.16000965	0.132	1.000
2009 vs 2012	1	0.04694764	**-**	1.8405752	0.15544644	0.141	1.000
2009 vs 2013	1	0.04930146	**-**	2.0035126	0.16691052	0.091	1.000
2009 vs 2019	1	0.07607301	**-**	3.5039515	0.25947601	0.024	0.360
2010 vs 2011	1	0.01176418	**-**	0.3806324	0.03666755	0.956	1.000
2010 vs 2012	1	0.02128464	**-**	0.6741538	0.06315759	0.756	1.000
2010 vs 2013	1	0.01726759	**-**	0.5629601	0.05329568	0.842	1.000
2010 vs 2019	1	0.05388361	**-**	1.9399361	0.16247458	0.045	0.675
2011 vs 2012	1	0.01701095	**-**	0.6055841	0.05710050	0.729	1.000
2011 vs 2013	1	0.02228486	**-**	0.8195791	0.07574963	0.543	1.000
2011 vs 2019	1	0.08492967	-	3.4959480	0.25903686	0.003	**0.045***
2012 vs 2013	1	0.02993426	**-**	1.0746047	0.09703324	0.380	1.000
2012 vs 2019	1	0.04844965	**-**	1.9411545	0.16256003	0.085	1.000
2013 vs 2019	1	0.08197076	-	3.4069792	0.25411982	0.003	**0.045***
Distance	2	0.21814	0.043628	1.69247	0.21326	**0.047***	
Pairs							
30 vs 80	1	0.03154106	**-**	1.2105801	0.05215639	0.274	0.822
30 vs 130	1	0.09419527	**-**	3.3272280	0.13136961	0.004	**0.012***
80 vs 130	1	0.02710991	**-**	0.9233425	0.04027958	0.497	1.000
Year: Distance	10	0.23884	0.023884	0.92651	0.23349	0.611	**-**
Residuals	18	0.46400	0.025778	**-**	0.45362	**-**	**-**
Total	35	1.02288	**-**	**-**	1.00000	**-**	**-**

3.1.3 Tagus functional diversity metrics

FRic is here defined as the volume of functional space delimited by traits filled by species and plotted as a convex hull (Fig. 7). FRic in the impact radials stations increased from **A** 2009 (0.143) to **B** 2010 (0.170), and this moderately rising trend continued in the next two years, 2011 (0.175) and 2012 (0.179). The steadiness of FRic scores during the period of 2010 to 2012 is not related to a strong taxonomic similarity among faunas as macrozoobenthic species composition was highly variable between years: 2010-2011, 64% and 2011-2012, 74% of the species were different (Table 7). However, in 2013, a minimum score of 0.140 was obtained, followed by a slight increase (0.148) in 2019 (Fig.7E). After the effluent treatment improvement in the Alcântara WWTP the total FRic in the impacted zones increased in the first three years as expected (Fig. 8), showing benthic macroinvertebrates assemblages with a wider range of traits. In 2013 the smallest volume of the trait space was occupied and six years later, in 2019, impacted stations had a very similar FRic value to 2009. These results suggests that the ecosystem had come to a new equilibrium, once more with lower functional richness, since these ecosystems are naturally stressed and have limited resources.
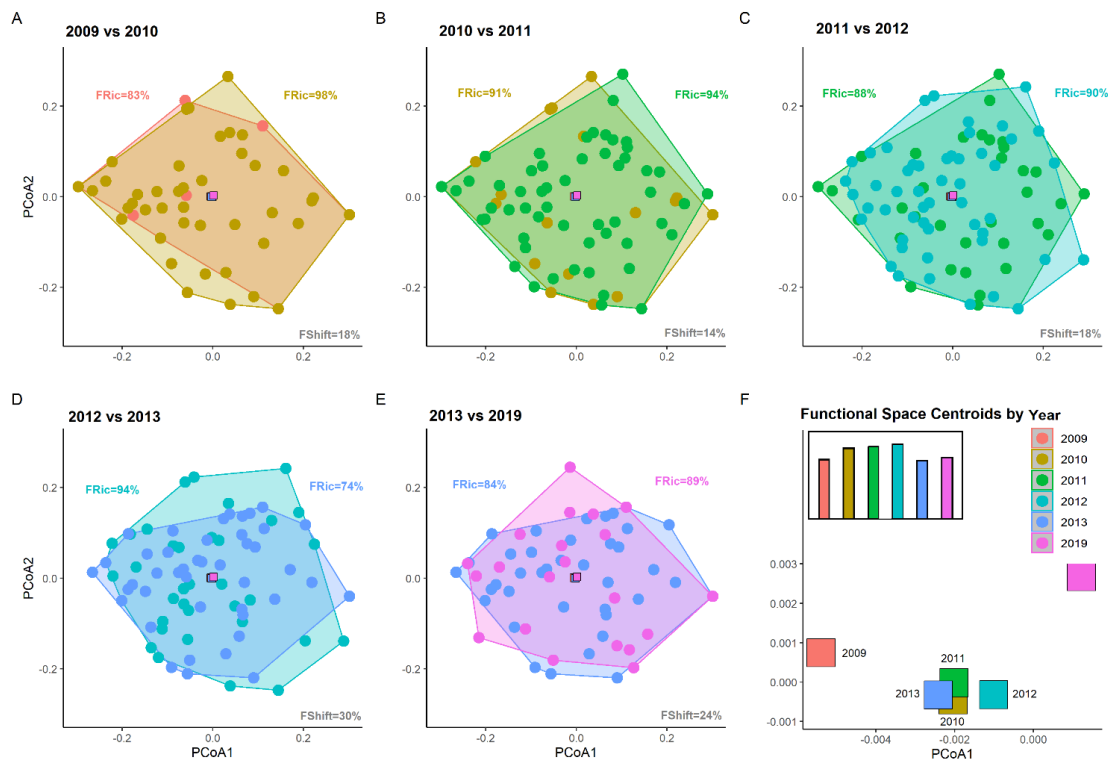
Figure 7: *Ordination of functional space of macrozoobenthic traits obtained through Principal Coordinates Analysis (PCoA). Species colored by year.* ***Total Functional Richness (FRic)*** *scores by year and shift in functional Richness (**FShift**) between each following year.* ***F*** *close up of functional centroids by year and histogram of absolute FRic by year.*

There was no substantial differences between each year functional space centroid ordination, although the 2009 (before WWTP improvement) and 2019 (last year of monitoring) centroids were the most distant from the remaining years. The traits of macrobenthic communities shift over the years, although most species had a set of trait-categories not too distant from each other.

In the first three years, the total shift in functional space (FShift) was relatively uniform (between 14% and 18%). This relative stability changed from 2012 to 2013 (Fig.7). In this period, the

proportion of the functional space occupied exclusively by one year assemblage accounts for 30% of their total volume, which corresponds to the highest number of distinct functional entities between years.

*Table 6: Number of species present in Alcântara's WWTP impact stations grouped by 2 years periods. Richness maintained (**Rmaintained**) – percentage of species that remain present from one year sampling to the next; Richness shift (**Rshift**) - percentage of species identified exclusively during one year of the sampling.*

Years	Nº of species present in both years	Rmaintained	Nº of S. present in just one year	Rshift	Total nº Species
2009-2010	13	28%	34	72%	47
2010-2011	26	36%	47	64%	73
2011-2012	22	26%	64	74%	86
2012-2013	17	22%	61	78%	78
2013-2019	8	14%	50	86%	58
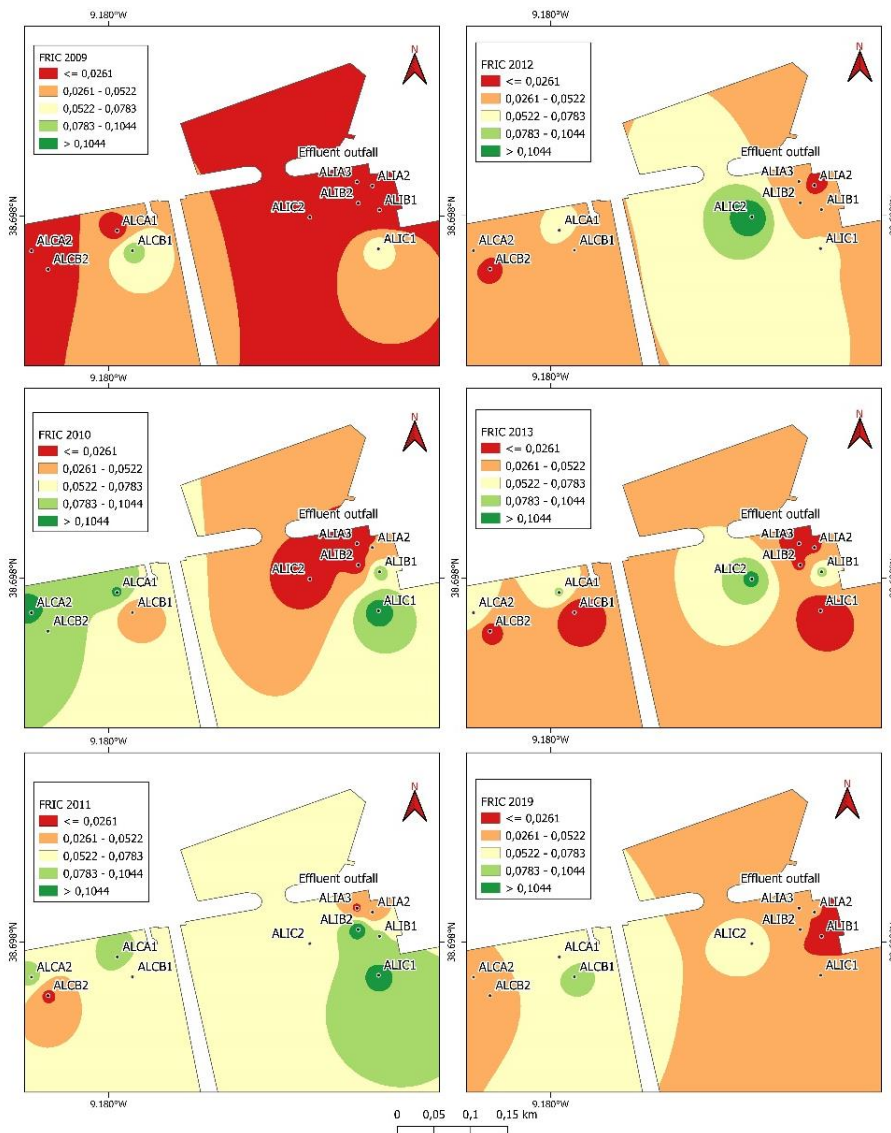
Figure 8: Map of the FRic index scores for all sampling stations (control and impact radials)of the Tagus estuary by year.

The only functional index that was capable of finding differences across stations, according to the statistical tests, was FRic. The IA stations significantly differed from the IC, according to Dunn's multiple comparison test (Table 8). The macrozoobenthic communities' weighted traits analysis had already showed these differences; however, the FRic results indicated that IA and C1 stations also differed. Functional richness values increased from 2009 to 2010 in all stations independently of the distance from the outfall. In 2010 there was a clear pattern along the outfall gradient, meaning that FRic increase with the distance from the outfall. IC has shown a consistent FRic rising trend across the years, except for 2019 (Fig. 8 and Fig. 10A). Although there have been fluctuations over the years, FRic never returned to pre-improvement values in the impacted stations. However, this increase in functional richness was very correlated to an increase in species richness as can be seen in Figure 9.

*Table 7: **Kruskal-Wallis** test and pairwise **Dunn's test** results on FD indices between **Year** (6 levels: 2009; 2010; 2011; 2012; 2013; 2019) and **Outfall Distance** (5 levels: 500 m; 350 m; 130 m; 80 m; 30 m) (df: degrees of freedom; Chi2: a scalar of the Kruskal-Wallis test statistic adjusted for ties; Z: vectors of Dunn statistics; p-value.adj: a vector of p-values corresponding to Z and adjusted for multiple comparisons using Holm's (1979) method. Significantly different groups in bold.*

	Factors	Chi²	df	p-value	p-value.adj	Z
FRic						
	Year	4.5861	5	0.4684	-	-
	Distance	12.69	4	**0.01289***	-	-
Dunn's Test	130-30	-	-	0.002434178	**0.02190760***	3.03140593
	30-350	-	-	0.002026488	**0.02026488***	-3.08632270
FEve						
	Year	9.8185	5	0.08054	-	-
	Distance	1.0258	4	0.9059	-	
FDiv						
	Year	7.854	5	0.1645	-	
	Distance	9.2068	4	0.0515	-	
FDis						
	Year	2.4997	5	0.7765	-	-
	Distance	3.9966	4	0.4065	-	-
RaoQ						
	Year	3.3521	5	0.6459	-	-
	Distance	4.3763	4	0.3575	-	-
FRed						
	Year	5.2362	5	0.3877	-	-
	Distance	9.401	4	0.05182	-	-
	Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					
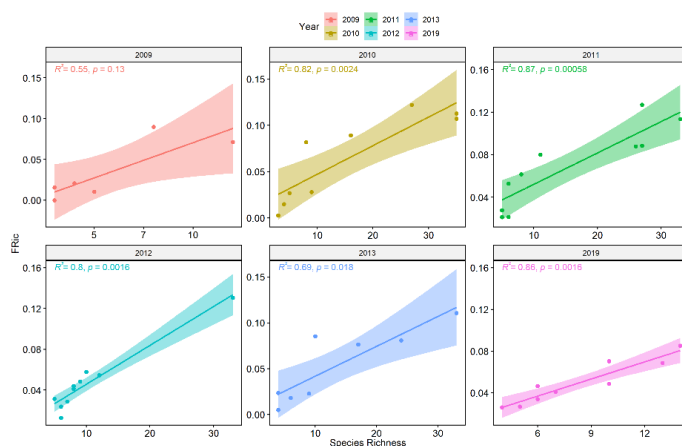
Figure 9: Kendall rank correlation coefficient between FRic and Species Richness for each year of the Tagus estuary dataset.

FEve increased from 2009 to 2010 in all impact radials and fluctuated during the subsequent years (Fig. 10B). This FEve increase in the first year after effluent treatment improvement could be related to better water quality conditions allowing species of different traits-combinations to be present, resulting in abundances being more uniformly distributed throughout trait-categories and a more evenly filled trait space.

Contrarily, FDiv decreased from 2009 across the years in IA and increased in IC (Fig. 10C). During 2009 in the IB stations only two species were collected being impossible to calculate this index. FDiv values were high throughout the years, these means that the most abundant species exhibit more extreme trait-categories even after the improvements. The species that inhabit this area remained to be under freshwater stress from the outfall and even from all the natural stress of an estuary, therefore the most adapted traits will probably continue to be prominent in these communities, despite improvements in water quality and an increase in species and traits.

FDis and RaoQ results were identical as they show high significantly positive correlation (Fig. 11) so were going to analyze them as one. Functional diversity (RaoQ and FDis as proxies) improved from 2009 to the following years in the impacted stations with 2013 IC being the only zone with a lower score than 2009. Although, this improvement was minimal and not significant (FDis range only from 0.00 to 0.15) (Fig. 10D).

Regarding FRed, in IA stations there was a decreased from 2009 to 2010 followed by an increase in the next years (Fig. 10F). IB zones exhibited an increasing functional redundancy until 2013, however there as a decline in redundancy in 2019, although still substantially higher than before improvements in the WWTP. FRed increases with outfall distance, this was a surprise as FRed is expected to increase with disturbance. However, a high FRed can also work as a measure of ecosystem resilience, since it protects against ecosystem function loss in the event that a species goes extinct, by increasing the chance that at least some of these species will endure environmental changes and continue to exist.
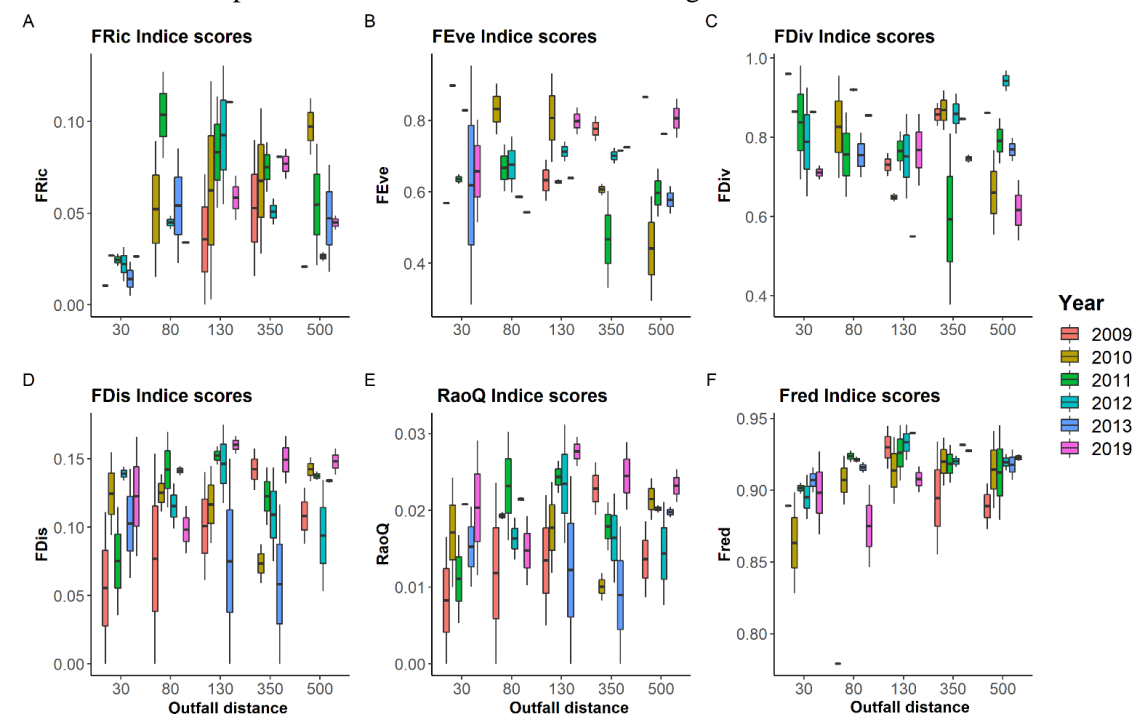
Figure 10: *Functional diversity indexes scores for the Tagus estuary Alcântara's WWTP stations by distance to outfall. Colored by year. **A** - Functional Richness; **B** – Functional evenness; **C** – Functional Divergence; **D** – Functional dispersion; **E** – Rao's quadratic entropy index; **F** – Functional redundancy. **IA – 30 m, IB – 80 m, IC – 130 m (Impact radials); C1 – 350 m, C2 – 500 m (Control stations).***

The majority of the indices were significantly correlated to at least another index. In addition to FRic being correlated with specific richness as already discussed, it is also positively correlated with FRed, RaoQ and FDis. FRed was also positively correlated to species richness, meaning that after the improvements the increase in the number of species led to a higher number of trait combinations but also to a higher number of species sharing the same traits.

Opposite to FRic, FDiv had a negative correlation with FDis and RaoQ. However, while these correlations with FDis and RaoQ were significant, their coefficients of determination were significantly smaller, which meant that the variation they explained was also substantially lower. These negative correlations were not expected as FDiv and FDis/RaoQ are all expected to decrease with disturbance. In this way, when the most abundant species have extreme trait-categories the amount of trait dissimilarity between two individuals in the community and the mean distance of an individual to the center of the trait space is lower.
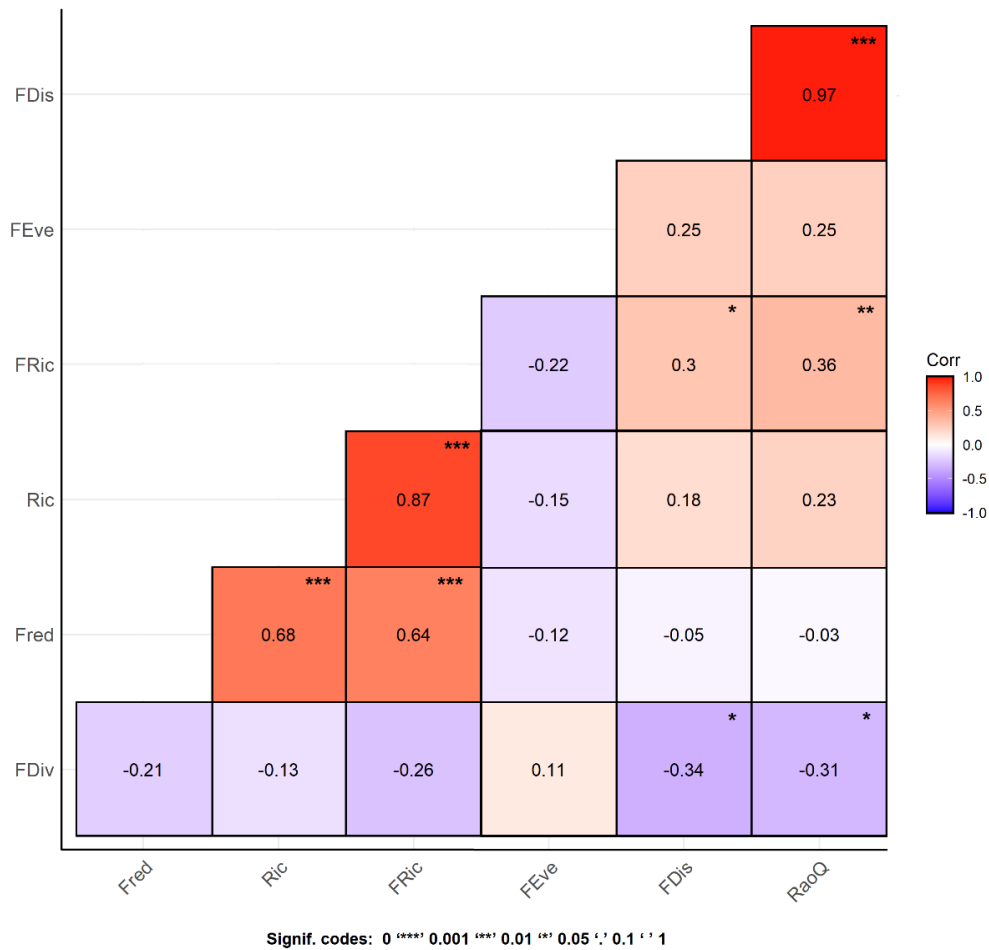
Figure 11: *Pearson correlation values between the functional indices and species richness of the Tagus estuary. Significantly correlated when p value is $\leq 0.05^{*}$)*

3.2 Mira dataset

3.2.1 Mira macrozoobenthic community distribution

A total of 143 taxa (Table A4, Supplementary data) were collected in the Mira estuary study area, along the two analyzed years. Euhaline benthic macroinvertebrate community of 1984 was dominated by *Spisula solida* (Linnaeus, 1758) and *Melita palmata* (Montagu, 1804), while the assemblage of 2019 in the same estuarine zone was dominated by *Abra alba* (W. Wood, 1802*)* and *Sthenelais boa* (Johnston, 1833). In the polyhaline area of the Mira estuary sampling, *Amphibalanus improvisus* (Darwin, 1854) and *Cerastoderma glaucum* (Bruguière, 1789) were the most abundant species in 1984, however in 2019 *Hilbigneris gracilis* (Ehlers, 1868) and *Neanthes nubila (*Savigny, 1822) became the dominant species. Nevertheless, *C. glaucum* continued to be a species with high abundance. *A. improvisus* and *Alitta succinea* (Leuckart, 1847) were the species presenting higher mean density in 1984 in the mesohaline sampling stations, while in 2019 the most abundant species were *Cerastoderma* spp. and *Dexamine spinosa (Montagu, 1813)* (Table 9).

Table 8: Mira macrozoobenthic most common species mean density (%) by year (1984 and 2019) and estuarine zone (euhaline, polyhaline and mesohaline).

	1984		2019	
	Taxa	x̄ density (%)	Taxa	x̄ density (%)
Euhaline	*Spisula solida*	26%	*Abra alba*	9%
	Melita palmata	22%	*Sthenelais boa*	8%
	Neanthes nubila	13%	*Bittium reticulatum*	7%
	Calyptraea chinensis	5%	*Melita palmata*	6%
	Glycera tesselata	5%	*Euspira catena*	5%
Polyhaline	*Amphibalanus improvisus*	36%	*Hilbigneris gracilis*	25%
	Cerastoderma glaucum	18%	*Neanthes nubila*	23%
	Corophium orientale	13%	*Cerastoderma glaucum*	13%
	Alitta succinea	7%	*Melita palmata*	7%
	Amphibalanus amphitrite	5%	*Dexamine spinosa*	3%
Mesohaline	*Amphibalanus improvisus*	53%	*Dexamine spinosa*	38%
	Alitta succinea	17%	*Cerastoderma edule*	21%
	Cerastoderma glaucum	11%	*Cerastoderma glaucum*	15%
	Dexamine spinosa	9%	*Alitta succinea*	13%
	Melita palmata	4%	*Amphibalanus improvisus*	4%

3.2.2 Mira community-weighted mean traits temporal and spatial patterns

The first two axes of the PCoA explained 50,9 % of the variance of community-weighted traits of the Mira macrozoobenthic assemblages. PERMANOVA using Gower dissimilarity distance showed significant differences among years ($p = 0.03$) and between estuarine zones ($p = 0.007$). There was no significant interaction between the Year and Estuarine zone factors. (Table 10).

A significant spatial heterogeneity in the density and composition of traits of the macrozoobenthic communities was observed along the Mira estuarine gradient. This shift in the traits of species along the estuarine gradient was more pronounced between the euhaline and mesohaline zones as seen by the segregation of the estuarine zone centroids along the first axis (Fig. 12B), which was supported by the pairwise PERMANOVA results (p adjusted=0.036). Furthermore, it was possible to detect differences between the trait composition of the macrozoobenthic communities from 1984 and those from 2019 along the second axis, which explained 20.7% of the variance (Fig. 12A).

*Table 9: Mira PERMANOVA analysis results on community-weighted traits (Gower similarity). Year (2 levels:1984; 2019) and Estuarine zone (3 levels: WB1; WB2; WB3) as fixed factors (df: degrees of freedom; MS: mean square). Pairwise PERMANOVA results with p-value adjusted by the Holm p-value correction method. Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05.*

Factor	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	P.adjusted
Year	1	0.08972	0.089724	2.1643	0.11063	**0.037***	-
Estuarine zone	2	**0.19038**	0.095192	2.2962	0.23474	**0.007****	-
Pairs							
WB1 vs WB2	1	0.09638694	-	2.089094	0.1728081	0.028	0.084
WB2 vs WB3	1	0.13116771	-	2.754212	0.2561054	0.015	**0.036***
WB1 vs WB3	1	0.04590513	-	1.045509	0.1155832	0.452	0.627
Year: Estuarine zone	2	0.11637	0.058186	1.4036	0.14349	0.157	-
Residuals	10	0.41456	0.041456	-	0.51115	-	-
Total	15	0.81104	-	-	1.00000	-	-

Of the 40 trait categories analyzed, 22 were significantly correlated with the first 2 PCoA axes (Fig. 13). The vectors representing the traits show a strong association of sensitive (EG_I) deposit feeders (F_DE) species to samples collected during 1984 in the euhaline zone of the Mira estuary. Contrarily, the 2019 samples from the same zone are associated with free living (LP_FREE) or burrow dwellers (LP_BD) predators (F_PRE), with planktotrophic development (DM_P) and a preference to temperate waters (T_W).

Regarding species temperature preference range there was a clear difference between estuarine zones for both years, with areas with higher salinity having communities more dominated by species with higher thermal preferences (Fig. 14). However, as can be seen in Figure 14, the percentage of temperature categories remained very similar when comparing the same zone between years, with 1984 even having a higher percentage of species with an affinity for high temperatures, albeit minimal.
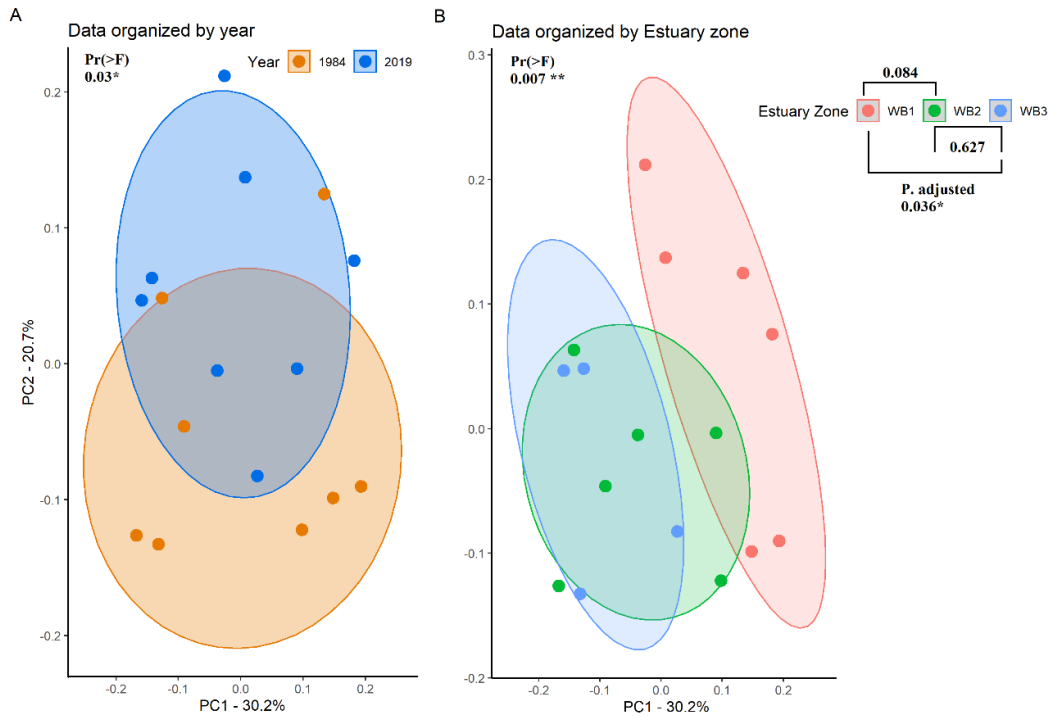
Figure 12: *Principal Coordinate Ordination (PCoA) of macrozoobenthic communities' weighted trait means at the Mira estuary based on Gower's similarity coefficient colored by year:1984 and 2019 (**A**) and by estuarine gradient/estuary zone: WB1; WB2; WB3 (**B**). PERMANOVA analysis statistically significant results (p<0.05) plotted for year (A) and estuarine zone (**B**) factors.*
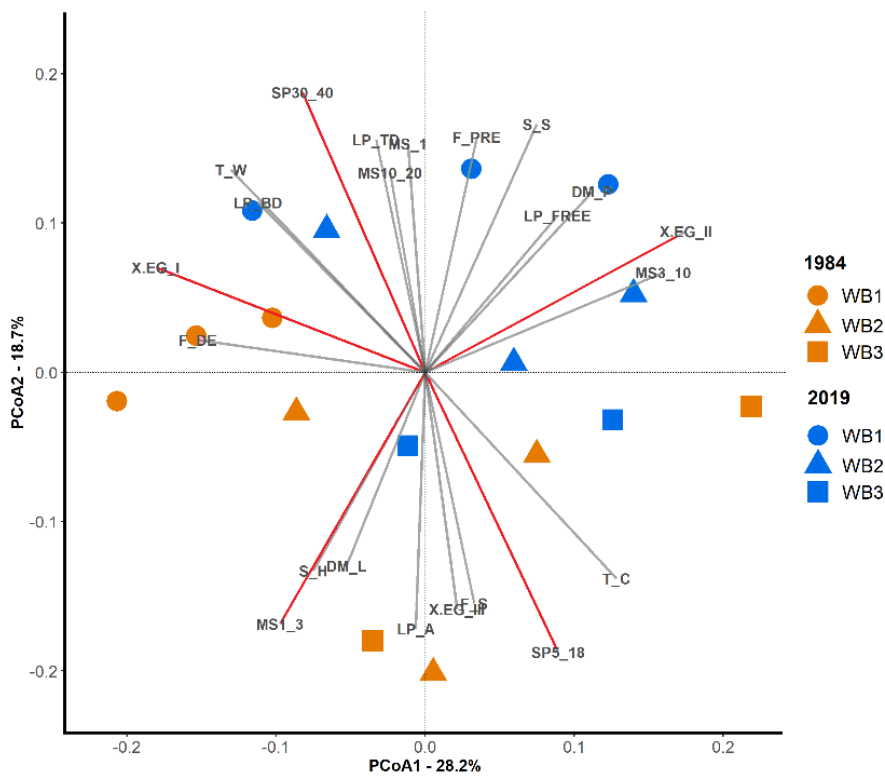
Figure 13: *Principal Coordinate Ordination (PCoA) of macrozoobenthic communities' weighted trait means at the Mira estuary based on Gower's similarity coefficient. Correlation coefficients of each trait modality (p < 0.05) with the two initial PCoA axes are indicated by the lengths of the overlaid vectors. Vectors in red are the ones whit R^2 values higher than 0.85: mesohaline (SP5_18); euhaline (SP30-40); very sensitive species (X.EG_I); indifferent species (X.EG_II); small size (MS1_3). See Table 2 for complete trait modalities labels.*

Communities were dominated by small to medium species in both years in all estuarine zones. However, the 1984 macrozoobenthic community had higher proportions of this trait categories, mainly in WB2 and WB3, where these classes reach from 92 to 100% of the community (Fig. 14). Large species were absent from the estuary in 1984 but were found in all zones in 2019, though in very small abundances. From a size variability perspective, the 2019 community is more diverse, with species of all class sizes present throughout the estuary.

Short-lived species dominated the mesohaline and polyhaline zones of the Mira estuary in 2019, while medium-lived species dominating the same zones in 1984. The opposite happened in the euhaline stations, however in 1984 the proportion of taxa of all longevity classes were very similar with high percentage of long-lived species.

Despite being present in residual proportions, opportunistic species were found in all stations of 2019, contrary to 1984 where these species were only found in the mesohaline zone. Very sensitive species (X.EG_I) were the prevailing group in the euhaline zone of the estuary in 1984. There was a decrease in the proportion of very sensitive species not only in this zone but also in the mesohaline area of the estuary (Fig. 14). The proportion of these type of species is expected to decrease with pollution/disturbance implying that 2019 was a more disturbed ecosystem.

The proportion of taxa with a planktotrophic larval development (high dispersal potential) increased between 1984 to 2019 in all estuarine zones. This increase ranged from 24% in the euhaline zone to only 6% in the mesohaline zone.

Regarding the living position trait in the euhaline zone, there was a decline in dwellers (tube and burrow) both categories normally associated with impacted areas. In the polyhaline and mesohaline zones, the opposite situation occurred, however maintaining high proportions of free-living and attached taxa.

There was a shift in substrate preference of the macrozoobenthic communities from 1984 to 2019. There was a decrease in the proportion of taxa more linked to coarser substrates (hard and gravel) and an increase in soft-sediments species (sand and mud) in all stations. Relatively to the salinity preference, in 2019 there was an increase in species with higher salinity preferences (Fig. 14).

Suspension feeders were the most dominant trait-category during 1984 suggesting lower levels of organic pollution and favorable currents with best conditions for suspension feeding. In 2019, predators were the dominant feeding guild in the Mira estuary, with the exception of the mesohaline area, that continue to be dominated by suspension feeders.
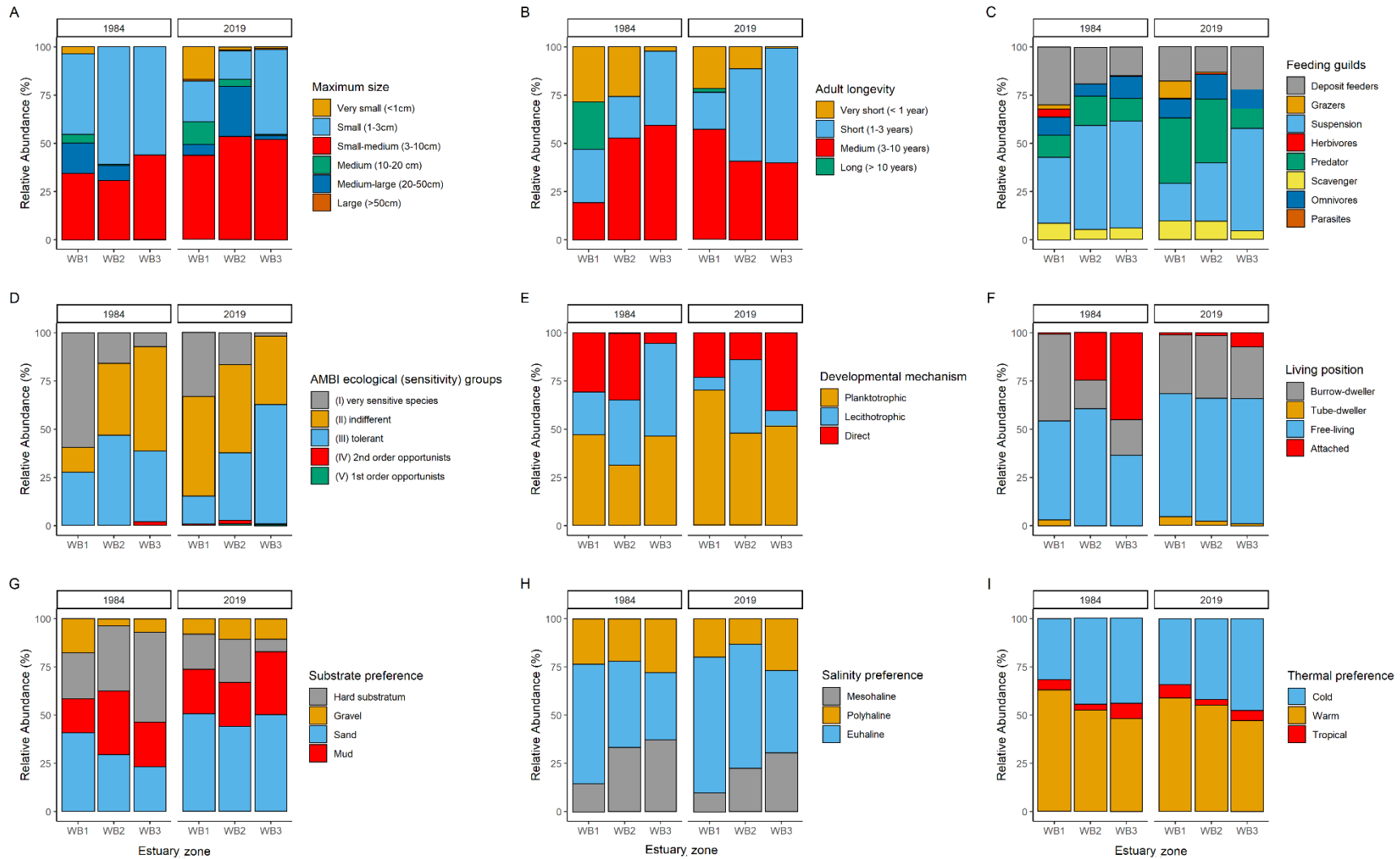
Figure 14: *Relative abundance (%) of a given trait category (A – maximum size; B –adult longevity; C – feeding guilds; D – AMBI ecological groups; E – Developmental mechanism; F – Living position; G – substrate preference; H – salinity preference; I – Thermal preference) in the three Mira estuarine zones (WB1, WB2, WB3) for 1984 and 2019. Mira estuary datasets.*

3.2.3 Mira functional diversity metrics

The results indicated an increase of total FRic from 1984 to 2019 as illustrated by the differences in the size of the convex hull gathering the species belonging to each community (Fig. 15A). There has been a shift in the total trait space occupied of about 16%, in other words, 16% of the functional space was only occupied by the 1984 or 2019 community combined. Additionally, the increase on functional richness was also noticeable in the euhaline and mesohaline zones of the Mira estuary from 1984 to 2019. The polyhaline zone maintained rather stable FRic values between the studied years, although with a slightly lower average value for 2019 (Fig. 15.D).
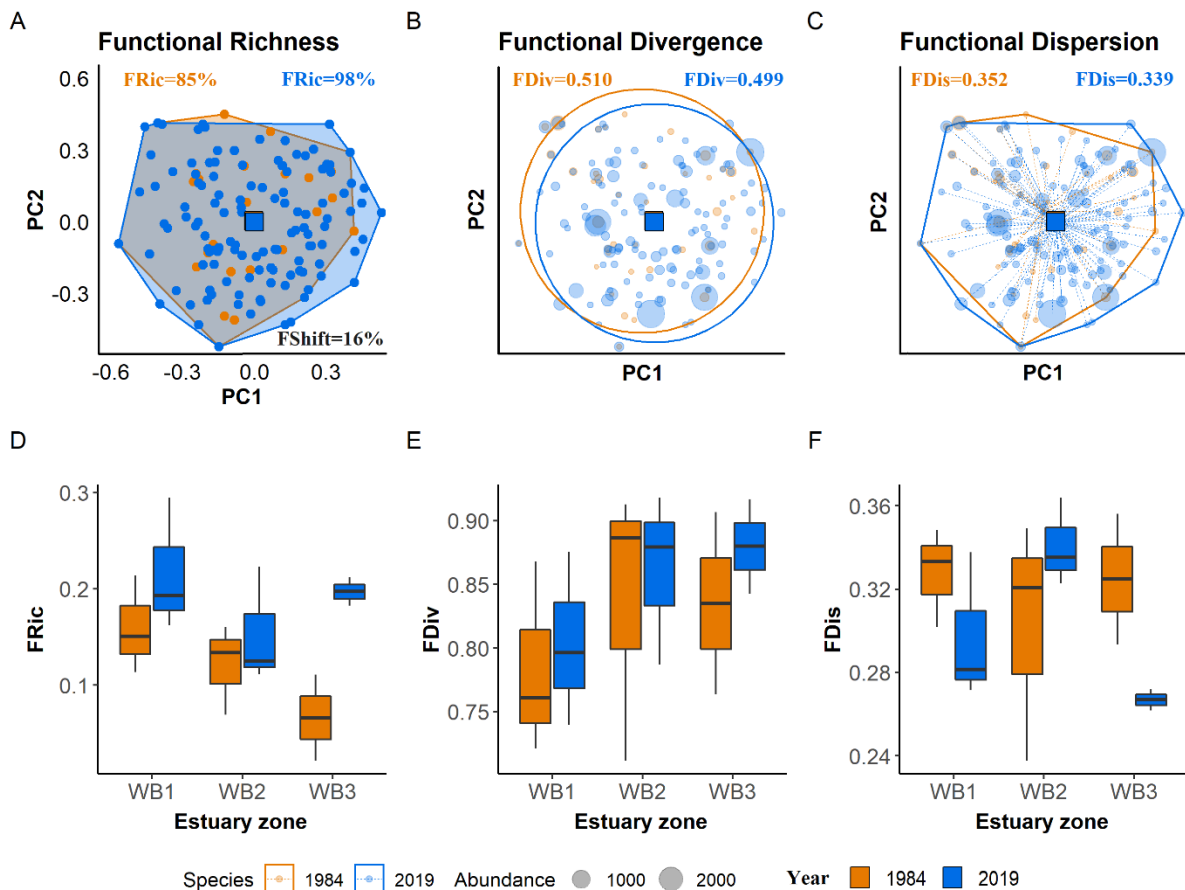
Figure 15: *Changes in different components of the functional structure of the Mira estuary macrozoobenthic communities from 1984 to 2019.* ***Axes PC1 and PC2 represent multiple traits extracted from PCoA****; species are represented by dots and plotted in a two-dimensional functional space according to their corresponding trait values. Size of the dots is proportional to the relative abundance of species during 1984 and in 2019 in orange and blue, respectively.* ***(A) Total FRic*** *(functional/trait space occupied by benthic macroinvertebrates communities);* ***(B) Total FDiv*** *represented in the functional space;* ***(C) Total FDis*** *changes in the abundance-weighted mean distance to the abundance weighted mean traits values of each community (1984 and 2019); (D) Boxplots of the FRic index results for each year and estuary zone; (E) Boxplots of the FDiv index results for each year and estuary zone; (F) Boxplot of the FDis index results for each year and estuary zone.*

The difference between the FRic from 1984 to 2019 illustrated in Fig.15A are supported by the Kruskal-Wallis test results (Table. 11).

Table 10: Kruskal-Wallis test results on FD indices between Year (2 levels: 1984;2019) and Estuarine Zone (3 levels: WB1; WB2; WB3) (df: degrees of freedom; Chi2: a scalar of the Kruskal-Wallis test statistic adjusted for ties; Significantly different groups in bold).

	Factors	**Chi²**	**df**	**p-value**
FRic	Year	3,9816	1	**0,046***
	Estuarine zone	2,3088	2	0,3152
FEve	Year	1,864	1	0,1722
	Estuarine zone	4,7059	2	0,09509
FDiv	Year	3,0184	2	0,2211
	Estuarine zone	0,70588	1	0,4008
FDis	Year	0,39706	1	0,5286
	Estuarine zone	1,0184	2	0,601
RaoQ	Year	0,39706	1	0,5286
	Estuarine zone	1,6618	2	0,4357
FRed	Year	6,3529	1	**0,01172****
	Estuarine zone	3,8125	2	0,1486
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Changes in species composition can maintain or change FRic, as different species may have the same trait modalities and therefore the same functions. However, FRic can also be correlated with Species Richness, as indicated by the significant Kendall rank correlation coefficient between these two indices (p=0.03). Since the 2019 benthic community has a much higher specific richness, the FRic index results should be assessed with caution (Fig. 16).
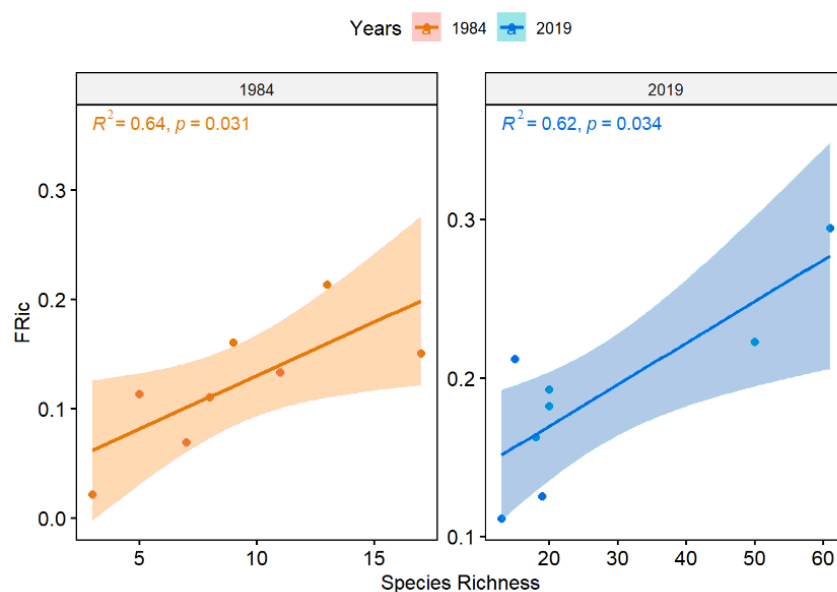
Figure 16: Kendall rank correlation coefficient between FRic and Species Richness for the 1984 and 2019. Mira estuary datasets.

Changes in the total FDiv were negligible between years (0.510 to 0.499), indicating that the total abundance of species with the rarest trait combinations (illustrated as the vertices of the functional space) were similarly distant from the centroid of the functional space (more common trait-modalities combination). That is, although the most abundant species have different trait-categories between years, these species have similar rarity of trait-modalities compared to each other average trait centroid. The two squares in the middle of the trait space in (A), (B), (C) represent the mean functional distance from the center of each assemblage (Functional centroid). Looking more closely at the changes in FDiv between years across the salinity gradient (Fig 15. E), again there was an increase in the average divergence in the euhaline and mesohaline areas and a slight decrease in the polyhaline section, but these changes were not significant. This index is expected to decrease after environmental disturbance. The Mira estuary showed a stable functional divergence between the years that were analyzed.

The total FDis outcome indicated a decrease in this index score from 0.352 in 1984 to 0.339 in 2019 reflecting that species of 1984 had a higher average distance to the abundance weighted centroid in the community trait space. This reduction in FDis happened in the higher and lower salinity zones of the Mira estuary, with the polyhaline area showing a higher FDis in 2019, contrarily to the other functional indices.

FEve values were lower in 2019 in the euhaline and mesohaline areas of the estuary, however they remained approximately equal in the polyhaline part of the estuary (Fig. 17). These differences were minimal and not significant; however these results suggests that the macrozoobenthic community of 1984 had a more regular distribution of species traits within the trait space. RaoQ and FDis were significantly correlated to each other presenting a high R^2 (Fig. 18). Both of these FDI values remained consistent, showing no differences between zones and years. Functional redundancy, on the other hand, was significantly higher in 2019 (Table 11) indicating that this community could be close to reaching an asymptote (maximum FRed) where species start to share more and more similar traits. FRed was positively correlated to FRic and Ric, but also negatively correlated to FEve.
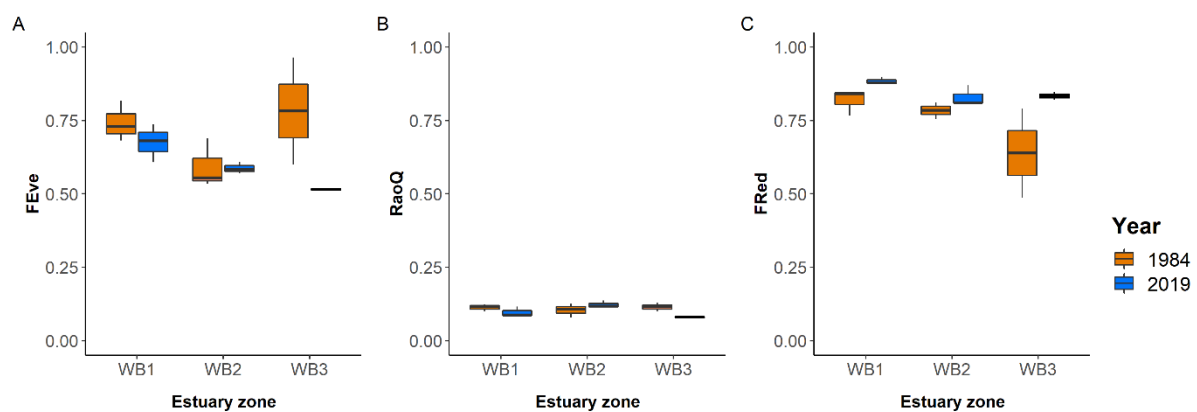
Figure 17: Changes in different components of the functional indices of Mira estuary macrozoobenthic communities from 1984 to 2019. (A) Boxplots of the FEve index results for each year and estuary zone; (B) Boxplots of the RaoQ index results for each year and estuary zone; (C) Boxplot of the FRed index results for each year and estuary zone.
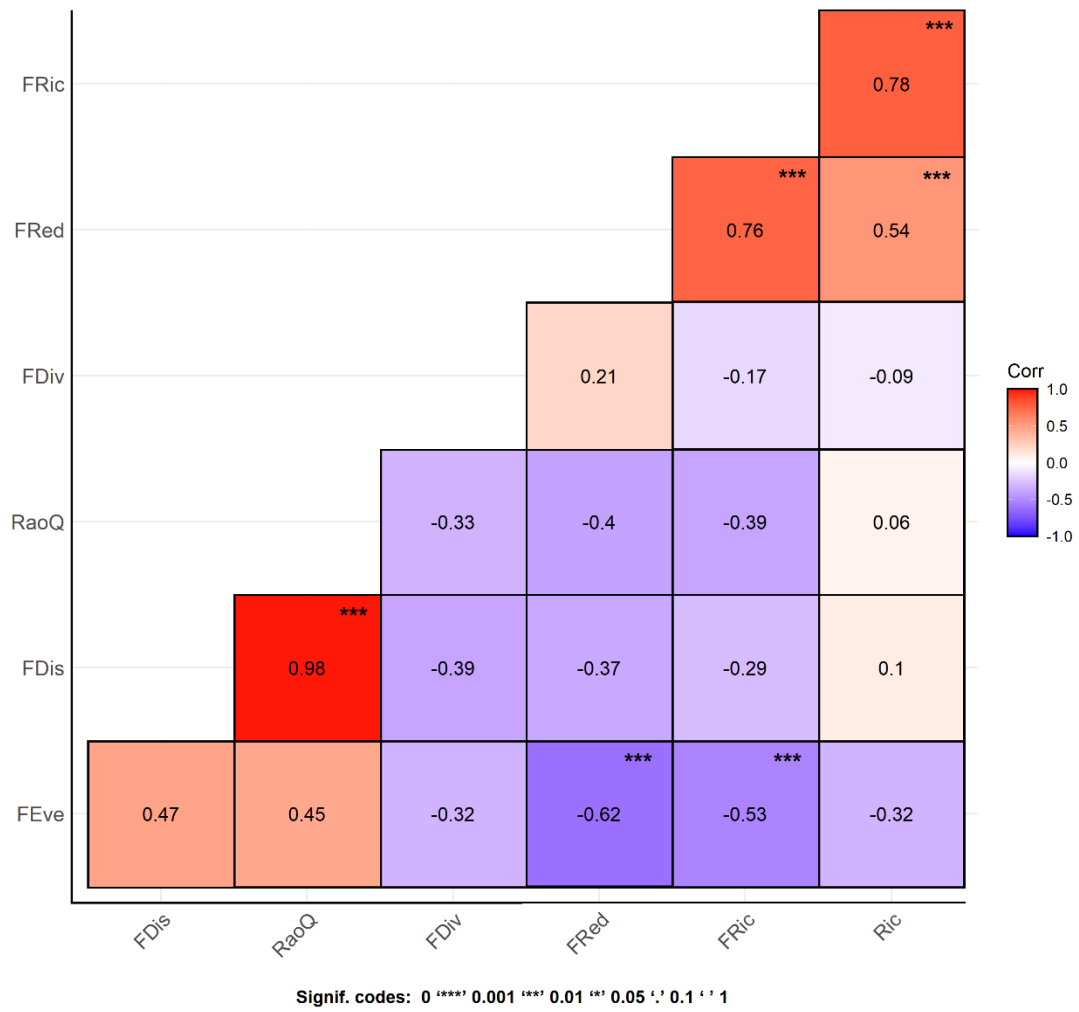
Figure 18: Pearson correlation values between the functional indices and species richness of the Mira estuary. Significantly correlated when p value is ≤ 0.05*).

4. Discussion

The main aim of this work was to assess the macrozoobenthic community functional response to local anthropogenic disturbance and climate change scenarios and to test potential differences in the ability of different trait-based approaches, namely BTA and numerous functional indices, to detect these pressures. The potential of these different trait-based approaches was tested in two dissimilar scenarios: in the first we tested the functional indices and BTA against the hypothesis that the macrozoobenthic communities have become less disturbed and thus more functionally diverse and stable after WWTP improvements in 2009 and also that communities further from the outfall were less disturbed; in the second one we tested the same approaches against the hypothesis that the macrozoobenthic communities of the Mira estuary in 2019 would be different from 1984 due to natural variations and climate change.

BTA and, more specifically, the community weighted-trait analysis was not very successful in detecting significant differences between communities before and after WWTP improvements in the Tagus estuary case study. However, previous studies have demonstrated the capacity of these method to detect different responses to disturbance levels between years (Pedelacq et al., 2022). The only differences detected were between 2019 and both 2011 and 2013, this may have occurred due to the drought in 2019 in which there was a higher average river flow in the summer than in the winter of 2018/2019 (Data from Almourol hydrometric station (17G/02H) (APA, 2023). BTA, on the other hand, was successful in showing changes along the distance from the outfall, mainly from the closest impact station (IA) and the furthest one (IC). These results are in agreement with the results of Gusmão et al. (2016), who reported that BTA is capable of detecting differences on functional traits along spatial pollution gradients in estuaries. The IC stations were primarily associated with traits related with lower levels of disturbance. The proportion of larger sized suspension feeder species that are attached to the substratum in a benthic community can be a proxy of lower levels of disturbance (van der Linden, 2017) since these species normally have a worse resilience capacity and are expected to decrease after disturbance by organic pollution (Pandey et al., 2022; Statzner and Bêche, 2010). However, the remaining traits associated to these stations, especially a short live span and tolerance to pollution (AMBI ecological groups) (Azeda et al., 2013), were expected to be more represented in the IA radial stations closer to the point of discharge, since species with these characteristics are better suited to live in areas impacted by environmental pressure (Oug et al., 2012), suggesting that this area is exposed to other pressures, as identified in Azeda et al. 2013, or still under pressure from the outfall, mostly to freshwater inflow.

Most of the IA samples for all years were correlated to 1^{sto} order opportunists, except for 2010 and 2019, showing that this attribute responded well to the spatial variation of organic-matter pollution, as species with this trait are expected to be more abundant in disturbed and polluted areas (Borja et al., 2000). The remaining samples from IA, mainly those from 2019, were associated to lower salinity preferences (SP5_18), which most likely is related to continuous freshwater discharges from the WWTP. IA samples were also associated to medium longevity species (L3_10), which has no particular response expected related to disturbance as these species normally have an intermediated resilience capacity to pollutants (van der Linden, 2017). After improvements in the sewage treatment, more precisely in 2010, 1^{sto} order opportunists were no longer associated with the IA stations, which seemed to be a good proxy of lower disturbance, yet in the following years these species were once more associated with the IA radial, with the exception of 2019. This pattern could be influenced by the natural instability of this transitional system, with high natural variations, which can cause stress levels that masks anthropogenic pressures. Ramajal et al. (2012) concluded that changes in the macrozoobenthic communities of the

downstream area of the Tagus estuary in 2009 and 2010 were most likely associated to the occurrence of floods in the Tagus estuary during winter (APA, 2023).

In a short spatial scale there were clear differences on traits along the organic pollution gradient/outfall distance, however, differences between years were not significant. The macrozoobenthic communities of the impacted radials continued to exhibit traits adapted to disturbance as a result of the WWTP outfall's prolonged role as an environmental filter. Despite the fact that there have been some ecological improvements, predominantly in the IA stations, the community in this area continues to have a reduced number of trait combinations (>higher than pre-improvement), indicating that the species present are able to tolerate environmental stress.

The results of the functional indices corroborated those from BTA since none of the indices was capable of detecting significant differences between years but were, once again, capable of detecting differences between impact stations. FRic was the only index that showed these differences, although with fluctuations over the years, FRic increased with the increasing distance from the outfall, as expected (Garrafo et al., 2018; Mouillot et al., 2013). Even though temporal variations were not significant there was an increase in functional diversity (RaoQ and FDis) and functional richness (FRic) in the impact zones after the improvements in water quality.

The results showed that, although there are strong seasonal and interannual variations in natural conditions (particularly salinity), associated with the river flow, the BTA and FRic were able to detect the impact gradient associated with the effects of the WWTP outfall. This indicates that there is still an impact of the WWTP outfall on benthic communities, however, this effect is strongly overlapped by other stress factors, such as natural variations in environmental conditions, which makes it difficult to clearly identify temporal trends associated with improvements in water quality. Although there are some improvements in functional diversity over the years, the area near the WWTP outfall remains under pressure, acting as a filter, with most of the species presenting traits that are suitable to disturbance. FEve, FDiv, and FRic can only be calculated with at least three species, and some impact station samples had fewer species than this number, in which case the indices could not be calculated. This happened at three replicates in 2009 (IA and IB) but also for one replicate in 2010 (IA), one in 2013 (IC) and one in 2019 (IB). This reduced number of species, in itself, can act as an indicator of low environmental quality, although it may also reflect the effect of the proximity to strong freshwater flows of the effluent, which for marine species acts as a pollutant, with the species present being mainly those with greater freshwater affinity. This makes it difficult to separate the effects of freshwater from the effects of organic pollution on macrozoobenthos communities. These facts supports the hypothesis that there is still a considerable level of disturbance in this area. Additionally, this inability to calculate FDI values for some stations, might be one reason why there were less significant differences in the results of these indices than expected.

In contrast to the Tagus estuary scenario, the BTA in the Mira estuary case study was able to identify differences of traits between years. The macrozoobenthic community identified in 2019 was mainly characterized by very small tube dwellers predators with a preference for sandy substrates. There was also an increase in the proportion of taxa with high dispersal potential, which are expected to increase after disturbance, because planktotrophic larval development species have higher dispersal potential and a lower extinction risk (McHugh and Fong, 2002). However, Garrafo et al. (2018) demonstrated that the control zones in a sewage affected area on the coast of Mar del Plata were dominated by planktotrophic species in contrast to the impacted stations, arguing that sites with more pelagic dispersal are theoretically more likely to recover from disturbance, and therefore, be considered to have higher environmental quality. The rise in the proportion of predators is probably connected to an increase in the availability of prey as 2019 community had higher species richness.

A spatial gradient was also identified, similarly to the Tagus estuary, but at different spatial scales, more specifically between the euhaline and mesohaline zones, which represent different ecological environments. The euhaline zone was correlated to a higher number of long lived but also very short-lived species, species sensitive to pollution, species with preference to higher salinity and with preference for warm waters. The high representativeness of the trait-categories Long Lived and Sensitive species (AMBI ecological groups) shows that this area was not very disturbed in both years (Pandey et al., 2022; van der Linden, 2017; Vinagre et al., 2017), although the longevity trait was more dubious with both extreme categories having high percentages. Regarding temperature and salinity preferences traits, they are aligned with environmental characteristics of the downstream area of estuaries, since species showed a thermal preference for lower temperatures and this area as the lowest temperatures in the estuary during summer (Chainho, 2008; Picado et al., 2020). Moreover, species with preference for higher salinity were also dominant in this area, which is in accordance with the characteristics of this estuarine zone. There was also strong association of very sensitive species (EG_I) and deposit feeders (F_DE) to samples collected during 1984 in the euhaline zone of the Mira estuary, which was not expected. The expression of this two traits in a community is anticipated to be contrasting, as deposit feeders are expected to be more abundant in disturbed environments with lower ecological quality, whereas very sensitive species (AMBI ecological groups) are predicted to be less abundant in impacted zones (Llanos et al., 2020; van der Lindn, 2017). However, this correlation to deposit-feeder species could possibly be an indication of a more reduced water movement and siltation as deposit-feeders prefer lower to medium hydrodynamic levels (Rosenberg, 1995). Furthermore, suspension feeders were the dominant group in all estuarine zones during 1984, indicating that the estuary in 1984 did not have high organic levels, in accordance with the presence of species very sensitive to pollution.

The mesohaline zone of the Mira, as expected, was dominated by species with preference to lower salinities and also by species tolerant to disturbance. This is predictable in this section, since it represents the intermediate zone of the estuary, being naturally more disturbed with lower levels of diversity, represented by species able to withstand high environmental fluctuations (Alves et al., 2009). Additional to these natural pressures, some of the Mira estuary most important pollution sources, such as sewage discharges and the irrigation perimeter of the Mira catchment, are located in this section of the estuary (Chainho, 2008). From 1984 to 2019 there was a considerable increase in the percentage of species with tolerance to pollution in this area of the estuary, and the appearance of opportunistic species in areas where they were absent before, suggesting that there has been an increase in the pressure levels in the area. Even though, the estuary continues to show very low disturbance when compared to the Tagus estuary.

The dominance of species with higher thermal preference could possibly function as a proxy to search for shift in species ranges related to climate change (Harvey et al., 2022), thus it was expected that there would be more species with affinity to tropical and subtropical waters in 2019. Thomas et al. (2021) demonstrated that there has been an increase of species which prefer subtropical climates in the shallower water of the Tagus estuary. This pattern was not detected in the Mira estuary, as there was no evidence of an increase in species with a stronger affinity to higher temperatures in 2019, since the representativeness of these trait-categories remained very constant. This trait was used as a proxy to assess changes in communities associated to thermal variations due to climate change, however as estuaries are highly complex mosaic environments (overlapping environmental stressors) (Lauchlan and Nagelkerken, 2020), with species adapted to thermally unstable environments and with high thermal tolerance (Madeira et al., 2012) it may be more challenging to detect these changes than in other coastal zones. In contrast to the results of the thermal preference trait, the salinity preference trait revealed differences between years, showing that there was an increase in species with higher salinity preferences in all zones of the estuary. This is probably due to hydromorphological changes that occurred during

this time period, such as a decrease in the river flow and an increase in the frequency of drought events in Portugal over the years. This hypothesis is in agreement with the findings of Portela et al. (2020), who demonstrated that the annual precipitation in southern Portugal has been continuously declining since the early 1980s. Although March values do not appear to be declining in recent years, current mean annual values are much lower than long-term historical averages. In January and February, on the other hand, is steadily decreasing. Additionally, based on four meteorological stations in the Mira river basin (SNIRH), the mean annual precipitation (mm), which may act as a proxy for river flow, decreased by more than half (from 729 mm to 289 mm) between the years studied (APA, 2023).

FDI were unable to detect differences between estuarine zones but were able to detect changes between years. FRic and FRed increased in 2019, very influenced by an increase in the number of species. These higher values indicate that the macrozoobenthic community of 2019 has the ecological niche space more occupied, having species with a greater variety of trait combination. Since FRic is considered a sensitive predictor of pressures (Pedelacq et al., 2022) expected to decrease in the occurrence of disturbance (Mouillot et al., 2013), the 2019 benthic macrozoobenthic communities is apparently less disturbed. On the other hand, the 1984 benthic community has a slightly more efficient use of resources, based on the results of FDis (Villéger et al., 2008), even though the 2019 community as a wider number of species with more diverse trait-categories combination as shown by the FRic results. Nevertheless, the 2019 community has also a greater number of functionally similar species, which in the long term may again lead to the disappearance of some species but with less severity, as the probability of some species with the same functions to remain is very high (Biggs et al., 2020).

Concisely FRic and FRed were the only indices able to highlight significant differences between both years in the Mira estuary. Both indices were significantly correlated to each other and to species richness (positive correlation) and functional evenness (negative correlation) (Fig. 18). Although there is clear evidence of a higher functional richness in 2019, this increase was mainly influenced by an increase in species richness. These results imply that, despite some functional disparities between communities in 1984 and 2019, these communities stayed mostly unchanged in terms of the majority of functional diversity's components (evenness and divergence). Climate change is causing sea level rise which in turn leads to an increase in marine water moving into estuaries and longer-lasting droughts (Mulamba et al., 2019; van Maanen and Sottolichio, 2018), especially in summers that are predicted to be warmer and drier, also resulting in a reduction in river discharge (Robins et al. 2016). These phenomenon is possibly led to an increase in the "migration" of "marine" species to the interior of the Mira estuary, influencing the rise in Ric and consequently in FRic. The species *Neanthes nubila,* for example, became one of the most abundant species in the mesohaline zone, whereas in 1984 it was very abundant in the euhaline zone. The increase in the percentage of species with affinity for soft sediments and of predator species that occurred in the Mira estuary in 2019, are also important factors molding the estuarine benthic community. Little et al. (2017) reinforced that although salinity is the main environmental driver of benthos composition in, the response of estuarine benthic communities to climate-driven increasing saline incursion should not be assumed to simply follow patterns of species salinity tolerance, but rather a hierarchy of factors followed by substratum type, with biotic competition and predator-prey interactions. Nevertheless, these new conditions in the Mira estuary may also had increased the functional redundancy, which throughout time may result in an increase in species that share analogous traits. Although the rise in sea temperature between 1984 and 2019 is clear, it was not possible to detect an upward trend of species with preference to higher temperatures. This may be due to natural stress and differences in the hydrological conditions of the estuary causing considerable temperature shifts along seasons and tidal water movements.

The combination of the BTA and the FRic index proved to be the more effective in showing how the macrozoobenthic communities might have been affected by different levels of disturbance, as both methods are complementary and have shortcomings. FRic was the index that most managed to find differences in both case studies, however, as in both cases it had a high correlation with species richness, it did not bring much more insights compared to traditional ecological methods. In cases where the number of species is very low, as is the case with the WWTP study before improvements, functional indices may not be the best choice since many of them have limitations and only show more reliable results with larger communities. RaoQ and FDis results were redundant, similar to what was explained by van der Linden et al. (2016a) and Festjens et al. (2023), therefore only one of them should be used in future research.

The Mira estuary remained mostly stable at a functional level, despite the increase in species and functional richness, showing a high resilience of benthic communities in an estuary strongly influenced by natural variations. The downstream area of the Tagus estuary located in the area near the Alcantara WWTP continues to be under pressure, however, there were improvements in functional and taxonomical diversity associated to improvements in sewage treatment.

5. Final remarks

This functional approach provided a framework capable of detecting different levels and types of disturbance affecting ecosystem functioning: local anthropogenic disturbance; and natural variations due to climate change. BTA and some of the FDI such as FRic provided similar levels of insight regarding the detection of these disturbances and should be used together for a better understanding of the ecological functioning within transitional ecosystems. In the first case FDI should be used with caution, when there are low levels of species richness. Furthermore, one of the main factors influencing functional traits was the occurrence of natural stress, most likely associated with fluctuations in the hydrological conditions, especially in the Mira estuary.

Although the results of the present study provide important information on the functional response of macrozoobenthic communities experiencing multiple stressors in temperate estuarine environments, there is still a need for future studies focused on the relationships between specific traits and ecosystem functions. Standard taxonomic methods cannot explain the causes and consequences of biodiversity change as well as BTA. However, these functional approaches still have limitations as gathering data on biological traits may be extremely time-consuming, especially for large community datasets, which limits its potential application. Despite the fact that several databases on macrobenthic features have recently been published (Clare et al., 2022; Costello et al., 2015; Degen and Faulwetter, 2019; Lam-Gordillo et al., 2023, 2020), information on some species' traits is still poor or nonexistent for many parts of the world. The next critical step would be to develop a comprehensive open access database of macrobenthic traits for the Atlantic Iberian Peninsula providing a reliable source of macrobenthic trait information. Additionally, laboratory-based ex situ experiments focusing on gathering trait information of specific species are important to update and address trait knowledge gaps facilitating further research using trait-based approaches in Iberian marine coastal waters.

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7. Appendix A. Supplementary data

Table A.1: *Species list of the benthic community sampled in the Tagus estuary in each year.*

Taxa	Years					
	2009	2010	2011	2012	2013	2019
Abludomelita gladiosa (Spence Bate, 1862)	X				X	
Abludomelita obtusata (Montagu, 1813)		X			X	X
Abra alba (W. Wood, 1802)	X	X	X	X	X	X
Abra nitida (O. F. Müller, 1776)			X			
Abra prismatica (Montagu, 1808)		X		X	X	
Abra sp.		X				
Acanthocardia echinata (Linnaeus, 1758)			X			
Achelia echinata Hodge, 1864					X	X
Actinia equina (Linnaeus, 1758)		X	X			
Ampelisca brevicornis (Costa, 1853)						X
Ampelisca diadema (Costa, 1853)				X		
Ampelisca typica (Spence Bate, 1856)		X				
Ampharete baltica Eliason, 1955				X		
Amphipholis squamata (Delle Chiaje, 1828)			X		X	
Amphiura chiajei Forbes, 1843				X		
Anomia ephippium Linnaeus, 1758				X		
Anoplodactylus petiolatus (Krøyer, 1844)			X	X		
Anoplodactylus pygmaeus (Hodge, 1864)		X			X	
Aonides oxycephala (Sars, 1862)	X	X	X	X	X	
Aphelochaeta sp.	X	X	X	X	X	X
Apolochus spencebatei (Stebbing, 1876)			X			
Arenicola marina (Linnaeus, 1758)					X	
Atelecyclus rotundatus (Olivi, 1792)	X	X				
Balanus crenatus Bruguiére, 1789		X				
Barnea candida (Linnaeus, 1758)		X	X	X	X	
Bittium reticulatum (da Costa, 1778)						X
Boccardiella ligerica (Ferronnière, 1898)					X	
Bodotria arenosa Goodsir, 1843		X	X	X		
Bodotria scorpioides (Montagu, 1804)		X	X	X	X	
Bolocera tuediae (Johnston, 1832)		X			X	
Capitella capitata (Fabricius, 1780)	X	X	X	X	X	
Caulleriella alata (Southern, 1914)				X	X	
Cerastoderma edule (Linnaeus, 1758)			X	X	X	
Cerastoderma glaucum (Bruguière, 1789)	X	X		X	X	X
Cerianthus lloydii Gosse, 1859						X
Chaetopleura angulata (Spengler, 1797)		X	X			
Chaetozone gibber Woodham & Chambers, 1994			X			
Chone sp.		X		X		
Cirriformia sp.						X
Clausinella fasciata (da Costa, 1778)					X	
Cossura coasta Kitamori, 1960		X		X		X
Cossura soyeri Laubier, 1964		X	X		X	

Species						
Crangon crangon (Linnaeus, 1758)		X	X	X	X	
Cuthona sp.		X				
Cyathura carinata (Krøyer, 1847)		X	X			
Cylista undata (Müller, 1778)						X
Cylista troglodytes (Price in Johnston, 1847)	X	X		X	X	X
Diadumene cincta Stephenson, 1925		X				
Diastylis rugosa Sars, 1865				X		
Diogenes pugilator (P. Roux, 1829)		X				
Diopatra micrura Pires, Paxton, Quintino & Rodrigues, 2010		X		X		
Diopatra neapolitana Delle Chiaje, 1841		X	X		X	X
Epitonium clathratulum (Kanmacher, 1798)		X				
Ericthonius punctatus (Spence Bate, 1857)		X	X	X	X	
Eulimella acicula (Philippi, 1836)		X				
Eulimella cerullii (Cossmann, 1916)		X				
Eunereis longissima (Johnston, 1840)	X	X	X	X	X	
Fimbriosthenelais minor (Pruvot & Racovitza, 1895)				X		
Galathea intermedia Lilljeborg, 1851		X				
Gammaropsis maculata (Johnston, 1828)		X				
Gattyana cirrhosa (Pallas, 1766)				X		
Glycera alba (O.F. Müller, 1776)		X	X		X	
Glycera sp.		X				
Glycera tridactyla Schmarda, 1861		X	X		X	
Glycera unicornis Savigny in Lamarck, 1818		X	X	X		
Gyptis propinqua Marion & Bobretzky, 1875	X	X	X	X	X	X
Harmothoe extenuata (Grube, 1840)		X				
Harmothoe imbricata (Linnaeus, 1767)						X
Harmothoe longisetis (Grube, 1863)		X				X
Harmothoe serrata Day, 1963		X	X			
Harpinia dellavallei Chevreux, 1910				X		
Hediste diversicolor (O.F. Müller, 1776)				X		X
Heteromastus filiformis (Claparède, 1864)		X	X	X	X	
Hilbigneris gracilis (Ehlers, 1868)		X	X			
Hormathia coronata (Gosse, 1858)		X	X			
Hyala vitrea (Montagu, 1803)						X
Idunella dentipalma (Dauvin & Gentil, 1983)		X				
Iphinoe tenella Sars, 1878		X	X		X	
Kurtiella bidentata (Montagu, 1803)	X	X	X	X		X
Lagis koreni Malmgren, 1866						
Lanice conchilega (Pallas, 1766)	X	X	X	X	X	X
Leptomysis gracilis (G.O. Sars, 1864)		X	X	X		X
Liocarcinus marmoreus (Leach, 1814 [in Leach, 1813-1815])		X				
Liocarcinus pusillus (Leach, 1816)				X		
Liocarcinus sp.				X	X	
Lumbrineris latreilli Audouin & Milne Edwards, 1833	X		X			
Magelona minuta Eliason, 1962		X	X	X		
Malmgrenia andreapolis McIntosh, 1874		X				
Malmgrenia lunulata (Delle Chiaje, 1830)				X		
Marphysa sanguinea (Montagu, 1813)		X				

Species						
Medicorophium aculeatum (Chevreux, 1908)			X	X		
Mediomastus fragilis Rasmussen, 1973				X	X	
Melinna palmata Grube, 1870	X	X	X	X	X	X
Melita palmata (Montagu, 1804)	X	X	X	X		X
Mesopodopsis slabberi (Van Beneden, 1861)		X				
Mimachlamys varia (Linnaeus, 1758)				X		
Modiolula phaseolina (Philippi, 1844)				X		
Monocorophium acherusicum (Costa, 1853)		X		X	X	
Monocorophium insidiosum (Crawford, 1937)		X	X	X	X	
Monocorophium sextonae (Crawford, 1937)		X				
Musculus subpictus (Cantraine, 1835)				X	X	
Myrianida sp.					X	
Mysta picta (Quatrefages, 1866)	X	X	X	X	X	
Mytilus galloprovincialis Lamarck, 1819				X		
Nassarius sp.				X	X	
Neomysis integer (Leach, 1814)		X				
Nephtys caeca (Fabricius, 1780)				X		
Nephtys hombergii Savigny in Lamarck, 1818				X		
Nepinnotheres pinnotheres (Linnaeus, 1758)	X	X	X	X	X	X
Nicolea sp.		X				
Notomastus latericeus Sars, 1851				X		
Nototropis guttatus Costa, 1853	X	X	X	X		
Nucula sp.		X		X		
Ocenebra erinaceus (Linnaeus, 1758)	X	X	X	X	X	X
Owenia fusiformis Delle Chiaje, 1844		X				
Parapionosyllis sp.				X	X	
Parthenina suturalis (Philippi, 1844)	X	X		X		
Parvicardium pinnulatum (Conrad, 1831)		X				
Peringia ulvae (Pennant, 1777)	X			X		X
Pharus legumen (Linnaeus, 1758)		X	X		X	X
Pholoe inornata Johnston, 1839	X					
Phyllodoce maculata (Linnaeus, 1767)		X			X	
Pilumnus hirtellus (Linnaeus, 1761)				X		
Pinnotheres pisum (Linnaeus, 1767)				X		
Pisidia bluteli (Risso, 1816)		X		X		
Polydora ciliata (Johnston, 1838)				X		
Polydora cornuta Bosc, 1802		X	X			
Polydora hoplura Claparède, 1868	X	X				
Prionospio cirrifera Wirén, 1883					X	
Prionospio fallax Söderström, 1920		X	X	X	X	X
Prionospio multibranchiata Berkeley, 1927				X		
Prionospio pulchra Imajima, 1990		X	X	X	X	
Prionospio sp.				X		
Prionospio steenstrupi Malmgren, 1867					X	
Psamathe fusca Johnston, 1836		X	X			X
Pseudopolydora paucibranchiata (Okuda, 1937)		X				
Pycnogonum litorale (Strøm, 1762)		X	X	X		
Ruditapes philippinarum (A. Adams & Reeve, 1850)		X				

Species						
Sabellaria spinulosa (Leuckart, 1849)				X		X
Scalibregma inflatum Rathke, 1843		X	X			
Sphaerosyllis hystrix Claparède, 1863						X
Sphaerosyllis sp.				X		
Sphaerosyllis taylori Perkins, 1981						X
Spio filicornis (Müller, 1776)			X	X	X	
Spirobranchus lamarcki (Quatrefages, 1866)			X			
Spisula elliptica (T. Brown, 1827)		X	X	X		
Spisula subtruncata (da Costa, 1778)					X	
Sthenelais boa (Johnston, 1833)	X	X	X	X	X	X
Streblospio shrubsolii (Buchanan, 1890)		X	X			
Subadyte pellucida (Ehlers, 1864)		X	X	X	X	X
Syllis gracilis Grube, 1840				X		
Tharyx sp.			X			
Timoclea ovata (Pennant, 1777)	X	X	X	X	X	
Tornus subcarinatus (Montagu, 1803)					X	
Tritia reticulata (Linnaeus, 1758)					X	
*Trophonopsis muricata (*Montagu, 1803)	X	X	X	X	X	X
Turbonilla lactea (Linnaeus, 1758)			X			
Unciola crenatipalma (Spence Bate, 1862)		X				
Upogebia stellata (Montagu, 1808)			X			
Urticina eques (Gosse, 1858)			X			
Varicorbula gibba (Olivi, 1792)	X					
Vaunthompsonia cristata Bate, 1858	X	X	X	X	X	X
Venerupis corrugata (Gmelin, 1791)						X
Venus casina Linnaeus, 1758	X	X	X	X	X	X
Venus verrucosa Linnaeus, 1758						X

Table A.2: *Total and cumulative abundance (N) and species richness (S) of the benthic community sampled in the Tagus estuary in each year.*

Year	Taxa	N (%)	S (cum%)
2009	*Aphelochaeta* sp.	20,17	20,17
	Capitella capitata	18,49	38,66
	Nephtys hombergii	15,97	54,62
	Tritia reticulata	9,24	63,87
	Mediomastus fragilis	7,56	71,43
	Venerupis corrugata	5,88	77,31
	Cerastoderma glaucum	3,36	80,67
	Spisula subtruncata	2,52	83,19
	Nucula sp.	1,68	84,87
	Tharyx sp.	1,68	86,55
	Abludomelita gladiosa	0,84	87,39
	Aonides oxycephala	0,84	88,24
	others	11,76	100,00
	(S) - 26		
2010	*Tharyx* sp.	54,48	54,48
	Streblospio shrubsolii	10,90	65,38
	Monocorophium acherusicum	5,47	70,86
	Mediomastus fragilis	4,59	75,44
	Polydora cornuta	3,70	79,14
	Nephtys hombergii	1,88	81,02
	Spisula subtruncata	1,36	82,38
	Ericthonius punctatus	1,30	83,68
	Venerupis corrugata	1,20	84,88
	Tritia reticulata	1,15	86,03
	Pseudopolydora paucibranchiata	1,09	87,12
	Barnea candida	0,89	88,01
	others	11,99	100,00
	(S) - 78		
2011	*Aphelochaeta* sp.	42,07	42,07
	Streblospio shrubsolii	12,44	54,51
	Cossura soyeri	6,16	60,67
	Capitella capitata	5,08	65,75
	Mediomastus fragilis	4,11	69,86
	Tharyx sp.	3,60	73,46
	Prionospio cirrifera	3,54	77,00
	Nephtys hombergii	3,42	80,42
	Lanice conchilega	2,80	83,22
	Tritia reticulata	1,43	84,65
	Heteromastus filiformis	1,37	86,02
	Venerupis corrugata	1,14	87,16
	others	12,84	100,00
	(S) - 74		

2012	*Nephtys hombergii*	27,06	27,06
	Tharyx sp.	10,09	37,16
	Spisula subtruncata	7,34	44,50
	Abra prismatica	7,11	51,61
	Aphelochaeta sp.	5,50	57,11
	Lagis koreni	3,67	60,78
	Abra alba	3,44	64,22
	Capitella capitata	2,98	67,20
	Streblospio shrubsolii	2,75	69,95
	Anoplodactylus petiolatus	2,06	72,02
	Bodotria scorpioides	1,83	73,85
	Monocorophium acherusicum	1,61	75,46
	others	24,54	100,00
	(S) - 61		
2013	*Tharyx* sp.	29,66	29,66
	Streblospio shrubsolii	16,14	45,80
	Monocorophium acherusicum	14,17	59,97
	Nephtys hombergii	6,17	66,14
	Heteromastus filiformis	4,72	70,87
	Mediomastus fragilis	3,94	74,80
	Cerastoderma glaucum	2,76	77,56
	Cossura soyeri	2,10	79,66
	Gyptis propinqua	1,84	81,50
	Barnea candida	1,71	83,20
	Abludomelita gladiosa	1,57	84,78
	Ericthonius punctatus	1,44	86,22
	others	13,78	100,00
	(S) - 60		
2019	*Nephtys hombergii*	20,20	20,20
	Aphelochaeta sp.	17,68	37,88
	Mediomastus fragilis	12,12	50,00
	Nucula sp.	7,58	57,58
	Vaunthompsonia cristata	7,58	65,15
	Kurtiella bidentata	5,56	70,71
	Ruditapes philippinarum	4,55	75,25
	Gyptis propinqua	2,53	77,78
	Streblospio shrubsolii	2,53	80,30
	Sphaerosyllis	2,02	82,32
	Glycera tridactyla	1,52	83,84
	Diopatra neapolitana	1,01	84,85
	others	15,15	100,00
	(S) - 37		

Table A.3: Tagus sediment grain size and mean total organic matter (TOM) at impact (IA, IB, IC) and control (C1, C2) areas sampled over six years (2009, 2010, 2011, 2012, 2013, 2019).

Sediment characteristics	Year	Impact radials			Control transects	
		IA	IB	IC	C1	C2
Gravel (>2 mm)	2009	0,7	1,1	22,3	1,2	1,8
	2010	2,6	4,2	0,3	1,8	0,9
	2011	12,7	4,6	34,5	1,0	2,5
	2012	24,7	0,0	0,1	0,0	1,6
	2013	0,0	3,5	0,3	0,4	0,2
	2019	0,0	0,1	4,0	2,6	1,1
Sand (<2->0.063mm)	2009	17,6	22,5	29,9	11,8	16,6
	2010	7,4	32,2	8,9	20,0	14,0
	2011	41,6	65,0	15,4	38,6	30,6
	2012	50,0	20,7	17,0	12,9	20,4
	2013	3,7	39,3	4,3	11,0	13,5
	2019	5,7	11,7	29,9	27,4	17,2
Mud (<0.063mm)	2009	81,7	76,4	47,8	87,1	81,6
	2010	90,0	63,7	90,9	78,2	85,2
	2011	45,8	30,4	50,2	60,4	66,9
	2012	25,3	79,3	82,9	87,0	78,0
	2013	96,3	57,2	95,4	88,6	86,2
	2019	94,3	88,2	76,8	70,0	81,6
Total organic matter (TOM)	2009	9,6	8,2	5,6	8,2	7,8
	2010	11,2	7,3	9,7	7,7	8,7
	2011	6,8	5,7	6,8	8,4	8,2
	2012	4,4	8,6	10,1	9,1	7,8
	2013	11,0	7,2	10,4	9,4	8,7
	2019	7,9	6,9	6,6	5,2	5,1

Table A.4: *Tagus scores of trait categories as fitted vectors. Trait categories with significant p-values in green. Correlation coefficient > 0.65 in red.*

Trait-categories	PC1	PC2	r2	Pr(>r)
MS_1	-0.93976	-0.34182	0.0026	0.930
MS1_3	0.89914	0.43767	0.1261	0.017*
MS3_10	0.82072	-0.57134	0.3648	0.001***
MS10_20	-0.93090	0.36527	0.4715	0.001***
MS20_50	-0.91719	0.39844	0.0634	0.136
MS_50	0.98640	-0.16434	0.0295	0.446
L_1	0.35689	0.93415	0.1310	0.018*
L1_3	0.64818	-0.76149	0.4234	0.001***
L3_10	-0.98382	0.17915	0.2702	0.002**
l_10	-0.58039	0.81434	0.0631	0.145
F_DE	0.78769	-0.61607	0.5564	0.001***
F_GRA	0.84789	0.53018	0.0599	0.164
F_S	0.70737	0.70684	0.1558	0.008**
F_HER	-0.11390	-0.99349	0.0406	0.315
F_PRE	-0.98687	0.16150	0.6894	0.005**
F_SCA	-0.99455	0.10424	0.6848	0.001***
F_OMNIP	-0.12188	0.99255	0.1657	0.001***
F_PARA	0.50362	0.86392	0.1334	0.005**
X.EG_I	0.32521	0.94564	0.0045	0.020*
X.EG_II	-0.99520	0.09788	0.6803	0.881
X.EG_III	0.22318	0.97478	0.1469	0.001***
X.EG_IV	0.99301	0.11805	0.3916	0.011*
X.EG_V	0.28041	-0.95988	0.4758	0.001***
DM_P	-0.80134	0.59821	0.6929	0.001***
DM_L	0.69651	-0.71755	0.7163	0.001***
DM_D	0.81331	0.58184	0.0343	0.001***
LP_BD	0.80019	-0.59975	0.2411	0.388
LP_TD	0.26051	0.96547	0.0193	0.001***
LP_FREE	-0.82919	0.55897	0.2150	0.003**
LP_A	-0.88628	0.46315	0.0466	0.235
S_H	0.49688	0.86782	0.6584	0.001***
S_G	0.60447	0.79663	0.1362	0.011*
S_S	-0.35420	-0.93517	0.4052	0.001***
S_M	-0.74016	-0.67243	0.2672	0.001***
SP5_18	-0.56096	0.82784	0.0816	0.113
SP18_30	0.15086	-0.98856	0.3904	0.001***
SP30_40	0.10325	0.99466	0.1033	0.055.
T_C	-0.99927	-0.03819	0.1995	0.002**
T_W	-0.77679	0.62976	0.3066	0.001***
T_T	0.92982	-0.36802	0.7546	0.001***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Permutation: free

Number of permutations: 999

Table A.5: *Species list of the Mira estuary dataset.*

Taxa	1984	2019
Abra alba (W. Wood, 1802)	-	X
Abra nitida (O. F. Müller, 1776)	X	-
Abra segmentum (Récluz, 1843)	-	X
Abra tenuis (Montagu, 1803)	-	X
Actinia equina (Linnaeus, 1758)	-	X
Acutigebia Sakai, 1982	-	X
Aeolidiella glauca (Alder & Hancock, 1845)	-	X
Alitta succinea (Leuckart, 1847)	X	X
Alkmaria romijni Horst, 1919	-	X
Ammothella longipes (Hodge, 1864)	-	X
Ampelisca brevicornis (Costa, 1853)	-	X
Amphibalanus amphitrite (Darwin, 1854)	X	X
Amphibalanus improvisus (Darwin, 1854)	X	X
Amphipholis squamata (Delle Chiaje, 1828)	-	X
Amphiura chiajei Forbes, 1843	-	X
Ampithoe rubricata (Montagu, 1808)	-	X
Anapagurus laevis (Bell, 1845)	-	X
Anomia ephippium Linnaeus, 1758	X	X
Aonides oxycephala (Sars, 1862)	-	X
Aora spinicornis Afonso, 1976	-	X
Apseudopsis latreillii (Milne Edwards, 1828)	-	X
Arcuatula senhousia (W. H. Benson, 1842)	-	X
Bathyporeia sarsi Watkin, 1938	-	X
Bittium reticulatum (da Costa, 1778)	-	X
Boccardiella ligerica (Ferronnière, 1898)	-	X
Callianassa Leach, 1814	-	X
Calyptraea chinensis (Linnaeus, 1758)	X	X
Capitella capitata (Fabricius, 1780)	-	X
Carcinus maenas (Linnaeus, 1758)	X	-
Cerastoderma edule (Linnaeus, 1758)	-	X
Cerastoderma glaucum (Bruguière, 1789)	X	X
Clausinella fasciata (da Costa, 1778)	X	X
Clymenura sp.	-	X
Cnemidocarpa mollis (Stimpson, 1852)	-	X
Corophium multisetosum Stock, 1952	X	-
Corophium orientale Schellenberg, 1928	-	X
Crangon crangon (Linnaeus, 1758)	-	X
Cucumaria de Blainville, 1830	X	-
Cyathura carinata (Krøyer, 1847)	X	X
Dexamine spinosa (Montagu, 1813)	-	X
Diogenes pugilator (Roux, 1829)	-	X
Diopatra neapolitana Delle Chiaje, 1841	X	X
Edwardsia claparedii (Panceri, 1869)	-	X
Eualus gaimardii (H. Milne Edwards, 1837)	-	X
Euclymene oerstedii (Claparède, 1863)	-	X

Euclymene palermitana (Grube, 1840)	-	**X**
Eurydice pulchra Leach, 1815	**X**	**X**
Euspira catena (da Costa, 1778)	-	**X**
Fabulina fabula (Gmelin, 1791)	-	**X**
Galathea bolivari Zariquiey Álvarez, 1950	**X**	-
Gari fervensis (Gmelin, 1791)	-	**X**
Gastrosaccus spinifer (Goës, 1864)	-	**X**
Glycera alba (O.F. Müller, 1776)	**X**	**X**
Glycera fallax Quatrefages, 1850	**X**	-
Glycera Lamarck, 1818	-	**X**
Glycera tesselata Grube, 1863	-	**X**
Glycera tridactyla Schmarda, 1861	**X**	**X**
Haplostylus normani (G.O. Sars, 1877)	**X**	-
Harmothoe spinifera (Ehlers, 1864)	-	**X**
Hediste diversicolor (O.F. Müller, 1776)	**X**	**X**
Heteroclymene robusta Arwidsson, 1906	-	**X**
Heteromastus filiformis (Claparède, 1864)	-	**X**
Hiatella arctica (Linnaeus, 1767)	-	**X**
Hilbigneris gracilis (Ehlers, 1868)	-	**X**
Hippolyte varians Leach, 1814	-	**X**
Iphinoe tenella Sars, 1878	-	**X**
Iphinoe trispinosa (Goodsir, 1843)	-	**X**
Irus irus (Linnaeus, 1758)	-	**X**
Jujubinus striatus (Linnaeus, 1758)	-	**X**
Kurtiella bidentata (Montagu, 1803)	-	**X**
Lagis koreni Malmgren, 1866	-	**X**
Leodamas chevalieri (Fauvel, 1902)	-	**X**
Leptocheirus pilosus Zaddach, 1844	**X**	-
Liocarcinus depurator (Linnaeus, 1758)	**X**	**X**
Liocarcinus navigator (Herbst, 1794)	-	**X**
Loripes orbiculatus Poli, 1795	-	**X**
Lucinella divaricata (Linnaeus, 1758)	-	**X**
Lumbrineris latreilli Audouin & Milne Edwards, 1833	-	**X**
Lutraria sp.	-	**X**
Lysidice ninetta Audouin & H Milne Edwards, 1833	-	**X**
Macomangulus tenuis (da Costa, 1778)	**X**	-
Malmgrenia lunulata (Delle Chiaje, 1830)	-	**X**
Malmgrenia McIntosh, 1874	-	**X**
Marphysa sanguinea (Montagu, 1813)	-	**X**
Mediomastus fragilis Rasmussen, 1973	-	**X**
Melinna palmata Grube, 1870	**X**	**X**
Melita palmata (Montagu, 1804)	**X**	**X**
Microcosmus claudicans (Savigny, 1816)	-	**X**
Microcosmus squamiger Michaelsen, 1927	-	**X**
Microdeutopus Costa, 1853	**X**	**X**
Moerella donacina (Linnaeus, 1758)	-	**X**
Monocorophium acherusicum (Costa, 1853)	**X**	**X**
Musculus discors (Linnaeus, 1767)	-	**X**

Musculus subpictus (Cantraine, 1835)	**X**	-
Mya Linnaeus, 1758	-	**X**
Neanthes fucata (Savigny, 1822)	**X**	-
Nephtys cirrosa Ehlers, 1868	-	**X**
Nephtys hombergii Savigny in Lamarck, 1818	-	**X**
Neanthes nubila (Savigny, 1822)	**X**	-
Nucula nucleus (Linnaeus, 1758)	**X**	-
Nucula sp.	-	**X**
Ophelia neglecta Schneider, 1887	-	**X**
Ophiocten affinis (Lütken, 1858)	-	**X**
Ostrea edulis Linnaeus, 1758	-	**X**
Owenia fusiformis Delle Chiaje, 1844	-	**X**
Paradoneis lyra (Southern, 1914)	-	**X**
Parvicardium pinnulatum (Conrad, 1831)	-	**X**
Peringia ulvae (Pennant, 1777)	-	**X**
Phtisica marina Slabber, 1769	-	**X**
Pisidia Leach, 1820	**X**	-
Platynereis dumerilii (Audouin & Milne Edwards, 1833)	**X**	-
Pleonexes helleri (Karaman, 1975)	-	**X**
Podarkeopsis capensis (Day, 1963)	-	**X**
Polydora ciliata (Johnston, 1838)	-	**X**
Polydora cornuta Bosc, 1802	-	**X**
Pontocrates arenarius (Spence Bate, 1858)	-	**X**
Prionospio sp.	-	**X**
Psammechinus microtuberculatus (Blainville, 1825)	**X**	-
Psammechinus miliaris (P.L.S. Müller, 1771)	-	**X**
Pseudopotamilla reniformis (Bruguière, 1789)	-	**X**
Ruditapes philippinarum (A. Adams & Reeve, 1850)	-	**X**
Scoloplos armiger (Müller, 1776)	-	**X**
Sigambra parva (Day, 1963)	-	**X**
Solen marginatus Pulteney, 1799	-	**X**
Sorgenfreispira brachystoma (Philippi, 1844)	-	**X**
Sphaeroma serratum (J. C. Fabricius, 1787)	-	**X**
Spiochaetopterus costarum (Claparède, 1869)	-	**X**
Spiophanes bombyx (Claparède, 1870)	-	**X**
Spirobranchus triqueter (Linnaeus, 1758)	**X**	-
Spisula solida (Linnaeus, 1758)	**X**	-
Spisula subtruncata (da Costa, 1778)	-	**X**
Sthenelais boa (Johnston, 1833)	-	**X**
Streblospio shrubsolii (Buchanan, 1890)	-	**X**
Streptosyllis websteri Southern, 1914	-	**X**
Tritia incrassata (Strøm, 1768)	-	**X**
Tritia reticulata (Linnaeus, 1758)	-	**X**
Trophonopsis muricata (Montagu, 1803)	-	**X**
Varicorbula gibba (Olivi, 1792)	-	**X**
Venerupis corrugata (Gmelin, 1791)	-	**X**
Venerupis geographica (Gmelin, 1791)	**X**	-
Virgularia mirabilis (Müller, 1776)	-	**X**

	X	-
Websterinereis glauca (Claparède, 1870)	X	-
Zeuxo holdichi Bamber, 1990	-	X

Table A.6: *Correspondence of sampling stations from MESCLA project to Andrade 1984.*

MESCLA	FA1986
MR11	**MiA1**
MR12	**MiA4**
MR13	**MiA7**
MR24	**MiA13**
MR25	**MiA19**
MR26	**MiA26**
MR37	**MiA30**
MR38	**MiA33**

Table A.7: Mira scores of trait categories as fitted vectors. Trait categories with significant p-values in green. Correlation coefficient > 0.75 in red.

Trait-categories	PC1	PC2	r2	Pr(>r)
MS_1	-0.07454	0.99722	0.4963	0.005**
MS1_3	-0.50016	-0.86593	0.7937	0.001***
MS3_10	0.92239	0.38626	0.6302	0.001***
MS10_20	-0.17197	0.98510	0.3865	0.032*
MS20_50	-0.69058	0.72326	0.1373	0.390
MS_50	-0.15610	0.98774	0.2941	0.084.
L_1	-0.75113	0.66015	0.3208	0.085.
L1_3	0.85800	-0.51364	0.0041	0.972
L3_10	0.84411	-0.53617	0.3906	0.044*
l_10	-0.95317	0.30244	0.2615	0.115
F_DE	-0.98997	0.14125	0.4966	0.016*
F_GRA	0.09723	0.99526	0.2485	0.130
F_S	0.20868	0.97798	0.5259	0.008**
F_HER	-0.96269	0.27061	0.1911	0.259
F_PRE	0.21506	0.97660	0.5473	0.005**
F_SCA	-0.10524	0.99445	0.1695	0.291
F_OMNIP	0.97423	0.22556	0.1253	0.385
F_PARA	-0.58007	0.81456	0.1565	0.365
X.EG_I	-0.93162	0.36342	0.7888	0.001***
X.EG_II	0.87974	0.47545	0.8295	0.001***
X.EG_III	0.13445	-0.99092	0.5377	0.006**
X.EG_IV	-0.38612	-0.92245	0.0427	0.799
X.EG_V	0.09500	0.99548	0.0783	0.597
DM_P	0.67964	0.73355	0.5725	0.005**
DM_L	-0.37697	-0.92623	0.4104	0.033*
DM_D	-0.96205	0.27286	0.1591	0.328
LP_BD	-0.70106	0.71310	0.5348	0.012*
LP_TD	-0.20600	0.97855	0.5320	0.007**
LP_FREE	0.64593	0.76339	0.3972	0.044*
LP_A	-0.03639	-0.99934	0.6186	0.004**
S_H	-0.49054	-0.87142	0.4878	0.009**
S_G	-0.72316	0.69068	0.1489	0.370
S_S	0.41090	0.91168	0.6979	0.001***
S_M	0.99889	0.04716	0.1640	0.307
SP5_18	0.42987	-0.90289	0.9038	0.001***
SP18_30	0.28555	-0.95836	0.2310	0.209
SP30_40	-0.40173	0.91576	0.8797	0.001***
T_C	0.68048	-0.73276	0.7447	0.001***
T_W	-0.69268	0.72125	0.7422	0.001***
T_T	0.83255	-0.55395	0.0417	0.760

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Permutation: free
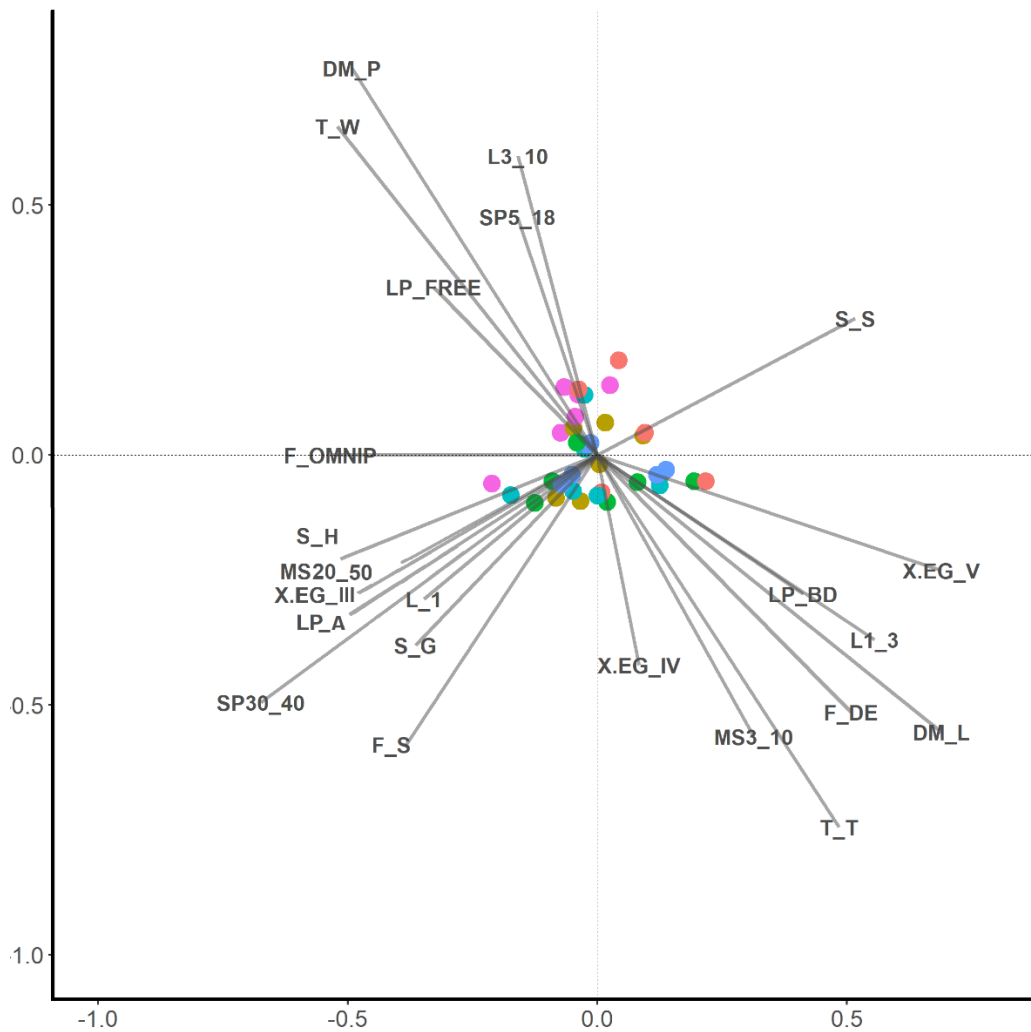
Number of permutations: 999

Figure A.1: *Principal Coordinate Ordination (PCoA) of macrozoobenthic communities' weighted trait means at the Tagus estuary Alcântara's WWTP based on Gower's similarity coefficient. Correlation coefficients of each trait modality with the two initial PCoA axes are indicated by the lengths of the overlaid vectors: planktotrophic larvae (DM_P); temperate waters (T_W); free-living (LP_FREE); mesohaline (SP5_18); medium longevity (L3_10); omnivores (F_OMNIP); medium size (MS20_50); very short longevity (L_1); attached (LP_A); tolerant (X.EG _III); euhaline (SP30-40); suspension feeder (F_S); gravel (S_G); 2ndo order opportunists (X.EG_IV); small-medium size (MS3_10); tropical temperatures(T_T); burrow-dweller (LP_BD); short longevity (L1_3); deposit feeder (F_DE); lecithotrophic larvae (DM_L).*