Taxonomic and functional macrofaunal diversity along a gradient of sewage contamination: A three-year study☆

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ABSTRACT

We investigated the structural and functional changes of the soft-bottom macrofaunal community following the improvement of a wastewater treatment-WWT plant. The macrofauna was collected at increasing distance from the main outfall in 2018, 2019, and 2021. Organic matter and nutrients were analysed in the water column near the outfalls to detect possible changes due to the improved treatment. We examined Functional Entities-FEs (i.e. a unique combination of species functional traits), species richness, Shannon-Wiener diversity-H′, and taxonomic and functional β-diversity. From 2018 (before the year of the treatment change), to 2021, we noted a gradual decrease of organic carbon in the water column. In contrast, sediment characteristics (i.e. grain-size) did not change before and after treatment enhancement, with the exception of redox potential. Species richness and FEs gradually increased moving far from the source of organic contamination and after wastewater treatment enhancement, especially near the outfall. We observed different phases of macrofaunal succession stage after the WWT amelioration. A 'normal stage', i.e. slightly lower species richness, was reflected in decreasing functional richness. Higher taxonomic β-diversity values with significant turnover components indicated that the community was subjected to broad changes in species composition. However, functional β-diversity did not follow the same pattern. After treatment improvement, modified environmental conditions led to the establishment of new species, but with the same functions. Towards 2021, the community improved its resilience by increasing functional redundancy and reduction of vulnerability, which enhanced community stability. The latter was also reflected in the well-balanced proportion of macrofaunal feeding habits after the WWT upgrade. Integrating the classical taxonomic approach with the analysis of FEs, and environmental characteristics can provide an accurate insight into macrofauna sensitivity to stressors that are likely to lead to changes in the ecological state of an area.

1. Introduction

Coastal areas are densely populated and therefore exposed to a variety of anthropogenic impacts, which are manifested in a wide range of inputs of organic matter, nutrients, and contaminants (Cabral-Oliveira et al., 2014). Among human pressures, wastewater discharges have long been recognized as one of the most widespread threats affecting coastal ecosystems (Calabretta and Oviatt, 2008). This calls for dedicated studies to assess how community structure and function changes under sewage discharge conditions.

It has been well established that the impacts of sewage discharge can result in severe degradation of macrozoobenthic communities in the marine environment, especially when discharges occur in relatively shallow and sheltered coastal areas (Mearns et al., 2015). Therefore, to protect and prevent damage to coastal ecosystems, there is an urgent need to improve the efficiency of wastewater treatment plants (WWT) to reduce the amount of organic matter and toxic material discharged into the coastal environment (Hallaji et al., 2018). Most studies on the

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ecological impacts of sewage loads on marine communities have focused on comparisons between reference and impacted sites (e.g. Vesal et al., 2021) or before and after the start of discharge (e.g. Díez et al., 2013) and along the temporal scale after the cessation of a long-standing release of sewage (e.g. Zubikarai et al., 2014). The influence of different wastewater systems has been studied, for example, on rocky meiofauna communities (Fraschetti et al., 2006), macrofauna invertebrates and seabirds (Chainho et al., 2010; Alves et al., 2012), and rocky macrofaunal assemblages (Bustamante et al., 2012; Cabral-Oliveira et al., 2014). However, as far as we know, there are no studies on the influence of WWT upgrade on sublittoral macrofaunal communities in coastal areas.

Benthic macrofaunal assemblages can be differently affected by sewage discharges depending on effluent treatments and flow rates (Del-Pilar-Ruso et al., 2010), but also on the environmental features of the area in which the effluent is discharged (Zubikarai et al., 2014). The effluent, diluted in the water column around the main outfall, is dispersed over a large area. Sedimentation of suspended organic particles to the bottom can be influenced by the intensity and direction of currents and the structural aspects of the pipeline (e.g. length of pipe and diffusion surface, number of turrets; Del-Pilar-Ruso et al., 2010).

Many studies have focused on structural aspects of macrofaunal communities, such as abundance, biomass, and diversity. In this context, a variety of valid biotic indices (e.g. AMBI and M-AMBI; Muxika et al., 2007) related to species composition and diversity, were used to assess the effects of organic enrichment on benthic communities. However, the structural approach alone rarely estimates functional variation of macrofaunal invertebrates in stressed environments. Macrofaunal organisms could be considered an important biological component of the benthic ecosystem. Changes in species composition, triggered by natural causes or anthropogenic activities, may adversely affect ecosystem processes (Gray et al., 2006). Notwithstanding, our knowledge and predictive ability of environmental change effects on ecosystem function and services are still limited, particularly in marine ecosystems (Loreau, 2001). Variations in functional traits can provide insight into the potential loss of ecosystem function in response to disturbance (Naeem et al., 2012). Moreover, studies of changes in the components of community functional diversity in response to environmental variation may link species extinctions to changes in ecosystem functions (Teixidó et al., 2018). Indeed, functional diversity could increase the resilience of communities to environmental fluctuations caused by human-induced changes across taxa and ecosystems. The high number of functions is closely related to many aspects of ecosystem functioning, as functions characterize the different ecosystem processes (Tilman et al., 2014). However, when systems lose species, it is often assumed that they are buffered against a decline in functional diversity by the ability of species-rich biota to exhibit high functional redundancy. In contrast, certain communities lack functional redundancy despite high species richness and exhibit only low functional redundancy among species, revealing their functional vulnerability (Díaz et al., 2013; Mouillot et al., 2014; see Glossary in Supplementary Text 1). Nevertheless, further research is needed to specifically examine the effects of human-induced species loss on the redundancy and vulnerability of species functions for ecosystem functioning.

Recently, an increasing number of studies have applied the functional traits approach to marine benthic assemblages in relation to various types of environmental stress (e.g. Villnäs et al., 2018; Sobczyk et al., 2021), chemical contamination (e.g. Nasi et al., 2018; Egres et al., 2019), riverine inputs (e.g. McGovern et al., 2020), and sewage-discharges (Gusmao et al., 2016; Krumhansl et al., 2016) using Biological Traits Analysis (BTA) introduced by Bremner et al. (2003, 2006). However, few studies emphasised the functional diversity, redundancy, and vulnerability of benthic communities in relation to environmental fluctuations (Mouillot et al., 2014; Teixidó et al., 2018). In this context, Mouillot et al. (2014) applied a functional-trait approach through the analysis of functional entities (FEs). FEs could be defined as

groups of species that share the same trait values within the investigated community and are methodologically represented as a unique combination of traits. The authors analysed the functional traits of a large number of fish species on different tropical reefs. Based on the extensive sample collections and relevant results of the study, we can conclude that this study has established the strength of this novel approach. Indeed, this approach allows us to investigate how species are distributed among functional groups and, in particular, which FEs are responsible for functional redundancy and vulnerability due to species variation. In this way, species and functional diversity and its components contribute to unravelling the mechanisms that structure species assemblages and their functions (Loiseau et al., 2017). Analyses of the components of diversity (β), turnover (i.e. replacement and loss or gain of species along spatial and/or temporal scales), and the nestedness-resultant components (i.e., those reflecting the difference in the number of species among communities; Villéger et al., 2013), together with biodiversity (α-diversity), are important to understand the variations of communities and their successional stages after an event (Baselga, 2012), such as the improvement of wastewater treatment.

We studied the changes in the community functional features following the ameliorated sewage abatement system of the wastewater treatment plant along a distance gradient from the principal source of contamination. We analysed species functional traits as unique combinations of traits (FEs) and combined taxonomic β-diversity with functional β-diversity analysis to: i) better define variations in functions due to species change and to ii) understand whether there is overlap in functions (i.e. different species perform the same functions) among species due to improvement in environmental quality conditions. Applying an approach based on functional traits to entire benthic marine assemblages influenced by sewage discharge, rather than just using taxonomic diversity, can provide new insights into resilience across taxa and functional changes before and after the treatment upgrade.

Given the importance of species and functional diversity in marine benthic communities, we sought to investigate patterns of change in species richness and functional diversity, answering the following questions: (1) Are the variations in FEs and diversity driven by specific sediment physical-chemical variables before and after treatment changes? (2) Do α- and β-biodiversity reflect the successional stage of the macrofaunal community after the wastewater upgrade, and along spatial scales? (3) Do functional diversity, vulnerability and resilience vary following the ameliorated sewage abatement system? (4) Which functional characteristics change as a result of improved wastewater treatment?

2. Material and methods

2.1. Study site and sampling design

The Gulf of Trieste is a shallow basin of about 600 km^2 with a coastline of 100 km, located in the northernmost part of the Adriatic Sea. The geographical, hydrological and sedimentological features are described in detail in Vesal et al. (2021). The Gulf of Trieste hosts several wastewater treatment-WWT plants, and the main one serving the city of Trieste is the Servola WWT plant that started operating in 1992. This plant is located in the north-eastern part of the Gulf, serving up to 190.000 equivalent inhabitants. The system is a mixed type collecting meteoric and domestic waters for about 35 million m^3 of water per year. In June 2018, the treatment plant was improved in each part (pumping station, primary sedimentation, UV disinfection, biofilters for pre-denitrification, nitrification/oxidation for post-denitrification, and phosphorus removal) and now has three phases of the treatment process. After the treatments, the plant carries the residual waters through two submarine pipelines (overall length of 7.5 km), equipped with specific diffusion turrets in the last 1.5 km to facilitate the dispersion of the effluent (AcegasApsAmga).

Sediments were collected in April 2018 (before WWT improvement),

December 2019, and January 2021 at 10 stations with an average depth of 22.7 ± 0.5 m. One station, 0D (LAT: 45°38′36.30′′; LON: 13°40′51.70′′; Fig. 1) was located near the main pipe (<5 m). The other stations were located along three transects arranged radially at increasing distance (25 m, 25D; 100 m, 1D; and 200 m, 2D) from the distal (D) part of the duct and followed the average annual seafloor current (NW350°). One transect was placed in front of the duct (25FD, 1FD, and 2FD), one along the direction of the average current (25D, 1D, and 2D), and one in the opposite direction (−25D, -1D, and -2D).

For each sampling period, the macrofaunal community was sampled with a van Veen grab (0.1 m^2) in three replicates at each station. In addition, to detect changes in water column chemical properties possibly due to the improved wastewater systems, water samples were collected every two months from February 2017 to February 2021 at the surface, intermediate (10 m), and bottom layers at the station near the main outfall (0D; 22 m depth) and at two stations along the diffusion zone (0P at the end of the first pipeline and 0M at an intermediate position between the outfall of the first and second pipeline; 22 and 20 m depth, respectively, Fig. 1).

2.2. Water, sediment and macrofauna sampling

Discrete water samples for total nitrogen (TN), total phosphorus (TP), and total organic carbon (TOC) were collected using 5-L Niskin bottles. TN and TP concentrations were determined colorimetrically (Hansen and Koroleff, 1999), while TOC was analysed by High Temperature Catalytic Oxidation method as described in Kralj et al. (2016). The Redox potential (Eh) of the sediments was measured to evaluate the physical-chemical state of the marine sediments and to provide evidence of oxidation-reduction processes that determine sediment oxic conditions (Colman and Holland, 2000). We also analysed the grain size, total organic carbon (TOC), total carbon (TC), and total nitrogen (TN) content of the sediments. Data are expressed as %. The molar ratio of organic carbon to total nitrogen (TOC/TN) was calculated and used as a proxy for the origin of the organic matter (Rumolo et al., 2011). For details of water column and sediment analyses, see Supplementary Text 2.

The sediments for the macrofaunal community were sieved on a 1.0 mm mesh to retain the fraction of macrofaunal organisms. The retained sediment and organisms were immediately fixed in ethanol 80%. Macrofaunal invertebrates were identified under a stereomicroscope (Zeiss Discovery V.12, 8–110 × final magnification) and counted. The keys listed in Morri et al. (2004) were used for taxonomic identification. The abundance number is expressed as individual per m^2.

2.3. Functional characterization of macrofaunal species

We characterized the ecology of 240 species using 9 functional traits A total of 188 different functional entities (FEs) were identified based on unique combinations of 9 categorical functional traits (as reported in Table 1). Functional traits for each species were derived from literature sources (i.e. Giangrande, 1997; Rouse, 2000; Jumars et al., 2015) and databases (i.e. MarLIN, 2006; Polytraits Team, 2022). We calculated three parameters: (i) species richness as number of taxonomic species, (ii) functional richness as the number of FEs present (functional diversity), and (iii) functional richness defined as a volume constructed from a computational geometry, namely the convex hull, that is an n-dimensional measure of the volume of functional space occupied by FEs in a community (Fig. S1). The larger the volume filled by each assemblage, the greater the number of functions performed by the species packed into different FEs. Species richness, number of FEs, and functional richness were expressed as a relative percentage of the global pool. The global pool represents the total number of species (n = 204) and FEs (n = 188) observed across the entire sampling area and in all periods. For the functional analyses, the total number of species of each year was divided into four distance zones (<5, 25, 100, and 200 m). The calculations and steps to obtain the functional diversity and richness are shown in Fig. S1 and follow Villéger et al. (2011); Mouillot et al. (2014); Maire et al. (2015).

We generated a null model to test if the observed values of functional richness reject the null hypothesis i.e. whether species are randomly distributed among the FEs (Gotelli and McGill, 2006). For each distance gradient and sampling year, we simulated random assignment (against 9999 random assignments of species to FEs consisting of at least one species), keeping the number of species and FEs constant. The functional
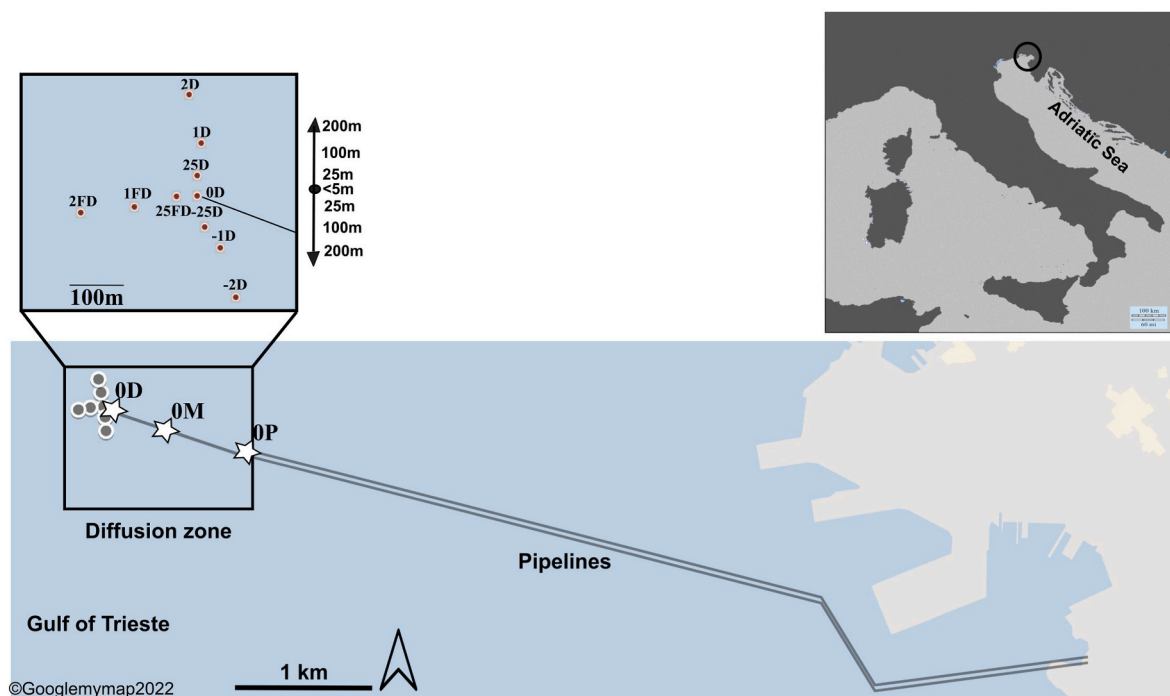

Fig. 1. Sampling area and stations in the Gulf of Trieste. The sampling stations in the water column are indicated with stars.

Table 1

Biological traits used to measure functional diversity of benthic species. The explanation of the selected modalities also in relation to the disposal of organic materials, is reported. N = number of categories.

Trait	Trait Type	N	Categories
1) Morphology *External characteristics of the taxon. Different morphologies vary in their relative susceptibility to damage from sediment disposal (Bolam et al., 2016).*	Categorical	6	a) Vermiform with no protection b) Dorso-ventral compressed with shell c) Dorso-ventral compressed with no protection d) Laterally compressed e) Case/shell protection f) Globose
2) Adult size *A measurement of the longest dimension of a body, typically between two distinct ends of the body. Smaller animals may be more able to take advantage of refugia from sedimentation at microhabitat scales.*	Ordinal	6	1) up to 5 mm 2) 5–15 mm 3) 15–35 mm 4) 35–60 mm 5) 60–80 mm 6) >80 mm
3) Longevity *The maximum length of time that any particular organism can be expected to live. Adult longevity indicates the time interaction with sediment disposal .*	Ordinal	4	1) ≤1 yr 2) 1–3 yrs 3) 3–6 yrs 4) 6–10 yrs
4) Reproductive frequency *Period and frequency of spawning in a population.*	Categorical	3	a) Semelparous b) Iteroparous c) Semi-continuous
5) Mechanism of development *Larval development is a proxy for recruitment success.*	Categorical	2	a) Direct b) Planktotrophic c) Lecithotrophic
6) Adult feeding habit *The common diet of an organism that includes the food items that it is enzymatically and behaviourally capable of using. The variety of feeding apparatus indicates the diversity of feeding types and can be considered a proxy of selective and not-selective feeding. Feeding habits indicate roles in the trophic pathway. They may be affected by increased suspended sediment (e.g., suspended feeders) (Thrush et al., 2004).*	Categorical	14	a) Suspension feeder b) Tentaculate suspension feeder c) Jawed suspension feeder d) Surface deposit feeder e) Tentaculate surface deposit feeder f) Jawed surface deposit feeder g) Subsurface deposit feeder h) Tentaculate subsurface deposit feeder i) Jawed subsurface deposit feeder j) Carnivores k) Tentaculate carnivores l) Jawed/toothed carnivores m) Omnivores n) Herbivores
7) Mobility The capability of an organism to move spontaneously and freely. Mobility may increase the possibility of evading burial following disposal (Vesal et al., 2021).	Categorical	6	a) Swimmer b) Crawler/creeping c) Burrower d) Tube-builder e) Temporary attachment f) Permanent attachment
8) Adult environmental position *Living location of the organism in relation to the sea floor and other organisms. Taxa will generally need to re-establish their position in sediments after or during continuous uploading of sewage*	Ordinal	4	1) Epibiont/epilithic 2) Epifauna 3) Demersal 4) Endofauna

Table 1 (*continued*)

Trait	Trait Type	N	Categories
materials to undertake their biological processes (Bolam et al., 2016).			
9) Ecosystem engineering *Organisms can be considered as ecosystem engineers when they directly or indirectly modulate the availability of resources to other species, by causing physical state changes in biotic or abiotic materials. In so doing they modify, maintain and/or create habitats (Jumars et al., 2015).*	Categorical	5	a) None b) Biodiffusor c) Conveyor d) Regenerator e) Habitat-building

richness data were compared with simulated values using a bilateral test ($\alpha = 5\%$). Since the functional richness can be affected by categorization of traits, to test the robustness of all results, we performed a sensitivity analysis. We reduced the number of categories for each trait and re-ran all analyses (functional diversity and richness). This new coarse categorization, with a total of 55 FEs, included two to four categories for each trait (Table S1).

We tested whether functional redundancy and vulnerability of FEs were affected by sewage inputs at the spatial scale, and before and after WWT improvement. Vulnerability is related to the lack of cover by functionally similar species in the community and it was expressed as the proportion of FEs in an assemblage that had redundancy of 1 (i.e. the proportion of FEs with only one species). For redundancy, we considered the abundance of species belonging to each FE to determine if the abundance was packed into specific functions.

2.4. Taxonomic and functional β-diversity

We assessed the taxonomic α- (Shannon-Wiener Log$_2$) and β-diversity of the benthic assemblages along the spatial scale, and before and after WWT improvement. The β-diversity (taxonomic and functional) portioning framework (Baselga, 2010; Baselga et al., 2012; Villéger et al., 2013) was based on Jaccard's dissimilarity index. β-Diversity equals 0 when communities are identical or when the portions of functional space filled by FEs completely overlap, and it equals 1 when communities or functions performed are maximally dissimilar. We examined whether taxonomic temporal and spatial β-diversity and functional β-diversity associated with sewage-derived material were mainly due to turnover (i.e. differences in species between sampling periods or along the wastewater gradient were due to a replacement) or to nestedness resultant processes (i.e. species between two sampling periods or species at gathered stations representing a subset of species found in previous years or in the other group of stations). Specifically, turnover equals 0 when communities are functionally nested and equals 1 when no species are shared by the communities (Baselga and Leprieur, 2015).

2.5. Statistical analyses

First, all data were tested for normality and collinearity, applying Shapiro Wilk's and Spearman's rank correlation test, respectively. The TOC, TN and TP values of the water samples were integrated over the water column and then linear regressions were calculated to show the variation of the values over time. In this way, the predictive variation of environmental parameters for each sampling year was distinguished by the coefficient of determination R_2 (linear regression).

Sediment characteristics (grain-size, TOC, TN, TC, and Eh), taxonomic diversity (i.e. macrofaunal density, number of species, α- and temporal β-diversity, and trait-categories occurrences) and species composition were tested among sampling periods and distance from the duct ('years' and 'distance from the duct' as factors) by a non-parametric

test (Kruskal-Wallis H test and post-hoc comparisons with Bonferroni correction) and multivariate analyses. For both analyses in the factor 'distance from the duct', we gathered the stations at <5 and 25 m along the distance gradient as unique level. We applied one- and two-way ('distance from the duct x year') PERMANOVA (Permutational multivariate analysis of variance) as multivariate analysis with an unrestricted permutation of raw data and 9999 permutations. To confirm that the differences among selected factors were not masked by higher within-group variability (within grouping factors), the heterogeneity of group dispersion (PERMDISP) analyses were performed based on distances to centroids, with 9999 permutations. Furthermore, non-metric multidimensional scaling (nMDS) analysis was performed to observe any spatial and temporal patterns of species' composition in the study area. To determine which species were mainly responsible for the variations in species composition along the spatial scale, and before and after the WWT upgrade, the SIMPER (SIMilarityPERcentange) analysis was used. A cut-off at 60% was applied. Before applying the multivariate analyses, we square root transformed the species composition matrix and applied the Bray-Curtis similarity. We also carried out a Principal Component Analysis (PCA) on physical-chemical sediment data, after normalizing the matrix, to visualize the temporal and spatial distribution of these abiotic samples. Due to the high negative correlation between sand and clay variables ($r_s= > -0.95$), the clay % was not included in the PCA.

For the statistical analyses performed in this study, we used different packages and software. The functional diversity analyses were computed using the R functions from the 'FD', 'tripack', 'geometry', 'matrixStats', and 'betapart' R packages (version R v 4.1.0; see Supplementary Text 3 for the R-codes). Shannon-Wiener diversity index and multivariate analyses were performed using PRIMER7 software. Kruskal-Wallis tests, linear regressions, and Spearman's rank correlations were calculated using STATISTICA™ software.

3. Results

3.1. Water column and sediments features

The chemical features of the water column varied over time. TOC concentration decreased significantly toward 2021 at station 0D ($R_2 = 0.16$ and $p < 0.05$; Fig. S2 and Table S2), while TP showed increasing values toward the end of the short-term series, especially at 0P ($R_2 = 0.19$ and $p < 0.05$; Fig. S4 and Table S1). At station 0M, similar trends were observed for all the parameters investigated in the water column (Fig. S3), except for TN, which decreased significantly after WWT upgrade ($R_2 = 0.16$ and $p < 0.05$; Table S2).

Sediments collected during the three sampling periods were mainly composed of silt (average value: $45.8 \pm 8.1\%$). However, in all sampling years, the sand fraction was predominant in the area closest to the pipe (25 and <5 m), contributing for $41.4 \pm 7.7\%$, while at the more distant stations (200 m) the sand fraction was only $8.3 \pm 2.5\%$ (Table S3).

We observed a significant trend along the distance gradient from the main outfall for all abiotic variables except for redox potential (Eh). Specifically, values of sand (H = 24.05; $p < 0.001$), TOC (H = 22.02; $p < 0.001$), TN (H = 13.64; $p < 0.05$), and TOC/TN (H = 22.02; $p < 0.001$) decreased with distance from the pipe, while silt content (H = 20.99; $p < 0.0001$) increased from the diffusion area toward the more distant stations. We found significant interannual variability only for Eh (H = 18.18; $p < 001$; post-hoc comparison: 2018 *vs.* 2021 z = 3.73; $p < 0.001$; 2019 *vs.* 2021, z = 3.05; $p < 0.01$).

The ordination plot of principal component analysis (PCA) explained 90.5% of the total variance (Fig. S5). TOC/TN and sand were the predominant elements of the positive part of the first axis, while silt was the major contributor to the negative axis. Eh was highly correlated with the second axes. In the principal component analysis, the stations nearby the main outfall in the sampling years were clearly related to TOC/TN, sand, TOC, and TN, while stations far from the duct were associated with silt.

In the analysis, all station sampled in 2021 were distinguished from other years based on increasing values of Eh.

3.1.1. Taxonomic diversity (α and β) and species composition

Macrofaunal density decreased from 2018 to 2021, as confirmed by K–W and post-hoc comparisons (H = 14.3; $p < 0.001$; 2018 *vs.* 2021, z = 3.8; $p < 0.005$, respectively). The number of species significantly increased from the stations nearby the outfall toward the farther ones in 2019 (H = 6.6; $p < 0.05$). The stations placed at 200 m significantly differed before and after WWT updates (H = 6.2; $p < 0.05$). The Shannon-Wiener value (H′) had the lowest value at 0D (1.89) in 2018, while the highest H′ value was found at 1D (5.40) in 2021 (Table 2). Higher H′ values were obtained in 2021, also confirmed by the K–W test, which revealed significant differences among years (H = 9.9; $p < 0.01$), especially between 2021 and 2018 (z = 3.1; $p < 0.01$). In 2021, we observed significant higher species richness values at <5 and 25 m than in previous years (H = 7.5; $p < 0.05$) and higher H′ values at 100 m compared to 2018 values (H = 5.9; $p < 0.05$).

Regarding taxonomic β-diversity over time, we found a large variation in species when analysing β-diversity values (Fig. 2d), mainly from 2019 to 2021. In particular, turnover evidenced that the higher β-diversity values from 2019 to 2021 were due to the increasing replacement of new species (Fig. 2e). In contrast, the value of β-diversity from 2018 to 2019 was mainly due to greater nesting results (i.e. richness differences) instead of turnover (Fig. 2).

The comparisons among sites for each sampling period revealed high values of the taxonomic β-diversity in 2018 (Table 3). In this year, the values of β-diversity were likely due to high nestedness-resultants between <5 *vs.* 25 m and <5 *vs.* 100 m. On the contrary, higher turnover (i. e. species replacement) characterized the value of β-diversity between 100 and 200 m. We observed a high turnover at <5 *vs.* 25 m and <5 *vs.* 100 m in 2019, whereas lower β-diversity was determined between 100 *vs.* 200 m. In 2021 similar results were observed. Overall, in 2021 major values of turnover components characterized the β-diversity from areas near the pipe to those farther away.

Statistical observations regarding variation in species composition confirmed what we observed by taxonomic β-diversity analysis. Throughout the sampling area, the species composition of the macrofauna varied significantly among years and along the distance gradient from the pipe (PERMANOVA main test: Pseudo-F = 3.67 and 4.28; $p < 0.001$, respectively). In addition, significant differences were measured in the two-way PERMANOVA test, year x distance (Pseudo-F = 3.67; $p < 0.05$). The PERMDISP analyses confirmed a homogeneity of dispersions within groups of factors 'year' and 'distance from the duct'. The nMDS analysis (Fig. 3) also corroborated the previous statistical results, by separating stations sampled in 2018 from those sampled in 2021. The nMDS also highlighted the variation in species composition along the spatial scale, by clearly separating the stations closest to the pipe from the farther ones.

The polychaetes *Capitella capitata* and *Hilbigneris gracilis*, and the

Table 2
Species richness (Sp) and α diversity values (H′: Shannon–Wiener diversity, $\log_2$) at sampling stations and years.

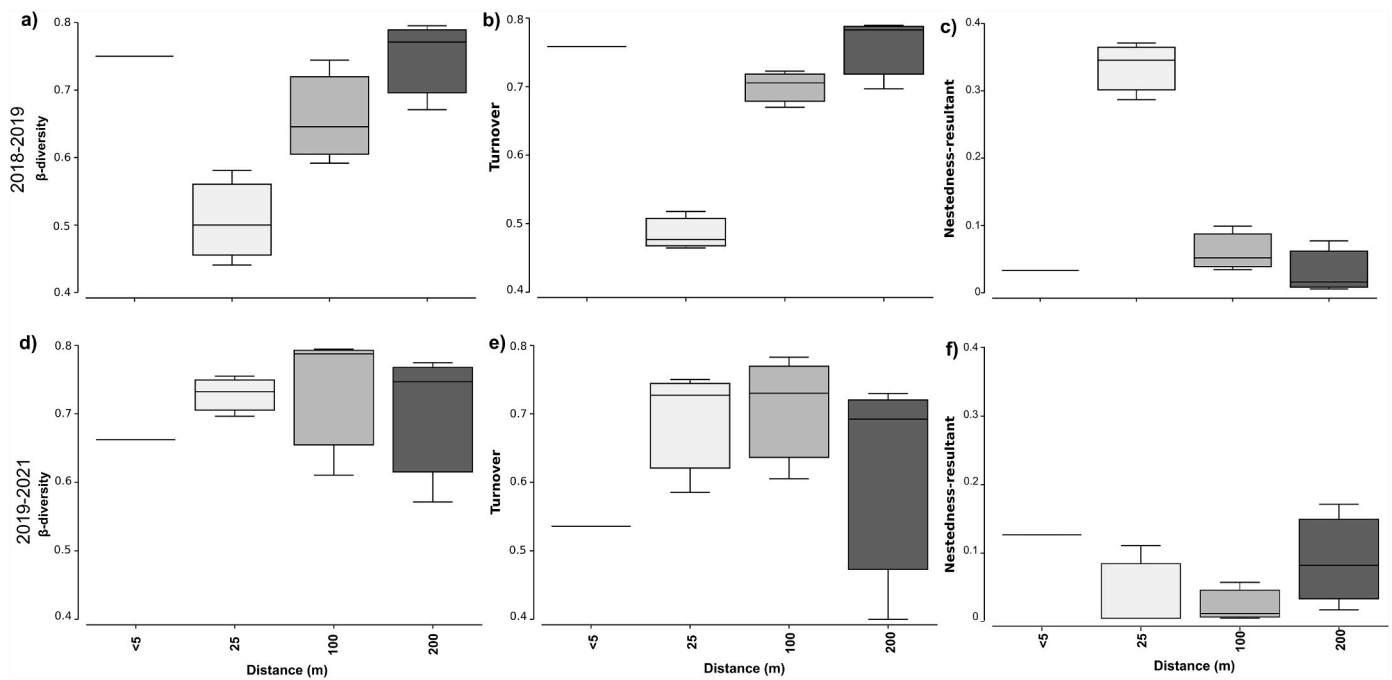|	2018		2019		2021	
	Sp	H'	Sp	H'	Sp	H'
0D	34	1.89	41	4.57	62	5.21
25D	58	3.78	30	4.14	31	4.43
25FD	56	4.37	36	4.68	35	4.76
−25D	52	2.72	29	4.07	44	4.83
1D	46	4.85	54	4.93	53	5.40
1FD	50	4.29	57	4.65	40	4.99
-1D	66	3.95	42	4.55	46	5.08
2D	45	4.15	60	5.09	40	4.56
2FD	47	4.82	53	4.87	34	4.40
-2D	50	4.50	52	4.21	47	5.02

Fig. 2. Boxplots showing the variability of temporal β-diversity (a, d) turnover (b, e), and nestedness-resultant (c, f) of macrofaunal community between 2018-2019 and 2019–2021. Stations were gathered based on the distance gradient from the pipe.

Table 3
Taxonomic β-diversity comparisons among sites along the increasing distance from the pipe (meters) for each sampling period. Values show β-diversity, its two components (turnover and nestedness-resultant), and relative contribution of turnover to β-diversity (%).

	2018			2019			2021		
	<5	**25**	**100**	**<5**	**25**	**100**	**<5**	**25**	**100**
Turnover									
25	0.34			0.51			0.59		
100	0.30	0.53		0.54	0.51		0.62	0.57	
200	0.52	0.59	0.43	0.61	0.60	0.52	0.77	0.71	0.47
Nestedness-resultant									
25	0.38			0.12			0.05		
100	0.42	0.01		0.23	0.16		0.09	0.05	
200	0.26	0.01	0.03	0.19	0.12	0.00	0.03	0.01	0.05
β-diversity									
25	0.72			0.63			0.64		
100	0.72	0.55		0.77	0.66		0.71	0.62	
200	0.78	0.60	0.46	0.80	0.72	0.52	0.80	0.71	0.52
Turnover/β-diversity (%)									
25	47.13			80.79			91.82		
100	41.51	97.33		69.96	76.28		87.71	91.89	
200	66.89	98.41	92.73	76.61	83.06	99.28	96.12	98.97	90.13

bivalve *Kurtiella bidentata* contributed to the major differences between 2018 *vs.* 2019 and 2021. Their densities decreased toward the end of the study period, and *C. capitata* disappeared in 2021. The difference between 2019 and 2021 was due to the higher density of the polychaete *Terebellides stroemii* in 2019. Considering the distance gradient, *C. capitata*, *H. gracilis* and the polychaetes *Lumbrineris latreilli* dominated the stations close to the pipe (<5 and 25 m), while *K. bidentata*, *T. stroemii* and the crustacean *Ampelisca intermedia* characterized the stations far from the main outfall. Species contributions to average dissimilarity among years and along the distance gradient highlighted by the SIMPER analysis are reported in Table S4.

3.2. Functional features and traits composition

The most striking result was the sharp increase in species diversity and functional richness (defined as the volume of functional space; volume 4D) over time near the main outfall (<5 m), indicating that the ameliorated WWT improved the environmental features. On the contrary, functional richness decreased, moving from 100 to 200 m, in 2019 and 2021, compared to the previous year (Fig. 4). In 2018, species collected at the station <5 m contributed only for 14.2% of the total pool of species (n = 240), whereas those collected at 100 and 200 m from the diffusion area represented the 39.6% and 44.2%. In 2021 the contribution of the 39 species collected at 200 m accounted for only 32.5% of the total pool. Consequently, we observed the highest FEs in 2019 at 100 and 200 m from the main outfall (50.0% and 46.7% of the total pool of FEs, respectively). In 2021 we noticed decreasing values of FEs, particularly at 200 m, where it accounted for 29.5% of the total pool of functional richness (Fig. 4). The latter result was remarkably noticed in the reduction of the volume of functional space (Vol. 4D: 53.5%).

The null model output (Fig. S6) revealed that the environmental condition forced the occurrence of certain trait values particularly at <5 m (in 2018) and 25 m from the duct (in 2019 and 2021), indicating that the observed values were significantly lower than expected by chance. The points were not placed within the confidence interval (95%) indicating that the area nearby the pipe before the WWT improvement did
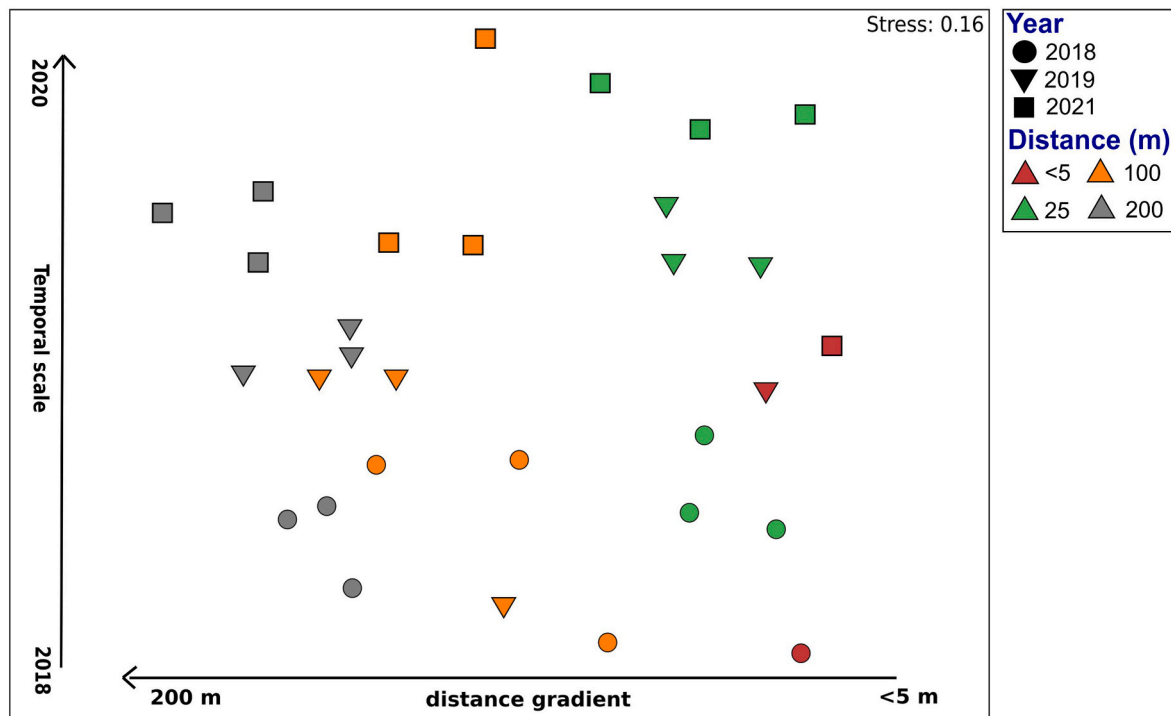
Fig. 3. NMDS ordination plot based on macrofaunal community for each sampling period (2018; 2019 and 2021) along distance gradient from the main outfall (<5, 25, 100, and 200 m). The black arrows indicate the position of the sites along the increasing distance gradient from the main outfall and the temporal scale. Sampling years and stations are specified with different shapes and colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
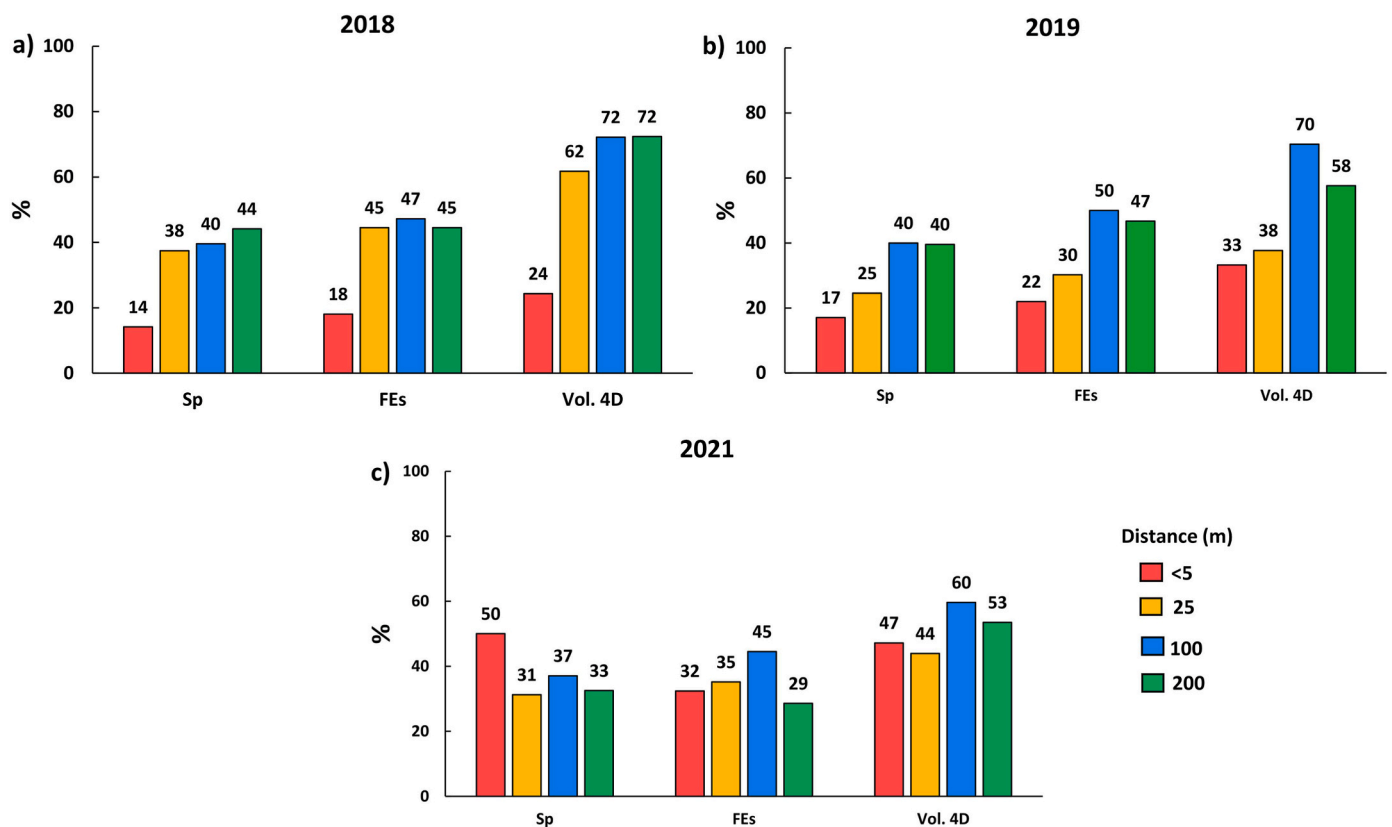

Fig. 4. Species and functional diversity changes among groups of stations along distance gradient from the main outfall (<5, 25, 100, and 200 m) for each sampling period. Bar plots show species richness (Sp), number of functional entities (unique trait combinations, FE), and functional richness (volume filled by each assemblage in the four dimensions of the functional space, Vol. 4D). Values are expressed as a relative percentage of the value for the total pool and are displayed above the bars.

not fall within the random scenario. In contrast, values of functional richness did not deviate from a random expectation in 100 and 200 m areas during the three-year study, except for stations at 200 m in 2019. From the sensitivity analyses, we found no significant changes to the reported results obtained with the finer categorization (Table S1 and Fig. S7).

The results obtained by functional β-diversity analyses over time were comparable to taxonomic β-diversity (Table 4). Overall, we observed a substantial decrease in functional β-diversity among sampling stations from 2018 to 2021. Higher levels of β-diversity were observed in 2018 and 2019; in 2018, β-diversity was driven by nestedness-resultants, whereas in 2019 it was driven by turnover. Similarly, in 2021, lower values of nestedness-resultants were observed among the sampling sites, so only the turnover component contributed to the values of β-diversity. The lower values of functional β-diversity observed between 100 and 200 m in 2019 and 2021 suggest that the portions of functional space filled by species communities are nearly overlapping, or that the functions performed in areas far from the pipeline are a subset of the functions performed in the closest area.

A total of 148 FEs with only one species were classified as vulnerable (Fig. 5). The change in environmental characteristics, especially at <5 m from 2018 to 2021, resulted in more new features being collected in the FEs classified as vulnerable. However, at this site the vulnerable FEs % values were lower than those obtained at the other sites. Similarly, higher values of vulnerable FEs were noticed along the distance gradient in the sampling years, while a reduction of vulnerability % was noticed at 200 m in 2021. These results and the balanced values of vulnerable FEs % among sampling sites toward 2021, confirmed what observed by functional β-diversity. The community analysed in 2021 was characterized by a subset of the functions performed in the closest sites, compared to previous years, this resulted in a reduction and balance among sites of vulnerable FEs %.

For the redundancy analysis, we included species abundance within the FEs to understand whether abundance is packed into specific functions and which functions are most vulnerable to abundance reduction. Regarding the FE abundances across the functional space in 2018 (Fig. S8), we observed that in most cases one FE dominated at <5 m from the pipe. Indeed, we noticed a high abundance value for only one FE, represented by the circle size, (circles represent FEs of the assemblages, and diameters are the relative abundances of species belonging to the different FEs). This result suggested that the ecological features of the area near the pipe favoured the presence of species with high number of individuals exhibiting certain functional traits rather than other functional attributes. Gradually moving toward the farther stations, and in particular after the WWT upgrade, we observed a balance between the numbers of FEs and macrofaunal densities. This was particularly

detected at 100 m in 2021, where different FEs had a similar circle size i. e. dimension (Fig. S8).

The functional attributes that characterized the dominant FE at <5 m in 2018 belonged to categories like *vermiform* (89.3%), *35–60 cm* (83.5%) and *≤ 1 year* (78.1%). The *subsurface deposit feeder* as 'feeding habits' trait, dominated the station at <5 m in 2018 (76.23%) as the *conveyor* modality (79.4%). On the contrary, after the WWT improvement, the prevailing feeding habits were *carnivorous* and *tentaculate suspension feeder* modalities (22.2% and 26.4%, respectively). As 'mobility' trait, the *tube-builder* gradually increased toward 2021. After the WWT improvement, both *conveyor* and *biodiffuser* decreased, and we observed a gradual increase in relative abundance (%) of *none* modality as 'ecosystem engineering' trait. Different functions characterized each FE both among stations and years (Fig. 6). A complete description of these results and the significant differences among years and along the distance gradient is detailed in Table S5 and Fig. S9.

4. Discussion

4.1. Influence of WWT amelioration on environmental features

The excess particulate matter that tends to accumulate in sediments also at deeper layers causes variations in structure and functions of the investigated community (Quijòn et al., 2008). We can infer, from our results, that the amelioration of the abatement system has resulted in a decrease of TOC in the water column toward the end of 2021. At the sediment level, the WWT upgrade led to higher redox potential values (Eh) near the main outfall whereas very low Eh values characterized sediments in 2018 and 2019. We deduce that the increased Eh values in 2021 were mainly due to the lower organic input from the water column that in turn enhanced the oxygen concentration in sediments. Sediment composition is a paramount factor in structuring the macrofaunal community and selecting dominant species (Hermand et al., 2008) but we observed the same pattern of grain size distribution in all sampling years. The taxonomic composition was in line with a typical community of sandy-mud bottoms and detrital sediments (Cozzi et al., 2008; Nasi et al., 2017), indicating that in our study case the organic matter variation and not the grain-size was the predominant element affecting the structure and functions of the macrofaunal community.

4.2. The successional stage of macrofaunal community after the WWT improvement

Following the improved effluent treatments, the reduced deposition enhanced the overall environmental condition leading to higher species richness (Bustamante et al., 2012) nearby the main outfall. The

Table 4

Functional β-diversity comparisons among groups of stations along the sewage distance gradient (meters) for each sampling period. Values show β-diversity, its two components (turnover and nestedness-resultant), and relative contribution of turnover to β-diversity (%).

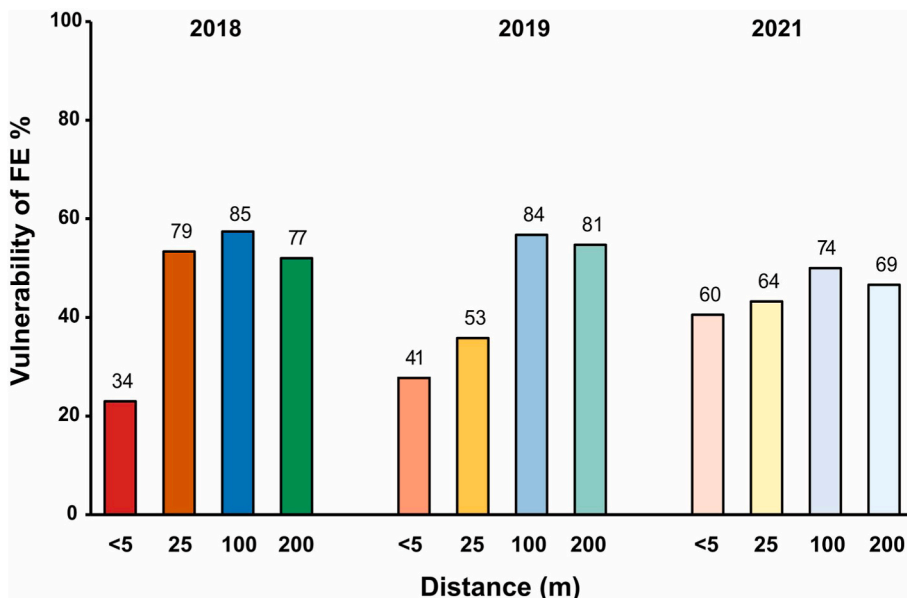|	2018			2019			2021		
Turnover	**<5**	**25**	**100**	**<5**	**25**	**100**	**<5**	**25**	**100**
25	0.12			0.37			0.26		
100	0.01	0.15		0.11	0.04		0.20	0.12	
200	0.09	0.17	0.18	0.23	0.10	0.07	0.39	0.28	0.07
Nestedness-resultant									
25	0.52			0.04			0.05		
100	0.65	0.11		0.47	0.49		0.19	0.16	
200	0.60	0.11	0.00	0.31	0.34	0.18	0.08	0.15	0.34
β-diversity									
25	0.64			0.41			0.31		
100	0.67	0.26		0.59	0.53		0.39	0.28	
200	0.68	0.28	0.18	0.54	0.44	0.26	0.47	0.43	0.42
Turnover/β-diversity (%)									
25	18.16			91.27			83.10		
100	1.68	56.54		19.38	8.12		51.12	43.58	
200	12.93	61.26	99.62	42.19	22.34	28.31	82.08	65.43	17.86

Fig. 5. Vulnerability of FEs (%, i.e. the proportion of FEs with only one species) for each sampling period (2018; 2019 and 2021) along distance gradient from the main outfall (<5, 25, 100, and 200 m). Numbers above bars represent the FEs per sites and years of the total number of vulnerable FEs (n = 148).
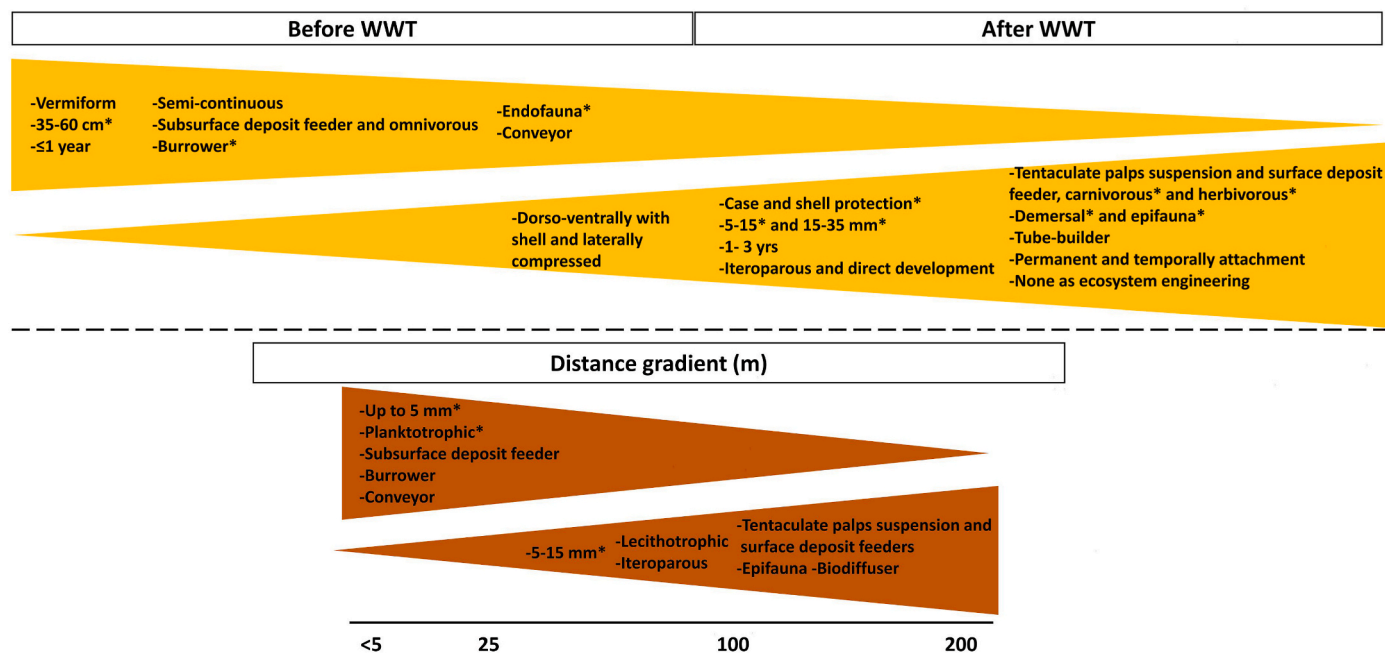

Fig. 6. Functional modalities mainly responsible for the differential characterization of FEs in time and along spatial scales. See Fig. S9 for variation in relative abundance (%) of functional trait categories for each sampling period and along a distance gradient from the main outfall. Categories with asterisks explain significant differences among sampling years and sites (Table S5).

improvement of the ecological status of assemblages was reflected in a decrease in the abundance of opportunistic/tolerant species, which increased α-diversity values. For instance, these higher values were likely due to the reduction or absence of r-strategist species (i.e. the polychaete *Capitella capitata*) that previously occurred at high densities (peak of opportunistic species) near the duct (primarily at <5 and 25 m) and completely disappeared after the WWT improvement. The higher species richness near the pipe after the improved WWT could also be related to the presence of an 'ecotone point' (Fig. 7). The presence of a punctual source of organic contamination implies the existence of an ecotonal zone (i.e. mixed zone between/among two or more biological communities), that in 2018 was observed between 100 and 200 m,

shifted closer to the main outfall after the WWT improvement. This zone lies in-between the natural environmental condition, with low organic matter, and the harsh environmental condition, with high organic matter. In the ecotonal zone, where the disappearance of sensitive species has not yet occurred, some opportunistic species have begun to appear (in the ecotone, we did not observe the peak of opportunistic species). Our findings are comparable to previous studies carried out in the same area (Solís-Weiss et al., 2007; Vesal et al., 2021) and others related to organic matter deposition worldwide (e.g. Calabretta and Oviatt, 2008; Hermand et al., 2008; Villnäs et al., 2011). Our results are comparable with the 'intermediate disturbance hypothesis' (Connell and Slatyer, 1977). The latter predicts that maximum biodiversity should be
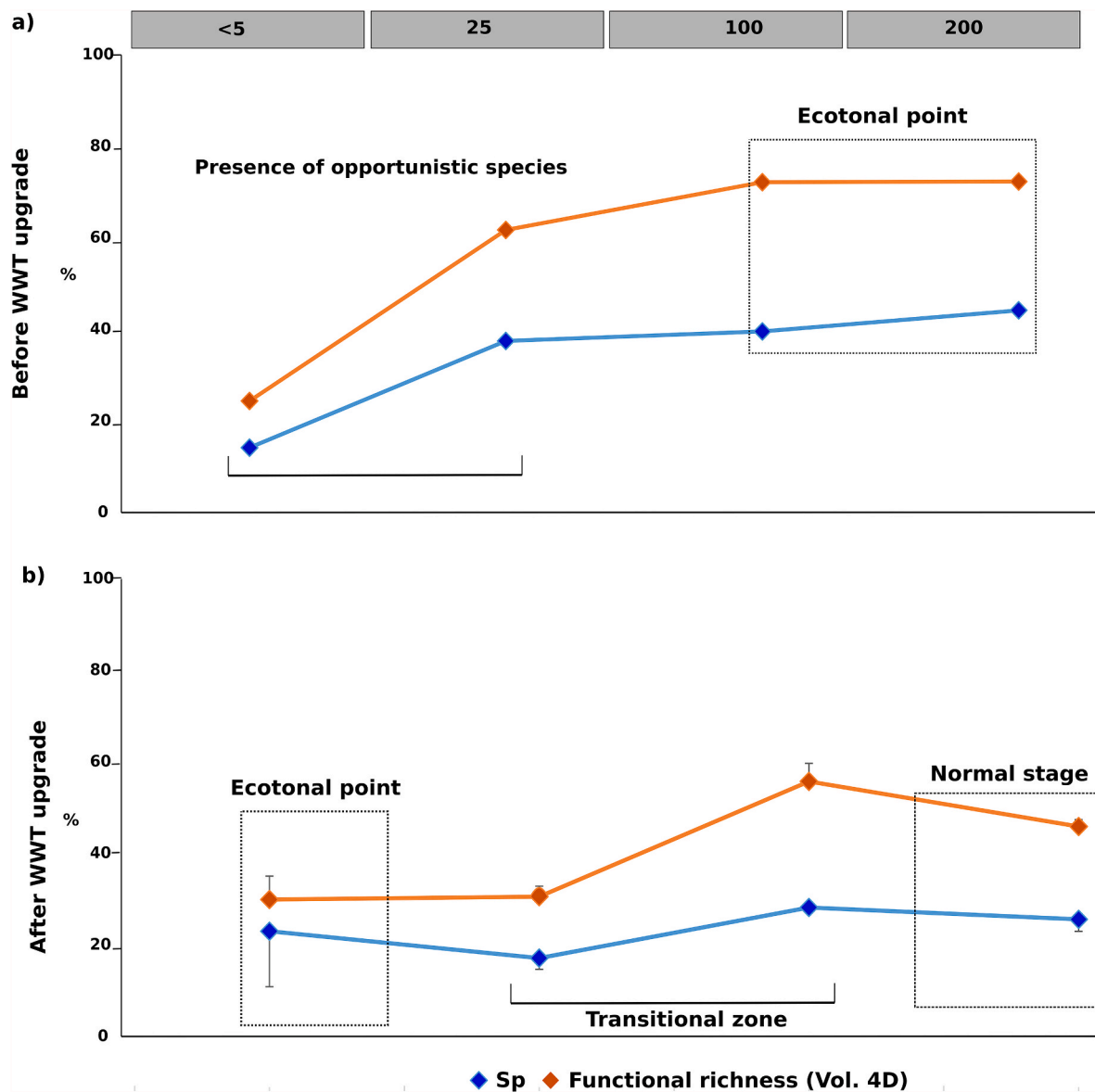
Fig. 7. Species and functional richness (expressed in the four dimensions of functional space, Vol. 4D) before (a) and after WWT improvement (b). Stations were gathered based on the distance gradient from the pipe. The plot evidences the different phases of macrofaunal successional stages in relation to sewage effluent amelioration. Values are expressed as a relative percentage of the value for the total pool. Graph (b) shows the average of the data for 2019 and 2021 with DS.

observed at intermediate disturbance frequencies because few species can tolerate very intense disturbance regimes, and few are able to compete successfully in habitats that receive little or no disturbance (Lorrillière et al., 2012). In 2019 and 2021, we observed both sensitive and tolerant species near the main outfall, which increased overall species richness. In 2021, the successional stage of the community, was first characterized by the 'ecotone zone', near the main outfall, followed by a 'transitional zone' with higher values of species richness (at 100 m), and by the 'normal stage', with slightly lower species richness observed at 200 m far from the duct (Fig. 7). Hence, after the WWT improvement, the recovery of the community and its progressive stabilization followed the successional stages within Pearson and Rosenberg's (1978) model of the organic pollution influence on benthic ecosystems (Del-Pilar-Ruso et al., 2010; Sanz-Lázaro and Marín, 2011; Zubikarai et al., 2014).

The successional stages of the macrofaunal community following the WWT improvement were further confirmed by the great variation in species composition throughout the sampling area, highlighted by the highest turnover (i.e. β-diversity values) of species, which gradually increased toward the end of the study period (see Table 3). These results

indicate that the community was not characterized by species that were subsets of other sites, but by the establishment of new species, especially at <5 m from the main pipeline. In the area most affected by sewage, the WWT improvement induced the turnover of species, as evidenced by the replacement of some species by others, resulting in a major change in the community (Baselga, 2012; Loiseau et al., 2017).

4.3. Functional diversity, vulnerability and resilience before and after improved wastewater treatment

Our results on functional diversity in 2018 are in accordance with those of Rao et al. (2021); Llanos et al. (2020); Krumhansl et al. (2016), who evidenced lower functional diversity at sites deeply influenced by high deposition of sewage material, and increasing moving far from the main source of pollution. However, in our study case, the most remarkable result was the sharp increase in time in FEs, and functional richness (expressed as volume 4D) in the zone near the outfall (<5 m). Functional richness instead decreased, moving far from the diffusion zone (25, 100, and 200 m) during 2019 and 2021. The variation of FEs

and functional richness followed the successional stage of macrofaunal community (see Fig. 7). The improved environmental conditions enhanced the variety of functions performed by the community, as reflected in the increasing values of FEs and functional richness, which has important implications for ecosystem processes. On the other hand, the gradual decline of FEs in 2019 and 2021 toward the more distant stations might be related to the successional stage of macrofaunal community. However, these results did not appear to profoundly alter the function that the community served. While we observed high species diversity during the study period and throughout the area, turnover of functions (functional β-diversity, see Table 4) was less pronounced in 2021. These results emphasise that the changed environmental conditions after WWT improvement led to the establishment of new species, but with the same functions. This is reflected in the lower percentage of functional vulnerability in 2021 across the study area (see Fig. 5). We infer that the establishment of new species was due to congeneric species (i.e., species belonging to the same genus, e.g. the crustaceans *Ampelisca intermedia* and *A. tenuicornis* and the polychaetes *Lumbrineris latreilli* and *L. lusitanica*) who contributed to the reduction in functional vulnerability. In principle, congeneric species are recent descendants of a common ancestor, so their current niche is the product of niche divergence and should have more overlap compared to more distantly related species (Sfenthourakis et al., 2006). We also observed the presence of co-occurring species (e.g. the polychaetes *Terebellides stroemii* and *Polycirrus aurantiacus*) that performed the same functions and thus belonged to the same FEs, that could have induced the reduction of function vulnerability and increased resilience among sampling areas. In fact, the higher the increase in diversity of species within the same functional group (i.e. FEs) with the ability to be resistant, the more likely the functional group will persist in the face of further disturbance (Gladstone-Gallagher et al., 2019). For example, in our study case, one of the functional attributes that belong to these groups (i.e. congeneric and co-occurrence species) and could make them resilient over time is 'iteroparity' as a reproductive frequency. Long-lived iteroparous organisms (K-strategists) are better protected against environmental stressors, and their competitive relationships are also thought to be more predictable and constant (Ranta et al., 2002).

The amount of sewage material settled to the sea bottom before the WWT improvement influenced the functional redundancy of the investigated macrofaunal community. From the comparison of the species richness, FEs diversity and FEs vulnerability, we could infer that in 2018 this community was not characterized by functional redundancy. In fact, we found lower numbers of species and functions near the main outfall and higher levels of vulnerable FEs (at 100 and 200 m from the pipe, see Fig. 5), suggesting that the community near the pipeline was likely more susceptible to loss of species and functions than those farther away (Gamfeldt et al., 2008). We found an additional mechanism for the loss of functional redundancy due to this specific human impact, emerging from abundance concentrated in a few FEs where effluent deposition increases (as at <5 and 25 m, see Fig. S8). Therefore, to this impact was attributable not only the loss of functional-trait combinations but also a redistribution of abundance; indeed, high abundance levels persisted only in a small subset of functional-trait combinations, and this potentially increased the vulnerability of low-abundance FEs. In a community, the degree of rarity of a functional group, characterized primarily by rare species, could be an indicator of the loss of the functional group itself under stress, as the rare species may be lost first (Hewitt et al., 2016). The increase in vulnerability reduces the long term-resilience of ecological communities to cope with other environmental stressors (e.g. heatwave, chemical contamination; Naeem et al., 2012), which can occur even at a small spatial scale. A similar pattern of low redundancy and higher vulnerability based on the same FEs approach has been previously reported for macrofaunal communities inhabiting naturally acidified environments (coastal CO_2 vents; Teixidó et al., 2018), where remarkably low redundancy was observed in areas characterized by extreme low pH. However, comparing our results with other studies

investigating the functional response of macrofaunal communities to allochthonous material (e.g. Gusmao et al., 2016 - wastewater discharges and Zhong et al., 2020 - river inputs), we can note that the general response of the macrofaunal community to this type of stress is characterized by few species with a high number of individuals, so that very few functions occur, making the community more vulnerable. After WWT improvement, we observed balanced values of vulnerable FEs % among sampling areas, leading to a gradual increase in functional redundancy and lower functional vulnerability. Changes in the disturbance regime (environmental stressors), in our case reductions in organic material, reduced rates of ecosystem degradation and increased ecosystem functions (Gladstone-Gallagher et al., 2019) over a large area rather than at small and fragmented patch scales. Before the WWT improvement, we observed a dominance of a few functions in front of the main outfall (patch scale), whereas toward 2021 we noticed an increasing variety of FEs (higher functional richness expressed as Vol 4D) across the sampling area.

4.4. Functional attributes variation after the WWT upgrade

Our results revealed which FEs were present before and after the WWT improvement. In 2018, in front of the main outfall, the dominant FEs included 'burrower' and 'conveyors' species; the invertebrates with these modalities are usually classified as surface deposit feeders. These animals ingest sediments containing organic matter, even of allochthonous origin, in large quantities and can therefore live in areas subject to continuous and heavy deposition of effluents (Gaston et al., 1998; Quijòn et al., 2008). However, even though bioturbation activities carried out by benthic species (Kristensen et al., 2012) promote remineralization of organic matter by transferring particles from deeper to surface layers and vice versa (Belley and Snelgrove, 2016), bioturbation was reduced after WWT improvement but did not disappear completely. In 2021, the dominance of 'tube builder', considered weak bioturbators, could have reduced the sediment organic matter mixing, mainly nearby the main outfall. On the other hand, in 2021, the increase of FEs due to 'tentaculate suspension feeders', in particular at 200 m, could have enhanced the benthic-pelagic coupling by capturing large quantities of suspended organic matter and phytoplankton and then incorporating them into the sediments through pseudo-faeces (Törnroos and Bonsdorff, 2012). These variations in modalities were previously observed in other studies investigating functional changes in relation to organic loads (Bolam et al., 2016; Nasi et al., 2020). The continuous and high loads of allochthonous material usually affect epifauna and tube-builder species (mainly suspension-feeding animals) by clogging their feeding structures, interfering with particle selection, and requiring the use of energy to clear away unvented particles. In our study case, the polychaete *T. stroemii* is an example of tube-builder species that we observed in increasing densities in 2019 and 2021. This species' settlement further confirmed that the community responded to the decrease in sewage-derived organic loads following the WWT upgrade.

The well-balanced proportion of macrofaunal feeding habits (i.e. suspension and deposit feeders, omnivores, and predators) indicated increasing community stability towards 2021. In 2018, a trophic pathway prevailed as deposit feeders dominated near the main outfall. Further, the greater abundance of carnivores and omnivores toward the end of the study confirmed that macrofauna community composition and functions changed following WWT improvement (see Fig. 6 and S9). Our results are consistent with those of Zubikarai et al. (2014), who observed an increase in omnivores and predators and a more even distribution of trophic groups following wastewater reduction, indicating healthier ecosystem functioning and thus improved environmental quality (Bremner et al., 2006).

Despite a large body of literature focusing on the structural aspects of WWT-induced changes, our study is the first attempt to examine the influence and attenuation of wastewater discharges on the structural and functional characteristics of the macrozoobenthic community by

applying Functional Entities and β-diversity (taxonomic and functional). This combined approach let us highlight functional variations of the soft-bottom macrofaunal community following the improvement of a sewage treatment plant, but it could be applied to investigate the effects of a plethora of environmental changes of natural or anthropogenic origin that are likely to result in modifications of the ecological state of an area. Our results highlight how important functional diversity and ecological redundancy are, alongside with species diversity, to assess and predict the effects of this kind of human impact and the induced environmental changes after the amelioration of sewage abatement system. Indeed, we observed high species numbers after the WWT improvement whereas the turnover of functions was less pronounced. Thus, this approach can give an accurate insight into the tight association between traits and their sensitivity to stressors and indicates that functional variations may be more revealing than taxonomic aspects alone.

Credit author statement

FN: manuscript preparation, conceptualization, data interpretation, statistical analysis, macrofaunal community analyses, field sampling. SEV: macrofaunal community analyses, field sampling, manuscript preparation. FR: total organic carbon water samples detection and chemical analyses of sediments, data interpretation, manuscript preparation. MB: grain-size analyses and interpretation, manuscript preparation. NT: data interpretation, manuscript critical revision. RA: sampling design, field sampling, manuscript revision. TC: manuscript critical revision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121022.

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