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Assessing the sewage discharge effects on soft-bottom macrofauna through traits-based approach

Seyed Ehsan Vesal[a,b], Federica Nasi[a,*], Jessica Pazzaglia[b,c], Larissa Ferrante[a], Rocco Auriemma[a], Federica Relitti[a], Matteo Bazzaro[a,d], Paola Del Negro[a]

[a] *National Institute of Oceanography and Applied Geophysics - OGS, via A. Piccard 54, I-34151 Trieste, Italy*
[b] *Department of Life Sciences, University of Trieste, 34127 Trieste, Italy*
[c] *Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, 80121 Napoli, Italy*
[d] *Dipartimento di Scienze Fisiche, della Terra e dell'Ambiente, Università degli Studi di Siena, Strada Laterina, 53100 Siena, Italy*

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ABSTRACT

We assessed the effect of sewage-derived materials on the structural and functional attributes of the soft-bottom macrofauna at an increasing distance from the entire diffusion area. Our results showed clear spatial changes of macrofaunal density and biomass along the distance gradient from the main outfall. High values of biodiversity, species composition, and species linked to organic enrichment near the duct suggested that moderate organic stress affected this community. The traits analysis abundance-based, compared to biomass-based one, distinguished most clearly sewage contamination conditions. Functional diversity displayed spatial patterns with higher values in the less impacted sites and was significantly related to species numbers and the biotic indices (like M-AMBI). This approach is ideal for detecting macrofaunal functional changes due to sewage contamination. Thus, we infer that traits analyses could offer great potential for environmental assessment and monitoring of coastal areas influenced by human activities.

1. Introduction

A large part of the world's population lives beside the sea. Moreover, the sea relies on the essential goods (e.g., fishing and aquaculture) and services (e.g., wastes assimilation) that the marine ecosystems provide (Costanza et al., 1997; Villnäs et al., 2018). However, the recent expansion of human activities in the marine domain has resulted in habitat alterations and biodiversity degradation (Lotze et al., 2018). Indeed, there has been a large increase of studies focused on marine communities inhabiting coastal areas during the last decades. Therefore, studying the effects of anthropogenic pressure on marine ecosystems is essential to conduct appropriate economic and environmental management with adequate monitoring programs (Katsanevakis et al., 2011).

Due to their ability to adapt their composition and structure in response to a different source of disturbance, soft macrofaunal communities are fundamental in assessing impacts from human activities (Pearson and Rosenberg, 1978; Oug et al., 2012). In this direction, most studies focused on the structural aspect of macrofaunal species 'assemblages, such as abundance, biomass, and diversity. A wide variety of valid biotic indices relating to species composition and biodiversity of macrobenthic communities, such as AMBI (AZTI's Marine Biotic Index; Borja et al., 2000), M-AMBI (multivariate AMBI; Bald et al., 2005), and BENTIX (Simboura and Zenetos, 2002), have been proposed as an attempt to address the objectives of many European Directives (e.g., Marine Strategy Framework Directive-MSFD, EU Biodiversity Strategy and Water Framework Directive-WFD; D'Alessandro et al., 2020). These indices focus on structural aspects of macrofaunal communities and rely on the taxonomic identification of species. They rarely assessed the functional variation of macrofaunal invertebrates to stressed environments. Species interact with and respond to the physical and chemical habitats with different patterns depending on their abilities (Díaz and Cabido, 2001). Every species can play a significant role in the various functions of ecosystems. Because of either natural causes or human activities, any changes in their composition can adversely affect ecosystem processes (Gray et al., 2006). Thus, the sole application of species composition may not be enough to explore the processes that sustain ecological systems (Díaz and Cabido, 2001). Following this issue, the

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* Corresponding author.
E-mail address: fnasi@inogs.it (F. Nasi).

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Biological Traits Analysis approach (BTA; Bremner et al., 2003) was developed, combining structural data of a macrofaunal community (species abundance or biomass) with the information on functional traits of every single species (e.g., Törnroos et al., 2015). Traits have been defined as a component of an organism's phenotype (e.g., body size, feeding structure, and life span) and represent the proxy of an individual's performance (Violle et al., 2007). Functional features of traits determine different responses to environmental factors and effects on ecosystem processes (Reiss et al., 2009). Therefore, BTA is nowadays considered a useful analytical method to understand better the relationships between organisms and ecosystem functioning (Solan et al., 2004; Bremner et al., 2006; Gagic et al., 2015). Besides, using this approach is possible to implement two functional aspects. Functional diversity represents the variety of functions settled by organisms in a community, comprising a component of biodiversity (Nasi et al., 2018). The other aspect is the functional identity, which indicates the role of a single species in the ecosystem. If a single trait is strongly linked to an ecosystem function, high occurrences of these traits may best predict the functioning (Gagic et al., 2015). Many marine studies applied BTA to benthic assemblages concerning several environmental variables (e.g., Paganelli et al., 2012; Weigel et al., 2016; Nasi et al., 2020) and human-induced impacts (e.g., Gusmao et al., 2016; Krumhansl et al., 2016; D'Alessandro et al., 2020). The growing of human pressure on marine environments is enhancing the need for ecosystem understanding and reliable indicators of environmental health (Bremner, 2008). Since traits represent the link between life history and habitat, the multiple traits approach could support the marine management and policies to curbing human impacts on coastal environments (Beauchard et al., 2017). However, the BTA in marine management is still challenging because the development of marine indicators based on functional approaches needs more studies and should be based on complete sets of magnitude traits (Beauchard et al., 2017). Further, few studies have focused on the relationship between functional diversity and principal biotic indices (e.g., AMBI and BENTIX), to evaluate functional attributes of macrofaunal communities as an indicator in coastal marine management (Gusmao et al., 2016; Krumhansl et al., 2016; D'Alessandro et al., 2020). Bremner et al. (2003) estimated the quantification of trait categories using abundance and biomass. In addition, Gusmao et al. (2016) compared the functional information obtained by the analyses on abundance and biomass data separately. However, compared to the abundance-based study, the use of biomass matrix in the BTA approach to underline the effect of human pressure on coastal environments remains less investigated.

In this study, we aim to understand which structural data (i.e., abundance or biomass) can be combined with traits information to describe best the interactions of organisms with one other and their physical and chemical environments. BTA enables us to link abundance or biomass data of species assemblages with information on the functional features of each species, which can then be used for analyses of their relationships with environmental variables, providing information on the connections among species, environments, and ecosystem processes (Bremner et al., 2003). In addition, we tested the applicability of BTA analyses in the framework of the monitoring program. Estimating how functional traits are related to specific environmental conditions can offer important insights into the mechanisms that determine species distributions and is an essential step in assessing ecological status (Peng et al., 2013).

In this study, we focus on the macrofaunal community inhabiting a coastal area influenced by sewage discharges. Several studies described the structural responses of macrofaunal invertebrates subjected to high organic loads and contaminants from wastewaters (e.g., Pearson and Rosenberg, 1978; Solís-Weiss et al., 2007; Souza et al., 2013), and others assessed the validity of different biotic indices, for the correct monitoring of this type of impact (e.g., Pinto et al., 2009; Ferrera et al., 2011). However, to the best of our knowledge, the influence of sewage discharges on the functional diversity of macrofaunal communities and trait composition in the sublittoral area of the Mediterranean Sea has not been investigated yet.

In this study, BTA was used: i) to explore and characterize the functional attributes of macrofaunal invertebrates nearby an area subject to wastewater loads; ii) to assess which matrices of structural data (i.e., species abundance and biomass) best explain the difference in functional diversity and identity of the community investigated iii) to assess the potential application of functional analyses to developing management practice for wastewater treatment systems. In particular, we aimed to answer the following questions: 1) Are the structural and functional patterns driven by specific sediment physical-chemical variables and contaminants? 2) Are there any differences in functional diversity and identity considering the structural abundance and biomass information? 3) Is it possible to include the BTA of macrofaunal communities in coastal monitoring programs?

2. Material and methods

2.1. Study site

The Gulf of Trieste is located at the northernmost part of the Adriatic Sea. It is a shallow embayment of about 600 km^2 and a coastline of 100 km (Brambati and Catani, 1988). The Gulf is connected to the rest of the Adriatic Sea by a sill (~22 m depth) located in the southern part of the basin (Ogorelec et al., 1991); 10% of its area is <10 m, and the maximum depth is about 25 m. The Gulf endures annual oscillations of temperature altering from 5 °C to >24 °C at the surface and from 6 °C to >20 °C at the bottom. Marine sedimentation is strongly influenced by local river plumes, particularly supplied by the Isonzo River (Covelli and Fontolan, 1997). The annual average sedimentation rate is about 1 mm y^{-1} in the middle of the Gulf and 2.5 mm y^{-1} in front of River Isonzo (Covelli et al., 1999). The Gulf is also affected by the Eastern Adriatic Current (EAC), flowing northwards along the Istrian coast and advecting warmer and saltier waters coming from the Ionian Sea (Poulain et al., 2001), which leads to general cyclonic circulation. However, the Gulf general pattern of currents may be quickly modified in response to intense local atmospheric forcing (winds) and river plume (Querin et al., 2007; Malačič and Petelin, 2009). In particular, the basin is highly influenced by the Bora, a north-easterly wind characterized by a strong intensity that can mix the entire water column also favoured by the shallow depth of the basin (Querin et al., 2007).

The city of Trieste discharges its main urban sewage in the centre of the Gulf by the Servola disposal plant, which serves up to 270,000 inhabitants, with a maximum flow of 6000 L sec^{-1} (Solís-Weiss et al., 2007). It is a mixed-type plant, collecting and treating both meteoric and wastewaters of about 50 million m^3 per year (Novelli, 1996). Since 1992, the disposal plant depurates wastewaters by chemical-physical treatment and finally releases them into the sea through two adjacent pipelines (6.5 and 7.5 km). 600 turrets characterize the last part of the pipes for a total diffusional zone length of 1.5 km (1 km the longest and 0.5 km the shortest duct, respectively). These conducts are located in the north-eastern part of the Gulf of Trieste at a depth between 20 and 23 m (45°38′36.30″N; 13°40′51.70″E).

2.2. Sampling design

Sediments were sampled during November 2016 and April 2018. To assess the influence of municipal wastewater discharge on macrofaunal community nearby the sewage outfalls and to best cover the entire diffusion zone, sediments were collected at 18 stations, gathered in three transects ("distal", "medial" and "proximal"). In each transect, the stations were placed at increasing distances from the duct (at 5, 100, and 200 m) (Fig. 1). Besides, a reference station (RS) was positioned 2 km far from the distal end of the pipeline in the opposite direction to the average annual current (SSE 170°).

Sediment samples for analyses of physical and chemical parameters

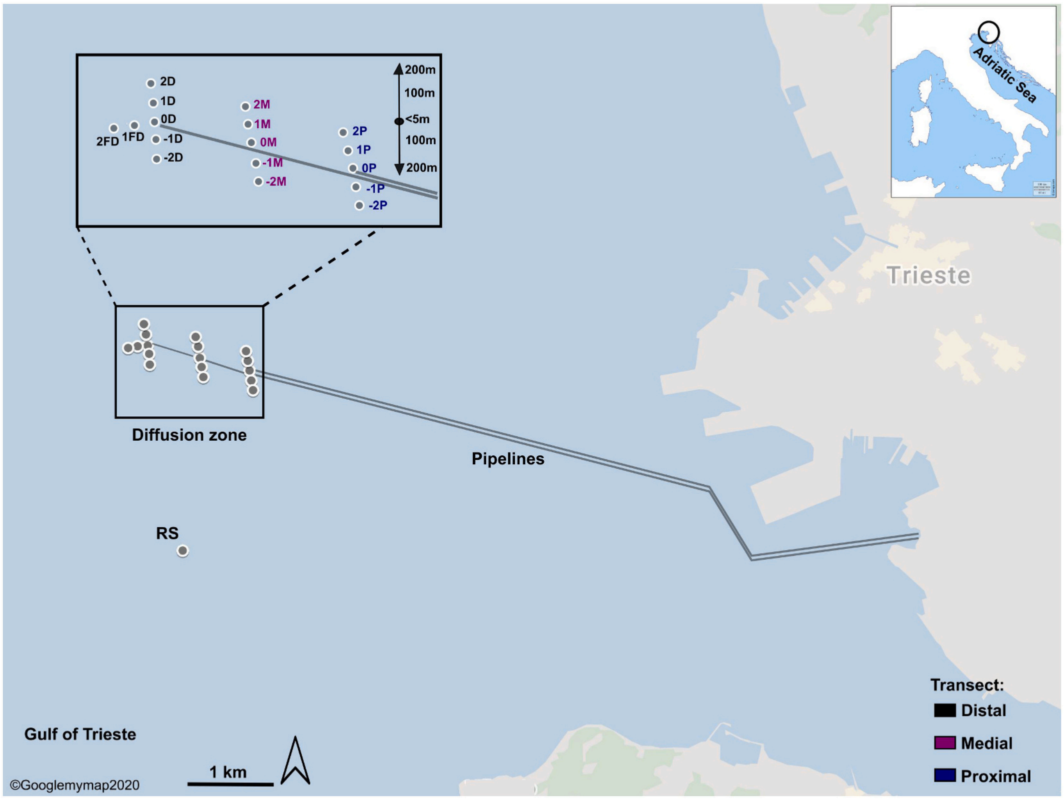

Fig. 1. Location of sampling sites in the Gulf of Trieste.

(grain-size, total Organic Carbon-Corg, Total Nitrogen-TN contents, and redox potential-Eh) were collected by a Box Corer (Haps Frame-Supported Bottom Corer, KC-Denmark) using PVC liners with a diameter of 13.5 cm and a height of 30 cm. For the macrofaunal community, sediments were retrieved with a van Veen grab (0.1 m^2) in three replicates of each station.

2.3. Redox potential, sediment grain-size, organic matter content and contaminants

Measures of redox potential (Eh) were carried out to assess the physical-chemical state of marine sediments, providing indications of oxidation-reduction processes that determine oxic sediment conditions (Colman and Holland, 2000). The Eh was estimated from undisturbed superficial sediment (0–1 cm), obtained immediately after collecting core liners. The analysis was performed with Metrohm 704 voltimet, after the platinum electrode standardization (CRISON 5265) in Light's solution (Clesceri et al., 1996).

For grain-size analysis, sediments were sieved at 2 mm and pre-treated with 10% hydrogen peroxide before being analysed with a BECKMAN COULTER LS 13 320 Laser Diffraction Particle Size Analyzer. Data are expressed as sand, silt, and clay percentages following the Udden-Wentworth grain-size classification (Wentworth, 1922). For chemical analyses, sediments were freeze-dried, homogenized, and ground to a fine powder. The contents of total organic carbon (Corg) and total nitrogen (TN) were measured using the elemental analyser CHNO-S Costech model ECS 4010, as described in Franzo et al. (2019). Corg and TN values were expressed as mg g^{-1}. The organic carbon to nitrogen molar ratio (C:N) was calculated and used as a proxy to infer the organic matter origin (Rumolo et al., 2011).

The mercury concentration was carried out by atomic absorption spectrophotometry by cold vapour (Analyst 100, PerkinElmer, USA). In addition, the concentrations of 16 USEPA priority pollutant Polycyclic Aromatic Hydrocarbons (PAHs) were also measured according to Cassin et al. (2018) were detected. For this study, we used the sum of the analysed PAHs.

2.4. Macrofaunal samples processing and biotic indices

The sediments for the macrofauna 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. For the taxonomic identification, the keys listed in Morri et al. (2004) were used. The abundance number is expressed as individual per m^2.

Macrofaunal biomass was performed for each sampling station and period. The average biomass of species was achieved by weighting all individuals belonging to the same taxon and sampling station to reduce the bias due to different developmental stages or size classes. Wet weight measurements were obtained after 30 s on absorbent paper. Where biomass was not available for measurements, we applied the conversion factor proposed by Ricciardi and Bourget (1998) and Brey (2001).

Biogenic Remain (BR) represents the fraction retained together with macrofaunal organisms after sieving. It comprises fragments of death molluscs' shells, echinoderm skeletal parts and polychaetes carbonate pipes. The volume of BR was measured after removing invertebrates alive at the time of sampling and is expressed as mL.

BENTIX index was estimated according to Simboura and Zenetos (2002). Besides, abundance (M-AMBI) and biomass (M-bAMBI) based indices were calculated using AMBI 5.0 software (Borja et al., 2000). For both indices, invertebrates are assigned a score for I to V, based on their tolerance, following AMBI library (Borja and Muxika, 2005). Both M-AMBI indicies are based on the following metrics: AMBI, Shannon diversity, and taxa richness (Mistri et al., 2018).

2.5. Biological traits analysis

The effect of sewage discharge on the benthic community was assessed using Biological Traits Analysis (BTA). BTA was applied on 276 species, considering 11 biological traits with 48 categories. The selected set of traits described important morphological, behavioural, and life history characteristics of marine benthic invertebrates potentially related to the deposal of sewage-derived materials. Traits were gathered in *response* (that concern the response to changes in the physical environment) and *effect* (that concern ecosystem properties; Table 1).

The taxa were coded based on their affinity for the chosen traits using the 'fuzzy coding' procedure. Through 'fuzzy coding', taxa can exhibit trait categories to different degrees taking into account the interspecific variations in trait expressions (Bremner et al., 2006). Traits for each species were derived from literature sources (Trainito and Doneddu, 2005; Hayward and Ryland, 2017) and databases (i.e. www.marlin.ac.uk/biotic; www.polytraits.lifewatchgreece.eu). In particular, for information on life histories, we followed the authors Giangrande (1997) and Rouse (2000). Polychaetes 'feeding habit' modes were obtained from Jumars et al. (2015). We detected information from the literature for 'adult activity' traits (Queirós et al., 2016; Kristensen et al., 2012). Lastly, information on 'tolerance' traits were attributed following the five ecological groups of the AMBI index's (Borja and Muxika, 2005). The taxonomic resolution was kept at species level whenever possible but adjusted to genus or family when the information on traits was available only at a higher taxonomic level.

Functional Dispersion (FDis) was calculated based on the fuzzy coding traits matrix and species abundance. FDis describes the abundance-weighted mean distance of individual species to their group centroid (all species community) in a multivariate functional space constructed by a principal coordinate analysis (PCoA) based on the Euclidian dissimilarity matrix of species traits (Scheiner et al., 2017; Villéger et al., 2008). FDis was calculated from the abundance matrix (FDis-abu) and biomass (FDis-biom). The functional identity was calculated as community level weighted mean (CWM) of trait category expression. CWM values widely reflect the traits strategies given by the species pool and environmental conditions of a site (Muscarella and Uriarte, 2016). CWM value represents the occurrence of a trait by species in each community. The values were weighted by species abundance (CWM-abu) and biomass (CWM-biom).

2.6. Data analysis

Firstly, all data were tested for normality and collinearity following Shapiro Wilk's and Spearman's rank correlation coefficient, respectively. Then, environmental parameters, biotic indices, and macrofaunal functional features (taxonomic diversity, functional diversity indices and CWM values based on abundance and biomass data) were compared among sampling stations and periods. Regarding smapling periods, as factor, for most part of species, stations were collected outside the breeding season. So we can infer that the differences observed were principally due to random variations of sewage discharges instead of seasonal differences. In this study, the sampling design and analyses aimed to evidence differences in spatial scales along a distance gradient from the main outfall using univariate (Mann-Whitney *U* test and Kruskal-Wallis H) and multivariate analyses (one-way PERMANOVA with an unrestricted permutation of raw data and 9999 permutations). For each test, the following fixed factors were applied: i) 'transect'; ii) 'distance from the duct' iii) 'sampling period'. Abiotic parameters and macrofaunal community values (i.e., structural and functional) of the reference site were not included in the previous analyses.

The relationships between each functional and diversity index were tested with linear regressions (R^2). We assumed that biotic indices, which describe the macrofaunal community's ecological status, must be considered as predictor variables. The linear regressions were previously tested without 'AMBI index' trait to avoid misleading results due to

Table 1

Traits and categories used in Biological Trait Analysis, divided in response and effect traits. Codes of categories are also presented.

Traits		Categories	Code	Relevant to sewage effluent discharges
Response	Body form	Vermiform Dorso-ventral compressed Laterally compressed Globose	Vrm Drs Lat Glb	External characteristics of the taxon. Different morphologies vary in their relative susceptibility to damage from sediments disposal (Bolam et al., 2016).
	Environmental position	Endofauna Epifauna Interface Epibiont	Endo Epif Inter Epib	Taxa will generally need to re-establish their position in sediments after or during continuous uploading of sewage materials to undertake their biological processes (Bolam et al., 2016).
	Mobility	Sessile Semi-motile Motile	Sess Smot Mot Mot	Mobility may increase the possibility to avoid contaminated sediments and evade burial following disposal. Species capable of movements are able of vertical migration through deposed sediments (Nasi et al., 2018; Bolam et al., 2016).
	Life habit	No mov. Swimmer Crawler Tube-builder Burrower	Nmo Swim Craw Tubl Bur	Indicates potential for the adult stage to evade, not to be exposed to physical disturbance.
	AMBI index	Group I (sensitive) Group II (indifferent) Group III (tolerant) Group IV (opportunistic) Group V (contamination indicating)	GrpI GrpII GrpIII GrpIV GrV	The sensitivity of species to organic enrichment, classified through the AMBI index.
Effect	Protection	No protection Tube Case Shell	Npr Tub Cas Shl	Proxy for palatability.
	Maximum size (mm)	<5 5–30 30–80 >80	S M M/L L	Smaller animals may be more able to take advantage of refugia from sedimentation at microhabitat scales.
	Longevity	≤1 yr 1–3 yrs 3–6 yrs 6–10 yrs	A11 A13 A16 A110	Maximum reported life span of the adult stage that indicates the relative investment of energy in rather than reproductive growth and the age of sexual maturity, i.e., proxy for *r*- or *K*-strategy (Ranta et al., 2002).
		Semelparous	Sem	

(*continued on next page*)

Table 1 (*continued*)

Traits		Categories	Code	Relevant to sewage effluent discharges
	Reproductive frequency	Iteroparous	Iter	Reproductive frequency indicates a role in community development throughout time scale.
		Semi-continuous	Scon	
Effect	Adult feeding habitat	Suspension feeder	Susp	Feeding habits indicate roles in the trophic pathway. Feeding habits may be affected by increased suspended sediment (e.g., suspended feeders) (Thrush et al., 2004).
		Surface deposit feeder	Sdep	
		Subsurface deposit feeder	Ssdep	
		Herbivore	Herb	
		Predation	Pred	
		Scavenger	Scav	
	Bioturbation	None	Non	Describes the ability of the organism to rework the sediments, influencing the oxygen concentration and nutrient cycling throughout sediment layers. Bioturbation mode has important implication for sediments-water exchanges and sediment biogeochemical proprieties (Kristensen et al., 2012).
		Superficial modifier	Smod	
		Biodiffuser	Bdif	
		Regenerator	Regr	
		Conveyor	Cnvy	

covariation among variables. Further, Spearman's rank correlation was performed to investigate the relationships between functional diversity, biotic indexes, and environmental parameters.

To test the significant relations between the different matrices, the RELATE routine was applied as follows i) abundance *vs* biomass; ii) CWM-abu *vs* CWM-biom.

Similarly, Principal Coordinates Analysis (PCoA) was performed on traits occurrences to highlight differences in functional modalities (CWM-abu and -biom, separately) among sampling stations and periods. Pearson' correlations with PCO axes were also tested.

Also, environmental parameters were used to perform the Distance-Linear Modelling (DistLM) to assess the variables that explain the differences ($p < 0.05$) in species abundance, biomass, and trait occurrences (CWM- abu and -biom). Before the analysis, the abiotic parameters were normalized and the option 'All specified' and R^2 were used as the selection procedure and criterion, respectively.

In this study, we applied the RLQ analysis (Dolédec et al., 1996) to look for relationships between trait-categories occurrences (abundance- and biomass-based, separately) and sediment variables. As a first step, we carried out the analysis separately on each of the following three tables: environmental variables (R), abundance (L), and traits (Q). For the abundance table, we applied Correspondence Analysis (CA) whereas, for the environmental tables, we applied Principal Component Analysis (PCA). Regarding fuzzy-coded trait data, a fuzzy correspondence analysis (FCA) was conducted. Afterward, we carried out RLQ analysis, considering the three components R, L, and Q simultaneously. This analysis estimates the correlation between functional traits and environmental components by computing a crossed array (cross-covariance matrix weighted by abundances). Next, the fourth-corner methods were applied to measure, one at a time, the associations between the species traits and the environmental variables. We combined two permutation models (one for the samples and the other one for the species; Dray et al., 2014), carrying out 49,999 permutations and applying the adjustment of *p*-values for multiple testing (False Discovery Rate [FDR] method; Benjamini and Hochberg, 1995). The correlations between traits and environmental variables were considered significant if the largest p-values of the permutation models (i.e., samples and species permutation models) were lower than α. Before the analyses, the Hellinger transformation was applied to abundance and biomass data.

Univariate and multivariate analyses were carried out using STATISTICA 7 and PRIMER 7 (PRIMER-E Ltd. Plymouth, UK), respectively. The two matrices (i.e., species composition and CWM values) were square root for the multivariate analyses, and the Bray-Curtis similarity was applied. Lastly, functional analyses, RLQ-fourth corner tests, and correlations were computed using the software program R, version 3.5.2, R packages: 'FD', 'ade4', and 'corrplot', respectively (R Core Team, 2018).

3. Results

3.1. Environmental variables

In the study area, sediments were mainly composed by silt (average value: 54.7 ± 6.8%) in both periods. Besides, higher percentages of silt were observed in 2016, compared to 2018 (*U* test: $z = 4.1$; $p < 0.01$). In the first sampling period, sand fraction was completely absent only at -2D, whereas the maximum was measured at −1 M (24.8%). Conversely, higher sand contents were noticed in 2018 (U test: $z = -3.1$ $p < 0.01$). Sand percentages varied between 5.7% at 2 M and 24.8% at −1 M. In both sampling periods, sand fraction significantly increased at stations nearby the duct ($H = 7.7$; $p < 0.01$). Clay percentages varied from a minimum of 25.3% at −1 M and 38.9% at -1D in 2016; whereas ranged between 22.4% at 0D and 42.5% at RS, in 2016 and 2018, respectively (Table 2). Overall, high volumes of BR were detected at stations near the duct compared to the farther ones.

In both sampling periods, higher values of Corg and TN were observed in the diffusion area compared to RS. In particular, Corg values at stations gathered in the 'distal' transect were significantly higher than stations in the 'medial' one (H test post-hoc comparison $z = 2.8$; $p < 0.05$). In April 2018 greater Corg and TN contents were noticed at 0D (60.5 and 3.8 mg g^{-1}), whereas the lowest value was observed at RS (10.6 and 1.2 mg g^{-1}, respectively). A similar pattern was noticed for C:N ratio. In both sampling periods, a higher ratio was observed in the diffusion area compared to RS. In particular, greater C:N values were detected at stations located nearby the duct (11.1 and 18.6 at 0D, in 2016 and 2018, respectively). As supported by the H test, significant differences in C:N ratio were observed at stations placed at 5 m from the duct compared to 200 m (post-hoc comparison $z = 2.6$; $p < 0.05$). Regarding Eh values, significant differences were detected among transects ($H = 11.6$; $p < 0.01$; 'distal' *vs* 'medial' $z = 2.7$; $p < 0.01$). In particular, very low Eh values were noticed at the station near the main outfall (−255 and −290 mV at 0D) compared to RS (95 and 54 mV) in 2016 and 2018, respectively. The highest amount of BR was estimated in 2016 than in 2018 (*U* test $z = 4.8$; $p < 0.01$). In both sampling periods, mercury concentrations significantly varied among stations located at increasing distance from the duct ($H = 7.7$; $p < 0.01$). In particular, Hg significantly increased at stations located 200 m from the pipe compared to the nearest ones (200 m *vs* 100 m $z = 2.7$; $p < 0.02$).

At stations 2FD (Hg: 0.77 mg kg^{-1}) and 1FD (0.74 mg kg^{-1}) the highest Hg contents were detected in 2016 and 2018, respectively. On the contrary, in both sampling periods, significant differences in PAHs concentrations were noticed among transects ($H = 12.0$; $p < 0.01$). In particular, higher PAHs contents were observed at stations gathered in the distal transect compared to the 'medial' one ('distal' *vs* 'medial' $z = 2.7$; $p < 0.01$), except for the maximum PAHs value observed at 0P (660 mg kg^{-1}) in 2018.

Table 2

Physical-chemical and contaminants values measured at sampling stations in November 2016 (white area) and April 2018 (gray area). Corg (organic carbon); TN (Total Nitrogen); C:N (carbon and nitrogen ratio); Eh (redox potential); BR (Biogenic remains); PAHs (Polycyclic Aromatic Hydrocarbons).

Station	Sand	Silt	Clay	Corg	TN	C:N	Eh	BR	Hg	PAHs
		%		mg C g^{-1}	mg N g^{-1}		mV	mL	mg Kg^{-1}	
–2D	0.0	67.5	32.5	16.6	2.0	9.6	-116.0	2400.0	0.7	189.0
–1D	5.3	55.8	38.9	17.4	1.9	10.4	-136.0	9166.7	0.5	118.0
0D	7.9	56.2	35.9	14.4	1.5	11.1	-255.0	12166.7	0.1	31.0
1FD	5.6	62.1	32.3	16.1	1.9	10.0	-124.0	3333.3	0.6	174.0
2FD	4.8	63.3	31.9	14.7	1.8	9.6	-176.0	3500.0	0.8	107.0
1D	7.6	60.9	31.5	17.4	1.9	10.9	-113.0	5333.3	0.6	58.0
2D	5.0	61.3	33.7	14.9	1.7	10.5	-69.0	2333.3	0.8	166.0
–2M	20.3	52.4	27.3	12.1	1.3	10.5	-132.0	3500.0	0.4	27.0
–1M	24.8	49.9	25.3	11.5	1.3	10.3	-82.0	9333.3	0.5	47.0
0M	6.5	56.9	36.6	12.8	1.3	11.1	-137.0	13333.3	0.2	57.0
1M	2.5	61.3	36.3	14.7	1.7	10.3	-67.0	3333.3	0.6	105.0
2M	5.6	61.7	32.7	16.4	1.9	9.8	-133.0	7000.0	0.5	101.0
–2P	5.0	60.9	34.1	16.0	1.9	9.8	-112.0	4500.0	0.8	89.0
–1P	22.5	53.2	24.3	18.2	1.8	12.0	-162.0	5000.0	0.4	89.0
0P	12.3	57.5	30.2	15.9	1.8	10.4	-117.0	14166.7	0.4	119.0
1P	4.6	60.3	35.1	16.4	1.9	10.3	-97.0	2416.7	0.6	127.0
2P	4.7	63.6	31.7	16.1	1.7	10.8	-77.0	3666.7	0.7	137.0
RS	3.6	58.8	37.6	12.1	1.4	9.8	95.0	2833.3	0.5	5.0
–2D	7.0	54.6	38.4	16.2	1.8	10.3	-210.0	223.3	0.5	170.0
–1D	10.5	54.2	35.3	21.9	2.5	10.2	-101.0	1533.3	0.5	297.0
0D	39.6	38.0	22.4	60.5	3.8	18.6	-290.0	3756.7	0.4	229.0
1FD	9.0	54.3	36.7	16.7	1.9	10.1	-150.0	373.3	0.7	207.0
2FD	5.9	52.7	41.4	15.2	1.8	10.0	-130.0	383.3	0.7	154.0
1D	13.6	53.3	33.1	16.8	1.8	10.8	-210.0	466.7	0.6	306.0
2D	7.0	54.1	38.9	16.1	1.9	10.0	-150.0	226.7	0.7	189.0
–2M	21.3	48.9	29.8	13.3	1.6	9.9	8.0	500.0	0.4	86.0
–1M	34.9	40.8	24.3	12.3	1.3	11.3	70.0	1483.3	0.4	114.0
0M	39.4	37.3	23.3	13.5	1.7	9.4	-25.0	1160.0	0.4	115.0
1M	11.4	54.6	34.0	15.9	1.9	10.0	-117.0	406.7	0.4	118.0
2M	5.7	55.0	39.3	16.0	1.9	9.7	35.0	526.7	0.6	156.0
–2P	7.8	53.2	39.0	14.1	1.8	9.3	-115.0	246.7	0.4	109.0
–1P	17.2	50.1	32.7	16.8	2.3	8.5	-117.0	1266.7	0.5	187.0
0P	25.7	46.6	27.7	27.8	2.5	13.0	-204.0	1600.0	0.5	660.0
1P	12.1	54.3	33.6	17.6	2.5	8.2	-57.0	636.7	0.5	155.0
2P	8.0	54.5	37.5	16.7	2.1	9.2	-15.0	176.7	0.5	143.0
RS	7.0	50.5	42.5	10.7	1.2	10.5	54.0	660.0	0.7	126.0

3.2. Macrofaunal abundance and biomass

Total abundance ranged from 326.6 ± 90.7 ind. m^{-2} at RS to 2546.7 ± 2281.0 ind. m^{-2} at 0D in 2016; whereas a minimum of 360.0 ± 157.2 at 0P and a maximum of 3436.6 ± 464.9 at -1D were observed in 2018. In 2016, the total values of biomass varied between 18.1 ± 10.2 (2D) to 539.9 ± 876.1 g m^{-2} (−2 M). Conversely, lower values were measured in 2018, with a minimum at RS (18.7 ± 13.1 g m^{-2}) and a maximum at 2P (3146.4 ± 3035.8 g m^{-2}). Overall, no significant differences in total abundances and biomass were noticed between sampling periods and among stations. However, the major macrofaunal densities were observed nearby the main outfall community. On the contrary, the higher values of biomass were measured at farthest stations (Fig. 2a and b). The PERMANOVA main-test performed on macrofaunal community highlighted the difference between years, transects, and distance from the pipeline (Pseudo-F = 5.18; 1.96; 2.64; $p < 0.01$). In particular, by PERMANOVA pair-wise tests, differences in species composition were observed between stations gathered in 'distal' and 'medial' transects ($t = 1.69$; $p < 0.01$) and among stations located at 5 m *vs* 100 m ($t = 1.53$; $p < 0.05$) and 5 m *vs* 200 m ($t = 2.06$; $p < 0.01$). These differences were principally related to the high dominance of polychaete *Capitella capitata* at stations gathered in 'distal' transect and at 5 m from the duct. In particular, great numbers of *C. capitata* were observed at 0D in 2016 (2116.6 ± 2317.5 ind. m^{-2}). Moreover, comparing the macrofaunal species abundance and biomass matrices, they were significantly related (RELATE test: $r_s = 0.62$; $p < 0.01$). Similarly, PERMANOVA main-test carried out on biomass matrix highlighted significant differences between sampling periods (Pseudo-F = 2.25; $p < 0.05$), as well as among the stations located at a different distance from the duct (Pseudo-F = 2.55; $p < 0.001$). On the contrary, no significant differences were noticed among transects. Besides, by pair-wise comparisons, significant differences were highlighted among stations located nearby the duct to the farther ones (5 m *vs* 100 m: $t = 1.39$; $p < 0.05$ and 5 m *vs* 200 m: $t = 2.11$; $p < 0.01$). The dissimilarity among station positions was linked to the highest biomass at stations located far from the duct, principally due to the bivalve *Atrina fragilis*. The highest weight of *A. fragilis* was measured at −1 M in 2018 (1809.6 ± 1566.3 g m^{-2}). The distance-based Linear Model (DistLM) performed on macrofaunal abundance highlighted sand, silt, and clay fractions, Corg, C:N, Eh, BR and Hg as important drivers in species distribution among stations and periods. Similarly, the same drivers were obtained with DistLM calculated on biomass, except for Corg, TN, Eh, and PAHs. The outputs of DistLM analyses are summarized in Table S1.

3.3. Biotic and functional indices

The calculation of BENTIX in 2018 at stations located in the 'distal' transect was characterized by lower values than the others. Overall, BENTIX for both sampling periods evidenced the lowest values at the RS station. High values of M-AMBI and M-bAMBI indices were calculated in the whole sampling area for both periods. However, the lowest values of M-AMBI were measured at 0D in 2016 (0.22) and 2018 (0.39). Moreover, as corroborated by the H test, significantly lower values of M-AMBI were noticed at stations gathered in the 'distal' transect compared to stations in 'medial' and 'proximal' one ('distal' *vs* 'medial' $z = 3.15$ $p < 0.01$; 'distal' *vs* 'proximal' $z = 2.62$ $p < 0.01$). Further, even M-bAMBI showed a lower value at 0D in 2016, whereas higher ones were noticed at 'medial' transect.

The number of species varied from a minimum of 24 (at 0D) to a maximum of 93 (at 1 M) in 2016 and 2018, respectively. Similarly, the lowest diversity values (H') were observed in both sampling periods at the distal station (0D) (1.2 in 2016 and 2.0 in 2018). On the contrary, the highest H' values were detected −1 M (5.3) in 2016 and 2018 (Fig. 2a). In particular, higher species richness and diversity values were noticed at stations gathered in the 'medial' transect compared to the 'distal' one ($z = 3.54$ and 2.73; $p < 0.01$, respectively). Overall, as confirmed by the *U* test, a higher number of species was observed in 2018 compared to 2016 ($z = -1.99$; $p < 0.05$). The FDis-abu followed the trends observed for the species richness in both sampling periods. The lowest values of FDis-abu were recorded at stations located nearby the main outfall (0D: 2.18 in 2016 and 3.86 in 2018), whereas the highest values were noticed at −2 M (6.91) and −1 M (7.58) in 2016 and 2018, respectively (Fig. 3). As species richness, FDis-abu showed significantly lower values at stations gathered in the 'distal' transects compared to the 'medial' one ($z = 2.77$; $p < 0.01$). FDis-biom varied from 0.56 (at 2P) to 6.98 (at 0 M). Moreover, different significant values were observed between station positions ($H = 7.00$; $p < 0.05$) (Fig. 3).

Regarding the relation between functional and biotic indices, M-AMBI and M-bAMBI showed a relation with FDis-abu (i.e., increased with environmental healthy). In particular, FDis-abu was significantly related to M-AMBI and the number of species with a higher value of R^2 (Fig. 4). On the contrary, for FDis-biom, no relations with biotic indices and number of species were noticed. Lastly, species and biotic indices (i.e., M-AMBI and M-bAMBI) were significantly correlated with sand

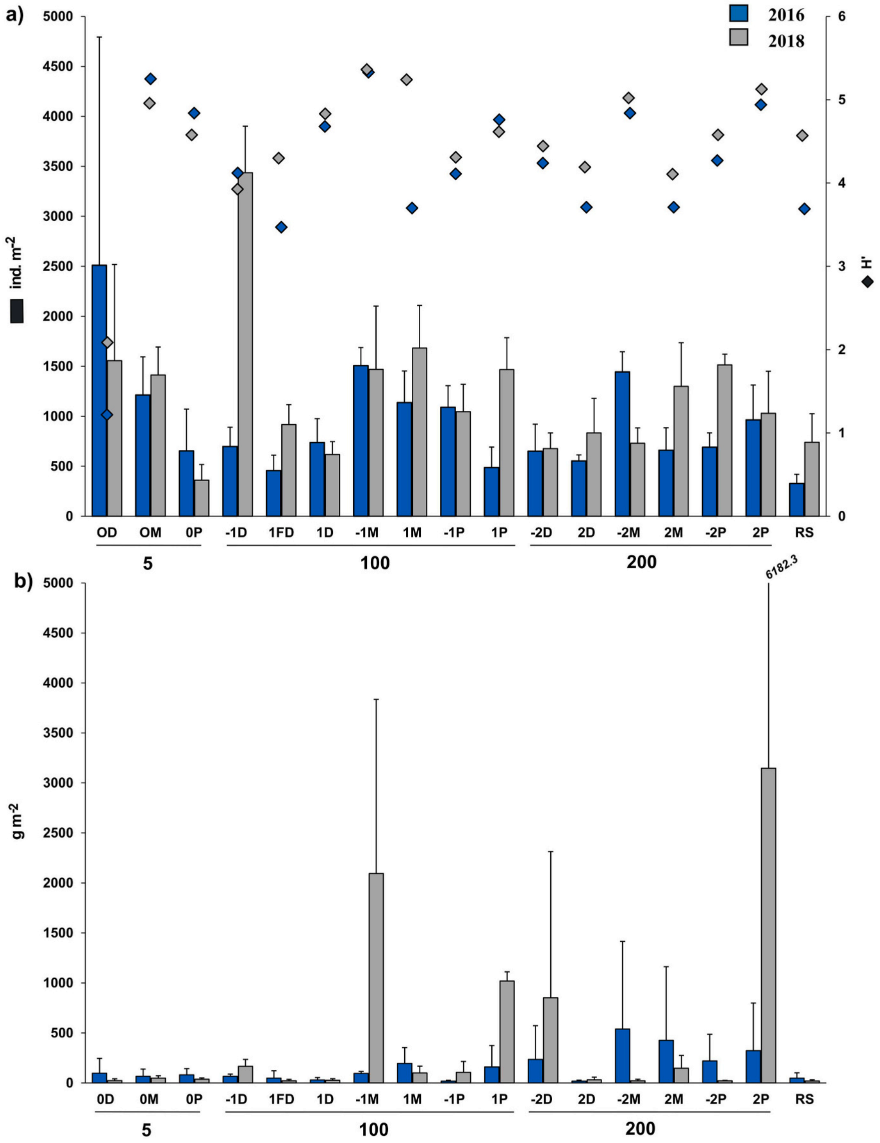

Fig. 2. Macrofaunal dencities, diversity (H'-a) and biomass (b) of sampling stations and periods. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

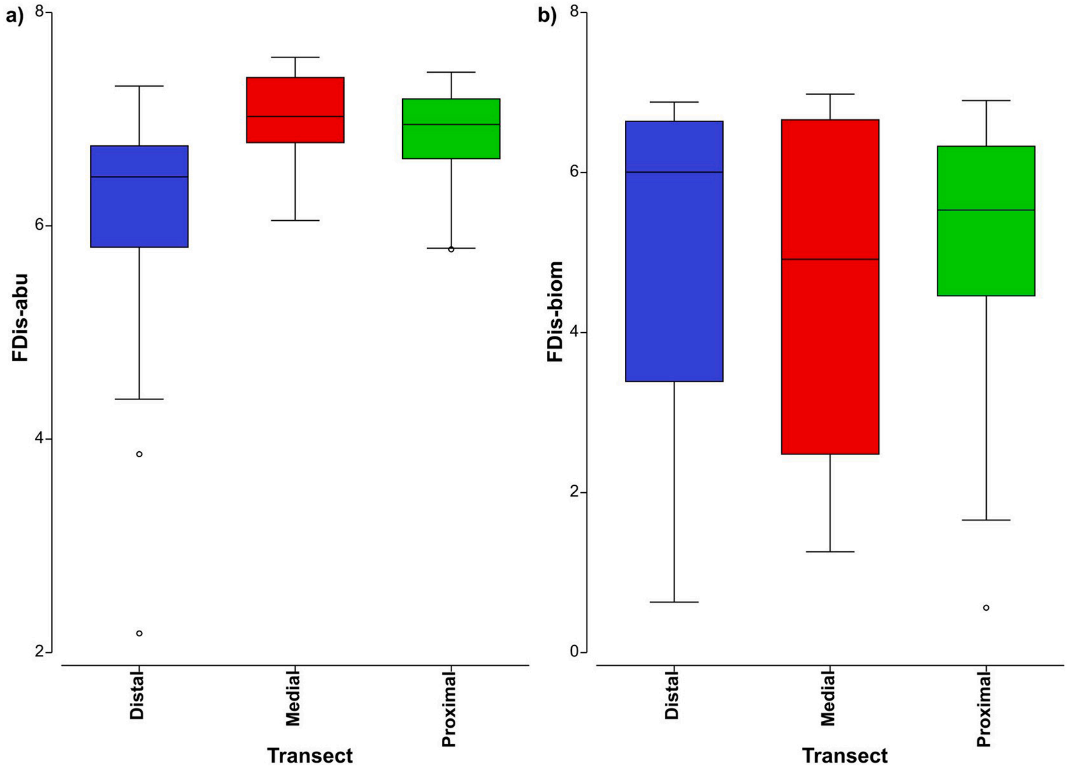

Fig. 3. Boxplots showing the variability of FDis-abu (a) and FDis-biom (b) at stations gathered by transects. The different groups are indicated with different colours.

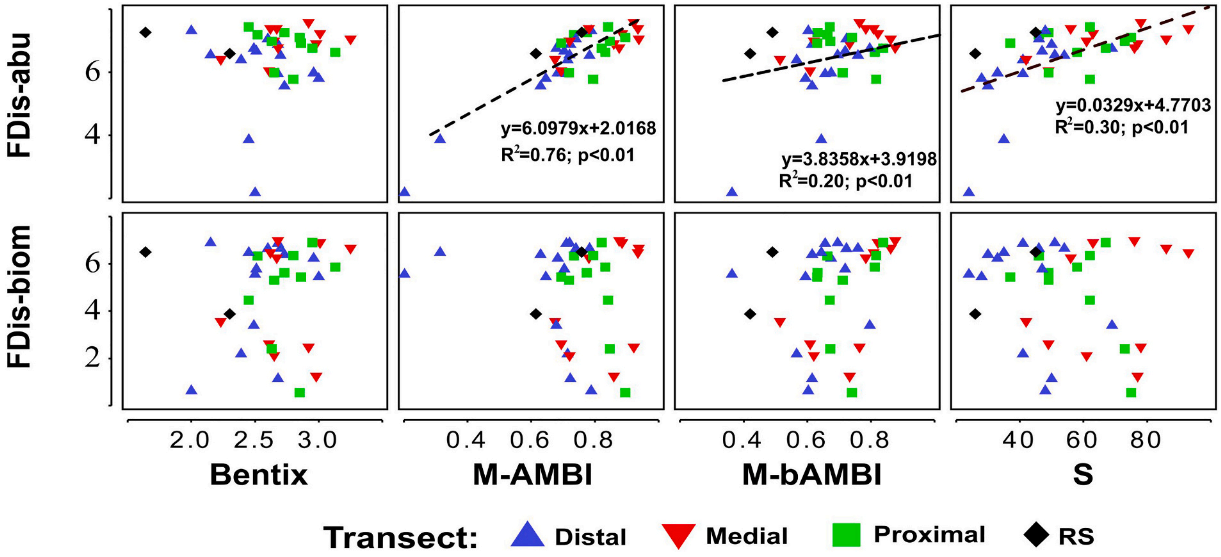

Fig. 4. Relationship between functional diversity (abundance- and biomass-based), number of species and biotic indices. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

percentage and Eh, but were negatively related with silt and Hg. On the other hand, FDis-abu highlighted a high positive correlation with Eh and a negative one with biogenic remains (BR) (Fig. 5).

3.4. Functional traits occurrences

The PERMANOVA tests on CWM values based on species abundance and biomass highlighted significant differences among stations located at 5, 100, and 200 m from the pipeline (Pseudo-F = 2.82 and 2.47 $p < 0.05$, respectively). Besides, significant variations in functional traits occurrences were noticed for both matrices between stations near the duct and farther ones (PERMANOVA pairwise tests: $t = 2.09$; $p < 0.01$ and 2.00; $p < 0.05$, respectively). On the contrary, CWM-abu significantly differed from sampling periods ($t = 3.27$; $p < 0.01$). The latter result confirmed the different traits occurrences described by CWM-abu and -biom among sampling stations and periods, since no match was tested by RELATE analysis.

The DistLM performed on CWM-abu highlighted sand and silt fractions, Corg, C:N, Eh, BR, and Hg act as important drivers in species distribution among stations and periods. On the contrary, none of the selected abiotic variables were the drivers for CWM-biom values. The outputs of DistLM analyses are summarized in Table S2.

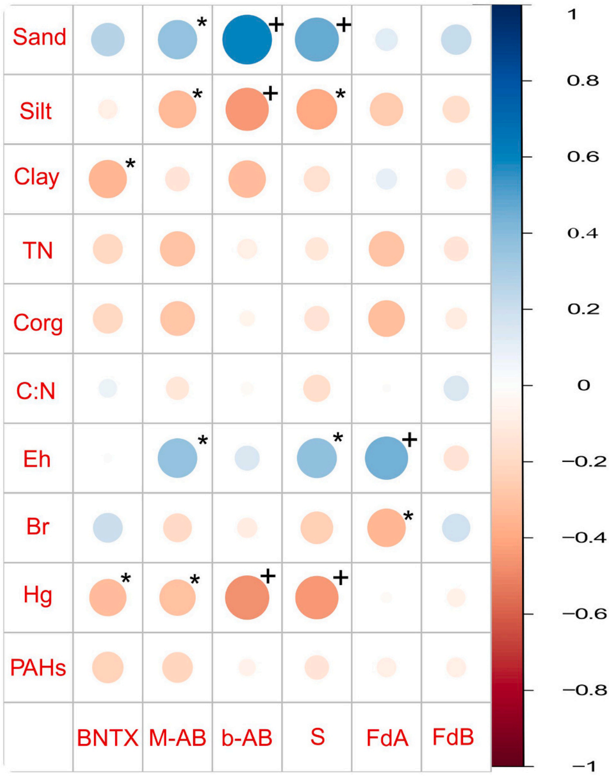

Fig. 5. Correlation matrix between functional diversity, benthic indices, and environmental variables. Positive correlations are displayed in blue while negative correlations in red colour. Colour intensity and the size of the circle are proportional to the correlation coefficients. Asterisk and cross indicate significant correlations (0.05 and 0.01, respectively). Corg (organic carbon); TN (Total Nitrogen); C:N (carbon and nitrogen ratio); Eh (redox potential); BR (Biogenic remains); PAHs (Polycyclic Aromatic Hydrocarbons); BNTX (BENTIX); MAMB (M-AMBI); MbAM (Mb-AMBI); S (number of species); FDA (FDis-abu); FDB (FDis-biom). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The Principal Coordinates Analyses (PCoA) performed on CWM species abundance showed clear separation in assemblage functional trait composition from the station nearby the duct (0D in 2016 and 2018) (Fig. 6a). This separation was associated mainly with the first axes (PCoA1, Table S3) for modalities belonging to the main group of traits as 'life histories', 'adult activity', and 'response to anthropogenic pressure'. In particular, at 0D station, there was a high occurrence of *conveyors*, *surface- subsurface-deposit feeders*, and *Group V* species. Besides, the PCoA2 plotted a group of stations positioned at 100, 200 and 2000 m from the duct (RS in 2018) characterized by higher occurrences of *sessile*, *epibiont*, *no movement*, and *no bioturbation* modalities. On the contrary, no clear pattern of distance gradient was observed at PCO performed on CWM-biom values. However, the great functional variation among stations was associated both for PCoA1 and PCoA2, with the main group of traits as 'adult size and shape', 'life history', and 'adult activity'. Lastly, a group of stations was plotted separately, resulting in a higher correlation with the negative axes PCoA1. These stations were characterized by high occurrences of modalities as *shell protection*, *adult longevity 6–10 yrs*, *suspension feeder*, *superficial modifier*, and *interface*. (Fig. 6b and Table S3).

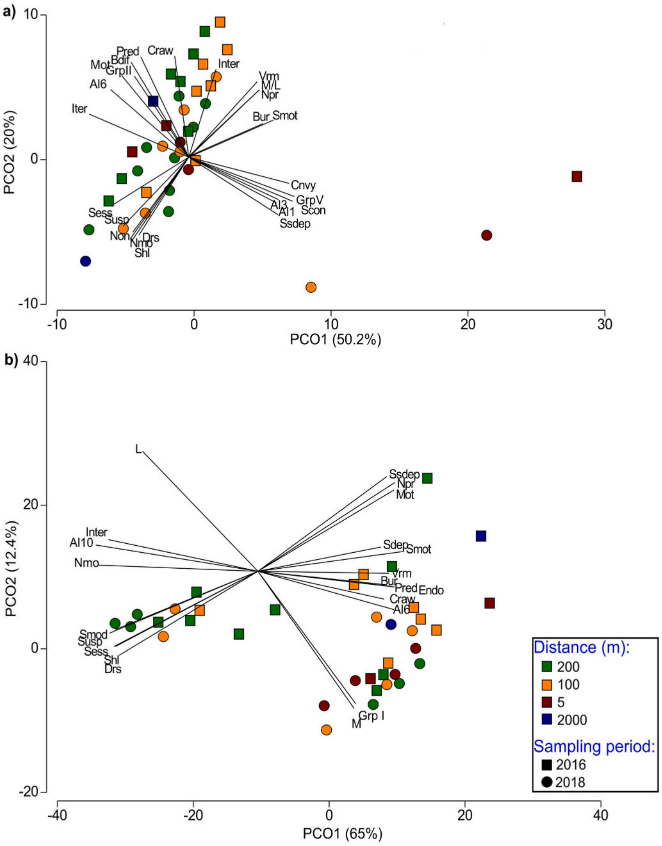

Fig. 6. PCO ordinations describing the variability in CWM values abundance- (a) and biomass-based (b) across sampling sites. Traits major correlated with PCO axes (>0.7) are overlaid. The distances from the pipe are indicated with different colours. See Table 1 for complete trait labels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The RLQ and fourth root analyses were carried out on traits occurrences, abundance and, biomass. However, since no significant relationships among traits occurrences biomass-based and environmental parameters were observed, the results were not reported. The RLQ analysis performed on species abundances accounted for 78.9% of the total variance (Fig. 7a and Table 3), and the sampling stations were plotted based on the distance from the pipeline. The station nearby the main outfall was plotted along RLQ1 axis (left- the hand of the plot), conversely, the farthest stations were positioned at the negative part of RLQ1 axis. Fig. 7 underlines the significant relationships between the RLQ environmental axes and individual traits (b), and between the RLQ trait axes and individual environmental variables (c). After the application of the FDR adjustment method, significant high correlations were recorded ($p < 0.01$). Significant positive correlations were also recorded between *semi-continuous* and AxcR1 (on the right of the diagram). Negative correlations were found for the same axis *vsiteroparous* (on the left of the chart). Regarding the second axis (AxcR2), positive correlations were recorded for the *dorso-ventally compressed* (on the top of the diagram). In contrast, negative correlations were recorded for the *30–80 mm adult size* and *vermiform* traits (on the bottom of the diagram). Concerning environmental variables, positive relations were recorded between AxcQ1 and TN, Corg and C:N, whereas negative correlations with AxcQ1 and Hg and Clay. The second AxcQ2 was positively related with Eh and sand and negatively with silt and BR.

4. Discussion

In this study, we explored the soft-sediment invertebrates' assemblages influenced by sewage-derived materials. We covered the entire diffusion area with an accurate sampling design that considered the sediments in front of the main outfall and the entire diffusion zone of the pipelines. Important information of macrofaunal community features were obtained as well, considering the stations placed at gradually increasing distances from the contamination sources. Doing that, we

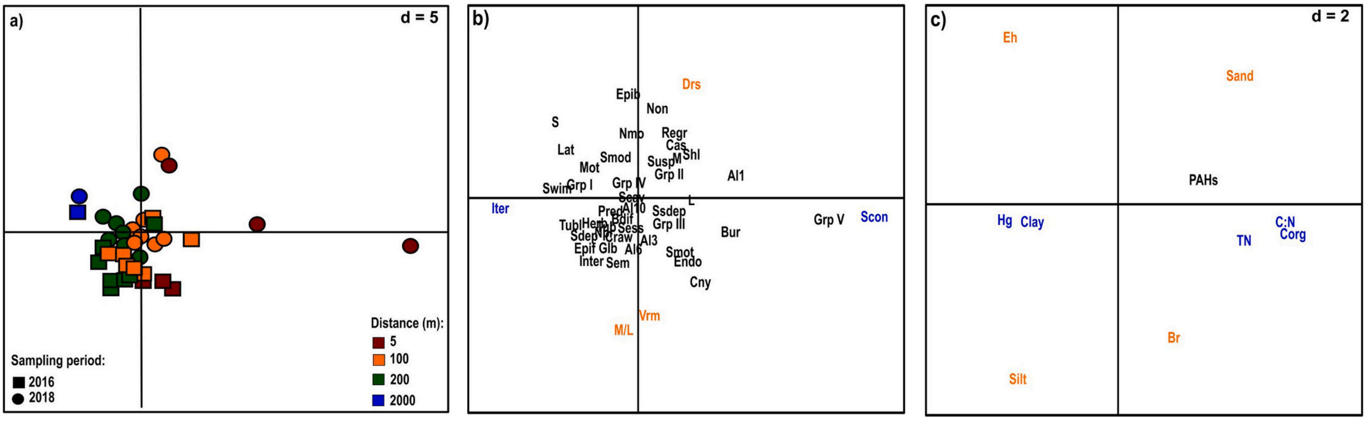

Fig. 7. Results of RLQ analyses performed on traits occurrences abundance (a) and relationships between the RLQ environmental axes and individual traits (b) and between the RLQ trait axes and individual environmental variables (c). The d value in the upper right of the corner is the scale of the graph (a). The distance from the pipe and the sampling periods are indicating with different colour and shape, respectively. Significant associations with the first axis are represented in blue, with the second axis in orange, while variables with no significant association are in black. Corg (organic carbon); TN (Total Nitrogen); C:N (carbon and nitrogen ratio); Eh (redox potential); BR (Biogenic remains); PAHs (Polycyclic Aromatic Hydrocarbons). See Table 1 for complete trait labels

Table 3
RLQ analysis based on species abundance. RLQ analyses and individual R-L-Q separate analysis.

Species abundance					
Cumulative projected inertia (%)					
Ax1	Ax1:2				
56.49	78.97				
Projected inertia (%)					
Ax1	Ax2				
56.48	22.49				
Eigenvalues decomposition					
	Eig	Covar	sdR	sdQ	Corr
eig1	0.58	0.76	1.82	1.60	0.26
eig2	0.23	0.48	1.38	2.07	0.16
Inertia & coinertia R (env)					
	Inertia	Max	Ratio		
eig1	3.31	3.46	0.95		
eig1 + 2	5.22	5.94	0.88		
Inertia & coinertia Q (traits)					
	Inertia	Max	Ratio		
eig1	2.56	7.59	0.34		
eig1 + 2	6.86	13.01	0.53		
Correlation L (CA on abundance)					
	Corr	Max	Ratio		
eig1	0.26	0.56	0.46		
eig2	0.16	0.53	0.31		

were able to highlight the structural and functional attributes of macrofaunal invertebrates in an area subject to wastewater discharges, determining, in particular, which structural data matrices (i.e., species abundance and biomass) can best explain the difference in functional diversity and identity of the community investigated. Furthermore, we tested the applicability of functional approaches in an area deeply influenced by anthropogenic pressures for the potential integration of BTA in monitoring activities.

4.1. Abiotic features

The wastewaters discharged into coastal environments contain considerable loads of organic matter. Moreover, an increase in the nutrients and suspended particles concentration is expected near the sewage-affected areas (Waldron et al., 2001). In this study, we did not find an evident pattern of Corg and TN contents depending on the distance from the pipeline, or rather, a decrease in Corg content was not observed at stations about 200 m from the duct. Waldron et al. (2001) suggested that the combined effect of wind and sea current actions might modify organic matter deposition throughout the sampling area. Cozzi et al. (2008) also demonstrated that meteorological characteristics and winds could have affected the material sedimentation nearby the outfall. The authors illustrated that the direction and intensity of all currents played an important role in the horizontal flow of wastewaters. Nevertheless, in agreement with Filgueiras et al. (2007), we found higher values of Corg and TN in the diffusion area compared to RS. Furthermore, we evidenced allochthones input of organic matter by sewage-derived materials near the main diffusion zone (higher C:N ratio). In both periods, the very low redox potential values (Eh) in the diffusion area compared to reference station suggested hypoxic conditions at sediments. In fact, under excessive nutrient load, the sedimentation of organic matter may exceed the rate of its degradation (Taylor et al., 1998), and microbial decomposition severely depletes dissolved oxygen, lacking sufficient oxygen to support most organisms (Arend et al., 2011). Also, the higher values of sand at stations adjacent to the duct could be linked to the presence of the sewage pipeline, as also confirmed by Melis et al. (2019). Moreover, the same distribution pattern of the sandy fraction was observed for biogenic remains (BR). High values of BR were measured at stations close to the pipeline in both sampling periods, especially that one located at the main outfall. The progressive increase of sand fraction and BR could be ascribable to the characteristics of the disposal plant. The sewage system is a mixed type, collecting not only wastewater but also meteoric ones. Therefore, in addition to shell fragments usually present in marine sediments (Díaz et al., 1995), a high amount of vegetal debris coarse sediments (i.e., gravel) is commonly observed at the stations close to the duct. In this study, we also analysed the concentrations of contaminants such as Hg and PAHs, as potentially brought by sewage sludge. Hg values significantly increased at stations located at 200 m from the duct, characterized by high clay percentages. Indeed, the concentrations of heavy metals generally increase as particle size decreases (Yao et al., 2015). However, it is well documented that the high values of Hg in the Gulf of Trieste are generally linked to the Isonzo River inputs. This river is known to be the main source of particulate Hg due to long-term cinnabar extraction activity at the Idrija mining district (Slovenia) (Covelli et al., 2007). On the contrary, higher PAHs contents were measured at stations gathered in the 'distal' transect compared to 'medial' one that could potentially be related to sewage outfall. Bolam et al. (2011) illustrated that the highest mean values of PAHs were detected close to the disposal site, which follows the same pattern found in our study.

4.2. Macrofaunal community structure and functional characteristics

The macrofauna community investigated is typical of coastal environments influenced by high and long-term deposition of sewage materials (Solís-Weiss et al., 2007; Souza et al., 2013). This community followed the model of Pearson and Rosenberg (1978) in both sampling periods. We observed significant differences in community structure among stations and transects. We noticed high macrofaunal densities and low biomasses at stations nearest the pipe, whereas the opposite was observed at the farthest stations (see Fig. 2). Also, a great variation in taxa composition following the distance gradient was detected. This difference is mainly ascribable to the dominance of the polychaete *Capitella capitata*. The presence of wastewater discharge is mirrored by the dominance of this opportunistic *r*-strategist species. Our results showed a high dominance of *C. Capitata* at stations gathered in a 'distal' transect, particularly at 0D in both periods. *C. capitata*, a mainly stress-tolerant species, is commonly observed in areas influenced by the large amount and long-lasting deposition of organic matter and oxygen depletion (Pearson and Rosenberg, 1978; Grémare et al., 1989).

In our study, species abundances and biomass are not affected by Hg and PAHs but rather by the amount and features of sewage-derived materials origin. For macrofaunal density, the grain-size distribution and organic matter with Eh and BR were the main drivers of species distribution. Sediment composition is a key element in structuring macrofauna community and the distribution of dominant species, also when related to organic enrichment (Rhoads and Boyer, 1982; Hermand et al., 2008). Species such as *Atrina fragilis* and *Maldane glebifex* were typically observed in the Gulf of Trieste muddy-sediments at these depths (Nasi et al., 2017). Conversely, typical sandy fraction species were observed at stations close to the duct, particularly the polychaete *Owenia fusiformis* (Pinedo et al., 2000). Eh levels also influenced the species distribution pattern. The hypoxic conditions measured mostly at 0D likely caused an increased presence of opportunistic and stress-tolerant species as observed in our study. Thus, our results agree with those Solís-Weiss et al. (2007) and Gray et al. (2002), pointing out that sewage discharges could influence the oxygen concentration within sediments, thus promoting the stress-tolerant macrofaunal species. To confirm this, in addition to *C. Capitata*, other polychaetes with opportunistic behaviour were observed at 0D (e.g., *Heteromastus filiformis* and *Lumbrineris latreilli*).

The influence of contamination on reducing species richness and diversity has been largely documented (e.g., Johnston et al., 2015; Mutlu et al., 2010). In conditions of moderate organic enrichment, an increase in species numbers can occur (Solís-Weiss et al., 2007), while in conditions of high organic load a decrease in species diversity is expected (Simonini et al., 2004; Auriemma et al., 2016). In fact, in our study, the highest number of species was found at the 'medial' transect, whereas 0D showed the lowest number of taxa. Besides, the condition of the mixed grain size with a high amount of sand in 'medial' transect resulted in additional ecological niches, increasing the possibility of a major number of species establishment (Ergen et al., 2007). This was also confirmed by the biotic indices (i.e., AMBI), which support moderate organic stress on the macrofaunal community. In our study, the FDis values similarly to species richness and diversity were higher if compared to heavily contaminated sites and relatively undisturbed habitats, except for the station in front of the main outfall, where the lowest FDis values were recorded for both sampling years (see Table 4). The major amount of sewage-origin materials seems to affect functional diversity, corroborating by the significant correlation between BR and Eh. Conversely, FDis was comparable to coastal areas not influenced by anthropogenic pressures (Weigel et al., 2016, Table 4). FDis-abu, in particular, showed spatial patterns with higher values in the non-contaminated condition and was significantly related with species numbers and M- and Mb-AMBI (see Fig. 4). The higher values of functional diversity occurred together with higher values of species richness, meaning that several species strictly perform different functions. Therefore, the community might be more susceptible to changes in ecosystem functions caused by species loss (Gladstone-Gallagher et al., 2019).

Table 4
Comparison of Functional dispersion values (FDis) between the current and (FDis-abundance based) and previous research.

Study areas	Disturbance types	FDis		References
		Min–Max	Average	
Valletta Grand Harbour (Malta-Mediterranean Sea)	Marine traffic	0–4.03	2.95	D'Alessandro et al., 2020
Augusta Harbour (Sicily-Mediterranean Sea)	Industrial plants	3.09–4.85	3.88	
Siracusa Harbour (Sicily-Mediterranean Sea)	Recreational activities	3.35–5.19	4.08	
Trieste Harbour (northern Adriatic Sea)	Industrial plants, marine traffic	4.05–4.74	4.64	Nasi et al., 2018
Mar Piccolo di Taranto (Ionian Sea)	Industrial plants, aquaculture	3.97–5.30	4.63	
Po River Prodelta (northern Adriatic Sea)	Riverine influence	4.05–4.74	4.02	Data not published
Gulf of Milazzo (Tyrrhenian Sea)	Artificial barrier	0–5.49	3.29	D'Alessandro et al., 2021
Aland Archipelago (Bothnian Sea)	Relatively undisturbed area	1.62–5.10	4.02	Weigel et al., 2016
Yangtze River Estuary (East China Sea)	Riverine influence	2.24–6.29	4.48	Zhong et al., 2020
Present study		2.18–7.58	6.43	

Regarding the functional identity, patterns in the distribution of modalities among sampling positions and transects were highlighted by CWM-abu values (see Figs. 6a and 7). As observed by Oug et al. (2012) and Krumhansl et al. (2016), the functional attributes were affected by organic effluents, showing distance-related gradients from the most contaminated stations. PCO and RLQ-fourth corner analyses have plotted high occurrances of *conveyor*, *surface-subsurface-deposit feeder*, and *group V* modalities nearby the stations directly influenced by sewage discharges. In particular, the traits as *30–80 mm maximum size*, *vermiform*, and *semi-continuous* were related with Eh, BR, and organic matter. The high expressions of *Vrm*, *M/L*, and *Ssdep* modalities was likely due to polychaetes *C. capitata*, *Naineris Laevigata*, and *Marphysa sanguinea*. These results agree with those of Gaston et al. (1998) and Oug et al. (2012), which found a preponderance of *Ssdep* modality in sediments subject to anthropogenic stress and low proportion of other trophic groups (e.g., predators, suspension feeders). Gaston et al. (1998) suggested that sub-surface deposit feeders, especially *C. capitata*, which is recognized as a general indicator of disturbance, would be the trophic habit most likely to develop pollution tolerance because they may regularly encounter allochthones organic matter released from sediments.

Furthermore, at stations more influenced by the sewage-derived materials, we observed variations in modalities belonging to 'mobility' and 'body size' *response* traits. The high expression of the *semi-motile* trait category was observed at 0D, whereas an increase in the abundance of sessile organisms was noticed toward the stations placed at 100 and, in particular, at 200 m from the duct (see Fig. 6a). At the same stations, the high dominance of *dorso-ventral compressed*, *shell-protection*, and *suspension feeder* modalities related to high contents of sands were observed. The latter traits belong principally to bivalve invertebrates (e.g., *Musculus subpictus*; *Atrina Fragilis*). The presence of suspension feeders, in particular bivalves, suggests that sediments might be affected by the low disposal of organic materials (Thrush et al., 2004). The dominance of these effect traits, performed by bivalves, make them fundamental players in benthic-pelagic coupling. They can enhance primary sediment productivity, capturing large quantities of suspended organic matter and sinking phytoplankton, and then incorporating them into sediments through pseudo-faces, stimulating the microbial loop (Jones et al., 2011; Törnroos and Bonsdorff, 2012). Conversely, nearby the main outfall conveyor species, such as *C. Capitata*, classified as head-down conveyor-belt feeder, can promote benthic cycling (Kristensen et al., 2012). In general, conveyors move sediment particles through their gut by ingestion and secretion, transferring particles from deeper to superficial layers and vice-versa, enhancing the organic matter remineralization (Belley and Snelgrove, 2016). This activity may modify sediment properties and promote microbial populations resulting in accelerated degradation of organic matter (Kinoshita et al., 2008; Wild et al., 2005).

Regarding the 'life history' modalities, the different reproductive frequencies represent survival strategy in the presence of a periodic disturbance, being therefore identified as typical of unstable environments (Paganelli et al., 2012). In this study, opportunistic species showed *semi-continuous* as reproduction modality, especially *C. capitata*, which tends to spread larvae (pelagic development) if food is supplied, rather than retained larvae (non-pelagic development) if the food is no longer available (Grémare et al., 1989). That confirms the high plasticity of these species to environmental constraints. Lastly, the *iteroparous* trait category was the dominant reproductive modality for the benthic invertebrates at stations located 200 m from the duct and at reference site. This kind of reproduction frequency generally belongs to *K*-strategists that breed several times during their lifetime. These species have delayed reproduction, slow growth, and a longer life span with a less pronounced numerical fluctuation than semelparous, such as semi-continuous invertebrates (Giangrande, 1997). The presence of iteroparous invertebrates confers more stability to the community throughout the time due to the low variation in species turnover (Törnroos and Bonsdorff, 2012).

4.3. Differences between abundance- and biomass-based analyses

In this study, the structural and functional features of the macrofaunal community followed the classical model of Pearson and Rosenberg (1978), which predicts the responses of abundance, biomass, and species richness for different levels of organic load (Pearson and Rosenberg, 1978; Rosenberg, 2001). However, the BTA results, derived from both abundance- and biomass-based functional analyses, show a clear inconsistency in functional trait composition among the stations in both study periods, corroborating by RELATE analyses. Besides, a relationship between functional diversity abundance-based and the M-AMBI and M-bAMBI was found, but no relation was highlighted with FDis-biom. The latter result was also confirmed by Gusmao et al. (2016). The authors underlined that results could differ depending on the use of abundance or biomass as predictive measures. Our results evidenced that BTA (CWM and RLQ-fourth corner) abundance-based highlighted differences in functional attributes along the distance gradient from the main outfall. This result is related to the numerical dominance of small-sized and thin stress-tolerant species at enriched conditions, which do not display an adequate part of the biomass. The opportunistic polychaete *C. capitata* was dominant at stations gathered in distal transect and at 5 m from the duct, representing the major drivers of abundance-based results in both sampling periods. The biomass-based followed the weight of bivalves, which seem to benefit from a moderate level of sewage inputs. However, in our case, the main driver of the biomass-based results is represented by the high weights of the bigger Mediterranean bivalve, *Atrina fragilis*.

The analyses performed with CWM abundance- and -biomass-based evidenced a strict relation with the matrices applied and the high numerical values that drove variation in traits occurrences among stations. On the other hand, the RLQ-fourth corner (abundance-based) showed clear trait-environment relationships along the distance gradient from the main outfall. Peres-Neto et al. (2017) indicated that the fourth-corner approach outperforms the CWM-based one regarding statistics' sampling accuracy and statistical power. Furthermore, since permutations were done for species, traits, and environmental variables separately, this analysis better highlighted the variation of traits along environmental gradients.

On a general basis, we infer that abundance-based analysis in functional assessments is more appropriate even in the presence of species numerically dominant or with higher weights expected to survive in a moderate environmental disturbance. Even if the biomass represents an essential descriptor of macrofaunal communities in response to anthropogenic organic enrichment (as described by Pearson and Rosenberg, 1978). By biomass-based analyses, we can infer that biomass information may not be helpful for studying functional variations of the macrofaunal communities in response to anthropogenic impacts, especially organic enrichment. This aspect could also be linked to the mathematical calculation behind BTA. Compared to the abundance data of each species in the community analysed, the biomass ones were characterized by lower values with minor variation among species, except for very few data out of range. Therefore, probably due to the numerical features of biomass data, the BTA could not evidence a clear gradient from the main point of contamination in traits composition biomass-based if compared to abundance one. Overall, considering these results and the long time consuming for species biomass measurements, we could infer that abundance information applied to BTA is probably the best way to understand communities' functions in the framework of environmental monitoring programs.

4.4. Functional analyses in monitoring programs

Macrofaunal is traditionally widely used in monitoring programs, in particular, to assess the influence of sewage discharges in the surrounding environments. Moreover, this aspect is extensively integrated into the monitoring programs due to the long-term variations of the sludge caused by the implementation and modification of treatments in the sewage plants (Calabretta and Oviatt, 2008). Since the ecosystem processes are influenced by the functional characteristics of the organisms, rather than by taxonomic identity (Grime, 1997), the use of structural features of macrofaunal communities alone might provide misleading information regarding the function performed by macrofaunal communities that sustain an ecological system (Díaz and Cabido, 2001). Therefore, in the framework of the monitoring programs, the ecosystem-based management needs to balance the expected outcomes due to various anthropogenic activities and requires metrics, indices, and systematic methods able to assess the variation of ecosystem functioning due to anthropogenic impacts (Mangano et al., 2017).

Our study applied the Functional Dispersion calculation since, among functional indices, it performs well for different kinds of matrices. FDis has no upper limit and requires at least two species to be computed. In addition, FDis calculation is unaffected by species richness. It can handle any number and type of traits (including more traits than species) and is not strongly influenced by outliers (Laliberté et al., 2014). In this research, functional diversity resulted significantly related to the biotic indices (i.e., AMBI and Mb-AMBI). Moreover, FDis-abu evidenced significant relationships with the abiotic variables strictly linked to sewage contaminations (i.e., Eh). Our results support the validity of FDis index in the study of environmental health, as also confirmed by other studies (see Table 4). Unfortunately, few studies consider the analyses of FDis of the macrofaunal community to confer threshold values, and more investigation is needed in this regard. However, if we compare previous data from sublittoral areas influenced by different impacts, we can assess the range of FDis values that indicate the status of the macrofaunal community based on the variety of functions performed by the organisms. For example, FDis index of macrofaunal community observed in areas deeply influenced by human impacts, like artificial structures (i.e., ports and barriers) and contaminated sediments, is around the values of FDis = 3, compared to less impacted sites where FDis is > of 4 (Table 4).

Overall, the interpretation of the BTA and the functional diversity indices depend on the selected traits. The *a priori* selection of traits and categories is fundamental when performing BTA because certain categories are more relevant in some circumstances than others (Bremner, 2008). Indeed, in this study, we used a huge set of response and effect traits for calculating macrofaunal traits occurrences. Even though we found a relationship between biotic indices and functional diversity, the traits related to the AMBI index did not evidence a pattern in distance gradient from the main outfall. The *Group V* modality resulted highly expressed at the 'distal' station because of the high density of *C. Capitata*, since this species belongs to Group V in the AMBI index. Notwithstanding these results and the type of traits (they do not represent a function exploited by the invertebrates), we infer not to consider this trait in the functional identities assignment. 'AMBI index' trait includes information on species sensitivities, which are related to the species traits in many ways. However, a BTA that considered detailed sets of traits linked both to sensitivity and ecosystem properties (e.g., bioturbation as *effective* traits) confer major information in the variety of functions performed by the macrofaunal communities investigated.

Similarly, we are not so confident to integrate for further analyses the traits belonging to 'body form' in BTA. These trait modalities are so closely linked to taxonomy that they become a proxy for taxonomy of the underlying community. For example, *vermiform* tend to cluster annelids together, and *laterally-ventral compressed* crustacean, and so on. In this study, as already discussed by Beauchard et al. (2017), we did not evidence any relation to fitness to explain species occurrences along distant gradient from the main source of contamination.

Along with traits relevant for sediment-related processes, as 'adult activities' (e.g., bioturbation and mobility) and 'feeding habits', we integrated the analyses with information regarding the 'longevity' and 'reproductive frequencies'. Life-cycle traits are related to the reproductive strategy of a species and its habit (Paganelli et al., 2012). This can give information about the development over time of communities influenced by human impacts.

Lastly, the above mentioned sets of traits, together with a sampling design that considered the effect of different levels of sewage contamination, evidenced their possible application in monitoring programs. The sampling along a distance gradient (evaluating the magnitude of organic loads) from the main source of pollution and considering the entire diffusion area, would represent a robust way to test macrofaunal functional features and their consistency of the patterns described in response to sewage contamination.

5. Conclusion

This study described spatio-temporal changes of the macrofaunal community's structural and functional attributes influenced by sewage discharge. We observed significant differences in community structure among station positions and transects. High densities and low biomasses were observed nearby the pipeline, whereas the opposite was observed at the farther stations. The abundance-based metric better evidenced variation patterns than those based on biomass. Functional abundance-based analyses highlighted that *vermiform*, *semi-continuous*, *conveyor*, and *subsurface-deposit feeder modalities* dominated at stations subject to continuous sewage discharges. On the contrary, major occurrences of *shell-protection*, and *suspension feeder* traits categories at stations far from the main outfall suggest that sediments might be affected by the low disposal of organic materials. Overall, the functional and structural analyses of the macrofaunal community indicated that surrounding environments are affected by moderate organic stress in both sampling periods. Moreover, the similar patterns observed between FDis (abundance-based) and biotic indices displayed functional diversity related to benthic environmental health. In this regard, we further confirm the applicability of abundance-based BTA (FDis and functional identity) as a reliable approach to detect the effects of sewage discharge on functional trait composition. Even if the assignment of traits for every species in the BTA could be considered a bit longer activity, functional features provide an accurate characterization of the association between traits and their sensitivity to environmental stressors. Thus, we infer that BTA could have a high potential for real-world assessments and monitoring environmental quality.

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CRediT authorship contribution statement

Seyed Ehsan Vesal: Methodology, Formal analysis, Investigation, Writing – original draft. **Federica Nasi:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Jessica Pazzaglia:** Methodology, Formal analysis, Investigation. **Larissa Ferrante:** Methodology, Formal analysis, Investigation. **Rocco Auriemma:** Methodology, Investigation, Conceptualization. **Federica Relitti:** Formal analysis, Investigation. **Matteo Bazzaro:** Formal analysis, Investigation. **Paola Del Negro:** Supervision, Funding acquisition.

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.

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