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Microbial communities as indicators of marine ecosystem health: Insights from coastal sediments in the eastern Adriatic Sea

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ABSTRACT

Considering the adaptability and responsiveness of microorganisms to environmental changes, their indicator potential is still not acknowledged in European directives. This comprehensive study examined the changes of microbial communities in sediments and a range of geochemical parameters from pristine and anthropogenically impacted coastal areas in the eastern Adriatic Sea. Various analytical methods found evidence of sediment contamination (high toxicity level, enrichments of metals, tributyltin) in certain areas, leading to the categorization of sediments based on the level of anthropogenic disturbance. Prokaryotes were identified as the most promising group of microbes for further research, with specific bacterial families (*Rhodobacteraceae*, *Ectothiorhodospiraceae*, *Cyclobacteriaceae*) and genera (*Boseongicola*, *B2M28, Subgroup 23, Sva0485, Thiogranum*) proposed as potential indicators of environmental status. Finally, predictive models were developed to identify key indicator variables for assessing anthropogenic impact in sediments. This research represents an essential step toward incorporating microbial communities into assessments of benthic environmental health.

1. Introduction

Coastal areas represent an ever-changing part of marine ecosystems that is strongly affected by various forms of contamination and eutrophication from human activities, as well as by human-induced climate change and the resulting ocean acidification (Šolić et al., 2016). It is estimated that more than half of the population in the Mediterranean region lives in coastal areas, which, together with the risks of climate change, makes semi-enclosed sea basins such as the Mediterranean and the Adriatic particularly vulnerable to perturbations (Drius et al., 2019; Caruso and Ziervogel, 2022). Increasing anthropogenic environmental degradation is expected to affect the dynamics of the entire food web, with consequences that are yet to be observed (Ferrera et al., 2020). The Marine Strategy Framework Directive (MSFD, 2008/56/EC) is a key instrument for the protection of marine ecosystems in the EU with the main objective to achieve and maintain the Good Environmental Status (GES) of marine areas. The concept of GES is defined in the MSFD by 11 Descriptors, such as the conservation of biodiversity or food webs, but also anthropogenic pressures on the marine environment including commercial fishing, eutrophication, marine litter, contaminants, or energy inputs.

The coastal benthic ecosystem is an important and complex environment with a high level of microbial biodiversity consisting of prokaryotes, protists, and fungi (Liu, 2013). As key drivers of the overall health of marine ecosystems, they contribute to numerous ecosystem services, such as primary production, maintenance of a stable global climate, natural attenuation of contamination, etc. (Šolić et al., 2016). Due to their rapid response and sensitivity to changing environmental conditions, both at structural and metabolic level (Caruso et al., 2022; Pawlowski et al., 2022), microorganisms are also potentially very good indicators of environmental perturbations (Caruso et al., 2022; Pinhassi et al., 2022). Despite that, their status, at the functional and diversity level, has been overlooked in the MSFD implementation which recommends continuous monitoring of endpoints associated with easily

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accessible and identifiable macroorganisms. Furthermore, microorganisms' vulnerability to pollution stress may be influenced by variations in their body size, metabolic activity, ecological role, and dispersion potential (Wu and Xu, 2018), which is why an assessment of the impact of human stress on coastal ecosystems at different levels (prokaryotes, fungi, and protists) is required. MSFD further emphasizes the importance of early detection of environmental changes in marine ecosystems. In this respect, marine microorganisms, as early responders, are considered promising early indicators of effects on marine ecosystem performance (Šolić et al., 2016). Studies by Borja (2018) and Aylagas et al. (2017, 2021) emphasized the importance of integrative assessments of marine ecosystems, including both high-throughput sequencing and *de novo* analyzes, and developed a novel index (*microgAMBI*) based on correlations between marine bacterial communities and sediment pollutants. The idea of using microbes as indicators of the ecological status of marine ecosystems was also proposed by Caruso et al. (2016) who, based on a literature review, suggested the inclusion of "total prokaryotic abundance", "fecal indicator bacteria" and "hydrocarbon-degrading bacteria" in the MSFD. Still, the authors stressed the need for targeted field studies that will identify consistent microbial response patterns in anthropogenically impacted vs. pristine ecosystems leading to GES criteria. One of the main obstacles to the inclusion of microorganisms into monitoring programs and further into ecological risk assessment is the lack of standardized protocols, especially for microbiota diversity analyzes (Sweeney et al., 2023).

We hypothesized that there is a general relationship between microbial community dynamics and anthropogenically induced contamination that could point to taxonomic units of the microbial community acting as early contamination indicators. To test this hypothesis, we collected sediment samples from bays and ports within the eastern Adriatic coast classified as "ecosystems at high risk"with bad or poor ecological status, but also from respective areas which are considered as reference sites based on the limited anthropogenic interventions they are exposed to. These areas were selected based on the results and reports of long-term monitoring activities carried out under the Croatian obligations according to the Water Framework Directive (WFD, 2000/60/EC), MSFD and Barcelona Convention (UNEP/MAP, 1976), indicating these locations as highly eutrophicated and polluted areas. We focused on sediments, which serve as a sink and long-term reservoir of terrigenous and aquatic particle-adsorbed pollutants, particularly in shallow coastal waters, as well as for cycling of biogenic elements (Fuhrman and Hewson, 2008; Guo et al., 2016). At the same time, as being tightly connected to the overlying water column, sediments reflect the long-term dynamics of the water layer (Chiaia-Hernández et al., 2022) providing a more accurate representation of the ongoing contamination impact on the "health"status of coastal areas, than the fast-changing water layer. Compared to the overlying water, sediments have not been studied as extensively in this context, which is reflected in poorly defined national and international regulations for polluted sediments (Chiaia-Hernández et al., 2022). In our study, we first analyzed the presence of pollutants (heavy metals and tributyltin), nutrient concentrations and sediment toxicity, which gave us an insight of environmental conditions in collected sediments. Besides, sediments were subjected to amplicon sequencing analysis to determine the taxonomic diversity of microbial communities with particular focus on prokaryotes (bacteria and archaea), fungi and protists. By bringing together and co-analyzing chemical and microbial parameters we aimed to identify microbial units that carry high potential as indicators of environmental disturbance in marine ecosystems. The further use of two different methods, DESeq2 and Classification and Regression Tree, which complement each other, allowed us to gain a more comprehensive understanding of microbial community dynamics in response to anthropogenic disturbances. Our findings could spark a debate over the significance of microorganisms as endpoints for measuring the environmental status of the benthic marine ecosystem.

2. Materials and methods

2.1. Study area and sediment sampling

Sampling campaigns were conducted in a two week period (April 2021) at seven marine areas designated as "ecosystems at high risk" and at three reference areas, all located on the eastern coast of the Adriatic Sea (Fig. 1). The past and present anthropogenic activities in the seven polluted areas are listed in Table 1. Four areas were selected in the northern Adriatic: port of Pula (PU), port of Rijeka (RI), Raša Bay (RA), Bakar Bay (BA), and three in the southern Adriatic: Šibenik Bay (SI), eastern part of Kaštela Bay - Vranjic Basin (VR), and port of Split (ST). The reference areas included: Cape Kamenjak (CK), Zlarin Island (CZ) and Vis Island (CV). The reference areas are locations where the only anthropogenic pressure is tourism during the summer (sailing, yachting and recreational fishing), while fisheries and weak agriculture on the islands are present throughout the year. In each sampling area, samples were taken from seven to eleven sites within the area, except for the reference areas where two samples were taken within the sampling area. The GPS coordinates and sample site details are provided in **Table S1**. Sampling of the surface sediments (0–5 cm, 200–500 mg of sediment) was conducted using a box corer or a Van Veen grab. Sampling was carried out at depths between 2.2 m and 40 m, with a mean sample depth of 14.6 m (± 9.1). The average temperature of the bottom seawater layer was 15.2 °C (± 2.7). After sampling, sediments were immediately stored on ice until arrival at the laboratory (within a few hours). In the laboratory, each sample collected at a specific site was thoroughly mixed and either frozen at −20 °C (for microbial analysis, grain size analysis, multi-element and toxicity analyzes, total nitrogen, phosphorus and tributyltin (TBT) content analyzes) or air-dried (for Hg content analysis). For grain size, multi-element and TBT analyzes samples were further prepared by freeze-drying (Freezone 2.5, Labconco), later two being additionally homogenized to a fine powder using a ball mill (Pulverisette 7, Fritsch). For phosphorus analysis freeze-dried sediment samples were ground and sieved ($\phi < 250$ μm).

2.2. Analytical methods

2.2.1. Grain size analysis

The sediment grain size was determined using a laser-based particle size analyzer (LS 13320, Beckman Coulter Inc.). Considering the ratio of the different grain-size fractions (clay, silt and sand), sediments were classified according to the Shepard classification (Shepard, 1954).

2.2.2. Multielement analysis of sediments

Prior to multielement analysis, the sediments were digested in a microwave oven (Multiwave 3000, Anton Paar, Graz, Austria) in a two-step total digestion procedure (I step: 5 mL HNO_3 (65 % p.a.) + 1 mL HCl (36 % s.p.) + 1 mL HF (48 % s.p.), and II step: 6 mL H_3BO_3 (40 g L^{-1}). Multielement analyzes were performed by high-resolution inductively coupled plasma mass spectrometry (HR ICP-MS; Element 2, Thermo, Germany) according to the method described in Fiket et al. (2017). For quantification, the external calibration method was used with diluted multielement standard solutions (in the range of 0.1–10 μg l^{-1}) prepared from the multielement or combined single reference standard solutions (Analytika, Prague, Czech Republic). Analytical quality control was performed by simultaneous analysis of procedural blanks and certified reference materials of marine sediments (MESS-3, NRC, Canada). The recoveries were in the range of 90 to 102 % (Fiket et al., 2017). The limit of detection (LOD) of the method, calculated as three times the standard deviation of ten consecutive measurements of the procedural blank, varied between 0.01 and 0.03 mg/kg. The limit of quantification (LOQ) was calculated as ten times the standard deviation and was approximately three times higher than the LOD values.

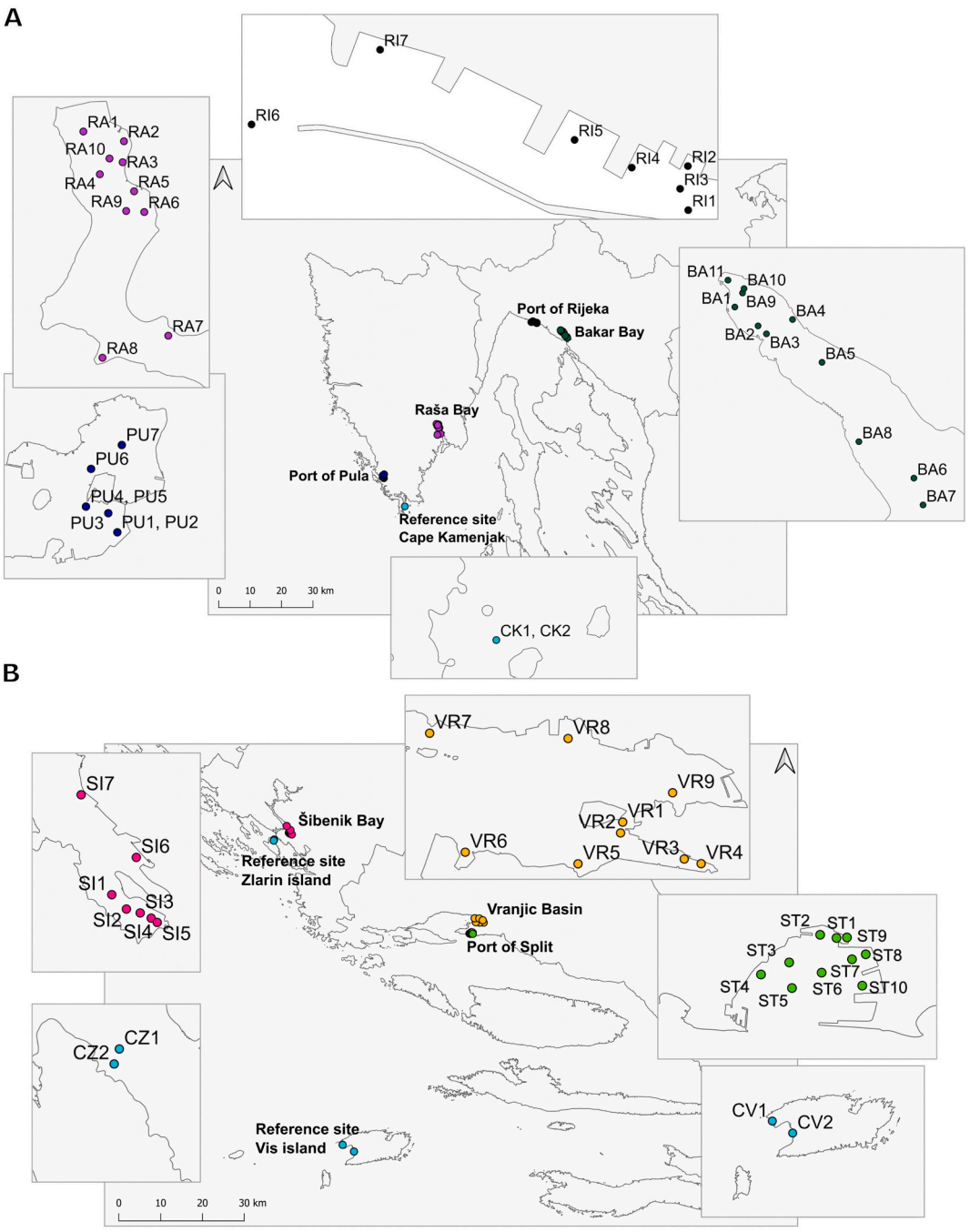

Fig. 1. Locations of the 67 sampling sites situated within seven polluted areas and three reference areas along the eastern Adriatic coast: Panel A = northern Adriatic; Panel B = southern Adriatic. Sites are marked as follows: polluted areas - port of Pula = PU, port of Rijeka = RI, Raša Bay = RA, Bakar Bay = BA, Šibenik Bay = SI, Vranjic Basin = VR, port of Split = ST; reference areas - Cape Kamenjak = CK, Zlarin Island = CZ and Vis Island = CV.

2.2.3. Determination of TBT in sediments

The analysis of TBT was performed according to the procedure described in Furdek Turk et al. (2020). Briefly, TBT was extracted by acetic acid and ultrasonic stirring, while derivatization was performed with $NaBEt_4$ in a sodium acetate-acetic acid buffer by mechanical shaking. The analysis was conducted on the gas chromatograph (GC, Varian CP3800) with pulsed flame photometric detector (PFPD, Varian). Quality control was performed by analyzing the standard reference materials (BCR-462, European Commission, JRC; BCR-646, European Commission, JCR).

2.2.4. Determination of total nitrogen and phosphorus in sediments

Total nitrogen (TN) in freeze-dried sediment was measured at the University of Zagreb, Faculty of Agriculture on a CHNS analyzer (Elementar, Germany) using ISO method 13878:1998 (Soil quality: Determination of total nitrogen content by dry combustion ("elemental analysis"). The results were expressed as a percentage of dry matter (dried at 105 °C to constant mass, i.e. lyophilized).

The contents of total phosphorus (TP) and inorganic phosphorus (IP) in freeze-dried sediment samples were determined according to Aspila et al. (1976). Phosphorus concentrations in the extracted solutions were measured using a Shimadzu UV-VIS Spectrophotometer. Certified reference sediments PACS-2 (Canadian Institute for National

Table 1
Anthropogenic activities in seven selected polluted sampling areas along the eastern Adriatic coast and the main groups of pressures defined according to ANNEX III of MSFD (2008/56/EC).
*Indicative lists of characteristics, pressures and impacts (referred to in Articles 8 (1), 9 (1), 9 (3), 10 (1), 11 (1) and 24) art.

		Anthropogenic activities	Pressures defined according to MSFD (2008/56/EC) ANNEX III*
Sampling area	Port of Pula (PU1-PU7)	Passenger port terminal (ferries and small boats) Shipyard (est. 1856) Marina Discharge from smaller recreational vessels and former municipal wastewater discharge (until 2015)	Systematic and/or intentional release of substances Nutrient and organic matter enrichment Contamination by hazardous substances Biological disturbance
	Raša Bay (RA1-RA10)	Port terminal (general cargo, timber and livestock) Small urban discharge and discharge from smaller recreational vessels Small marina, small port Aquaculture Runoff from agricultural areas (estuary)	Systematic and/or intentional release of substances Nutrient and organic matter enrichment Biological disturbance
	Port of Rijeka (RI1-RI5)	The third biggest city in Croatia Passenger port terminal (cruise ships, ferries and small boats) Biggest national cargo port Ballast water discharges and small urban discharges Former phosphorous transshipment operations and industries discharges	Nutrient and organic matter enrichment Contamination by hazardous substances Biological disturbance
	Bakar Bay (BA1-BA11)	Former coke plant Petroleum refinery Tanker berth Terminal for bulk cargo (iron ore, coal and other bulk cargoes) Urban discharges from wider area	Systematic and/or intentional release of substances Contamination by hazardous substances Biological disturbance Other physical disturbance (underwater noise; marine litter)
	Šibenik Bay (SI1-SI7)	Passenger port terminal and marina Discharge from smaller recreational vessels Shipyard (est. 1992) Terminal for bulk cargo Former phosphorous transshipment operations and former industry ferrous alloy production factory (until 1994) Former municipal wastewater discharge (until 2007)	Nutrient and organic matter enrichment Contamination by hazardous substances Biological disturbance Other physical disturbance (underwater noise; marine litter)
	Vranjic Basin (VR1-VR9)	Former industrial and municipal wastewater discharge (until 2005) Former chemical industry (until 1991) Shipyard and multipurpose container cargo terminal Grain terminal Dry cement manufacturing Small marina, discharge from smaller recreational vessels Freshwater inflow contamination by a number of sewage outfalls Septic tanks and small urban discharges Runoff from agricultural areas	Nutrient and organic matter enrichment Contamination by hazardous substances Systematic and/or intentional release of substances Biological disturbance Other physical disturbance (underwater noise; marine litter)
	Port of Split (ST1-ST10)	The second largest city of Croatia Passenger port terminal (cruise ships, ferries, small boats, yachts) and multipurpose cargo ports Marina and discharge from smaller recreational vessels Municipal wastewater discharge (city overflow) Third largest passenger port in the Mediterranean and largest in Croatia Shipyard (est. 1932) Natural sulfur spring	Nutrient and organic matter enrichment Contamination by hazardous substances Biological disturbance Other physical disturbance (underwater noise; marine litter)

Measurement Standards NRC-CNRC) and estuarine NIST 1646a (National Institute of Standards and Technology) were used for the method evaluation.

2.2.5. Determination of mercury in sediments

The concentration of mercury in air-dried sediment samples (expressed in mg/kg d.m.) was analyzed by the Institute of Public Health of Primorje-Gorski Kotar County according to the "in-house" accredited method M 146–200 (edition 1, 15.11.2019) (Adapted method according to the producer manual: AMA 254 Advanced Mercury analyzer Operation manual, 2002) on an instrument AMA254 Mercury Analyzer. The accuracy control was performed with each set of samples, using certified reference material IAEA 405 (International Atomic Energy Agency, Vienna, Austria) and reference material IAEA-MEL-2017-01-TE (International Atomic Energy Agency, Vienna, Austria). Recovery ranged from 85 to 105 %.

2.3. Determination of the local enrichment factors (LEF)

To estimate the level of contamination in terms of metal(*oid*)s in the sediments, the concept of the local enrichment factor (LEF) was used (Álvarez-Vázquez et al., 2023; Lučić et al., 2023). This concept distinguishes between natural and anthropogenic origin of elements as it proved to be a good tool to reduce the natural (background) factors that can significantly influence the element concentrations in sediments such as grain-size effect, effect of dilution by a predominant matrix phase and the different provenances (Matys Grygar and Popelka, 2016; Birch, 2017; Lučić et al., 2023). The background used for the calculation of LEF came from the huge dataset measured in the Adriatic Sea since 2012 as a part of the monitoring program that Croatia is carrying out in connection with the implementation of the WFD and MSFD and have been published as a part of different studies (Cukrov et al., 2011; Cukrov et al., 2014; Cukrov et al., 2024; Felja et al., 2016; Fiket et al., 2021; Surricchio et al., 2019; Ujević et al., 2000). The minimum, median and maximum concentrations of the measured elements in the reference stations used as background are provided in the Supplementary material (**Table S2**). The LEF method was calculated according to the formula: $LEF = E/E_{BN}$; $E_{BN} = f(E_{REF})$, where E_{BN} stands for background normalization and E_{REF} for the reference element. In this way, the LEF was determined by the empirical background function $f(E_{REF})$, which best describes the relationship between the target (As, Ba, Bi, Cd, Co, Cu, Cr, Mn, Ni, Pb, Sb, Sn, U and Zn) and the predictive element (Al). Aluminum was chosen as an important constituent of the main carriers of potentially toxic elements (PTEs) and the element with which they correlate

best (**Fig. S1**).

2.4. Microtox acute toxicity test

The potential toxicity of sediment organic extracts was determined by Microtox© bioassay (Bihari et al., 2007; Fafanđel et al., 2015). Organic sediment (50 g) extracts were prepared according to Bihari et al. (2007) and dissolved in DMSO (50 μL). The potential toxicity was measured as the decrease of the bacterial (*Aliivibrio fisheri*) luminescence after exposure to a series of 1:2 dilutions of organic extract according to the BioFix_Lumi procedure prescribed by manufacturer Macherey-Nagel, Germany, in Microtox_Model 500 luminometer (AZUR, Environmental, U.S.A.). Estimates of EC_{50} (mg) were obtained using MicrotoxOmniTM Software package and toxicity was expressed as $1/EC_{50}$ x 1000.

2.5. Determination of the different levels of anthropogenic disturbance

An unsupervised k-means method was applied to the obtained dataset (parameters: LEFs of As, Ba, Bi, Cd, Co, Cu, Cr, Mn, Ni, Pb, Sb, Sn, U, Zn, TBT, TN, TP, Hg and toxicity level) to identify potential groupings of different sediment samples based on the presence and level of measured contamination within these samples (Xu et al., 2021). K-means clustering aims to divide the dataset into k non-overlapping clusters and assign each observation to the nearest center to maximize the between-cluster variance and minimize the within-cluster variance. Due to limitation of k-means clustering, i.e. poor performance when the variables differ in absolute frequency by several orders of magnitude and the data are highly skewed, data were log-transformed before k-means clustering is applied. To determine the optimal number of clusters, we used the Silhouette method. Details about the k-means algorithm can be found elsewhere (Xu et al., 2021; Ikotun et al., 2023). Groups of sediments obtained in this way were designated as representing sediments with different disturbance level (hereafter referred to as DL).

2.6. Taxonomic profiling of microbial communities in the sediment

2.6.1. DNA extraction and sequencing

Total DNA was extracted from 0.3 to 0.5 g of a wet sediment using the DNeasy® PowerSoil® Pro kit (QIAGEN), according to the manufacturer's protocol. Quantity of the extracted DNA was determined with the QUBIT fluorometer (Thermo Fisher Scientific) and quality both by Biospec Nano (Shimadzu) and agarose gel electrophoresis. Three different marker genes were selected for amplicon sequencing using extracted total DNA as a template: (i) the V4 region of the 16S rRNA gene (300 bp, primers 515F 5′-GTGCCAGCMGCCGCGGTAA-3′ and 5′-806R GGACTACHVGGGTWTCTAAT-3′) targeting bacteria and archaea, (ii) the V7-V8 region of the 18S rRNA gene (390 bp, primers FF390 5′-CGATAACGAACGAGACCT-3′, FR1 5′-ANCCATTCAATCGGTANT-3′) targeting fungi (Banos et al., 2018) and (iii) the V9 region of the 18S rRNA gene (200 bp, primers 1391F 5′-GTACACACCGCCCGTC-3′, EukB 5′-TGATCCTTCTGCAGGTTCACCTAC-3′) targeting protists in sediments (Stoeck et al., 2010). The derived amplicons were sequenced by Novogene Bioinformatics Technology Co., Ltd., Beijing, China using the Illumina NovaSeq PE250 platform.

2.6.2. Amplicon data analysis

Amplicon data analysis was performed using the 'Quantitative Insights Into Microbial Ecology 2' (QIIME2) software (Bolyen et al., 2019), release 2022.2. Raw, demultiplexed paired-end fastq files were imported into QIIME2 using the manifest file. The imported sequences were denoised, dereplicated and filtered for chimeras using the DADA2 plugin (Callahan et al., 2016). The resulting amplicon sequence variants (ASVs) were aligned using mafft and used to construct a phylogenetic tree using fasttree2 via the q2-phylogeny plugin. Taxonomy was assigned to ASVs using a pre-trained Naïve Bayes classifier. As a referent taxonomic database RESCRIPt (Robeson II et al., 2021) processed Silva v138 (Quast et al., 2013) database clustered at 99 % sequence similarity was used.

Visualization of the sequencing data was performed in RStudio using R software (version 4.2.2). Alpha and beta diversity were analyzed using the *phyloseq* package (McMurdie and Holmes, 2013) and the results were visualized using the *ggplot2* package (Wickham, 2016). The average relative abundance was calculated for each disturbance level and used for further analysis and visualization using the *ggplot2* in RStudio using R software (version 4.2.2). To investigate community-wide differences in taxonomy and abundance, we used a non-metric multidimensional scaling analysis (NMDS) based on a Bray-Curtis dissimilarity distance calculated with the package *vegan* (Oksanen et al., 2019). Ordinations were performed at the family level and analyzes of variance were performed using distance matrices with the ADONIS function from the *vegan* package to test for differential abundances of microbial composition between different sampling sites in the northern and southern Adriatic sampling areas (Anderson, 2001).

2.7. Identification of potential indicators in sediments

To uncover potential microbial indicators, we employed DESeq2 package (version 1.14.1) in R (Love et al., 2014) and Classification and Regression Tree (CRT) methodologies (Alkhasawneh et al., 2014). DESeq2 offers a more nuanced view of abundance changes at a finer taxonomic resolution, while CRT provides a broader, model-driven understanding of environmental impacts on microbial communities. DESeq2 uses a model based on negative binomial distribution, which is particularly suited for counting data like those from sequencing experiments, identifying changes in microbial abundance related to different environmental conditions. We employed DESeq2 to analyze the differential abundance of ASVs across different sampling sites that allowed us to identify specific microbial families or ASVs that are distinctly abundant (i) within different DLs and (ii) between contaminated (integrating mild, medium, high and extreme DL) and non-contaminated samples (low DL). CRT, on the other side, focuses on creating predictive models for classifying and prioritizing data as "key indicator variables". CRT employs a decision tree approach, partitioning the dataset based on informative environmental variables to create a hierarchical structure of decision rules (Alkhasawneh et al., 2014). We used CRT to identify specific taxonomic groups as "key indicator variables" that show ability to indicate different DLs.

3. Results

3.1. Grouping of sediment samples into different levels of anthropogenic disturbance

All environmental data measured in the 67 sediments are published as original numerical data at Mendeley Data (Ramljak and Petric, 2024). In addition, results of the sediment grain size analysis is shown in **Fig. S2**. The k-means clustering was performed on all data to group the 67 analyzed sediment samples into different categories of anthropogenic disturbance levels. Values of the measured geochemical parameters are presented depending on the five defined anthropogenic disturbance levels (given in ranges, from minimum to maximum values) in **Table S3**. Prior to clustering procedure, we first calculated local enrichment factors (LEFs) for potentially toxic elements (As, Ba, Bi, Cd, Co, Cu, Cr, Mn, Ni, Pb, Sb, Sn, U and Zn) and obtained results were compiled with other chemical parameters such as TBT, TN, TP, Hg and toxicity level. These input parameters revealed that the sediments could be classified into five distinct categories according to k-means clustering, which we consider to represent five different anthropogenic DLs (Fig. 2): low (16 samples), mild (19 samples), medium (18 samples), high (10 samples) and extreme (4 samples). Samples from Raša Bay were classified as samples under low DL, while the majority of samples from Bakar and

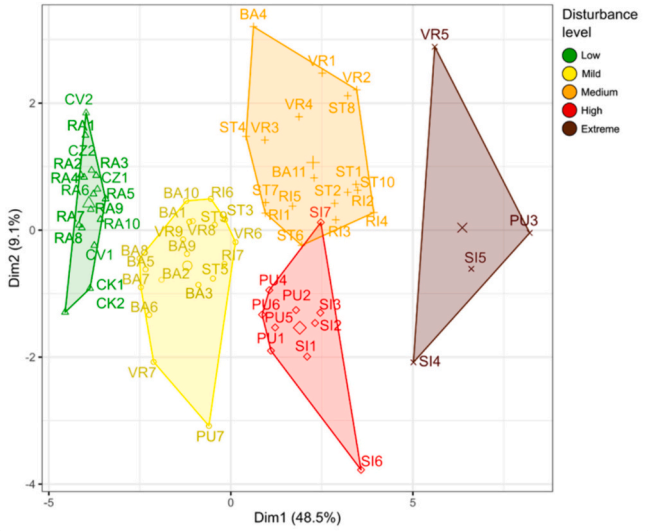

Fig. 2. K-means clustering analysis showing groupings of 67 collected sediments into five different levels of anthropogenic disturbance: low, mild, medium, high and extreme. Sediments are marked as follows: port of Pula = PU, port of Rijeka = RI, Raša Bay = RA, Bakar Bay = BA, Šibenik Bay = SI, Vranjic Basin = VR, port of Split = ST, Cape Kamenjak = CK, Zlarin Island = CZ and Vis Island = CV.

Split clustered as mild and medium DL, respectively. Samples from Vranjic Basin showed no clear geographical grouping, while samples from the port of Pula and Šibenik Bay were assigned as under high or extreme DL.

3.2. Characterization of the benthic microbial community

Of the total 67 sediments collected, DNA was successfully extracted from 55 sediment samples. High-throughput sequencing on the Illumina NovaSeq PE250 sequencing platform yielded a total of 10,464,885 high-quality reads which were assigned to 93,914 ASVs with details provided in **Table S4**. Raw sequence reads were deposited into European Nucleotide Archive (ENA) under project PRJEB72621.

3.2.1. Community composition of prokaryotes, protists and fungi

Community composition of prokaryotes (bacteria and archaea), protists and fungi was analyzed at the genus (**Fig. S3A, B and C**), family (**Fig. S4A, B and C**) and phylum level (**Fig. S5A, B and C**). A complete list of identified organisms at the family level can be found in **Table S5**.

For prokaryotes, we identified 53 genera and 68 families belonging to 19 different phyla. The most abundant phylum was Proteobacteria (relative abundance of avg. 34.5 % ± 5.2) followed by Desulfobacterota (14.6 % ± 3.29), Actinobacteriota (9.2 % ± 2.6), Acidobacteria (5.72 % ± 1.88) and Chloroflexi (5.69 % ± 1.65). The unclassified prokaryotes at the genus level accounted for 31.85 % (± 5.89) in addition to 53 identified genera. The most dominant genera were *B2M28* (3.53 % ± 1.48), *Sulfurovum* (2.15 % ± 3.44), *Sva0081* sediment group (2.14 % ± 0.61) and *Woeseia* (3.54 % ± 1.49). The highest relative abundances at the family level were observed for families B2M28, Desulfocapsaceae, Desulfosarcinaceae, Unclassified Actinomarinales, Unclassified Gammaproteobacteria, and Woeseiaceae. Families showing increasing or decreasing pattern of relative abundance between the DLs were singled out (Fig. 3, **Table S6**). Bacillaceae, Burkholderiaceae, Lachnospiraceae, Pseudomonadaceae and Xanthobacteraceae showed a decreasing pattern toward the more severe anthropogenic disturbance i.e. being presented in highest relative abundance in low DL. Conversely, Ectothiorhodospiraceae, Rhodobacteraceae and Cyclobacteriaceae showed highest relative abundance in extreme DL.

Regarding protists, 50 genera along with 46 families belonging to 17 phyla were identified. Unassigned sequences (designated as "Unclassified Eukaryota") represented the highest percentage of the community and accounted up to 33.87 % (± 12.95) of the total community. Dinoflagellata and Diatomea represented the most dominant phyla with relative abundances of avg. 20.28 % (± 12.51) and 14.75 % (± 9.5), respectively. At the family level, in which ASVs represented with <1 % accounted for up to 23 % of the total community, most abundant community members included Bacillariophyceae, followed by Unclassified Dinophyceae, Thoracosphaeraceae, Gymnodinium clade, Mediophyceae and Suessiaceae. The "Unclassified Eukaryota" at the genus level accounted for avg. 34.05 % (± 13.02) with additional avg. 20.97 % (± 6.89) of protist sequences unidentified ("Unclassified" protists (data not shown)). Genera *Scrippsiella* (avg. 3.52 % ± 2.12), *Novel Apicomplexa Class 2* (avg. 2.64 % ± 4.55), *Gymnodinium* (avg. 2.41 % ± 3.55) and *Skeletonema* (avg. 2.23 % ± 4.64) dominated the protist community. Even though we observed increasing relative abundances for families Phyllopharyngea and Unclassified Chrysophyceae at the extreme DL (Fig. 3), detailed analysis showed inconsistency, confirmed with statistical analyses (*p*-values 0.182 and 0.053), with high abundances detected only in one sample (sample from PU and SI, respectively) belonging to this category (data not shown). A similar observation was evident for Eugregarinorida (Fig. 3) being highly enriched only in one reference sample from Zlarin island (data not shown, *p*-value 0.373).

Regarding fungi, a total of 53 genera and 51 different families were detected in the sediment samples belonging to 9 different phyla. Again, unassigned eukaryote sequences made up most of the community (36 % - 56 % relative abundance). The two most abundant phyla were Ascomycota and Basidiomycota, which accounted for avg. 25.58 % (± 11.62) and avg. 9.58 % (± 7.59) of the identified sequences, respectively. At the family level, Metschnikowiaceae, Incertae Sedis Cryptomycota and Unclassified Pleosporales prevailed. Families associated with different DLs are presented in Fig. 3 and included Aigialaceae, Aspergillaceae, Unclassified Polyporales and Unclassified Sordariomycetes, all showing highest relative abundance in the low DL samples, while Chytridiomycetes and Gromochytriaceae showed highest relative abundance in the extreme DL samples. In average 46.68 % (± 20.40) of eukaryote sequences at the genus level were not classified. Additionally, avg. 17.08 % (± 10.36) of fungal sequences were not classified, with the most dominant genera being *Metschinkowia* (7.11 % ± 5.85) and *Paramicrosporidium* (2.98 % ± 3.02).

3.2.2. Diversity of microbial communities in the sediment samples

Alpha and beta diversity were determined separately for prokaryotes, protists and fungi. Alpha diversity indices including taxonomic richness index (observed ASVs), Shannon's diversity index, and Pielou's evenness are presented in **Tables S7A, B and C.** For each of the three microbial communities, the Shannon's diversity index was used to compare the diversity in the different levels of anthropogenic disturbance (Fig. 4). For bacteria and archaea (Fig. 4A), the Shannon's diversity index showed no significant difference between all five DLs (Kruskal-Wallis: *p*-value = 0.2553). Among protists (Fig. 4C), a significant difference in Shannon's diversity index was found between the following categories: low and mild (Kruskal-Wallis: *p*-value = 0.00017), low and medium (Kruskal-Wallis: *p*-value = 0.02201) and low and high (Kruskal-Wallis: *p*-value = 0.00038). Finally, a significant difference in the alpha diversity of the fungal community was recorded only between the mild and medium DL categories (Fig. 4E) (Kruskal-Wallis: *p*-value = 0.042).

PCA analysis for prokaryotes, protists and fungi, indicated an overall significant effect (ADONIS: *p*-value = 0.001) of the five DLs on their beta diversity (Fig. 4B, D and F). The highest percentage of variance explained by DLs was evident for prokaryotes ($R^2 = 0.22$), followed by fungi ($R^2 = 0.17$) and protists ($R^2 = 0.16$). However, pairwise PERMANOVA analysis revealed that only prokaryotes showed a direct correlation with the defined DLs, with the low DL, and the high DL differing significantly from all other DLs (**Tables S8A, B and C**).

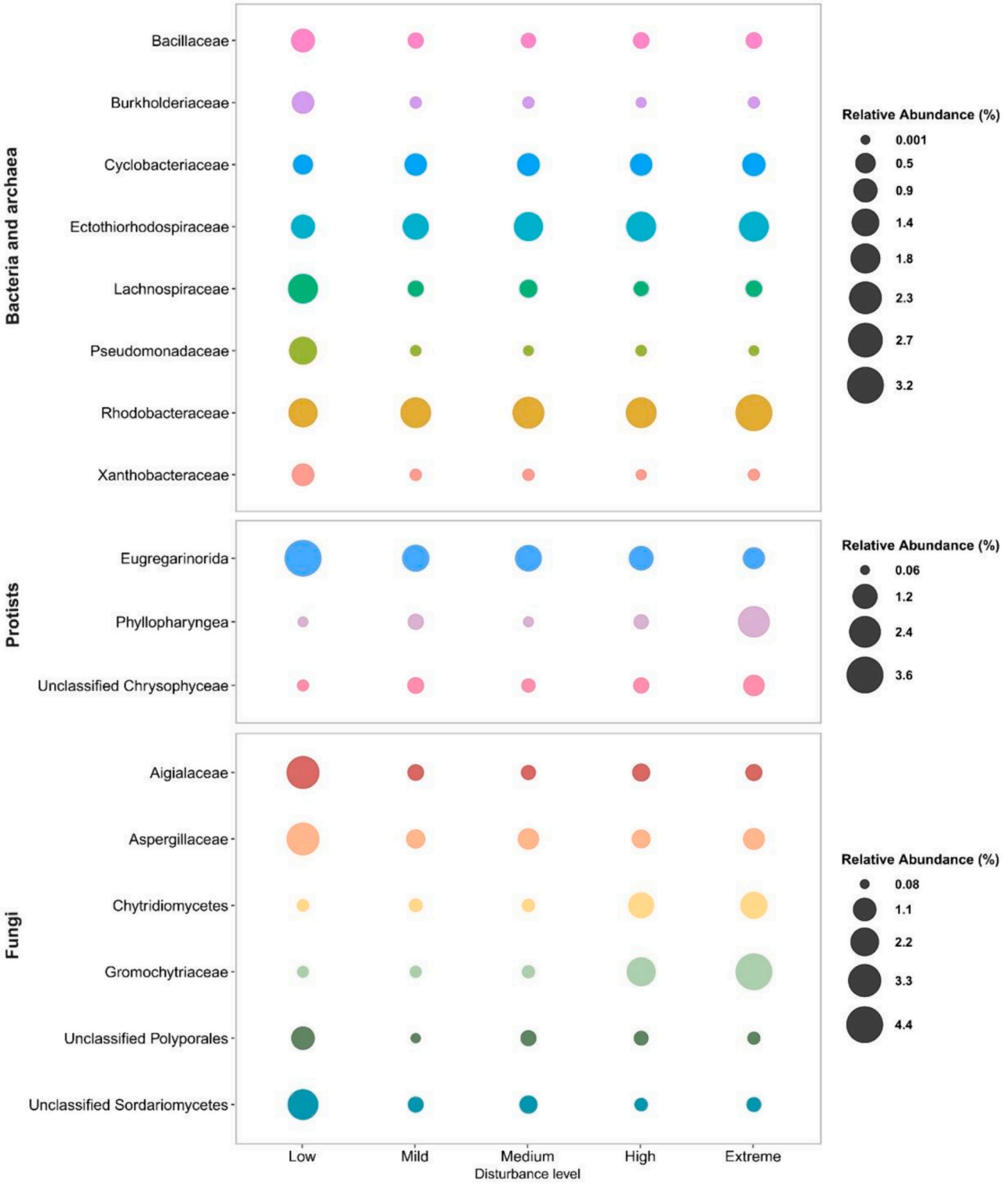

Fig. 3. Bubble plot showing relative abundances (%) of selected families of prokaryotes (bacteria and archaea), protists and fungi showing patterns of increasing or decreasing relative abundance in sediment samples across different disturbance levels (DLs): low, mild, medium, high and extreme.

3.2.3. Similarities among sediment samples based on the sampling area

We further assessed if the sediment microbiota showed geographical signatures. NMDS ordination biplots for each of the three groups of microbial communities in seven sampling areas (Bakar Bay, port of Pula, Raša Bay, port of Rijeka, Šibenik Bay, port of Split and Vranjic Basin) and three reference areas clustered according to the sampling area (ANOSIM: prokaryotes - $R = 0.558$, $p < 0.0001$; protists - $R = 0.572$, $p < 0.0001$; fungi - $R = 0.645$, $p < 0.0001$) (Fig. 5). A further look at the analysis revealed further clustering of the samples at a wider geographic level with samples from the northern (Bakar, Pula, Rasa and Rijeka) and the southern (Šibenik, Split and Vranjic) Adriatic region showing clear groupings (ANOSIM: prokaryotes - $R = 0.239$, $p < 0.0001$; protists - $R = 0.375$, $p < 0.0001$; fungi - $R = 0.452$, $p < 0.0001$).

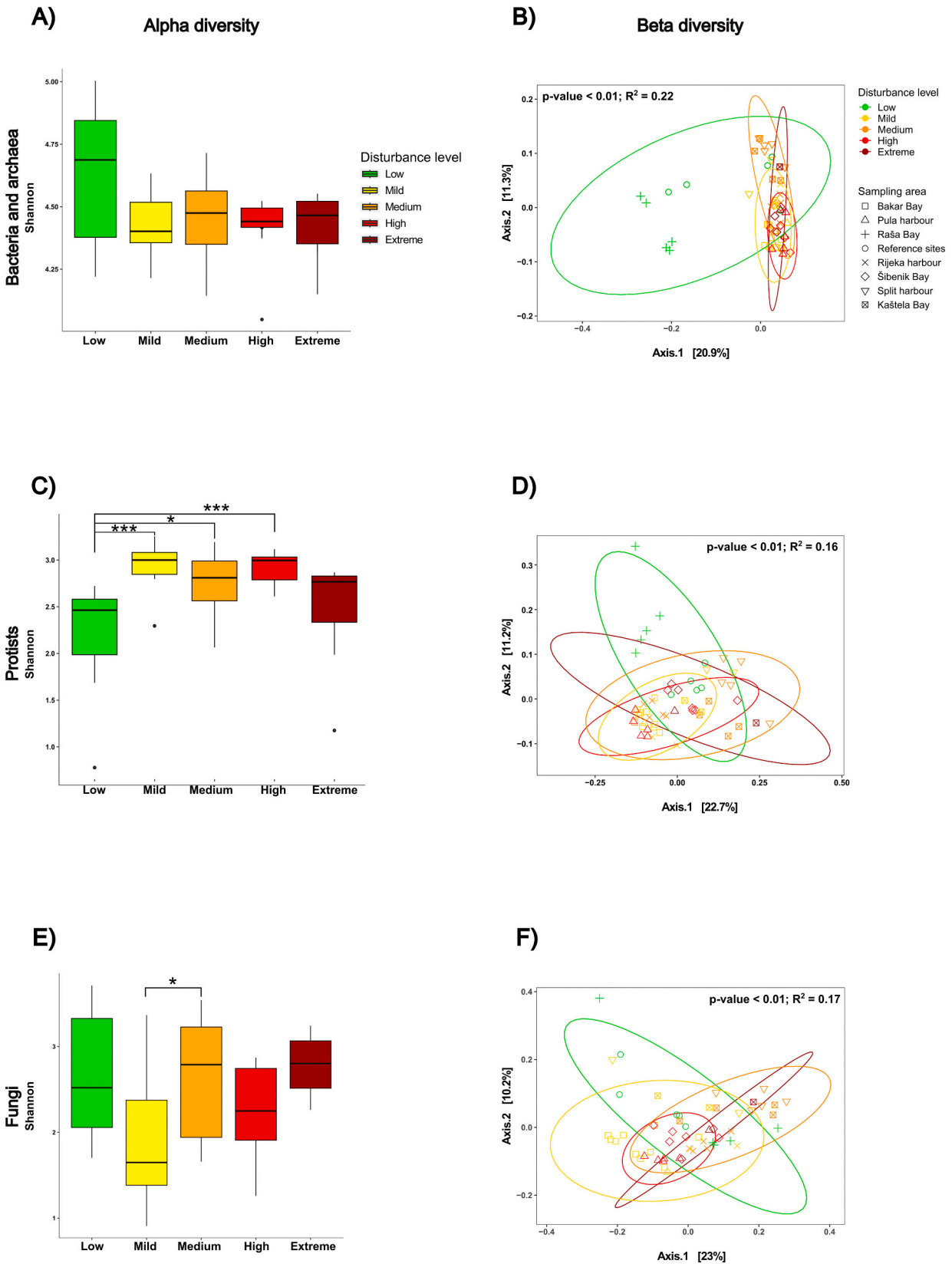

Fig. 4. Analysis of the alpha (Shannon diversity index) and beta diversity of prokaryotes (A and B), protists (C and D), and fungi (E and F) in the different sediment samples according to their disturbance level (DL). The Kruskal-Wallis non-parametric test was used to determine significant differences between the different DLs and the resulting *p*-values were corrected for multiple testing using Benjamini-Hochberg correction.

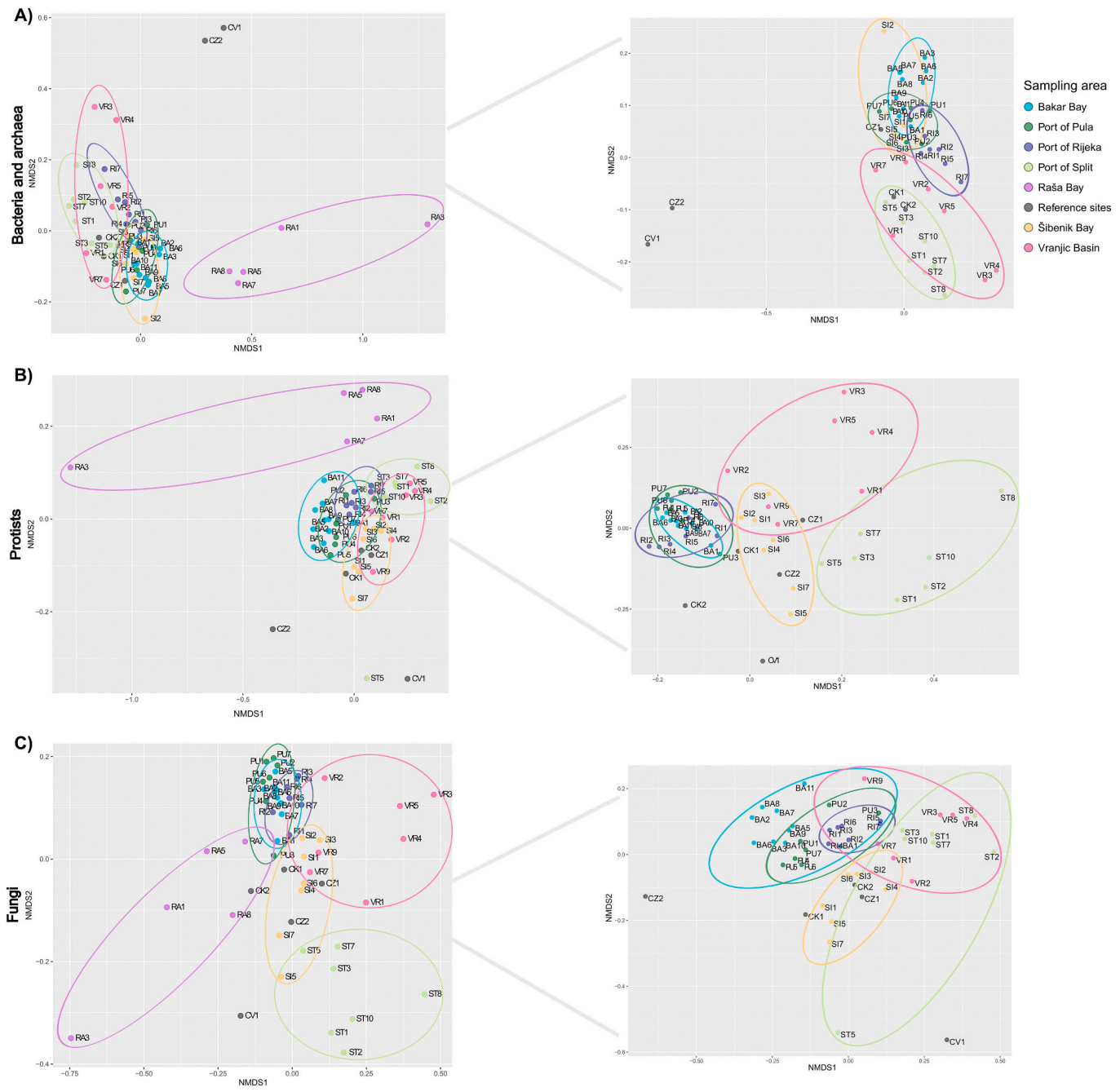

Fig. 5. Non-metric multidimensional scaling (NMDS) ordinations based on Bray-Curtis dissimilarity showing clustering of the prokaryotic (bacteria and archaea) (A), protist (B) and fungal (C) communities according to the sampling areas monitored. On the right side of the panel additional NMDS for all three communities are shown but without samples from Raša Bay area, for better clarity.

3.3. Proposal of specific indicators using DESeq2

By using the DESeq2 method we identified the 20 most significant ASVs per studied microbial domain that could differentiate sediment samples of the different DLs, as well as those contaminated vs. non-contaminated (Fig. 6A and B**, Fig. S6**). In the case of protists, the analysis showed no clear distinction regarding neither different DLs nor contamination vs. non-contamination. For the prokaryotic community (Fig. 6A) differentiation was observed between low DL and all other samples i.e. non-contaminated and contaminated samples. We selected seven ASVs as the potentially most important indicators that included bacteria belonging to the genera *Thiogranum* (ASV5044), *B2M28* (ASV4772 and ASV4766), *Sva0485* (ASV5571), *Boseongicola* (Rhodobacteraceae) (ASV4491), *Subgroup 23* (Thermoanaerobaculaceae) (ASV347) and uncultured gammaproteobacterium (ASV5345), shown to be abundant exclusively at contaminated sites and five ASVs including genera *Lactobacillus* (ASV2587), *Ralstonia* (ASV4806), *Burkholderia* (ASV4796), *Pseudomonas* (ASV5274) and an unidentified member of the family *Lachnospiraceae* (ASV2830), detected in the majority of the non-contaminated sediment samples. Differentiation for the fungal community was observed for three ASVs belonging to genera *Paramicrosporidium* (ASV17), uncultured *Basidiobolus* (ASV332) and *Chytridiomycetes* (ASV270) identified only at contaminated sites. On the other hand, three ASVs were recorded almost exclusively at non-contaminated sites and included genus *Aigialus* (ASV755), an unidentified genus belonging to class *Sordariomycetes*

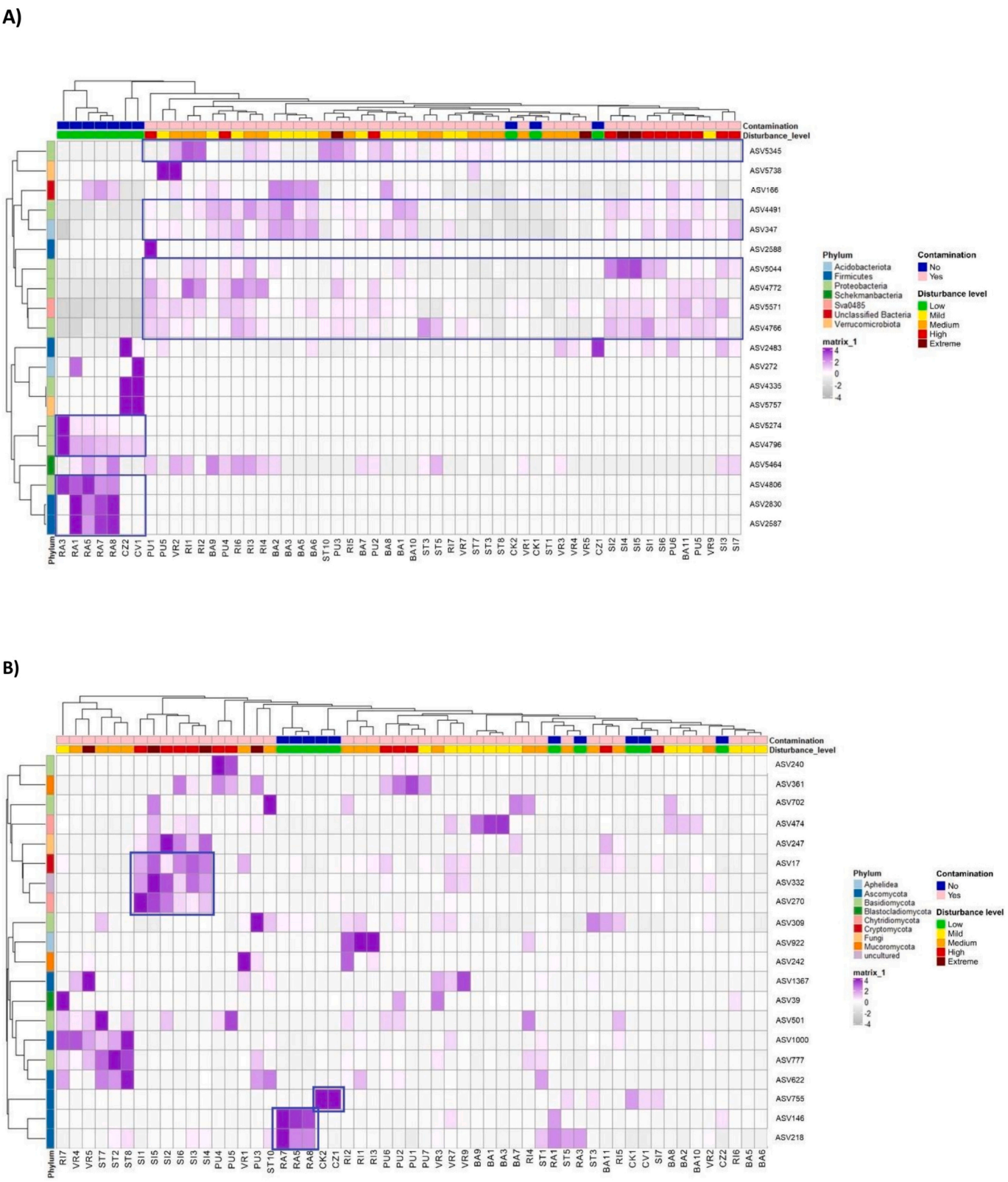

Fig. 6. Heatmap of the 20 most important amplicon sequence variants (ASVs) selected for prokaryotic (A) and fungal (B) communities showing clustering between contaminated and non-contaminated samples and between samples grouped in five different disturbance levels. Heatmap data were generated using DESeq2 analysis ($p_{adj} < 0.05$). Each row represents the taxonomic assignment for each ASV (details in Tables S8A, B and C). The color scheme ranges from gray (low abundance) to purple (high abundance). ASVs selected as important are highlighted in squares. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(ASV146) and genus *Aspergillus* (ASV218) (Fig. 6B). The complete list of taxa belonging to each ASV with their taxonomic affiliations for all three microbial communities can be found in **Tables S9A, B and C**.

3.4. Proposal of the groups of indicator microorganisms using CRT

CRT analysis led to the identification of 'key indicator variables' i.e. specific taxonomic groups whose presence and relative abundance can be correlated with the previously defined DLs. The taxonomic groups highlighted in Table 2 are those that define the specific DL with 100 % certainty and occur in >50 % of the samples belonging to the specific DL. Detailed results of the analysis can be found in **Fig. S7A, B and C**. For the bacterial and archaeal dataset, the CRT analysis identified seven families (*Bacillaceae, Sulfurovaceae, Thiotrichaceae, Thermoanaerobaculaceae, Marine Benthic Group D and DHVEG-1, Saprospiraceae* and *Nitrosopumilaceae*) as the most significant for classifying samples into the five DLs. Results showed that each of the five DLs can be described with a combination of up to four of the above-mentioned families (i.e. key indicator variables) that need to be present in sediment in defined relative abundances. Even though among protists, nine most significant families were singled out as key indicators (*Dinophysiales, Suessiaceae, Trebouxyiophyceae, Heteronematina, Prostomatea, Pseudoperkinsidae, Euglyphida,* unclassified *Cercozoa* and Unclassified *Apicomplexa*) detailed examination revealed difficulties in data interpretations, with no logical grouping of protist families (i.e. key indicator variables) depending on the DLs. For example, similarity between key indicator variables was found for low, medium and extreme DL categories (having three mutual familes), when compared to high and mild DL categories. For fungi, CRT analysis identified key indicator variables only for low, mild and medium DL and therefore this was not further explored.

4. Discussion

To establish a link between benthic environmental conditions and microbial community structure and diversity, we analyzed both the chemical and microbiological components of sediment samples collected along the Adriatic coast in areas impacted by various anthropogenic activities (marina, industry, tanker berth and terminals, shipyards, urban discharges, wastewater discharge, etc.). Compared to similar studies focused on a specific location (Chen et al., 2019; Dell'Anno et al., 2021; Caruso et al., 2022; Wu et al., 2023), our approach aimed to identify a generalized response of benthic communities to anthropogenic pressures that could be used as a universal tool to determine the ecological status of the marine environment. Our findings of high and extremely high concentrations of heavy metals, TBT, and an unknown mixture of toxic organic pollutants in the sediment samples from certain sites (defined in the Norwegian Environmental Quality Classification System (Bakke et al., 2010) confirmed that these sites were highly polluted environments. As the focus of our research was on the microbial communities, the chemical dataset obtained was mainly used as a basis to cluster the collected sediments into categories reflecting different anthropogenic DLs in the selected areas. The microbial sequencing data were plotted against the DLs to investigate the relationship between the degree of anthropogenic disturbance and the changes in the benthic microbial communities. In addition to chemical factors, complex interplay between microorganisms belonging to either similar or different trophic levels also influences microbial diversity (Worm and Duffy, 2003). Considering the microbial interactions in polluted sediments our study included three benthic microbial communities – prokaryotes, protists, and fungi. Although bacteria, protists and fungi are known to play an important role in the microbial food web, biogeochemical cycling, biological carbon pump and pollutant removal, the latter two have rarely been studied in the context of marine sediment pollution. To date, we have not found a study that includes all three communities in their investigation, particularly in relation to sediment pollution and the MSFD.

Surprisingly, our findings show a high diversity of benthic microbial communities regardless of the level of anthropogenic disturbance. This has already been suggested in previous studies where microbial communities were unaffected by sediment contamination (Gillan et al., 2005., Dell'Anno et al., 2021), with neutral or even positive effects on community diversity (Chen et al., 2019; Johnston and Roberts, 2009).

Table 2
Key indicator variables, identified based on the Classification and Regression Tree analysis, for each of the three microbial communities (Bacteria and Archaea, Protists and Fungi). Presence and relative abundance of a group of key indicator variables define specific level of anthropogenic disturbance within the sediment (low, mild, medium, high and extreme). NI – 'key indicator variables' not identified in >50 % of samples. Key indicator variables shown in bold represent those common for more than two DLs.

		Bacteria and Archaea		Protists		Fungi	
		Key indicator variable	Defined limit of relative abundance	Key indicator variable	Defined limit of relative abundance	Key indicator variable	Defined limit of relative abundance
Disturbance level	Low	***Thiotrichaceae***	≤ 1.964	***Dinophysiales***	≤ 0.548	*Rynchogastremataceae*	≤ 0.282
		Bacillaceae	**> 0.353**	***Suessiaceae***	≤ 4.539	unclassified	> 8.666
				Trebouxyiophyceae	≤ 0.389	*Pleosporales*	
		Thermoanaerobaculaceae	≤ 2.610	*Heteronematina*	≤ 1.952		
	Mild	***Thiotrichaceae***	≤ 1.964	***Dinophysiales***	≤ 0.548	*Rynchogastremataceae*	≤ 0.282
		Bacillaceae	≤ 0.353	***Suessiaceae***	≤ 4.539		
		Sulfurovaceae	**> 0.813**			unclassified	≤ 8.666
		Marine Benthic Group D and DHVEG-1	≤ 0.096	*Prostomatea*	≤ 1.264	*Pleosporales*	
	Medium	***Thiotrichaceae***	**> 1.964**	***Dinophysiales***	≤ 0.548	*Rynchogastremataceae*	> 0.282
				Suessiaceae	≤ 4.539		
		Saprospiraceae	> 0.138	***Trebouxyiophyceae***	> 0.389		
				Pseudoperkinsidae	> 0.231	*LKM15*	≤ 3.315
				Euglyphida	≤ 0.275		
	High	***Thiotrichaceae***	≤ 1.964	***Dinophysiales***	≤ 0.548	NI	
		Bacillaceae	≤ 0.353				
		Sulfurovaceae	**≤ 0.813**	unclassified *Cercozoa*	≤ 0.514		
		Thermoanaerobaculaceae	≤ 4.453				
	Extreme	***Thiotrichaceae***	≤ 1.964	***Dinophysiales***	≤ 0.548	NI	
				Suessiaceae	≤ 4.539		
		Bacillaceae	≤ 0.353	***Trebouxyiophyceae***	> 0.389		
		Sulfurovaceae	**> 1.766**	*Pseudoperkinsidae*	≤ 0.231		
		Nitrosopumilaceae	> 0.039	Unclassified *Apicomplexa*	≤ 0.099		

On the contrary, an earlier study by Korlević et al. (2015) indicated a decrease in bacterial diversity in oil-contaminated sediments from the northern Adriatic Sea. This underlines the significance of our study which included several sites with different anthropogenic pressures, not just for the research in the Adriatic, but internationally, in order to gain a more comprehensive understanding of the diversity changes in benthic microbial communities. Overall, the highest diversity was recorded for prokaryotes, followed by protists and fungi. The lower diversity could be attributed to the poor database representation for both protists and fungi compared to the prokaryotic ones (Schoenle et al., 2021; Rojas-Jimenez et al., 2020). A significant change in alpha diversity was only observed in protists when contamination was considered, while no significant changes were observed for the defined DLs. Due to their short life cycle and quick response to environmental changes, many protists (mainly diatoms, foraminifera, and amoebae) are widely studied as bioindicators of contamination (Potapova and Charles, 2007). Still, invertebrates remain the most commonly used target organisms for benthic quality assessments (Hosokawa et al., 2021).

Considering that the biogeographic aspect of variability in microbial community composition has been proposed previously, although no consensus has been reached on the primary underlying processes (Li et al., 2021; Hortal, 2011), we found that benthic microbial communities exhibit strong geographic signatures. These observations can be partly linked to the geological characteristics of the sediments (grain sizes), which are mainly grouped by geographical location. However, studies also suggest that, as with macroorganisms, increasing geographic distance between microbial populations may contribute to the development of genetically distinct populations (Hortal, 2011). The effect of geographic distance, in addition to local environmental conditions, is often associated with historical events in the area (e.g. dispersal limitation), which could be the factor influencing the differences in microbial communities in our study (Plante et al., 2021). Xiong et al. (2014) found that geographical distance between the sites strongly impacted the benthic microbial structure, along with geochemical factors, although much of the variation remained unexplained.

In general, the microbial communities were dominated by Proteobacteria, Actinobacteriota and Desulfobacterota (bacteria), Ascomycota and Basidiomycota (fungi) and Bacillariophyceae (protists), which have been shown to predominate in benthic microbial assemblages (Sarma, 2019; Hoshino et al., 2020; Dell'Anno et al., 2021). To further determine the possible link between the degree of anthropogenic disturbance and the changes in the taxonomic structure, we filtered out the microbial families that showed a decrease or increase in their relative abundance depending on the defined DL. Accordingly, these families could be classified as sensitive or tolerant. Eventually, we found that rare (less abundant) families showed a better potential for indicating anthropogenically impacted sites, with some of them even proliferating in the disturbed environments. The importance of rare taxa in the context of contamination has already been suggested with the argument that members of the rare microbial community act as buffers against environmental disturbance and promote the stability of bacterial networks (Wu et al., 2023). Our study implies that microbes can only serve as indicators of contamination, i.e. when comparing contaminated (including mild, medium, high and extreme DL) and non-contaminated (including low DL) sediments, but not as indicators of a specific level of disturbance in the sediments. Rare families highlighted as possible indicators include the bacterial families *Rhodobacteraceae*, *Ectothiorhodospiraceae* and *Cyclobacteriaceae*, as potentially disturbance-tolerant populations, while *Bacillaceae*, *Burkholderiaceae, Pseudomonadaceae* and *Xanthobacteraceae* represent disturbance-sensitive populations. The selected tolerant bacterial families include sulfate and nitrate reducers, which are known to thrive in nutrient-rich marine environments under hypoxic or anoxic sediment conditions (Aylagas et al., 2017) and can also be associated with various pollutants (polyaromatic hydrocarbons, microplastics) (Garcés-Ordóñez et al., 2022; Wang et al., 2022).

DESeq2 analysis, which provides a more nuanced view of changes at a finer taxonomic resolution, allowed us to propose additional specific bacterial genera with a bioindicator potential. From the *Ectothiorhodospiraceae* and *Rhodobacteraceae* families mentioned above, members of *Thiogranum* and *Boseongicola* were selected as disturbance-tolerant bacteria, together with *Sva0485*, *Subgroup 23* and *B2M28*. The family *Thermoanaerobaculaceae* (genus *Subgroup 23*) includes anaerobic chemoheterotrophs found in hot freshwater springs, while *Sva0485* and *B2M28* have sulfate/iron-reducing and sulfate-oxidizing role in sediments, respectively. Rare fungal families sensitive to disturbance and showing potential indicator traits were *Aigialaceae*, *Aspergillaceae*, *Sordariomycetes* and *Polyporales*, and correspondingly the genera *Aigialus*, *Aspergillus* and unidentified *Sordariomycetes*. In addition, disturbance-tolerant families *Chytridiomycetes* and *Gromochytriaceae* along with the three uncultured genera *Paramicrosporidium*, *Basidiobolus* and *Chytridiomycetes* appeared in 40 % of highly contaminated samples. Given the lack of relevant studies on marine fungi and their relationship to disturbance in the benthic environment, identification at the genus level is not as reliable as for prokaryotes and the proposed fungal indicators should be treated with caution. Contrary to our expectations and their frequent use as contamination indicators (Desrosiers et al., 2013), the highly abundant protists *Bacillariophyceae* (diatoms) did not demonstrate indicator potential in our study. The studied sites have a long-term history of contamination, which could indicate that the communities have already adapted and are resistant to the various stressors in their environment or that they are highly resilient communities whose structure is maintained or restored even under prolonged disturbance (Nogales et al., 2011; Yin et al., 2015; Philippot et al., 2021; Shade, 2023).

Additionally, we used CRT on the sequencing data as a *de novo* testing approach to predict microbial community behavior in disturbed environments. Compared to DESeq2, CRT analysis provides a broader understanding of environmental influences on benthic microbial communities, but it requires a larger sample size for a reliable analysis (>1000 inputs). Still, using CRT we predicted a specific group of microbes that must be present (in precise relative abundance) to determine the level of sediment disturbance. This approach opened an additional perspective on the complexity of microbial communities while searching for bioindicators. The analysis predicted suppression of *Bacillaceae* in highly and extremely disturbed sediments with *Sulfurovaceae* being specifically enriched in extremely disturbed sediments. In addition, key indicator variables for highly and extremely disturbed sediments include *Thermoanaerobaculaceae* (highly suppressed) and *Nitrosopumilaceae* (enriched), respectively. Conversely, identifying *Bacillaceae* enrichment should indicate no disturbance in sediments. Interestingly, key indicator variables defining medium disturbance differed from others, both in relative abundance (*Thiotrichaceae*) and taxonomy (*Saprospiraceae*), which could possibly be explained by intermediate disturbance theory (Santillan et al., 2019). CRT-identified populations serve as a robust indicator of ecological impact, signifying that their precise relative abundance is strongly associated with a particular intensity of anthropogenic disturbance.

5. Conclusion

Our findings, which revealed a link between benthic environmental conditions and changes in microbial community structure, raised the question of microbes' potential contribution as a complementary tool to the currently used biotic indices for assessing the health of the benthic marine environment. We discovered specific microorganisms belonging to the "rare" community pools with altered abundances in areas subjected to long-term contamination pressure when compared to reference areas, with bacteria showing a greater potential as indicators. Finally, we proposed specific bacterial populations as potential benthic health indicators at two different taxonomic levels - family (Rhodobacteraceae, Ectothiorhodospiraceae, Cyclobacteriaceae) and genus (*Boseongicola*, *B2M28*, *Subgroup 23*, *Sva0485* and *Thiogranum*). Even though fungal

populations have also demonstrated their potential as disturbance indicators, the current lack of knowledge about marine fungi calls for new research studies to confirm this. Additionally, by employing predictive modeling and classification, we identified seven prokaryotic families: *Bacillaceae, Sulfurovaceae, Thiotrichaceae, Thermoanaerobaculaceae, Marine Benthic Group D and DHVEG-1, Saprospiraceae* and *Nitrosopumilaceae,* as the most significant key indicator variables for disturbed benthic environment. Nevertheless, further investigations are planned to determine whether, in addition to observed structural changes, disturbance has affected the functionality of the targeted benthic communities, whose lack of ecosystem services may have a domino effect on the entire marine environment.

CRediT authorship contribution statement

A. Ramljak: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **J. Žučko:** Writing – review & editing, Software, Methodology, Formal analysis. **M. Lučić:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **I. Babić:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis. **Z. Morić:** Writing – review & editing, Writing – original draft, Visualization, Software, Formal analysis. **M. Fafanđel:** Writing – review & editing, Methodology, Formal analysis. **M. Furdek Turk:** Writing – review & editing, Methodology, Formal analysis. **S. Matijević:** Writing – review & editing, Methodology, Formal analysis. **D. Karpouzas:** Writing – review & editing. **N. Udiković-Kolić:** Writing – review & editing. **I. Petrić:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2024.116649.

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