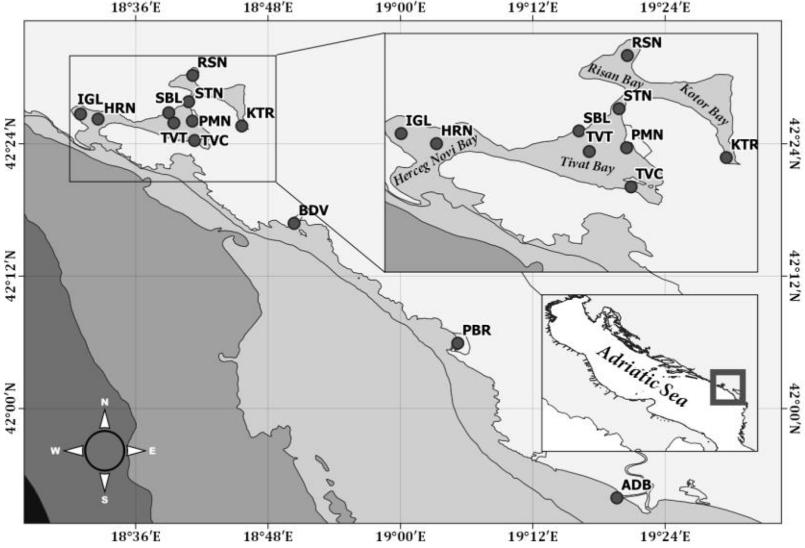
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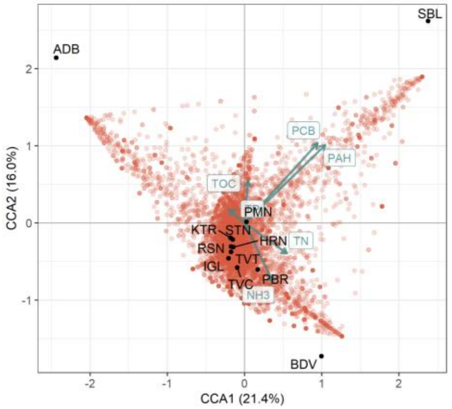
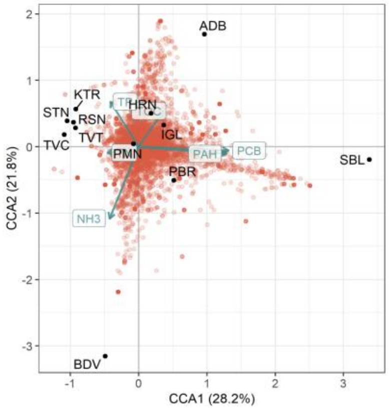
RSN
STN
SBL
IGL
HRN
PMN
KTR
TVT
TVC
BDV
PBR
ADB
Risan Bay
Kotor Bay
Herceg Novi Bay
Tivat Bay
Adriatic Sea
N
W
E
S


SBL
ADB
PCB
PAH
TOC
PMN
KTR
STN
RSN
HRN
TN
IGL
TVT
TVC
PBR
NH3
BDV
CCA1 (21.4%)
CCA2 (16.0%)
-2
-1
0
1
2


ADB
KTR
STN
RSN
TVT
TVC
HRN
IGL
PMN
PAH
PCB
SBL
PBR
NH3
BDV
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CCA2 (21.8%)
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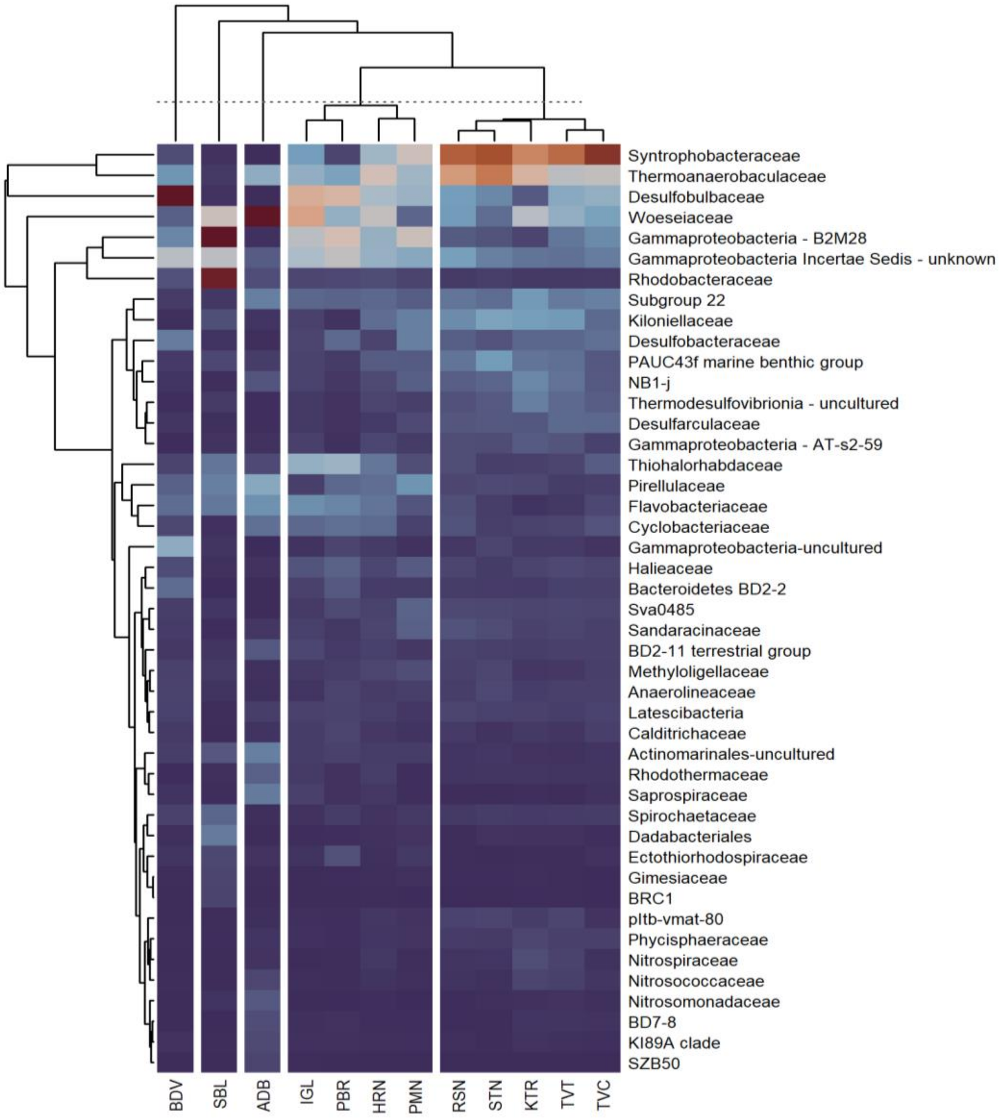
-2
-1
0
1
2
3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Desulfobulbaceae
Woeseiaceae
Gammaproteobacteria - B2M28
Gammaproteobacteria Incertae Sedis - unknown
Rhodobacteraceae
Subgroup 22
Kiloniellaceae
Desulfobacteraceae
PAUC43f marine benthic group
NB1-j
Thermodesulfovibrionia - uncultured
Desulfarculaceae
Gammaproteobacteria - AT-s2-59
Thiohalorhabdaceae
Pirellulaceae
Flavobacteriaceae
Cyclobacteriaceae
Gammaproteobacteria-uncultured
Halieaceae
Bacteroidetes BD2-2
Sva0485
Sandaracinaceae
BD2-11 terrestrial group
Methyloligellaceae
Anaerolineaceae
Latescibacteria
Calditrichaceae
Actinomarinales-uncultured
Rhodothermaceae
Saprospiraceae
Spirochaetaceae
Dadabacteriales
Ectothiorhodospiraceae
Gimesiaceae
BRC1
pltb-vmat-80
Phycisphaeraceae
Nitrospiraceae
Nitrosococcaceae
Nitrosomonadaceae
BD7-8
KI89A clade
SZB50
BDV
SBL
ADB
IGL
PBR
HRN
PMN
RSN
STN
KTR
TVT
TVC

Anthropogenic influence on the environmental health along Montenegro coast based on the bacterial and chemical characterization

Jokanović Sandra[a#], Kajan Katarina[b,c#], Perović Svetlana[d], Mačić Vesna[a], Orlić Sandi[b,c,*]

a - Institute of marine biology, University of Montenegro, 85 330 Kotor, Montenegro

b - Ruđer Bošković Institute, Division of Materials Chemistry, 10 000 Zagreb, Croatia

c - Center of Excellence for Science and Technology-Integration of Mediterranean Region (STIM), University of Split, 21 000 Split, Croatia

d - Faculty of Science and Mathematics, University of Montenegro, 81 000 Podgorica, Montenegro

[#] these two authors contributed equally

*Author for correspondence

Dr. Orlić Sandi

e-mail: sorlic@irb.hr

Abstract

Coastal marine sediments are particularly exposed to human activities. The function of a coastal ecosystem is largely affected by eutrophication, wastewater discharges, chemical pollution, port activities, industry and tourism. Bacterial classification can be used as a measure in assessing the harmful effects on the ecosystem. This study provided insight into the environmental health of the coastal region of Montenegro analyzing the possible impact of PAHs and PCBs upon the bacterial community diversity and function as well as nutrients. Two stations at the shipyards were defined as very high PAH polluted together with PCB concentration exceeding threshold values. The bacterial community at the OTU level clustered together all stations except the most polluted site (SBL), the main tourist destination in Montenegro (BDV) and estuary site (ADB) forming the independent clusters. Lowest richness and diversity were indicated at the site with the highest concentration of PAHs and PCBs with the highest abundance of *Alphaproteobacteria* followed by *Gammaproteobacteria*. OTUs affiliated to phyla *BRC1*, *Dadabacteria* and *Spirochaetes* were present with a total abundance higher than 1% only at the most polluted site indicating their persistence and possible potential for degradation of aromatic compounds. To compare functional capabilities potentially related to biodegradation of aromatic compounds and active transport systems, PICRUSt was used to predict metagenomes of the sediments. From our data, we identified specific bacterial community and predicted metabolic pathways that give us a picture of the environmental health along the coast of Montenegro, which provides us a new insight into human-induced pollution impacts on the coastal ecosystem.

Key words: marine sediment, pollutants, sequencing, PICRUSt, coastal health

Introduction

Nearly one-third of the world's population inhabits coastal areas and the near-coastal zone (Kummu et al., 2016). The function of the coastal ecosystem is largely affected by urban development, maritime transport, tourism, chemical pollution, wastewater discharge, fishing, aquaculture, industry (Rodriguez et al., 2018). The highest risk posed by nutrient enrichment is in enclosed bays and seas with limited water exchange, in shallow waters and estuaries (Nogales et al., 2010).

Microbial communities constitute the most abundant fraction of the benthic biomass (Zoppini et al., 2019). In the aquatic environment, microbes play a crucial role in regulating and transforming major bioactive elements, recycling organic matter to benthic food webs, as well as in degradation of organic pollutants (Duran and Cravo-Laureau, 2016). Marine microbes are highly affected and sensitive to environmental changes. They have fast growth rates, genomic plasticity and promptly respond to environmental perturbations. Therefore, bacterial diversity can be used to assess the harmful effects on sediment health (Ager et al., 2010; Zoppini et al., 2019). Pollutants contribute to the reduction of biodiversity, shifts in community composition, the removal of sensitive species and the selection of the more tolerant ones (Ager et al., 2010; Zoppini et al., 2019).

Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), as a group of carcinogenic and toxic pollutants having a potential to bioaccumulate in the food chains, have attracted significant research interest (Ghosal et al., 2016; Yang et al., 2020). The main sources of PAHs are petrogenic and pyrogenic, resulting from anthropogenic activities, including direct inputs of petroleum and emissions from the incomplete combustion of fossil fuels, organic material, wood (Louvado et al., 2015; Duran and Cravo-Laureau, 2016). Regardless of the contamination origin, PAHs finally reach the sediments representing the main sink and constitute PAH reservoirs (Duran and Cravo-Laureau, 2016). PCBs are aromatic, synthetic chemicals that do not occur naturally in the environment. They are produced commercially by catalytic chlorination of biphenyls providing a complex mixture of multiple isomers. PCB concentrations are generally higher near human activities and shorelines persisting for a long time in the environment before breaking down and entering the food chain (Zhao et al., 2016). Anaerobic reductive dechlorination is the only process known to convert PCBs into less chlorinated congeners with lower toxicity (Matturro et al., 2005).

The Montenegrin coast (south Adriatic) covers a narrow strip with a total coastline length of 294 km, of which 105.7 km belong to the Boka Kotorska Bay. The Boka Kotorska Bay represents a unique fjord where the sea penetrates in the continent deeply and naturally divides into smaller bays. The coast is influenced by excessive and uncontrolled urbanization, untreated municipal wastewaters, increasing maritime activities, fishing derived activities and shipbuilding overhaul facility, and a significant increase in population during summer (tourist season) (Mandić et al., 2016). Although covering a relatively small coastline length compared to other Mediterranean countries, in this coast is possible to identify all the typical environmental perturbations.

Our research represents the first baseline assessment of sediment microbial communities along the Montenegrin coast. This study provided insight into the potential impact that PAHs and PCBs may have on the microbial community as well as nutrients (total nitrogen, total phosphorous, ammonia), using a molecular approach. In this research, we have investigated the influence of the abiotic environment on microbial diversity and their functional capabilities potentially related to biodegradation of aromatic compounds and active transport systems, considering both toxic pollutants and natural substances from various origins. Can we identify a typical microbial community for the heavily polluted sites or nutrients and whether it affects their complexity and function?

Materials and methods

Sampling sites

The surface layers of sediment samples (0-2 cm) were collected from 12 locations by Ponar grab along the Montenegrin coast in September 2015. The study area with the location of the sampling sites is shown in Figure 1, while details of the sampling points in Table S1. Sampling in the Boka Kotorska Bay included: Port of Kotor (KTR; 15m depth), Risan (RSN; 22m depth), St. Nedelja (STN; 36m depth), Port of Montenegro (PMN; 15.5m depth), Tivat cove (TVC; 12.5m depth), Tivat center (TVT; 40m depth), Shipyard Bijela (SBL; 26m depth), Herceg Novi center (HRN; 40m depth) and Igalo (IGL; 10m depth) while outside the Bay included: Budva (BDV; 10m depth), Port of Bar (PBR; 10m depth) and Ada Bojana (ADB; 10m depth). According to Bortoluzzi et al. (2016), clay is typically found at the sea bottom in the Kotor and Risan Bay, clayey-loamy sand is found in the Tivat Bay while the sea bottom of the Herceg Novi is covered by clay, loamy clay and clayey sand. Central parts of the Bay are covered by fine terrigenous mud. Investigated seabed area of Budva and Bar is mostly covered with silty clay (Del Bianco et al., 2014) while the seabed of Ada Bojana is covered by sandy sediment (Petković and Sekulić, 2018).

Sediment collection

Sediment samples for analysis of total organic carbon (TOC), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyl (PCBs) were sealed in aluminum foil. Samples for analysis of total nitrogen (TN), total phosphorus (TP) and ammonia (NH_3) were sealed in the plastic bags while samples for DNA analysis of bacterial diversity were collected in sterile plastic cups. Samples were stored at -70 ºC until analysis.

Chemical analysis

Total organic carbon was analyzed according to ISO/TS 13137 (2001) using the TOC 5000A instrument with model SSM 5000A for solid samples. TOC was obtained from the difference between total carbon (TC) and inorganic carbon (IC). Total phosphorus content was performed following the method described by EPA 3051A (2007) on inductively coupled plasma emission spectrometry (Icap 6300, Thermo, USA). Total nitrogen content in the sediment samples was determined using Kjeldahl digestion according to Persson et al. (2008) on Kjeltec 2300 (Foss Tecator, Denmark) equipped with a block system for mineralization. The concentration of ammonia was analyzed according to ISO/TS 14256-1 (2003) filtering the extraction solution and analyzing the nitrogen fractions on spectrometry UV-1800 (Shimadzu, Japan).

The analysis of PAHs and PCBs were performed according to EPA 8270 (1998). Samples were defrosted to room temperature prior to analysis. Freeze-dried, homogenized sediment samples were extracted in a Soxhlet extractor with a mixture of hexane and dichloromethane (1:1). The extracts were concentrated on a rotatory evaporator. Clean up treatments were achieved by neutral, activated silica column for PAHs and a combination of acid and base Silica for PCBs. Elution was performed using a mixture of hexane and dichloromethane (1:1) for PAHs, and hexane for PCBs. The sulfur clean up procedure was performed according to EPA 3660B. Evaporation of clean extracts was carried out under a stream of nitrogen. Quantification was done by gas chromatography-mass spectrometry (GC-MS 2010 QP plus, Shimadzu, Japan) attached to SLB-5 ms capillary GC column (Supelco Inc; 30x 0.25mm, 0.25µm thickness).

The ascertained components of PAHs were: naphthalene (Naph), acenaphtylene (Aceph), acenaphtene (Ace), fluorene (Fl), phenanthrene (Phen), anthracene (Ant), fluoranthene (Flu), pyrene (Pyr), benzo[a]anthracene (BaA), chrysene (Chr), benzo[a]pyrene (BaP), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), indeno[1,2,3-c,d]pyrene (InP), benzo[g,h,i]perylene (BgP) and dibenzo[a,h]anthracene (DBgP). The ascertained PCBs were: PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153 and PCB 180.

Assessment of PAHs and PCBs concentration was made against the OSPAR Background Assessment Concentrations (BAC) and Effects Range Low (ERL). Sediments with concentrations below BAC were considered to have high environmental status, while concentrations significantly below ERL were considered to have good, and those above, bad environmental status (Webster et al., 2009).

PAH pollution ratios

The level of PAH pollution was classified based on the sum of PAH concentration, indicating low (<0.1 mg kg^{-1}), moderate (0.1-1 mg kg^{-1}), high (1-5 mg kg^{-1}) and very high (>5 mg kg^{-1}) pollution. Four different ratios according to Yunker et al. (2002) were examined: (i) the Ant/Ant+Phen ratio, a value <0.10 indicates petroleum contamination, while a value >0.10 combustion processes; (ii) the BaA/BaA+Chr ratio, a value <0.20 indicates petroleum input, mixed sources for values between 0.20 and 0.35, and values >0.35 indicate combustion sources; (iii) the Flu/Flu+Pyr ratio, a value <0.40 is characteristic of petroleum input, values between 0.40 and 0.50 indicate the combustion of liquid fossil fuels and crude oil whereas values >0.50 indicate the combustion of grass, wood or coal; and (iv) the Inp/Inp+BgP ratio, values <0.20 imply petroleum input, values between 0.20 and 0.50 the combustion of liquid fossil fuels and crude oils, and values >0.50 indicate combustion of grass, wood and coal. According to Soclo et al. (2000), the sum of low molecular weight PAHs (∑LPAH; Phen, Ant, Flu and Pyr) and the sum of high molecular weight PAHs (∑HPAH; BaA, Chr, BbF, BkF, BaP, InP, BgP and DBgP) was calculated. The ratio of ∑LPAH/∑HPAH <1 implies a pyrogenic source of PAH, whereas the ratio >1 a petrogenic source of PAH.

DNA extraction, PCR and amplicon sequencing

Total DNA was extracted from 0.5 g of sediment with the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The hypervariable region V4/V5 of the bacterial 16S rRNA genes was amplified with the primer pair 515F and 926R. Samples were further processed and sequenced by the SeqIT GmbH & Co. KG (Kaiserslautern, Germany). Purified V4/V5 amplicons were paired-end sequenced on an Illumina MiSeq platform. The sequences generated for this study were deposited in the European Nucleotide Archive (ENA) under the accession number _.

Bioinformatics and functional prediction of genes

Paired-end reads were quality trimmed using the *bbduk* function and merged using *bbmerge* function of the BBMap package (v38.71; https://sourceforge.net/projects/bbmap/). Merged reads were quality-filtered using QIIME v1.8.0 (Caporaso et al., 2010). Reads with exact barcodes and primers, unambiguous nucleotides, and a minimum length of 250 base pairs were retained. Chimera check was done using UCHIME (Edgar et al., 2011). Non-chimeric reads were clustered with SWARM v3.0.0 (Mahé et al., 2015) with default settings into Operational Taxonomic Units (OTUs). The bacterial reads were blasted against the SILVA database (release 132; December 13, 2017) using *blastn* (BLAST v2.9.0; Altschul et al., 1990). Nontarget OTUs (chloroplasts, mitochondria), as well as singletons and doubletons, were excluded. Resulting OTUs were filtered by the quality of the blast results (≥ 98% identity). Standardization among samples was performed by randomly subsampling the table of OTUs to the minimum read level of 18,057 using the *rrarefy* function of the R package *vegan* (Oksanen et al., 2019) to minimize biases associated with sequencing and allow comparison between the samples.

The software PICRUSt2 (v2.3.0 beta; Douglas et al., 2020) was used to predict the metagenome of the bacterial community identified based on 16S sequences and their relative proportions in each sediment sample. The rarefied OTU table was normalized in PICRUSt2 with further analysis performed at the gene-level (KEGG orthologs, KO) and the pathway-level (MetaCyc) to determine whether the observed taxonomic diversity in microbial communities accompanies their functional capabilities. To assess the effect of PAHs and PCBs on bacterial community, functional categories related to xenobiotics biodegradation and metabolism, and environmental information processing were selected. The functional categories include predicted genes from degradation pathways of aromatic compounds, polycyclic aromatic hydrocarbon, chloroalkane, chloroalkene, naphthalene and aminobenzoate, and a large family of membrane proteins - ABC transporters.

Statistical analysis

All statistical analyses and visualizations were performed in R version 4.0.0 (R Core Team, 2020) using multiple R packages. Bacterial communities were characterized in terms of alpha diversity by calculating OTU richness, Shannon, Simpson, non-parametric ACE, Chao index and Good's coverage. Principal Component Analysis (PCA) was conducted to cluster samples according to chemical parameters. Beta diversity of the microbial community on the OTU level was calculated using Bray-Curtis dissimilarity and clustered based on Ward's hierarchical clustering method (Ward.d2; Murtagh and Legendre, 2014). Differences occurring between clusters of samples identified on the OTU level were assessed with permutational multivariate analysis of variance (PERMANOVA). Spearman's rank correlation analysis was used to examine the response of the bacterial community to chemical parameters. Heatmap was constructed considering taxonomic classification and abundance on the family level. Canonical

Correspondence Analysis (CCA) was carried out to determine potential bacterial community composition drivers on the OTU level. A separate CCA was carried out on the KO level to compare trends of functional capabilities of microbial communities with observed OTUs. Results of CCAs were visualized using *ggplot2* (Wickham, 2016). ANOVA with the HSD Tukey test was carried out to test the significant differences of predicted genes associated with degradation pathways and ABC transporters between the categories of samples.

Results

Assessment of chemical parameters of sediment samples

Results of chemical analysis from the investigated sediment samples are presented in Figure S1 and Table S2. The sum of PAH concentration ranged from 0.04 mg kg^{-1} to 15.6 mg kg^{-1}. Only station ADB was characterized by low PAH pollution, six stations (RSN, TVC, TVT, HRN, IGL, PBR) had moderate PAH pollution, three (KTR, STN, BDV) had high PAH pollution and two (PMN, SBL) were defined as very high PAH polluted.

The ∑LPAH/∑HPAH ratio varied from 0.42 (PBR) to 0.90 (SBL) at investigated sites implying the pyrolytic origin (Table 1). The Ant/Ant+Phe and BaA/BaA+Chr ratio varied between 0.134 (PBR) to 0.5 (ADB) and 0.478 (PBR) to 0.543 (KTR), respectively, indicating the dominant contribution of combustion sources. The InP/InP+BgP ratio varied between 0.467 (STN) to 0.525 (SBL). Combustion of liquid fossil fuels and crude oils prevail at KTR, RSN, STN, IGL, BDV, PBR, while combustion of grass, wood and coal prevail at PMN, TVC, TVT, SBL, HRN and ADB site. The Flu/Flu+Pyr ratio ranged from 0.5 (ADB) to 0.562 (SBL), indicating grass, wood or coal combustion at all investigated sites. PAH components with values higher than threshold ERL were observed at KTR, PMN and SBL. At station STN, one PAH component benzo[g,h,i]perylene exceeded ERL value. Total PCB content ranged from 0.001 mg kg^{-1} to 0.830 mg kg^{-1}. PCB components at stations PMN and SBL have exceeded threshold values. The value of PCB 118 at station KTR was higher than the threshold values as well.

The TOC content in the sediment surface revealed values in the range of 7,700 (RSN) up to 24,900 mg kg^{-1} (KTR) (Figure S1). US EPA (2002) recommended the following assessment categories for TOC in sediment: low impact (≤10,000 mg kg^{-1}), intermediate impact (10,000 mg kg^{-1} to 30,000 mg kg^{-1}) and high impact (≥30,000 mg kg^{-1}). TOC levels in the analyzed sediments were in the range from low to medium organic carbon levels. The values of total nitrogen ranged between 303 mg kg^{-1} (ADB) to 1,697 mg kg^{-1} (KTR). The TOC/TN ratio has been widely used to infer organic matter origin and sources: >15 the organic carbon (OC) is terrestrial, and <10 predominance of marine organic matter influx (Gälman et al., 2008). In our study four stations: STN, PMN, SBL, IGL had a TOC/TN ratio greater than 15, and stations RSN, TVC, PBR had ratio lower than 10. The sampling sites KTR, TVT, HRN, BDV, ADB with the ratio of TOC/TN ranging from 10 to 14 indicated the combined source (algal and terrestrial). The concentration of ammonia was in the range from 8.5 (PMN) to 70.8 mg kg^{-1} (TVT). The values of total phosphorus varied between 273 (PMN) and 838 mg kg^{-1} (KTR). TOC/TP ratio ranged from 3.57 (PBR) to 56.5 (PMN).

PCA plot of chemical parameters revealed with separation of SBL and PMN station according to PAH and PCB components from other stations (Figure 2). The first two axes of PCA represent 75.9% of the total variance. Different parameters appeared to shape station KTR with the maximum value of TOC, TN and TP, while four stations TVT, STN, BDV and PBR grouped based on the higher content of NH_3.

Microbial community profiling of sediment samples

A total of 376,167 valid reads were obtained and clustered into 10,521 OTUs with taxonomic identity ≥98%. The OTU richness, Shannon and Simpson diversity indices, as well as Chao1, ACE index and Good's coverage, are summarized in Table 2. Based on the OTU richness, TVC had the highest diversity (3,021), followed by HRN (2,995) and KTR (2,917). SBL site had the lowest number of OTU richness (1,652) followed by ADB (1,881). The nonparametric richness indices showed the highest value at TVC site (ACE = 3,812; Chao1 = 4,659) while samples from SBL had the lowest one (ACE = 1,851; Chao1 = 2,125). The highest sediment bacterial diversity (Shannon = 6.5) was found at the PBR site followed by the HRN site (Shannon = 6.455) while the lowest one was at SBL (Shannon = 5.418). The Simpson index varied between 0.971 (SBL) to 0.994 (PBR). Good's coverage showed values between 92.3% to 97.5%.

In the overall bacterial community 47 phyla were identified, from which 13 phyla were present with ≥1% of relative abundance at least at one station (Figure S2). Based on average relative abundance, *Gammaproteobacteria* (29.8%), *Deltaproteobacteria* (25.2%) and *Alphaproteobacteria* (7.6%) of the *Proteobacteria* phylum, *Thermoanaerobaculia* (8.1%) of the *Acidobacteria* phylum (12%) and *Bacteroidia* (6.3%) of the phylum *Bacteroidetes* (7.4%) were the five largest classes in the bacterial community of sediments. *Gamma*- and *Deltaproteobacteria* were the most abundant classes in all samples except at the station SBL, where *Alphaproteobacteria* followed *Gammaproteobacteria* in relative abundance taking together more than 60% of the total. In the candidate phyla, members of division *BRC1*, *Dadabacteria* (*Dadabacteriales*) and *Spirochaetes* were present only in SBL with an abundance higher than 1% (1.1%, 3.5% and 2.6%). OTUs affiliated to *Latescibacteria* were present in all stations with abundance higher than 1% except at the station PMN, SBL and ADB, while OTUs affiliated to *Chloroflexi* at the station SBL, IGL and ADB. *Gammaproteobacteria* was the most abundant class at stations: SBL, HRN, IGL, PBR and ADB, with 46.3%, 31.5%, 41.4%, 41.3% and 44.1% respectively. Within the order *Steroidobacterales*, family *Woeseiaceae* accounted for the maximum abundance (27.6%) at ADB, followed by IGL station (12.6%) (Figure 3). At other investigated stations, their abundance varied between 2.4% to 9.2%. In contrast, *Deltaproteobacteria* was the most abundant class in other seven stations: KTR, RSN, STN, PMN, TVC, TVT, BDV with 28.9%, 32.3%, 32%, 31.1%, 36.2%, 34.7% and 38.6% respectively (Figure S2). Within this class, family *Desulfobulbaceae* and *Syntrophobacteraceae* were the most abundant. *Desulfobulbaceae* varied between 0.1% to 30.4% with the highest abundance accounted in the BDV sample (Figure 3). *Syntrophobacteraceae* showed values between 0.1% to 18.6% with the maximum at TVT. The percentage of *Alphaproteobacteria* ranged from 3.8% to 23.5%, with maximum abundance at station SBL. Family *Rhodobacteraceae* counted 19.5% at SBL station while at the other investigated stations varied between 0.6% to 1.7%. The abundance of *Bacteroidia* varied from 2.7% to 12.5% with a maximum at station ADB.

The community structure of the sediment samples based on the family-level composition showed a clear separation of BDV, SBL and ADB from other stations (Figure 3). At the inner and middle part of the Boka Kotorska Bay (KTR, RSN, STN, TVT, TVC) community has displayed a similar taxa dominance clustering together on the family-level. The main representatives of the bacterial community were uncultured *Synthrophobacteraceae*, with a percentage of 14.3%, 16.5%, 17.3%, 18.6% and 15.9%, respectively. Apart from the dominance of *Synthrophobacteraceae*, *Woeseiaceae*, *Thermoanaerobaculaceae* and *Desulfobulbaceae* also were present in two-fold lower abundance. In the area of Kotor Bay and Tivat Bay class *Thermoanaerobaculia* reached abundance above 10% with a maximum of 15.1% at the station STN. Family *Thermoanaerobaculaceae* showed maximum values at the STN site (15.1%), followed by RSN (13%) and KTR (11%). Stations of the outer part of the Boka Kotorska Bay (HRN, IGL) clustered together with the station PBR and PMN with the dominant taxa consisted of *Woeseiaceae* (8.7%, 12.6%), *Desulfobulbaceae* (7.3%, 11.6%) and *Thermoanaerobaculaceae* (9.8%, 6.3%). PMN was characterized by B2M28 (9.3%; *Gammaproteobacteria*) and *Synthrophobacteraceae* (9.4%), while the SBL station with B2M28 (20.9%) and *Rhodobacteraceae* (19.5%) in a noticeable abundance.

The abundant bacterial groups like *Alphaproteobacteria* showed a positive correlation with PAH and PCB (Figure S3). In contrast, *Bacteroidia* showed a negative correlation with PAH, PCB and TN. Groups present only in SBL had a significant positive correlation, where *Dadabacteria* highly correlated with PAH and PCB, while *BRC1* only with PCB. Phyla with abundance lower than 1% such as *Hydrogenedetes*, *Dependentiae* and *Tenericutes* have also shown a significantly positive correlation with PAH and PCB, while *Armatimonadetes* negative to PAH and *Zixibacteria* negative to PAH and PCB. *Acidobacteria* was found to be negatively correlated with TN, while *Deltaproteobacteria* was negatively correlated with TOC. Positively correlated phyla to TN and TP were *Nitrospirae* and *BRC1*. The most abundant bacteria at the family level, like *Woeseiaceae* and *Desulfobulbaceae,* showed a positive correlation with TOC and negative correlation with TP, respectively (Figure 4).

Beta diversity based on Ward's minimum variance cluster analysis of Bray-Curtis dissimilarity distances calculated on the OTU level had identified five clusters showing a similar clustering based on the family-level (Figure S4). The bacterial community at TVT, KTR, RSN, STN, TVC (inner and middle part of the Boka Kotorska Bay) clustered together, IGL, HRN, PMN clustered into the second group (outer part of the Boka Kotorska Bay with Port of Montenegro station), and BDV with PBR (area outside the Bay) clustered into the third group. In contrast, SBL (the most polluted site) and ADB (estuary sandy sediment area) formed independent clusters. This was confirmed by PERMANOVA with clusters of samples identified on the OTU level having a strong, significant difference (pseudo-F, 6.46; $P < 0.05$).

The predictive functional profile of samples, deciphered by PICRUSt2, resulted in identifying 6,530 KOs, which were collapsed to 401 MetaCyc microbiome metabolic pathways. The Canonical Correspondence Analysis (CCA) ordination of established KOs has shown similar sample separation as the CCA based on all OTUs with no significant correlation to environmental parameters (Figure 5). In consistence with the CCA results, observed taxonomic diversity on the OTU level in microbial communities accompanied their functional capabilities. In order to capture the impact of PAHs and PCBs on the proportion of predicted genes potentially involved in the degradation of aromatic compounds and related to ABC transporter proteins, the stations were grouped into five groups based on the results of the cluster analysis of all OTUs (Figure S4). The proportion of predicted genes in functional profiles showed a statistically significant difference between the groups (PERMANOVA, $p \leq 0.001$). Selected KOs had a significantly higher frequency in Cluster 4 (SBL) than the remained clusters. Overall, only KOs that significantly differed in relative abundance compared to Cluster 4 ($p \leq 0.05$) are shown in frequency heatmaps (Figure 6; Figure 7). SBL hosted a higher frequency of genes coding for the degradation of different aromatic compounds such as catechol, phenol, naphthalene, terephthalate, phenanthrene, benzoate, etc. It is important to mention that ADB station assigned as a Cluster 5 had the significantly highest frequency of genes involved in phenol/toluene degradation (data not shown). Genes coding for the ABC transporter proteins were significantly higher in SBL station associated to biotin, urea, glycerol, polysaccharide, phosphate transport system, etc. No significant correlations were found between the frequency of the genes involved in the degradation of aromatic compound and PAH and PCB content which opposed to genes related to ABC transporter proteins. The frequency of the genes coding phosphate, glycerol and methionine transport system proteins were positively correlated to PAH and PCB content ($p \leq 0.05$), while genes coding urea transport system proteins correlated positively only to PCB content ($p \leq 0.05$).

Discussion

Prokaryotic communities in the sediment can be shaped by various natural and anthropogenic perturbations, e.g., chemical pollution, WWTP, eutrophication, etc. (Wang et al., 2016; Rodriguez et al., 2018; Zoppini et al., 2019, Di Cesare et al., 2020). Along the Montenegrin coast different pollution sources, all connected with the tourism or industry, could be identified: unresolved issues of wastewater discharges or maritime activities in ports and shipyards. Tourism and maritime activities represent the major income in Montenegro and are also the most significant threat to the environment. This is due to the tourist season when the number of inhabitants multiplies several times and the maritime activity significantly intensifies. In this research, sediments environmental health through chemical and microbiological characterization along the Montenegrin coast were determined.

Chemical characterization of sediment

Chemical analysis confirmed that shipyard Bijela (SBL) and luxury yacht port of Montenegro (PMN) were the most polluted sites in the investigated area (Kljajić et al., 2011). These results are in agreement with the PAH source characterization in other Mediterranean ports (e.g., Korlević et al., 2015; Vitali et al., 2019). However, it is important to note that the higher PAH concentrations were observed at sites located in harbor and shipyard where shipping traffic is intense, demonstrating that the harbors are the major sources of sediment contamination (Korlević et al., 2015; Frena et al, 2017). The origin of PAHs could be attributed to the nutrient enrichment, incomplete combustion of fuels (i.e., gasoline, diesel oils) in ports (PBR, KTR), luxury yacht marina (PMN), as well as recreational marina (BDV). Activities during shipbuilding processes at the SBL site contribute to pyrolytic PAH origin since high molecular weight PAHs (HPAHs >3 rings) dominated. The potential source for contamination could be from the disposal of the waste grit as well (Mandić et al., 2016). Petroleum hydrocarbon concentration was higher at station PBR (military, cargo port) (Kljajić et al., 2011), while in the present research did not exceed the limits. The lowest PAH and PCB content was detected at estuary station ADB with sandy sediment. These might be attributed to the smaller surface area of mass in a sandy fraction, which decreases the adsorption capacity (GIPME, 2000). PCB are persistent and ubiquitous organic pollutants widely used in industrial applications. Even if the industrial production of PCBs has been banned since the late 1970s, the chronic toxicity of these recalcitrant hazardous substances still remains. A higher value of PCB was detected in two investigated sites: PMN and SBL. These values are similar to other Mediterranean ports (Cardellichio et al., 2007; Merhaby et al., 2015).

The processes controlling sediment PAH and PCB concentration are complex and their distribution is affected by numerous factors, including the chemical composition of the sediment (organic matter, clay content, etc.) and the currents (Araghi et al., 2014; Frena et al., 2017). Specifically, PAH concentrations tend to be higher in sediments with higher TOC content due to the high sorption capacity of organic matter (Wang et al., 2014). However, a weak correlation was found between TOC and PAH concentration in our research. In our study, PAH content was highly correlated with TOC/TN ($p \leq 0.05$, $r = 0.536$) (Ladakis et al., 2006). Some authors (Wang et al., 2014) suggest that PAHs preferentially attach to terrestrial organic matter (OM) than to marine algal OM. Terrestrial OM contains more aromatic moieties than marine OM (Wang et al., 2014), so the association of terrestrial OM with the aromatic PAHs was expected. We have observed the highest values of PAHs and PCBs in PMN and SBL locations with terrestrial input of TOC.

Variability in the TOC/TN ratio, which we noticed in our study, prevails in semi-enclosed and shallow environments due to the mixed nature of the organic matter sources (Frena et al., 2017). Organic carbon, nitrogen and ammonia in sediment are derived by plants, animals, plankton or anthropogenic sources such as chemical contaminants, organic-rich waste. Organic carbon level was in the range from low to medium in analyzed sediment samples. Due to increased inflow of nutritive substances from the land through rivers, runoff caused by precipitation and untreated wastewater, Kotor and Risan Bay were exposed to the process of eutrophication during the last few decades (Krivokapić et al., 2018). Since the sampling was carried out in September when the freshwater intake is minimized, the inner part of the Boka Kotorska Bay (KTR, RSN) was probably under pressure of wastewater during the intensive touristic season or mussels farms, which resulted in increased TOC, TP, TN.

Bacterial community characterization

Numerous studies have shown the predominance of *Proteobacteria* in sediments of various marine ecosystems with a difference in the composition of major classes and relative proportions (Wang et al., 2015; Sun et al., 2013; Korlević et al., 2015; Di Cesare et al., 2020). At the most polluted site (SBL), the presence of the members of *Gammaproteobacteria* can be correlated to the early stages of oil hydrocarbon degradation (Acosta-González et al., 2013; Quero et al., 2015). The increasing pollution level was likely to affect the occurrence of *Alphaproteobacteria* (positive correlation in our study) which comprised bacteria possessing the biochemical and ecological capacities to degrade organic pollutants as was detected in other studies (Korlević et al., 2015). Uncultured *Rhodobacteraceae* were noticed at SBL site ten-folder times compared to the other investigated stations, but they did not significantly correlate with PAH and PCB. The family *Rhodobacteraceae* is among the nine most widely distributed bacterial lineages in marine habitats (Pohlner et al., 2019) and one genus from this family (*Polymorphum*) has been identified for their ability to degrade crude oil in a saline environment (Nie et al., 2012).

Our data highlighted the presence of family *Woeseiaceace* within *Steroidobacterales,* especially at ADB station (sand sediment station). Rod-shaped or coccoid *Woeseia* cells populated most sand grains (Probdant et al., 2018). This family covers a broad physiological spectrum that could provide adaptations to various biogeochemical settings and possibly explains their success in marine sediments worldwide (Mußmann et al., 2017).

Members of *Deltaproteobacteria*, the second abundant class in our study, were mainly affiliated to uncultured *Syntrophobacteraceae* and uncultured *Desulfobulbaceae* that belong to sulfate-reducing bacteria (SRB). In marine sediments, sulfate reduction is the major pathway for anaerobic degradation of organic matter where interfered, especially *Desulfobacterales* (Mahmoudi et al., 2015; Zhang et al., 2019). *Desulfobulbaceae* comprised the most representatives at the BDV site and they are mainly chemoorganoheterotroph (Zhang et al., 2019). The production of higher amounts of hydrogen sulfide by sulfate reduction with a decrease in oxygen might lead to the inhibition of important microbial processes such as nitrification (Hicks et al., 2018). That could explain the higher content of ammonia in the BDV sediment sample. Evidence of SRB together with abundant aerobic sulfur oxidizers, i.e., *Gammaproteobacteria*, suggested a rapid transition between oxic and anoxic conditions at surface sediment, probably related to seasonal conditions (Zoppini et al., 2019). Members of B2M28 (*Gammaproteobacteria*) occurred in a higher percentage at the most polluted SBL and PMN site. This group is related to sulfur-oxidizing bacterial endosymbiont (Cifuentes et al., 2000). Family *Syntrophobacteraceae* (*Deltaproteobacteria*) counted higher relative abundance at the inner (KTR, RSN) and middle part (STN, TVC, TVT) of the Boka Kotorska Bay. Increasing water temperature with a higher abundance of *Syntrophobacteraceae* can also indicate lower oxygen concentrations and promote the formation of anoxic "hotspots" within the surface sediment (Mahmoudi et al., 2015; Hicks et al., 2018), although this was not measured.

In the phylum *Acidobacteria, Thermoanaerobaculaceae* (subgroup 10 and subgroup 23) were identified in all sediment samples. These groups were represented in the highest percentage at KTR, RSN and STN. Previous research (Jokanović et al., 2016) classified this part of the Bay as a mesotrophic and eutrophic area with moderately productive water with hypoxia episodes. Certain subgroups of *Acidobacteria* are known to prefer environments with increased available nutrients. In several cases, *Acidobacteria* has appeared to tolerate various pollutants, such as PCBs and petroleum compounds (Sanchez-Peinado et al., 2010). Due to the still low number of sequenced genomes and difficulties associated with cultivation, the ecological role of this phylum remains unknown (Kielak et al., 2016).

Uncultured *Cyclobacteriaceae* and *Flavobacteriaceae,* within class *Bacteroidia* (*Bacteroidetes*), appeared in the estuary (ADB) station, which is influenced by the Bojana river. *Bacteroidetes* demonstrated the ability of the hydrolysis and utilization of complex carbon sources (Lapébie et al., 2019). Some of the species of the family *Cyclobacteriaceae* are known to degrade a number of polysaccharides and other macromolecules like casein and lipids (Pinnaka and Tanuku, 2014).

Despite to low percentage, members of *BRC1*, *Dadabacteria* and *Spirochetes* were present at the most polluted site the SBL suggesting the potential selection of PAH and PCB degrading communities. Reconstructing the central metabolism of *BRC1* has revealed pathways involved in fermentation and complete oxidation of organic substrates through aerobic and anaerobic respiration (Kadnikov et al., 2019), while *Dadabacteria* has shown the potential to degrade microbial particulate organic matter, specifically peptidoglycan and phospholipids (Graham and Tully, 2020). Detected *Spirochaetes* did not correlate with PAH and PCB content, contrasting to recent studies indicating their capability to degrade hydrocarbons or chlorinated compounds under anoxic conditions, such as toluene, naphthalene, alkanes, terephthalate and trichloroethene (Dong, 2018). According to indicator species SRB bacteria (*Sterioidobacterales* and *Desulfobacterales*) were present in the most polluted sites. This finding is in accordance with other studies (Acosta Gonzales et al., 2013; Korlević et al., 2015), where the presence of the role of sulfur cycle microorganisms in oil-polluted coastal marine sediments with focus on SRB bacteria was highlighted.

Many studies have indicated the significance of PAH and PCB (Wang et al., 2015; Quero et al., 2015; Vitali et al., 2019) as potential drivers of bacterial community structure changes. Although the high concentration of PAHs and PCBs measured at stations SBL and PMN, the bacterial community was characterized by diverse composition and significantly different gene frequencies involved in the degradation of aromatic compounds and ABC transporter proteins. This might be due to the domination of high molecular weight PAHs (HPAHs >3 rings) at the SBL station, having longer persistence and resistance toward biodegradation in the environment altering the structure of microbial community (Ghosal et al., 2016). However, both the physio-chemical characteristics of compounds as well as the physical, chemical and biological properties of sediments can have a high influence on the degradation capacity of microorganisms. Having regard to PCBs, only two genes with low frequency were found to be related to biphenyl degradation encoding biphenyl 2,3-dioxygenase and also genes related to pathways of chloroalkane and chloroalkene degradation, but with no significantly different gene frequencies between the samples (Jing et al., 2018). Predicted genes with the capability to utilize aromatic compounds, with a significant difference between the groups, were related to oxidoreductase enzymes such as salicylate hydroxylase, p-hydroxybenzoate 3-monooxygenase, hydrolases 3-oxoadipate enol-lactonase, catechol 2,3-dioxygenase, 3,4-dihydroxybenzoate indicating aerobic degradation pathways at the SBL station (Ghosal et al., 2016). Genes coding for the ABC transporter proteins were associated with biotin, urea, glycerol, polysaccharide, phosphate transport system, methionine transport system with higher frequency at the SBL station in order to fulfill metabolic requirements for growth and persistence of microbe growing in the polluted sediment.

Conclusion

This research provided insight into the potential impact of PAHs, PCBs and nutrients on bacterial community in sediments along the Montenegrin coast. Total PAHs have a statistically significant influence on bacterial phyla diversity. The influence of PAH and PCB in the most polluted SBL site was manifested as an increased abundance of *Alphaproteobacteria*. OTUs affiliated to phyla *BRC1*, *Dadabacteria* and *Spirochaetes*, are indicating their persistence and possible potential for degradation of aromatic compounds. Members of *Dadaproteobacteria* significantly correlated with PAH and PCB, while members of SRB bacteria (*Syntrophobacteraceae*) and *Thermoanaerobaculaceae* counted higher relative abundance at the inner and middle part of the Boka Kotorska Bay. Our data highlighted the presence of family *Woeseiaceace* within *Steroidobacterales*, especially at ADB station (sand sediment station) highly correlated with TOC. The functional capabilities potentially related to biodegradation of aromatic compounds and active transport systems showed a high difference between the SBL and other stations, indicating that microbial community was not only affected by PAHs and PCBs, but also with concertation of nutrients and physio-chemical characteristics of compounds. Further research can reveal the potential of bacterial community found at the SBL station contributing to the new insights into the biodegradation and bioremediation. At the environmental health level, the molecular and chemical characterization gives us a useful baseline for future monitoring programs.

CRediT author statement

Sandra Jokanović: Writing - original draft, Investigation. **Katarina Kajan:** Formal analysis, Writing - original draft, Software. **Svetlana Perović:** Funding acquisition, Supervision, Conceptualization. **Vesna Mačić:** Funding acquisition, Resources, Conceptualization. **Sandi Orlić:** Funding acquisition, Writing - review & editing, Conceptualization, Methodology.

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Figure captions

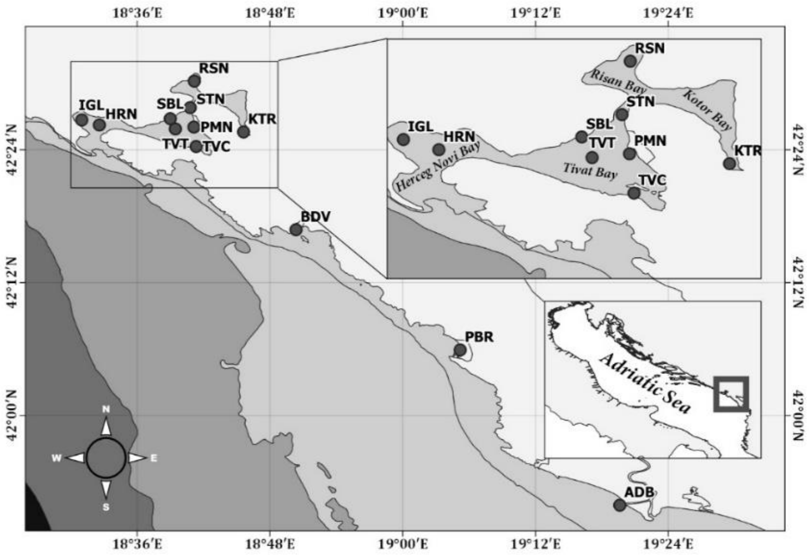


Figure 1. A map of Boka Kotorska Bay with marked sampling locations.

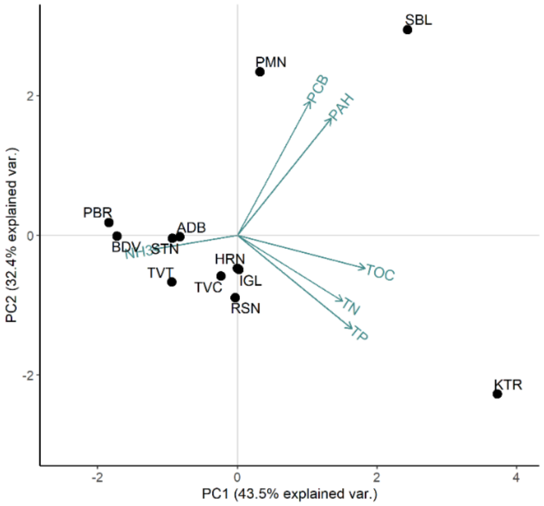


Figure 2. Principal component analysis (PCA) of chemical parameters.

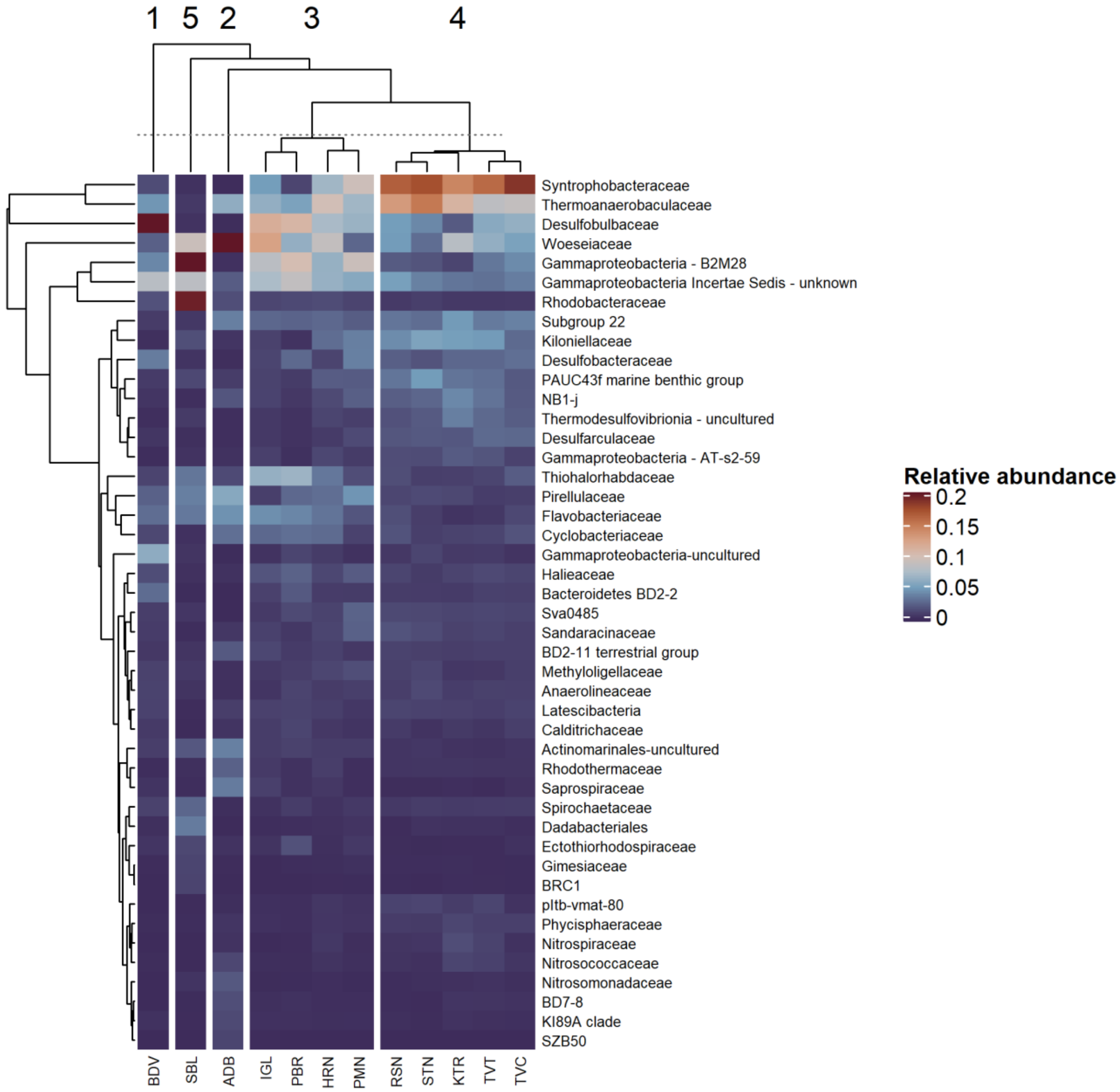
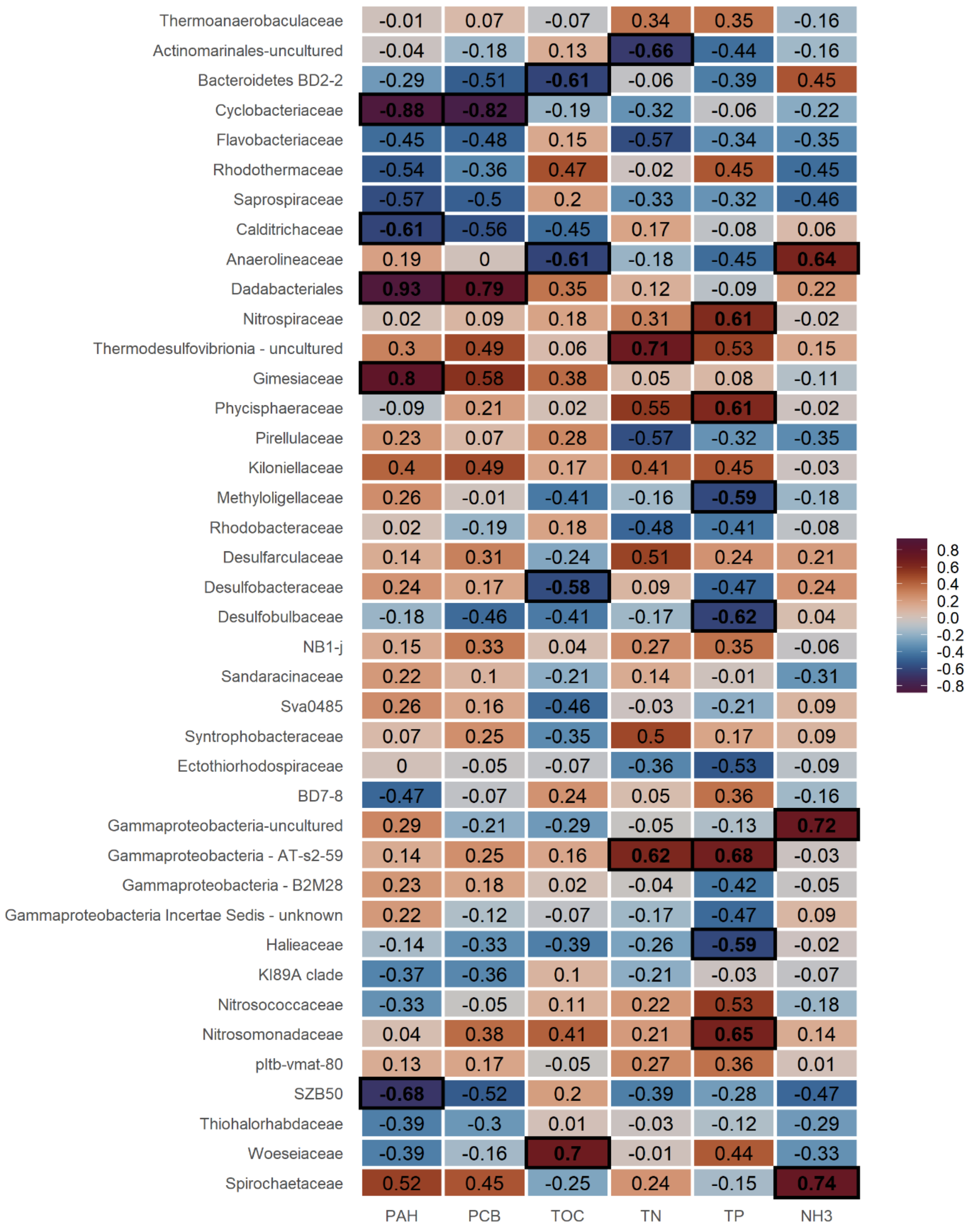


Figure 3. Family-level hierarchical clustering of bacterial distribution from twelve samples based on Bray-Curtis dissimilarity. Rows represent the relative abundance of abundant bacterial family (≥0.01) and column represents different sample. The relative abundance for each family is indicated by colour key legend.



	PAH	PCB	TOC	TN	TP	NH3
Thermoanaerobaculaceae	-0.01	0.07	-0.07	0.34	0.35	-0.16
Actinomarinales-uncultured	-0.04	-0.18	0.13	**-0.66**	-0.44	-0.16
Bacteroidetes BD2-2	-0.29	-0.51	**-0.61**	-0.06	-0.39	0.45
Cyclobacteriaceae	**-0.88**	**-0.82**	-0.19	-0.32	-0.06	-0.22
Flavobacteriaceae	-0.45	-0.48	0.15	-0.57	-0.34	-0.35
Rhodothermaceae	-0.54	-0.36	0.47	-0.02	0.45	-0.45
Saprospiraceae	-0.57	-0.5	0.2	-0.33	-0.32	-0.46
Calditrichaceae	**-0.61**	-0.56	-0.45	0.17	-0.08	0.06
Anaerolineaceae	0.19	0	**-0.61**	-0.18	-0.45	**0.64**
Dadabacteriales	**0.93**	**0.79**	0.35	0.12	-0.09	0.22
Nitrospiraceae	0.02	0.09	0.18	0.31	**0.61**	-0.02
Thermodesulfovibrionia - uncultured	0.3	0.49	0.06	**0.71**	0.53	0.15
Gimesiaceae	**0.8**	0.58	0.38	0.05	0.08	-0.11
Phycisphaeraceae	-0.09	0.21	0.02	0.55	**0.61**	-0.02
Pirellulaceae	0.23	0.07	0.28	-0.57	-0.32	-0.35
Kiloniellaceae	0.4	0.49	0.17	0.41	0.45	-0.03
Methyloligellaceae	0.26	-0.01	-0.41	-0.16	**-0.59**	-0.18
Rhodobacteraceae	0.02	-0.19	0.18	-0.48	-0.41	-0.08
Desulfarculaceae	0.14	0.31	-0.24	0.51	0.24	0.21
Desulfobacteraceae	0.24	0.17	**-0.58**	0.09	-0.47	0.24
Desulfobulbaceae	-0.18	-0.46	-0.41	-0.17	**-0.62**	0.04
NB1-j	0.15	0.33	0.04	0.27	0.35	-0.06
Sandaracinaceae	0.22	0.1	-0.21	0.14	-0.01	-0.31
Sva0485	0.26	0.16	-0.46	-0.03	-0.21	0.09
Syntrophobacteraceae	0.07	0.25	-0.35	0.5	0.17	0.09
Ectothiorhodospiraceae	0	-0.05	-0.07	-0.36	-0.53	-0.09
BD7-8	-0.47	-0.07	0.24	0.05	0.36	-0.16
Gammaproteobacteria-uncultured	0.29	-0.21	-0.29	-0.05	-0.13	**0.72**
Gammaproteobacteria - AT-s2-59	0.14	0.25	0.16	**0.62**	**0.68**	-0.03
Gammaproteobacteria - B2M28	0.23	0.18	0.02	-0.04	-0.42	-0.05
Gammaproteobacteria Incertae Sedis - unknown	0.22	-0.12	-0.07	-0.17	-0.47	0.09
Halieaceae	-0.14	-0.33	-0.39	-0.26	**-0.59**	-0.02
KI89A clade	-0.37	-0.36	0.1	-0.21	-0.03	-0.07
Nitrosococcaceae	-0.33	-0.05	0.11	0.22	0.53	-0.18
Nitrosomonadaceae	0.04	0.38	0.41	0.21	**0.65**	0.14
pltb-vmat-80	0.13	0.17	-0.05	0.27	0.36	0.01
SZB50	**-0.68**	-0.52	0.2	-0.39	-0.28	-0.47
Thiohalorhabdaceae	-0.39	-0.3	0.01	-0.03	-0.12	-0.29
Woeseiaceae	-0.39	-0.16	**0.7**	-0.01	0.44	-0.33
Spirochaetaceae	0.52	0.45	-0.25	0.24	-0.15	**0.74**

Figure 4. The correlation of bacteria - Spearman's rank correlation analysis at the family level. Colour indicates whether the correlation is positive (red) or negative (blue). Bold values showed significant correlation of bacterial family and chemical parameter ($p \leq 0.05$).

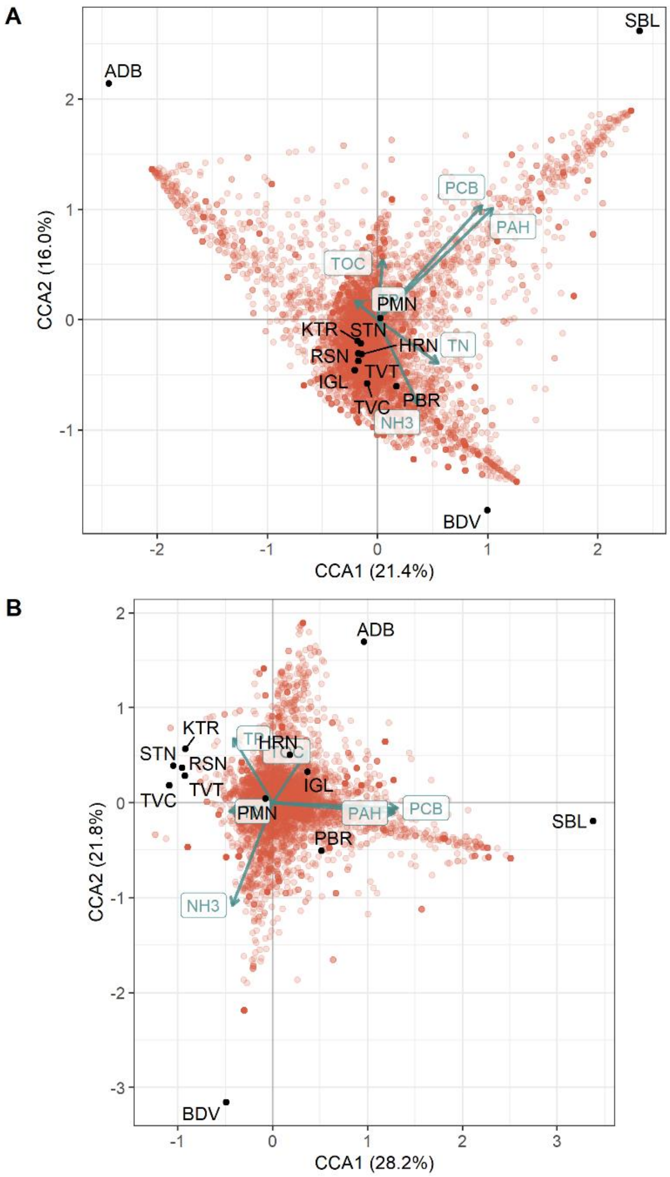


Figure 5. Canonical correspondence analyses (CCAs) ordination diagram. Relationship between chemical parameters presented as vectors (PAH, PCB, NH_3, TOC, TN, TP) and the taxonomic composition at the OTU level (A) and the KO level (B). Black dots represent sampling sites; red dots represent OTUs (A) and KOs (B). The chemical factors are represented as arrows: the length of the arrows indicates the relative importance of that environmental factor in explaining the variation in the bacterial communities, whilst the angle between each arrow and the nearest axis indicates the closeness of the relationship between each other.

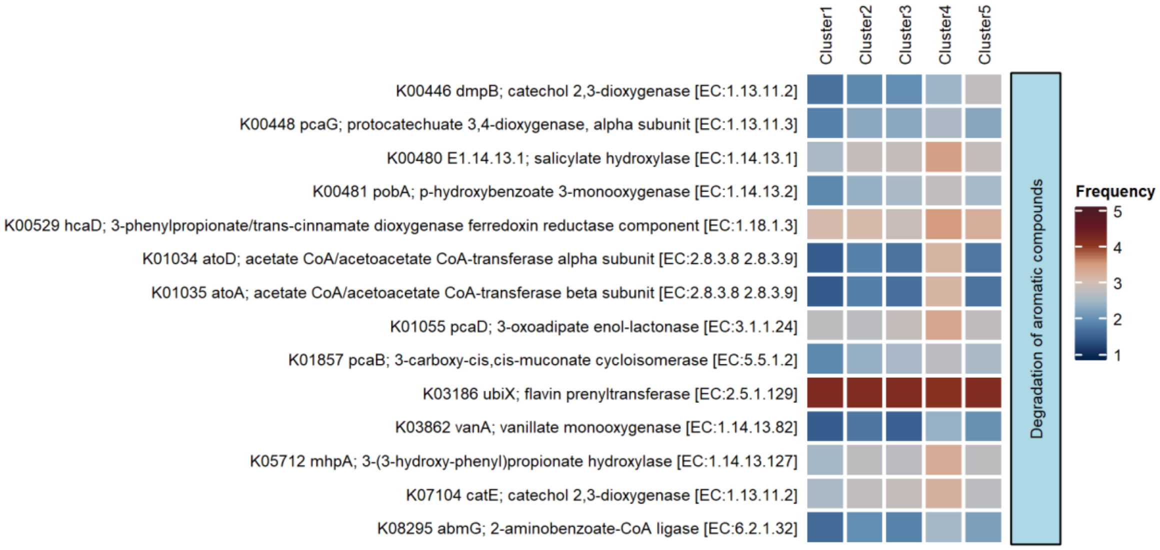


Figure 6. Frequency heatmap of the significantly different KOs related to the degradation of aromatic compounds between the clustered samples ($p \leq 0.05$). The frequency data has been transformed in log10 for better visualization of the differences between the frequencies. Clusters represent the average of gene frequencies: Cluster 1 (TVT, KTR, RSN, STN, TVC), Cluster 2 (IGL, HRN, PMN), Cluster 3 (BDV, PBR), Cluster 4 (SBL) and Cluster 5 (ADB).

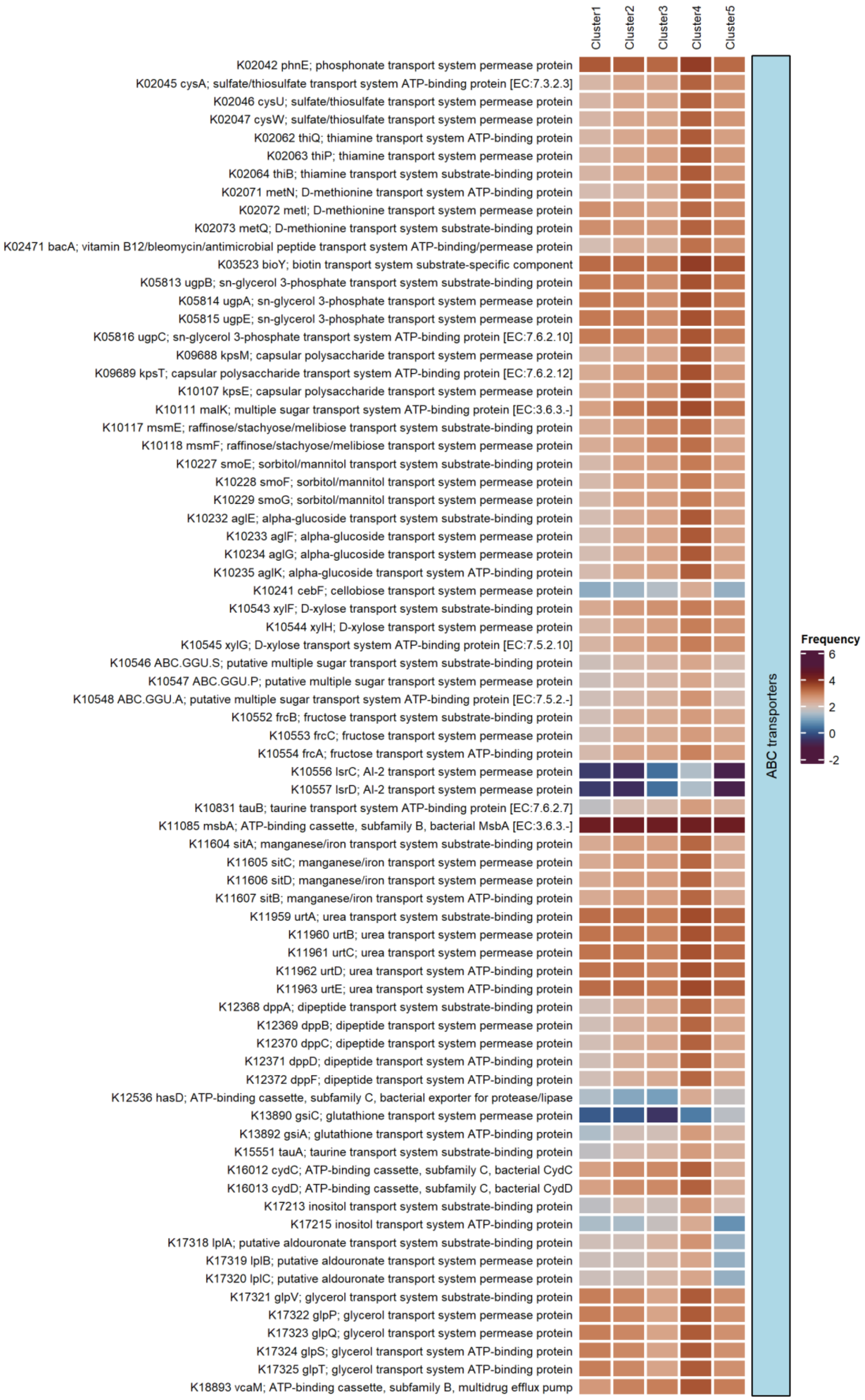

Cluster1
Cluster2
Cluster3
Cluster4
Cluster5
K02042 phnE; phosphonate transport system permease protein
K02045 cysA; sulfate/thiosulfate transport system ATP-binding protein [EC:7.3.2.3]
K02046 cysU; sulfate/thiosulfate transport system permease protein
K02047 cysW; sulfate/thiosulfate transport system permease protein
K02062 thiQ; thiamine transport system ATP-binding protein
K02063 thiP; thiamine transport system permease protein
K02064 thiB; thiamine transport system substrate-binding protein
K02071 metN; D-methionine transport system ATP-binding protein
K02072 metI; D-methionine transport system permease protein
K02073 metQ; D-methionine transport system substrate-binding protein
K02471 bacA; vitamin B12/bleomycin/antimicrobial peptide transport system ATP-binding/permease protein
K03523 bioY; biotin transport system substrate-specific component
K05813 ugpB; sn-glycerol 3-phosphate transport system substrate-binding protein
K05814 ugpA; sn-glycerol 3-phosphate transport system permease protein
K05815 ugpE; sn-glycerol 3-phosphate transport system permease protein
K05816 ugpC; sn-glycerol 3-phosphate transport system ATP-binding protein [EC:7.6.2.10]
K09688 kpsM; capsular polysaccharide transport system permease protein
K09689 kpsT; capsular polysaccharide transport system ATP-binding protein [EC:7.6.2.12]
K10107 kpsE; capsular polysaccharide transport system permease protein
K10111 malK; multiple sugar transport system ATP-binding protein [EC:3.6.3.-]
K10117 msmE; raffinose/stachyose/melibiose transport system substrate-binding protein
K10118 msmF; raffinose/stachyose/melibiose transport system permease protein
K10227 smoE; sorbitol/mannitol transport system substrate-binding protein
K10228 smoF; sorbitol/mannitol transport system permease protein
K10229 smoG; sorbitol/mannitol transport system permease protein
K10232 aglE; alpha-glucoside transport system substrate-binding protein
K10233 aglF; alpha-glucoside transport system permease protein
K10234 aglG; alpha-glucoside transport system permease protein
K10235 aglK; alpha-glucoside transport system ATP-binding protein
K10241 cebF; cellobiose transport system permease protein
K10543 xylF; D-xylose transport system substrate-binding protein
K10544 xylH; D-xylose transport system permease protein
K10545 xylG; D-xylose transport system ATP-binding protein [EC:7.5.2.10]
K10546 ABC.GGU.S; putative multiple sugar transport system substrate-binding protein
K10547 ABC.GGU.P; putative multiple sugar transport system permease protein
K10548 ABC.GGU.A; putative multiple sugar transport system ATP-binding protein [EC:7.5.2.-]
K10552 frcB; fructose transport system substrate-binding protein
K10553 frcC; fructose transport system permease protein
K10554 frcA; fructose transport system ATP-binding protein
K10556 lsrC; AI-2 transport system permease protein
K10557 lsrD; AI-2 transport system permease protein
K10831 tauB; taurine transport system ATP-binding protein [EC:7.6.2.7]
K11085 msbA; ATP-binding cassette, subfamily B, bacterial MsbA [EC:3.6.3.-]
K11604 sitA; manganese/iron transport system substrate-binding protein
K11605 sitC; manganese/iron transport system permease protein
K11606 sitD; manganese/iron transport system permease protein
K11607 sitB; manganese/iron transport system ATP-binding protein
K11959 urtA; urea transport system substrate-binding protein
K11960 urtB; urea transport system permease protein
K11961 urtC; urea transport system permease protein
K11962 urtD; urea transport system ATP-binding protein
K11963 urtE; urea transport system ATP-binding protein
K12368 dppA; dipeptide transport system substrate-binding protein
K12369 dppB; dipeptide transport system permease protein
K12370 dppC; dipeptide transport system permease protein
K12371 dppD; dipeptide transport system ATP-binding protein
K12372 dppF; dipeptide transport system ATP-binding protein
K12536 hasD; ATP-binding cassette, subfamily C, bacterial exporter for protease/lipase
K13890 gsiC; glutathione transport system permease protein
K13892 gsiA; glutathione transport system ATP-binding protein
K15551 tauA; taurine transport system substrate-binding protein
K16012 cydC; ATP-binding cassette, subfamily C, bacterial CydC
K16013 cydD; ATP-binding cassette, subfamily C, bacterial CydD
K17213 inositol transport system substrate-binding protein
K17215 inositol transport system ATP-binding protein
K17318 lplA; putative aldouronate transport system substrate-binding protein
K17319 lplB; putative aldouronate transport system permease protein
K17320 lplC; putative aldouronate transport system permease protein
K17321 glpV; glycerol transport system substrate-binding protein
K17322 glpP; glycerol transport system permease protein
K17323 glpQ; glycerol transport system permease protein
K17324 glpS; glycerol transport system ATP-binding protein
K17325 glpT; glycerol transport system ATP-binding protein
K18893 vcaM; ATP-binding cassette, subfamily B, multidrug efflux pump
ABC transporters
Frequency
6
4
2
0
-2

Figure 7. Frequency heatmap of the significantly different KOs related to ABC transporter proteins between the clustered samples ($p \leq 0.05$). The frequency data has been transformed in log10 for better visualization of the differences between the frequencies. Clusters represent the average of gene frequencies: Cluster 1 (TVT, KTR, RSN, STN, TVC), Cluster 2 (IGL, HRN, PMN), Cluster 3 (BDV, PBR), Cluster 4 (SBL) and Cluster 5 (ADB).

Table 1. PAH ratio descriptors.

	Stations											
	KTR	RSN	STN	PMN	TVC	TVT	SBL	HRN	IGL	BDV	PBR	ADB
Ant/Ant+Phen	0.198	0.211	0.178	0.187	0.145	0.186	0.152	0.159	0.160	0.180	0.134	0.500
BaA/BaA+Chr	0.543	0.500	0.518	0.528	0.500	0.500	0.536	0.523	0.500	0.516	0.478	0.500
Flu/Flu+Pyr	0.537	0.537	0.542	0.554	0.531	0.544	0.562	0.533	0.552	0.548	0.544	0.500
InP/InP+BgP	0.494	0.485	0.467	0.515	0.507	0.503	0.525	0.508	0.489	0.483	0.486	0.500
∑LPAH/∑HPAH	0.730	0.570	0.591	0.740	0.614	0.600	0.900	0.600	0.564	0.800	0.420	0.500

Table 2. Alpha diversity indices estimated using Richness, Shannon, Simpson, ACE, Chao1 and Good’s Coverage.

	Stations											
	KTR	RSN	STN	PMN	TVC	TVT	SBL	HRN	IGL	BDV	PBR	ADB
Richness	2917	2796	2692	2847	3021	2800	1652	2995	2728	2353	2862	1881
Shannon index	6.333	6.049	5.990	6.222	6.138	6.197	5.418	6.455	6.207	5.946	6.525	6.143
Simpson index	0.985	0.983	0.980	0.990	0.983	0.982	0.971	0.993	0.991	0.984	0.994	0.986
ACE	3526.59	3516.64	3415.79	3549.25	3812.31	3439.62	1851.33	3700.00	3429.59	2766.15	3438.29	2032.21
Chao1	4085.64	4232.06	4133.78	4133.20	4658.66	4006.08	2124.91	4344.26	4110.98	3127.19	4022.00	2180.56
Good's coverage (%)	93.38	92.94	93.08	92.97	92.27	93.38	97.18	92.76	93.08	95.13	93.58	97.52

Supplementary Material

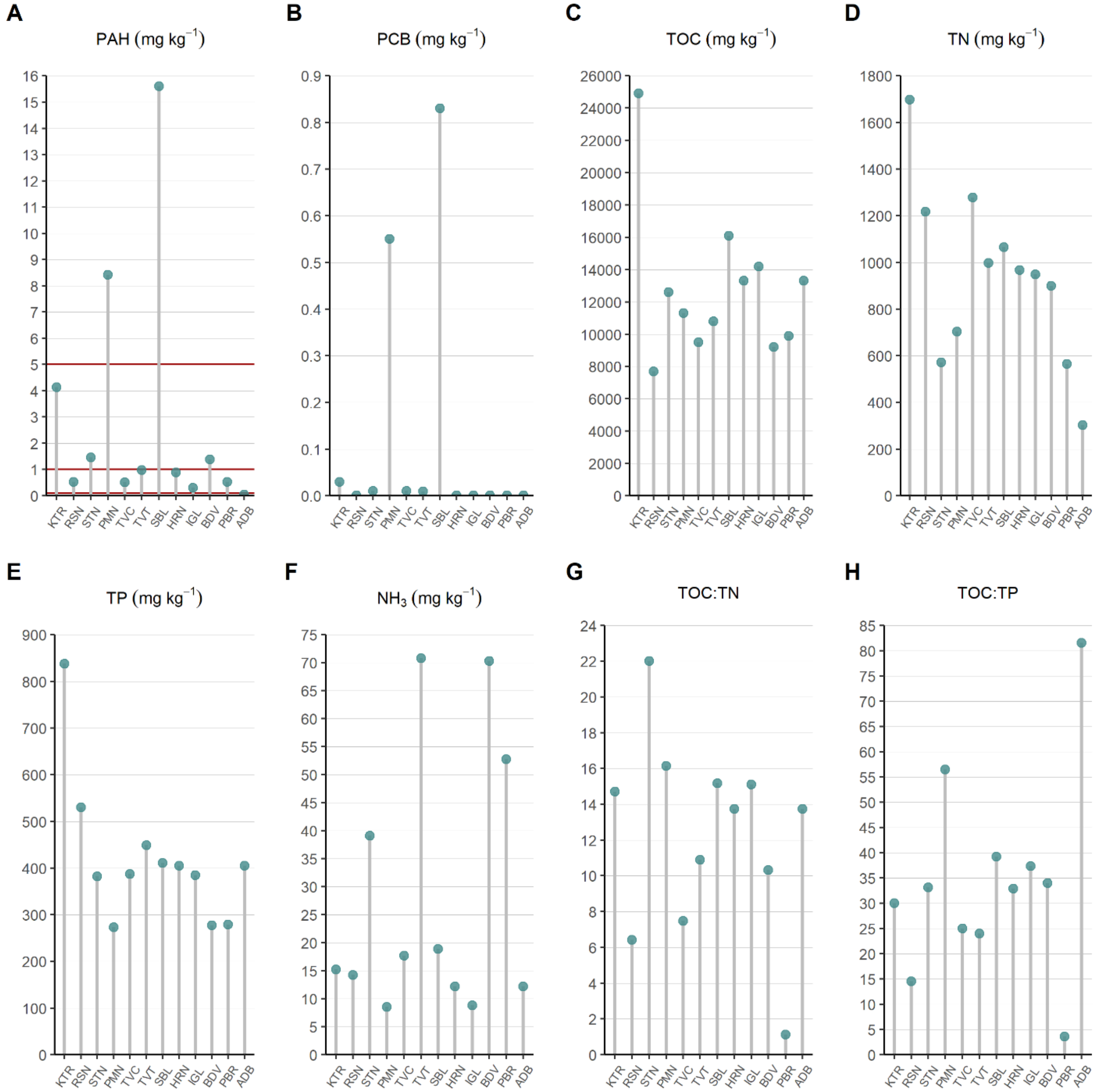


Figure S1. The concentration values and ratios of chemical parameters: (A) PAH (mg kg^{-1}), (B) PCB (mg kg^{-1}), (C) TOC (mg kg^{-1}), (D) TN (mg kg^{-1}), (E) TP (mg kg^{-1}), (F) NH$_3$ (mg kg^{-1}), (G) TOC/TN and (H) TOC/TP. In (A) the red lines indicate PAH pollution levels: low <0.1 mg kg^{-1}, moderate 0.1-1 mg kg^{-1}, high 1-5 mg kg^{-1} and very high >5 mg kg^{-1}.

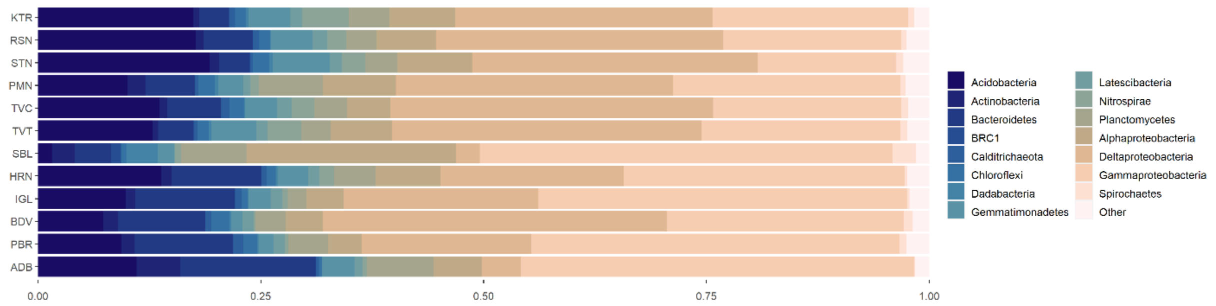


Figure S2. Relative abundance of the most abundant phylum or classes (for Proteobacteria) (≥0.01) based on the classified OTUs in investigated samples. Phyla with relative abundance <0.01 were aggregated into the group reported as “Other”.

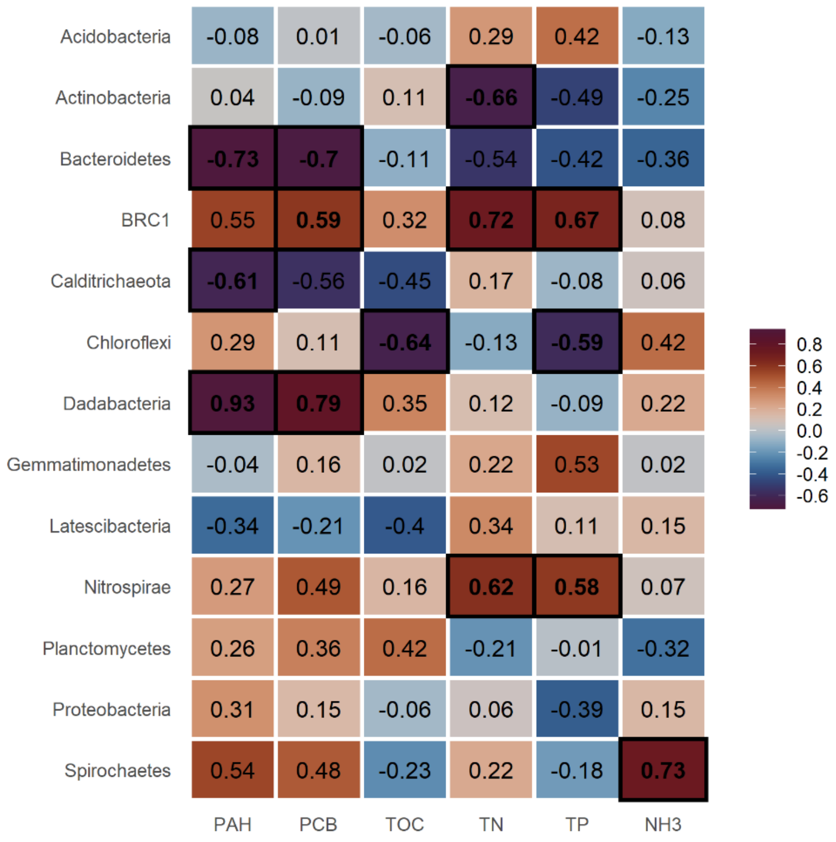


Figure S3. The correlation of bacteria - Spearman's rank correlation analysis at the phylum level. Colour indicates whether the correlation is positive (red) or negative (blue). Bold values showed significant correlation of bacterial phylum and chemical parameter (p≤0.05).

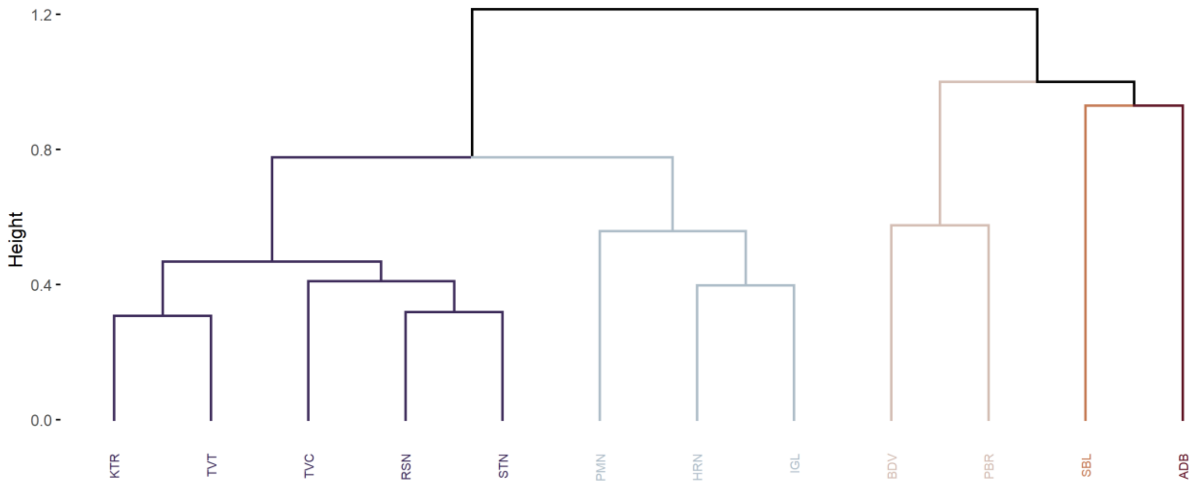


Figure S4. Ward's minimum variance cluster analysis of bacterial community among twelve sediment samples based on Bray-Curtis dissimilarity distance calculated on the OTU level.

Table S1. Description of locations selected as sampling sites.

Station	Station label	Latitude	Longitude	Location description
Port of Kotor	KTR	42.426967	18.765733	The inner part of the Boka Kotorska Bay, located close to the old town Kotor. The great influence of freshwater by river Škurda, wellspring Gurdić, as well as the wastewater from urban effluents. Risk of oil spills due to intensive maritime activities. The third Adriatic cruising port, increased in the number of cruise ships in 2015 by 33% compared to 2010.
Risan	RSN	42.503417	18.686167	Influence of freshwater and organic matter by submerged springs Sopot and Morinj. Exposed to eutrophication.
St. Nedelja	STN	42.463567	18.679950	The narrow part of the Boka Kotorska Bay. Pollution risks due to maritime activities.
Port of Montenegro	PMN	42.434317	18.685583	Former shipyard Arsenal. Nowadays a 450-berth superyacht marina and tourist resort. Affected by the discharges from yachts.
Tivat Cove	TVC	42.404983	18.677933	The shallowest part of the Tivat Bay. Two mussel farming sites. Influence of freshwater by Široka and Gradiošnica river. Their activity is of seasonal character.
Tivat center	TVT	42.431483	18.657550	The middle part of the Boka Kotorska Bay. Influence of Shipyard Bijela.
Bijela Shipyard	SBL	42.446917	18.649933	The largest shipyard in the Southern Adriatic established in 1927, situated within the urban area of settlement Bijela. The potential source for contamination from the disposal of the waste grit.
Herceg Novi	HRN	42.437367	18.543183	The outer part of the Boka Kotorska Bay. Greater influence of the open sea. Affected by urban effluents.
Igalo	IGL	42.444933	18.516700	Influence of the Sutorina river with smaller tributaries flow into it. Mineral water source Slatina is situated in the southern-western part of the alluvial plain of Igalo.
Budva	BDV	42.279800	18.839283	300-berth capacity yacht marina situated in the vicinity of old town Budva, the main tourist destination in Montenegro. Affected by the discharges from small motorboats and yachts.
Port of Bar	PBR	42.098933	19.086583	Montenegro's main port (commercial, cargo ship) situated in the city center of Bar. Capacity about 500-berth for commercial ships and 200-berth for non-commercial boats. The greatest risks are associated with cargo handling, cargo damage, spillage or breakage.
Ada Bojana	ADB	41.865233	19.326900	Estuary area, influenced by the Bojana river, the second tributary of the Adriatic Sea.

Table S2. Assessment criteria for ascertained components of PAHs and PCBs in sediment. Bold shaded cells indicate components with values higher than Effects Range Low (ERL). Concentrations above ERL values could be considered to have bad environmental status (Webster et al., 2009).

mg kg^{-1} d.w.	Stations												Ospar treshold	
	KTR	RSN	STN	PMN	TVC	TVT	SBL	HRN	IGL	BDV	PBR	ADB	BAC	ERL
Naphthalene (Naph)	0.114	0.009	0.033	0.097	0.018	0.022	**0.594**	0.020	0.004	0.005	0.010	0.004	0.008	0.160
Acenaphtylene (Aceph)	**0.015**	0.004	0.004	0.017	0.004	0.004	0.037	0.004	0.004	0.004	0.004	0.004	-	-
Acenaphtene (Ace)	0.024	0.004	0.011	0.090	0.004	0.006	0.283	0.005	0.004	0.018	0.006	0.004	-	-
Fluorene (Fl)	0.034	0.004	0.013	0.102	0.005	0.006	0.259	0.005	0.004	0.021	0.005	0.004	-	-
Phenanthrene (Phen)	**0.327**	0.041	0.120	**0.908**	0.041	0.070	**1.560**	0.058	0.021	0.213	0.045	0.004	0.032	0.240
Anthracene (Ant)	0.081	0.011	0.026	**0.209**	0.007	0.016	**0.281**	0.011	0.004	0.047	0.007	0.004	0.005	0.085
Fluoranthene (Flu)	**0.653**	0.078	0.197	**1.270**	0.067	0.136	**2.150**	0.129	0.047	0.226	0.067	0.004	0.039	0.600
Pyrene (Pyr)	0.563	0.067	0.166	**1.020**	0.059	0.114	**1.670**	0.113	0.038	0.186	0.056	0.004	0.024	0.665
Benzo[a]anthracene (BaA)	**0.331**	0.038	0.112	**0.687**	0.032	0.069	**1.250**	0.066	0.022	0.108	0.034	0.004	0.016	0.261
Chrysene (Chr)	0.278	0.037	0.104	**0.612**	0.032	0.069	**1.080**	0.060	0.022	0.101	0.037	0.004	0.020	0.384
Benzo[a]pyrene (BaP)	0.360	0.049	0.138	**0.746**	0.042	0.086	**1.410**	0.081	0.029	0.111	0.047	0.004	0.030	0.430
Indeno[1,2,3-c,d]pyrene (InP)	0.238	0.034	0.093	**0.494**	0.035	0.070	**0.994**	0.063	0.023	0.057	0.037	0.004	0.103	0.240
Benzo[g,h,i]perylene (BgP)	**0.243**	0.036	**0.106**	**0.464**	0.034	0.089	**0.898**	0.061	0.024	0.061	0.039	0.004	0.080	0.085
Benzo[b]fluoranthene (BbF)	0.513	0.076	0.212	1.07	0.074	0.143	2.00	0.133	0.051	0.144	0.076	0.004	-	-
Benzo[k]fluoranthene (BkF)	0.192	0.034	0.078	0.385	0.028	0.059	0.73	0.050	0.020	0.055	0.029	0.004	-	-
Dibenzo[g,h,i]perylene (DBgP)	0.045	0.006	0.017	0.098	0.006	0.012	0.189	0.011	0.004	0.013	0.007	0.004	-	-
PCB 28	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.00022	0.0017
PCB 52	0.001	0.001	0.001	**0.008**	0.001	0.001	**0.006**	0.001	0.001	0.001	0.001	0.001	0.00012	0.0027
PCB 101	0.003	0.001	0.001	**0.044**	0.001	0.001	**0.054**	0.001	0.001	0.001	0.001	0.001	0.00014	0.003
PCB 118	**0.003**	0.0005	0.0005	**0.028**	0.001	0.001	**0.032**	0.001	0.001	0.001	0.001	0.001	0.00017	0.0006
PCB 138	0.007	0.001	0.003	**0.119**	0.001	0.001	**0.200**	0.001	0.001	0.001	0.001	0.001	0.00015	0.0079
PCB 153	0.012	0.001	0.007	**0.140**	0.001	0.006	**0.211**	0.001	0.001	0.001	0.001	0.001	0.00019	0.04
PCB 180	0.005	0.001	0.003	**0.109**	0.001	0.001	**0.179**	0.001	0.001	0.001	0.001	0.001	0.00010	0.012

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: