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Anthropogenic influence on the environmental health along Montenegro coast based on the bacterial and chemical characterization
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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.
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Response to Reviewers:	

Sandi Orlić

Ruđer Bošković Institute

Zagreb, 15 December 2020

Dear Editor

Please find enclosed the revised manuscript entitled "**Anthropogenic influence on the environmental health along Montenegro coast based on the bacterial and chemical characterization**" for submission to Environmental Pollution, by authors Jokanović, Kajan, Perović, Ivančić, Mačić, Orlić.

We have answered on your and reviewer comments. In the following documents you can find also the new highlights and the revised manuscript.

We are looking forward to a positive replay on this revised manuscript for publication in Environmental Pollution.

Yours sincerely,
Sandi Orlić and co-authors

Response to Editor's and Reviewer's comments

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

Jokanović Sandra, Kajan Katarina, Perović Svetlana, Ivanić Maja, Mačić Vesna, Orlić Sandi

The authors would like to take a chance to express their gratitude for the reviewer and Editor constructive comments.

For the detailed answers to the Reviewer, please refer to the responses as follows: comments of the Reviewers are in black color, and the authors' responses are in red. All accepted remarks are incorporated directly in the final text version marked with the red color.

Editor and Reviewer comments:

Editor   and   Reviewer   comments:

Reviewer #2: The revised manuscript have a great improve by adding a factor: sand content of sediment. Generally, the results of chemical PCA (Figure 2) were consistent in some degree with those the results of bacterial community analysis (Figure 3). For example, SBL, ADB, and BDV were separated from other sites in figure 2. Similarity, the bacterial community composition in these site were also different from other sites as showed in Figure 3. On the another hand, the results of chemical PCA also exhibited some degree inconsistent with the results of bacterial community analysis. For example, PMN was separated from other sites when perform PCA analysis using chemical characterization but it clustered with other sits if considering bacterial community composition. This results indicated that other factor which did not know was also important in determine the bacterial community composition. More discussions should provided on other potential factors in the text such as temperature, salinity, DO parameters, metal concentrations. These parameters should mentioned in the text.

Answer: We have modified and added in the discussion section a more detail analysis of different physical chemical parameters and theirs influence on the bacterial diversity in sediments.

The spatial distribution and environmental constraints of microbial diversity in marine sediment are poorly investigated, despite the ecological and evolutionary significance of marine sedimentary life. At global level (299 samples from 40 worldwide sites) oxygen presence or absence and organic carbon concentration were identified as key environmental factors for defining taxonomic composition and diversity (Hoshino et al., 2020). Recently few papers (Lu et al., 2019; di Cesare et al., 2020; Lee et al., 2020; Rodríguez et al., 2021; Huang et., in press; Ramirez et al., in press) are describing the strong influence of different parameters on microbial diversity in costal

anthropologically influenced sites. We have identified that along the coast of Montenegro TOC, PAHs and silt content are the drive force of bacterial diversity. In our previous research (di Cesare et al., 2020) in a more then 100 - year polluted site (Pula Bay) the diversity was primarily driven by water column depth and nutrient (carbon and nitrogen) availability. Similar results were identified in the North West Gulf of Mexico (Rodríguez et al., 2021) (depth, distance from the shoreline, temperature, dissolved oxygen and aluminum). In Bohai Bay organic matter, in Laizhou Bay the inflow of Yellow River (Lu et al., 2019), in Hangzhou Bay the total phosphorus (Su et la., 2018) influenced the bacterial diversity. At the contrary when a broader area is investigated (South Korean west coast) the bacterial community was influenced by PAHs, alkylphenols and cadmium and mercury (Lee et al., 2020). Similar results were detected in the marginal seas of the Western Pacific region, where influenced by environmental factors, including pressure, depth, seawater density, salinity, organic matter content, nutrient, and heavy metal. The difference that we have identified in our study between the chemical and bacterial diversity and the potential drivers in the PMN site could be attributed to the non-identified factors.

Highlights

- Total PAHs, TOC and silt are potential drivers of the bacterial community on the OTU level.

- The high concentration of PAHs and PCBs influenced the lowest richness and diversity.

- High differences in the functional capabilities of bacterial communities were recorded.

Graphical Abstract
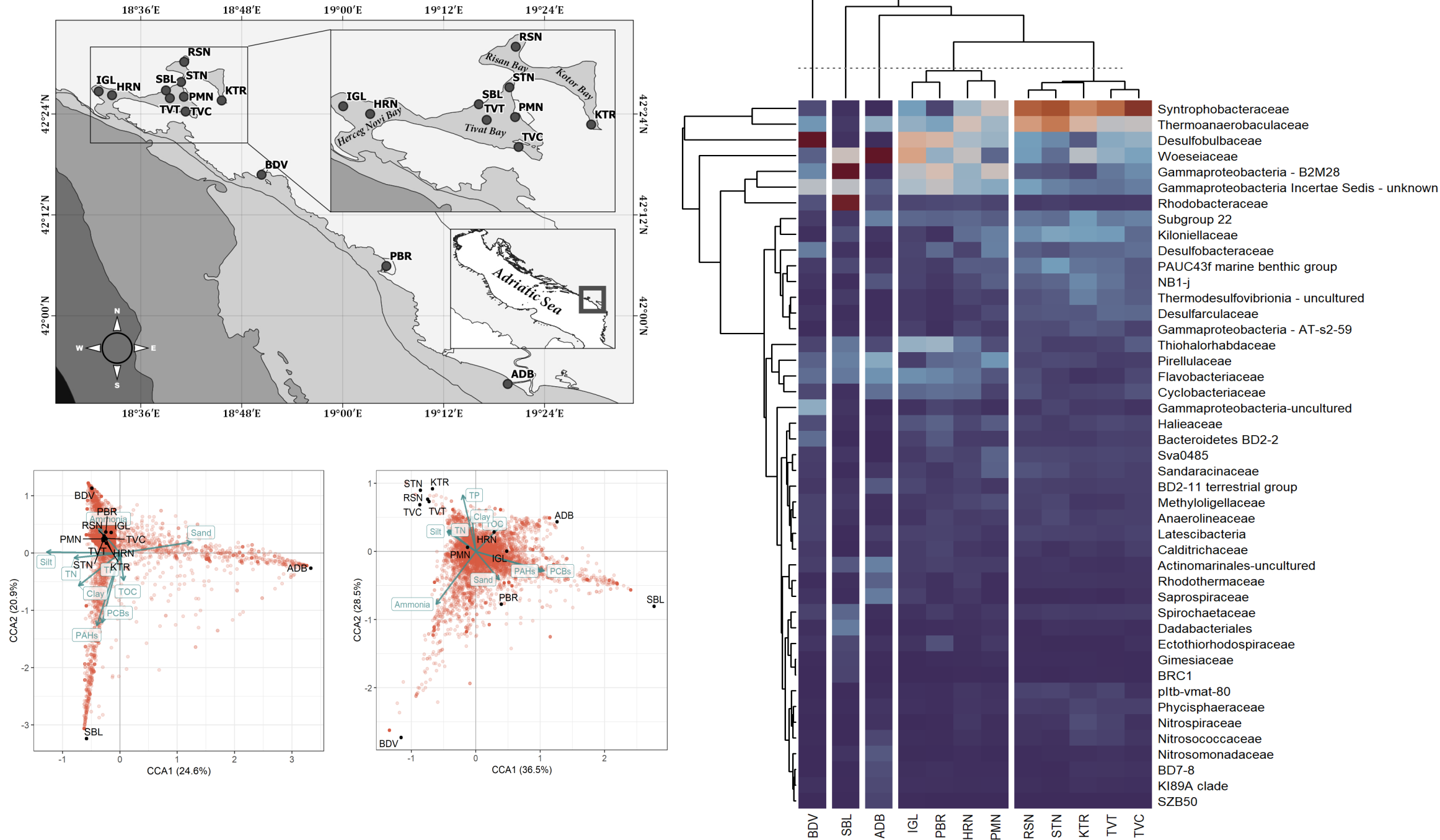
1   Anthropogenic influence on the environmental health along Montenegro coast based on the

2   bacterial and chemical characterization

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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 the estuary site (ADB) forming the independent clusters. Bacterial community based on the OTU level was driven by PAHs, TOC and silt content. The 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

60

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).

92   The Montenegrin coast (south Adriatic) covers a narrow strip with a total coastline length of
93   294 km, of which 105.7 km belong to the Boka Kotorska Bay. The Boka Kotorska Bay
94   represents a unique fjord where the sea penetrates in the continent deeply and naturally divides
95   into smaller bays. The coast is influenced by excessive and uncontrolled urbanization,
96   untreated municipal wastewaters, increasing maritime activities, fishing derived activities and
97   shipbuilding overhaul facility, and a significant increase in population during summer (tourist
98   season) (Mandić et al., 2016). Although covering a relatively small coastline length compared
99   to other Mediterranean countries, in this coast is possible to identify all the typical
100 environmental perturbations.
101 Our research represents the first baseline assessment of sediment microbial communities along
102 the Montenegrin coast. This study provided insight into the potential impact that PAHs and
103 PCBs may have on the microbial community as well as nutrients (total nitrogen, total
104 phosphorous, ammonia) and sediment structure, using a molecular approach. In this research,
105 we have investigated the influence of the abiotic environment on microbial diversity and their
106 functional capabilities potentially related to biodegradation of aromatic compounds and active
107 transport systems, considering both toxic pollutants and natural substances from various
108 origins. Can we identify a typical microbial community for the heavily polluted sites together
109 with the contribution of nutrients and whether it affects their complexity and function?
110
111   **Materials and methods**
112 **Sampling sites**
113 The surface layers of sediment samples (0-2 cm) were collected from 12 locations by Ponar
114 grab along the Montenegrin coast in September 2015. The study area with the location of the
115 sampling sites is shown in Figure 1, while details of the sampling points in Table S1. Sampling
116 in the Boka Kotorska Bay included: Port of Kotor (KTR; 15m depth), Risan (RSN; 22m depth),
117 St. Nedelja (STN; 36m depth), Port of Montenegro (PMN; 15.5m depth), Tivat cove (TVC;
118 12.5m depth), Tivat center (TVT; 40m depth), Shipyard Bijela (SBL; 26m depth), Herceg Novi
119 center (HRN; 40m depth) and Igalo (IGL; 10m depth) while outside the Bay included: Budva
120 (BDV; 10m depth), Port of Bar (PBR; 10m depth) and Ada Bojana (ADB; 10m depth).

121 **Sediment collection**
122 Sediment samples for analysis of total organic carbon (TOC), polycyclic aromatic
123 hydrocarbons (PAHs), polychlorinated biphenyl (PCBs) were sealed in aluminum foil.
124 Samples for analysis of total nitrogen (TN), total phosphorus (TP) and ammonia (NH_3) were

125 sealed in the plastic bags while samples for grain-size and DNA analyses of bacterial diversity

126 were collected in sterile plastic cups. Samples were stored at -70 ºC until analysis.

127 **Analyses of grain-size and chemical parameters**

128 The grain-size analyses were performed using a laser diffraction particle size analyzer LS

129 13320 (Beckman-Coulter, USA). Sediments were classified according to Shepard's

130 classification scheme (1954).

131 Total organic carbon was analyzed according to ISO/TS 13137 (2001) using the TOC 5000A

132 instrument with model SSM 5000A for solid samples. TOC was obtained from the difference

133 between total carbon (TC) and inorganic carbon (IC). Total phosphorus content was performed

134 following the method described by EPA 3051A (2007) on inductively coupled plasma emission

135 spectrometry (Icap 6300, Thermo, USA). Total nitrogen content in the sediment samples was

136 determined using Kjeldahl digestion according to Persson et al. (2008) on Kjeltec 2300 (Foss

137 Tecator, Denmark) equipped with a block system for mineralization. The concentration of

138 ammonia was analyzed according to ISO/TS 14256-1 (2003) filtering the extraction solution

139 and analyzing the nitrogen fractions on spectrometry UV-1800 (Shimadzu, Japan).

140 The analysis of PAHs and PCBs were performed according to EPA 8270 (1998). Samples were

141 defrosted to room temperature prior to analysis. Freeze-dried, homogenized sediment samples

142 were extracted in a Soxhlet extractor with a mixture of hexane and dichloromethane (1:1). The

143 extracts were concentrated on a rotatory evaporator. Clean up treatments were achieved by

144 neutral, activated silica column for PAHs and a combination of acid and base Silica for PCBs.

145 Elution was performed using a mixture of hexane and dichloromethane (1:1) for PAHs, and

146 hexane for PCBs. The sulfur clean up procedure was performed according to EPA 3660B.

147 Evaporation of clean extracts was carried out under a stream of nitrogen. Quantification was

148 done by gas chromatography-mass spectrometry (GC-MS 2010 QP plus, Shimadzu, Japan)

149 attached to SLB-5 ms capillary GC column (Supelco Inc; 30x 0.25mm, 0.25µm thickness).

150 The ascertained components of PAHs were: naphthalene (Naph), acenaphtylene (Aceph),

151 acenaphtene (Ace), fluorene (Fl), phenanthrene (Phen), anthracene (Ant), fluoranthene (Flu),

152 pyrene (Pyr), benzo[a]anthracene (BaA), chrysene (Chr), benzo[a]pyrene (BaP),

153 benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), indeno[1,2,3-c,d]pyrene (InP),

154 benzo[g,h,i]perylene (BgP) and dibenzo[a,h]anthracene (DBgP). The ascertained PCBs were:

155 PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153 and PCB 180.

156 Assessment of PAHs and PCBs concentration was made against the OSPAR Background

157 Assessment Concentrations (BAC) and Effects Range Low (ERL). Sediments with

158   concentrations below BAC were considered to have high environmental status, while

159   concentrations significantly below ERL were considered to have good, and those above, bad

160   environmental status (Webster et al., 2009).

161

162   **PAH pollution ratios**

163   The level of PAH pollution was classified based on the sum of PAH concentration, indicating

164   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})

165   pollution. Four different ratios according to Yunker et al. (2002) were examined: (i) the

166   Ant/Ant+Phen ratio, a value <0.10 indicates petroleum contamination, while a value >0.10

167   combustion processes; (ii) the BaA/BaA+Chr ratio, a value <0.20 indicates petroleum input,

168   mixed sources for values between 0.20 and 0.35, and values >0.35 indicate combustion sources;

169   (iii) the Flu/Flu+Pyr ratio, a value <0.40 is characteristic of petroleum input, values between

170   0.40 and 0.50 indicate the combustion of liquid fossil fuels and crude oil whereas values >0.50

171   indicate the combustion of grass, wood or coal; and (iv) the Inp/Inp+BgP ratio, values <0.20

172   imply petroleum input, values between 0.20 and 0.50 the combustion of liquid fossil fuels and

173   crude oils, and values >0.50 indicate combustion of grass, wood and coal. According to Soclo

174   et al. (2000), the sum of low molecular weight PAHs ($\sum$LPAH; Phen, Ant, Flu and Pyr) and

175   the sum of high molecular weight PAHs ($\sum$HPAH; BaA, Chr, BbF, BkF, BaP, InP, BgP and

176   DBgP) was calculated. The ratio of $\sum$LPAH/$\sum$HPAH <1 implies a pyrogenic source of PAH,

177   whereas the ratio >1 a petrogenic source of PAH.

178   **DNA extraction, PCR and amplicon sequencing**

179   Total DNA was extracted from 0.5 g of sediment with the DNeasy PowerSoil kit (Qiagen,

180   Hilden, Germany) according to the manufacturer's instructions. The hypervariable region

181   V4/V5 of the bacterial 16S rRNA genes was amplified with the primer pair 515F and 926R.

182   Samples were further processed and sequenced by the SeqIT GmbH & Co. KG (Kaiserslautern,

183   Germany). Purified V4/V5 amplicons were paired-end sequenced on an Illumina MiSeq

184   platform. The sequences generated for this study were deposited in the European Nucleotide

185   Archive (ENA) under the accession number _.

186   **Bioinformatics and functional prediction of genes**

187   Paired-end reads were quality trimmed using the *bbduk* function and merged using *bbmerge*

188   function of the BBMap package (v38.71; https://sourceforge.net/projects/bbmap/). Merged

189   reads were quality-filtered using QIIME v1.8.0 (Caporaso et al., 2010). Reads with exact

190   barcodes and primers, unambiguous nucleotides, and a minimum length of 250 base pairs were

191   retained. Chimera check was done using UCHIME (Edgar et al., 2011). Non-chimeric reads

192   were clustered with SWARM v3.0.0 (Mahé et al., 2015) with default settings into Operational

193   Taxonomic Units (OTUs). The bacterial reads were blasted against the SILVA database

194   (release 132; December 13, 2017) using *blastn* (BLAST v2.9.0; Altschul et al., 1990).

195   Nontarget OTUs (chloroplasts, mitochondria), as well as singletons and doubletons, were

196   excluded. Resulting OTUs were filtered by the quality of the blast results ($\geq 98\%$ identity).

197   Standardization among samples was performed by randomly subsampling the table of OTUs

198   to the minimum read level of 18,057 using the *rrarefy* function of the R package *vegan*

199   (Oksanen et al., 2019) to minimize biases associated with sequencing and allow comparison

200   between the samples.

201   The software PICRUSt2 (v2.3.0 beta; Douglas et al., 2020) was used to predict the metagenome

202   of the bacterial community identified based on 16S sequences and their relative proportions in

203   each sediment sample. The rarefied OTU table was normalized in PICRUSt2 with further

204   analysis performed at the gene-level (KEGG orthologs, KO) and the pathway-level (MetaCyc)

205   to determine whether the observed taxonomic diversity in microbial communities accompanies

206   their functional capabilities. To assess the effect of PAHs and PCBs on bacterial community,

207   functional categories related to xenobiotics biodegradation and metabolism, and environmental

208   information processing were selected. The functional categories include predicted genes from

209   degradation pathways of aromatic compounds, polycyclic aromatic hydrocarbon, chloroalkane,

210   chloroalkene, naphthalene and aminobenzoate, and a large family of membrane proteins - ABC

211   transporters.

212   **Statistical analysis**

213   All statistical analyses and visualizations were performed in R version 4.0.0 (R Core Team,

214   2020) using multiple R packages. Bacterial communities were characterized in terms of alpha

215   diversity by calculating OTU richness, Shannon, Simpson, non-parametric ACE, Chao index

216   and Good's coverage. Principal Component Analysis (PCA) was conducted to cluster samples

217   according to chemical and granulometric parameters. Beta diversity of the microbial

218   community on the OTU level was calculated using Bray-Curtis dissimilarity and clustered

219   based on Ward's hierarchical clustering method (Ward.d2; Murtagh and Legendre, 2014).

220   Differences occurring between clusters of samples identified on the OTU level were assessed

221   with permutational multivariate analysis of variance (PERMANOVA). Spearman's rank

222   correlation analysis was used to examine the response of the bacterial community to chemical

223 and granulometric parameters. Heatmap was constructed considering taxonomic classification

224 and abundance on the family level. Canonical Correspondence Analysis (CCA) was carried out

225 to determine potential bacterial community composition drivers on the OTU level. A separate

226 CCA was carried out on the KO level to compare trends of functional capabilities of microbial

227 communities with observed OTUs. ANOVA test was applied to test the statistical significance

228 of each parameter. Results of CCAs were visualized using *ggplot2* (Wickham, 2016). ANOVA

229 with the HSD Tukey test was carried out to test the significant differences of predicted genes

230 associated with degradation pathways and ABC transporters between the categories of samples.

231

232 **Results**

233 **Granulometric and chemical characterization of sediment**

234 The grain-size analyses showed a predominance of the silt fraction in all sediments of Boka

235 Kotorska Bay, ranging from 66% to 82%, whereas the share of clay fraction varied between

236 8% and 36%. According to Shepard's classification, sediments were defined as silts, clayey and

237 sandy silts (Table S3). Outside the Bay, the sediment content of BDV and PBR was mostly silt

238 and sand, while the ADB was classified as sandy sediment with a total of 92% sand fraction.

239 Results of chemical analysis from the investigated sediment samples are presented in Figure

240 S1 and Table S2. The sum of PAH concentration ranged from 0.04 mg kg^{-1} to 15.6 mg kg^{-1}.

241 Only station ADB was characterized by low PAH pollution, six stations (RSN, TVC, TVT,

242 HRN, IGL, PBR) had moderate PAH pollution, three (KTR, STN, BDV) had high PAH

243 pollution and two (PMN, SBL) were defined as very high PAH polluted.

244 The ∑LPAH/∑HPAH ratio varied from 0.42 (PBR) to 0.90 (SBL) at investigated sites implying

245 the pyrolytic origin (Table 1). The Ant/Ant+Phe and BaA/BaA+Chr ratio varied between 0.134

246 (PBR) to 0.5 (ADB) and 0.478 (PBR) to 0.543 (KTR), respectively, indicating the dominant

247 contribution of combustion sources. The InP/InP+BgP ratio varied between 0.467 (STN) to

248 0.525 (SBL). Combustion of liquid fossil fuels and crude oils prevail at KTR, RSN, STN, IGL,

249 BDV, PBR, while combustion of grass, wood and coal prevail at PMN, TVC, TVT, SBL, HRN

250 and ADB site. The Flu/Flu+Pyr ratio ranged from 0.5 (ADB) to 0.562 (SBL), indicating grass,

251 wood or coal combustion at all investigated sites. PAH components with values higher than

252 threshold ERL were observed at KTR, PMN and SBL. At station STN, one PAH component

253 benzo[g,h,i]perylene exceeded ERL value. Total PCB content ranged from 0.001 mg kg^{-1} to

254 0.830 mg kg^{-1}. PCB components at stations PMN and SBL have exceeded threshold values.

255 The value of PCB 118 at station KTR was higher than the threshold values as well.

256 The TOC content in the sediment surface revealed values in the range of 7,700 (RSN) up to

257 24,900 mg kg^{-1} (KTR) (Figure S1). US EPA (2002) recommended the following assessment

258 categories for TOC in sediment: low impact ($\leq$10,000 mg kg^{-1}), intermediate impact (10,000

259 mg kg^{-1} to 30,000 mg kg^{-1}) and high impact ($\geq$30,000 mg kg^{-1}). TOC levels in the analyzed

260 sediments were in the range from low to medium organic carbon levels. The values of total

261 nitrogen ranged between 303 mg kg^{-1} (ADB) to 1,697 mg kg^{-1} (KTR). The TOC/TN ratio has

262 been widely used to infer organic matter origin and sources: >15 the organic carbon (OC) is

263 terrestrial, and <10 predominance of marine organic matter influx (Gälman et al., 2008). In our

264 study four stations: STN, PMN, SBL, IGL had a TOC/TN ratio greater than 15, and stations

265 RSN, TVC, PBR had ratio lower than 10. The sampling sites KTR, TVT, HRN, BDV, ADB

266 with the ratio of TOC/TN ranging from 10 to 14 indicated the combined source (algal and

267 terrestrial). The concentration of ammonia was in the range from 8.5 (PMN) to 70.8 mg kg^{-1}

268 (TVT). The values of total phosphorus varied between 273 (PMN) and 838 mg kg^{-1} (KTR).

269 TOC/TP ratio ranged from 3.57 (PBR) to 56.5 (PMN).

270 PCA plot of chemical and granulometric parameters revealed with separation of SBL and PMN

271 station from other stations according to PAH and PCB components (Figure 2). The first two

272 axes of PCA represent 66.2% of the total variance. Different parameters appeared to shape

273 station KTR with the maximum value of TOC, TN, TP, clay and silt content, while stations

274 ADB, PBR and BDV separated based on the higher content of sand.

275 **Microbial community profiling of sediment samples**

276 A total of 376,167 valid reads were obtained and clustered into 10,521 OTUs with taxonomic

277 identity $\geq$98%. The OTU richness, Shannon and Simpson diversity indices, as well as Chao1,

278 ACE index and Good's coverage, are summarized in Table 2. Based on the OTU richness, TVC

279 had the highest diversity (3,021), followed by HRN (2,995) and KTR (2,917). SBL site had the

280 lowest number of OTU richness (1,652) followed by ADB (1,881). The nonparametric richness

281 indices showed the highest value at TVC site (ACE = 3,812; Chao1 = 4,659) while samples

282 from SBL had the lowest one (ACE = 1,851; Chao1 = 2,125). The highest sediment bacterial

283 diversity (Shannon = 6.5) was found at the PBR site followed by the HRN site (Shannon =

284 6.455) while the lowest one was at SBL (Shannon = 5.418). The Simpson index varied between

285 0.971 (SBL) to 0.994 (PBR). Good's coverage showed values between 92.3% to 97.5%.

286 In the overall bacterial community 47 phyla were identified, from which 13 phyla were present

287 with $\geq$1% of relative abundance at least at one station (Figure S2). Based on average relative

288 abundance, *Gammaproteobacteria* (29.8%), *Deltaproteobacteria* (25.2%) and

289   *Alphaproteobacteria* (7.6%) of the *Proteobacteria* phylum, *Thermoanaerobaculia* (8.1%) of
290   the *Acidobacteria* phylum (12%) and *Bacteroidia* (6.3%) of the phylum *Bacteroidetes* (7.4%)
291   were the five largest classes in the bacterial community of sediments. *Gamma-* and
292   *Deltaproteobacteria* were the most abundant classes in all samples except at the station SBL,
293   where *Alphaproteobacteria* followed *Gammaproteobacteria* in relative abundance taking
294   together more than 60% of the total. In the candidate phyla, members of division *BRC1*,
295   *Dadabacteria* (*Dadabacteriales*) and *Spirochaetes* were present only in SBL with an
296   abundance higher than 1% (1.1%, 3.5% and 2.6%). OTUs affiliated to *Latescibacteria* were
297   present in all stations with abundance higher than 1% except at the station PMN, SBL and
298   ADB, while OTUs affiliated to *Chloroflexi* at the station SBL, IGL and ADB.
299   *Gammaproteobacteria* was the most abundant class at stations: SBL, HRN, IGL, PBR and
300   ADB, with 46.3%, 31.5%, 41.4%, 41.3% and 44.1% respectively. Within the order
301   *Steroidobacterales*, family *Woeseiaceae* accounted for the maximum abundance (27.6%) at
302   ADB, followed by IGL station (12.6%) (Figure 3). At other investigated stations, their
303   abundance varied between 2.4% to 9.2%. In contrast, *Deltaproteobacteria* was the most
304   abundant class in other seven stations: KTR, RSN, STN, PMN, TVC, TVT, BDV with 28.9%,
305   32.3%, 32%, 31.1%, 36.2%, 34.7% and 38.6% respectively (Figure S2). Within this class,
306   family *Desulfobulbaceae* and *Syntrophobacteraceae* were the most abundant.
307   *Desulfobulbaceae* varied between 0.1% to 30.4% with the highest abundance accounted in the
308   BDV sample (Figure 3). *Syntrophobacteraceae* showed values between 0.1% to 18.6% with
309   the maximum at TVT. The percentage of *Alphaproteobacteria* ranged from 3.8% to 23.5%,
310   with maximum abundance at station SBL. Family *Rhodobacteraceae* counted 19.5% at SBL
311   station while at the other investigated stations varied between 0.6% to 1.7%. The abundance of
312   *Bacteroidia* varied from 2.7% to 12.5% with a maximum at station ADB.

313   The community structure of the sediment samples based on the family-level composition
314   showed a clear separation of BDV, SBL and ADB from other stations (Figure 3). At the inner
315   and middle part of the Boka Kotorska Bay (KTR, RSN, STN, TVT, TVC) community has
316   displayed a similar taxa dominance clustering together on the family-level. The main
317   representatives of the bacterial community were uncultured *Synthrophobacteraceae*, with a
318   percentage of 14.3%, 16.5%, 17.3%, 18.6% and 15.9%, respectively. Apart from the
319   dominance of *Synthrophobacteraceae*, *Woeseiaceae*, *Thermoanaerobaculaceae* and
320   *Desulfobulbaceae* also were present in two-fold lower abundance. In the area of Kotor Bay and
321   Tivat Bay class *Thermoanaerobaculia* reached abundance above 10% with a maximum of
322   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, *Bacteroidetes* showed a negative correlation with PAH, PCB, TN and clay content, while the positive correlation with sand content. Groups present only in SBL had a significant positive correlation, where *Dadabacteria* highly correlated with PAH and PCB, while *BRC1* with PCB, TN, TP and clay content. Phyla with abundance lower than 1%, such as *Hydrogenedetes*, *Dependentiae* and *Tenericutes* have also shown a significant positive correlation with PAH, PCB and clay content, while *Armatimonadetes* negative to PAH and *Zixibacteria* negative to PAH, PCB and clay content. *Actinobacteria* was found to be negatively correlated with TN and clay content, while *Deltaproteobacteria* was negatively correlated with TOC. Positively correlated phyla to TN and TP was *Nitrospirae*. 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). 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 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. According to the PERMANOVA, the clustering of sediment samples based on the Bray-Curtis dissimilarity was confirmed having a strong, significant difference based on the PAH status, clay, silt and sand content, respectively (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 (Figure 5). CCA based on the OTUs showed

357 that fundamental parameters including PAHs, TOC and silt content impact the variation of
358 bacterial community. In consistence with the CCA results, observed taxonomic diversity on
359 the OTU level in microbial communities accompanied their functional capabilities. In order to
360 capture the impact of PAHs and PCBs on the proportion of predicted genes potentially involved
361 in the degradation of aromatic compounds and related to ABC transporter proteins, the stations
362 were grouped into five groups based on the results of the cluster analysis of all OTUs (Figure
363 S4). The proportion of predicted genes in functional profiles showed a statistically significant
364 difference between the groups (PERMANOVA, p ≤0.001). Selected KOs had a significantly
365 higher frequency in Cluster 4 (SBL) than the remained clusters. Overall, only KOs that
366 significantly differed in relative abundance compared to Cluster 4 (p ≤0.05) are shown in
367 frequency heatmaps (Figure 6; Figure 7). SBL hosted a higher frequency of genes coding for
368 the degradation of different aromatic compounds such as catechol, phenol, naphthalene,
369 terephthalate, phenanthrene, benzoate, etc. It is important to mention that ADB station assigned
370 as a Cluster 5 had the significantly highest frequency of genes involved in phenol/toluene
371 degradation (data not shown). Genes coding for the ABC transporter proteins were significantly
372 higher in SBL station associated to biotin, urea, glycerol, polysaccharide, phosphate transport
373 system, etc. No significant correlations were found between the frequency of the genes
374 involved in the degradation of aromatic compound and PAH and PCB content which opposed
375 to genes related to ABC transporter proteins. The frequency of the genes coding phosphate,
376 glycerol and methionine transport system proteins were positively correlated to PAH and PCB
377 content (p ≤0.05), while genes coding urea transport system proteins correlated positively only
378 to PCB content (p ≤0.05).
379
380

381 **Discussion**
382 Sediment prokaryotic communities can be shaped by various natural and anthropogenic
383 perturbations, e.g., chemical pollution, WWTP, eutrophication, etc. (Wang et al., 2016;
384 Rodriguez et al., 2018; Zoppini et al., 2019, Di Cesare et al., 2020). Along the Montenegrin
385 coast different pollution sources, all connected with the tourism or industry, could be identified:
386 unresolved issues of wastewater discharges or maritime activities in ports and shipyards. These
387 activities represent the major income in Montenegro but are also the most significant threat to
388 the environment. This is specially noticed during the tourist season when the number of
389 inhabitants multiplies several times and the maritime activity significantly intensifies. In this

390 research, sediments environmental health through chemical and microbiological

391 characterization along the Montenegrin coast were described.

Sediments chemical characterization

393 Chemical analysis confirmed that shipyard Bijela (SBL) and luxury yacht port of Montenegro

394 (PMN) were the most polluted sites in the investigated area (Kljajić et al., 2011). These results

395 are in agreement with the PAH source characterization in other Mediterranean ports (e.g.,

396 Korlević et al., 2015; Vitali et al., 2019). However, it is important to note that the higher PAH

397 concentrations were observed at sites located in harbor and shipyard where shipping traffic is

398 intense, demonstrating that the harbors are the major sources of sediment contamination

399 (Korlević et al., 2015; Frena et al, 2017). The origin of PAHs could be attributed to the nutrient

400 enrichment, incomplete combustion of fuels (i.e., gasoline, diesel oils) in ports (PBR, KTR),

401 luxury yacht marina (PMN), as well as recreational marina (BDV). Activities during

402 shipbuilding processes at the SBL site contribute to pyrolytic PAH origin since high molecular

403 weight PAHs (HPAHs >3 rings) dominated. The potential source for contamination could be

404 from the disposal of the waste grit as well (Mandić et al., 2016). Petroleum hydrocarbon

405 concentration was higher at station PBR (military, cargo port) (Kljajić et al., 2011), while in

406 the present research did not exceed the limits, because the port is not in use anymore as was in

407 the past. The lowest PAH and PCB content was detected at estuary station ADB. These might

408 be attributed to the smaller surface area of mass in a sandy fraction, which decreases the

409 adsorption capacity (GIPME, 2000). PCB are persistent and ubiquitous organic pollutants

410 widely used in industrial applications. Even if the industrial production of PCBs has been

411 banned since the late 1970s, the chronic toxicity of these recalcitrant hazardous substances still

412 remains. A higher value of PCB was detected in two investigated sites: PMN and SBL. These

413 values are similar to other Mediterranean ports (Cardellichio et al., 2007; Merhaby et al., 2015).

414 The processes controlling sediment PAH and PCB concentration are complex and their

415 distribution is affected by numerous factors, including the chemical composition of the

416 sediment (organic matter, clay content, etc.) and the currents (Araghi et al., 2014; Frena et al.,

417 2017). Specifically, PAH concentrations tend to be higher in sediments with higher TOC

418 content due to the high sorption capacity of organic matter (Wang et al., 2014). However, a

419 weak correlation was found between TOC and PAH concentration in our research. In our study,

420 PAH content was highly correlated with TOC/TN ($p \leq 0.05$, $r = 0.536$) (Ladakis et al., 2006).

421 Some authors (Wang et al., 2014) suggest that PAHs preferentially attach to terrestrial organic

422 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

The spatial distribution and environmental constraints of microbial diversity in marine sediment are poorly investigated, despite the ecological and evolutionary significance of marine sedimentary life. At global level (299 samples from 40 worldwide sites) oxygen presence or absence and organic carbon concentration were identified as key environmental factors for defining taxonomic composition and diversity (Hoshino et al., 2020). Recently few papers (Lu et al., 2019; di Cesare et al., 2020; Lee et al., 2020; Rodríguez et al., 2021; Huang et., in press; Ramirez et al., in press) are describing the strong influence of different parameters on microbial diversity in costal anthropologically influenced sites. We have identified that along the coast of Montenegro TOC, PAHs and silt content are the drive force of bacterial diversity. In our previous research (di Cesare et al., 2020) in a more then 100 - year polluted site (Pula Bay) the diversity was primarily driven by water column depth and nutrient (carbon and nitrogen) availability. Similar results were identified in the North West Gulf of Mexico (Rodríguez et al., 2021) (depth, distance from the shoreline, temperature, dissolved oxygen and aluminum). In Bohai Bay organic matter, in Laizhou Bay the inflow of Yellow River (Lu et al., 2019), in Hangzhou Bay the total phosphorus (Su et la., 2018) influenced the bacterial diversity. At the contrary when a broader area is investigated (South Korean west coast) the bacterial community was influenced by PAHs, alkylphenols and cadmium and mercury (Lee et al., 2020). Similar results were detected in the marginal seas of the Western Pacific region,

Different type of environments or pollution will give a different response on the bacterial diversity and activity. Further, the interactions of different conditions in similar environments could have a specific response at microbial level. It is almost impossible to measure all the factors that can have an influence on the biotic community. In the present study we were focused on the PAH, PCB and nutrient component in the surface sediments.

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.,

490 2019). The production of higher amounts of hydrogen sulfide by sulfate reduction with a
491 decrease in oxygen might lead to the inhibition of important microbial processes such as
492 nitrification (Hicks et al., 2018). That could explain the higher content of ammonia in the BDV
493 sediment sample. Evidence of SRB together with abundant aerobic sulfur oxidizers, i.e.,
494 *Gammaproteobacteria*, suggested a rapid transition between oxic and anoxic conditions at
495 surface sediment, probably related to seasonal conditions (Zoppini et al., 2019). Members of
496 B2M28 (*Gammaproteobacteria*) occurred in a higher percentage at the most polluted SBL and
497 PMN site. This group is related to sulfur-oxidizing bacterial endosymbiont (Cifuentes et al.,
498 2000). Family *Syntrophobacteraceae* (*Deltaproteobacteria*) counted higher relative abundance
499 at the inner (KTR, RSN) and middle part (STN, TVC, TVT) of the Boka Kotorska Bay.
500 Increasing water temperature with a higher abundance of *Syntrophobacteraceae* can also
501 indicate lower oxygen concentrations and promote the formation of anoxic "hotspots" within
502 the surface sediment (Mahmoudi et al., 2015; Hicks et al., 2018), although this was not
503 measured.

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

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

519 Despite to low percentage, members of *BRC1*, *Dadabacteria* and *Spirochetes* were present at
520 the most polluted site the SBL suggesting the potential selection of PAH and PCB degrading
521 communities. Reconstructing the central metabolism of *BRC1* has revealed pathways involved
522 in fermentation and complete oxidation of organic substrates through aerobic and anaerobic
523 respiration (Kadnikov et al., 2019), while *Dadabacteria* has shown the potential to degrade

524 microbial particulate organic matter, specifically peptidoglycan and phospholipids (Graham
525 and Tully, 2020). Detected *Spirochaetes* did not correlate with PAH and PCB content,
526 contrasting to recent studies indicating their capability to degrade hydrocarbons or chlorinated
527 compounds under anoxic conditions, such as toluene, naphthalene, alkanes, terephthalate and
528 trichloroethene (Dong, 2018). According to indicator species SRB bacteria
529 (*Sterioidobacterales* and *Desulfobacterales*) were present in the most polluted sites. This
530 finding is in accordance with other studies (Acosta Gonzales et al., 2013; Korlević et al., 2015),
531 where the presence of the role of sulfur cycle microorganisms in oil-polluted coastal marine
532 sediments with focus on SRB bacteria was highlighted.

533 Many studies have indicated the significance of PAH and PCB (Wang et al., 2015; Quero et
534 al., 2015; Vitali et al., 2019; Rodriguez et al., 2021) as potential drivers of bacterial community
535 structure changes. In our study, bacterial community was potentially driven by PAH, TOC and
536 silt content. Although the high concentration of PAHs and PCBs measured at stations SBL and
537 PMN, the bacterial community was characterized by diverse composition and significantly
538 different gene frequencies involved in the degradation of aromatic compounds and ABC
539 transporter proteins. This might be due to the domination of high molecular weight PAHs
540 (HPAHs >3 rings) at the SBL station, having longer persistence and resistance toward
541 biodegradation in the environment altering the structure of microbial community (Ghosal et
542 al., 2016). However, both the physio-chemical characteristics of compounds as well as the
543 physical, chemical and biological properties of sediments can have a high influence on the
544 degradation capacity of microorganisms. Having regard to PCBs, only two genes with low
545 frequency were found to be related to biphenyl degradation encoding biphenyl 2,3-dioxygenase
546 and also genes related to pathways of chloroalkane and chloroalkene degradation, but with no
547 significantly different gene frequencies between the samples (Jing et al., 2018). Predicted genes
548 with the capability to utilize aromatic compounds, with a significant difference between the
549 groups, were related to oxidoreductase enzymes such as salicylate hydroxylase, p-
550 hydroxybenzoate 3-monooxygenase, hydrolases 3-oxoadipate enol-lactonase, catechol 2,3-
551 dioxygenase, 3,4-dihydroxybenzoate indicating aerobic degradation pathways at the SBL
552 station (Ghosal et al., 2016). Genes coding for the ABC transporter proteins were associated
553 with biotin, urea, glycerol, polysaccharide, phosphate transport system, methionine transport
554 system with higher frequency at the SBL station in order to fulfill metabolic requirements for
555 growth and persistence of microbe growing in the polluted sediment.

556

557 **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 and TOC have a statistically significant influence on bacterial diversity on the OTU level. 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 the 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 where a high correlation with TOC were determined. 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 nutrients concentrations, physio-chemical characteristics and sediment granulometric composition. Further research can reveal the potential of bacterial community found at the SBL in new biodegradation and bioremediation pathways. At the environmental health level, the molecular and chemical characterization of the selected sites gives us a useful baseline for future monitoring programs, especially with the cost reduction of metagenomic analysis.

CRediT author statement

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

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866   Figure captions
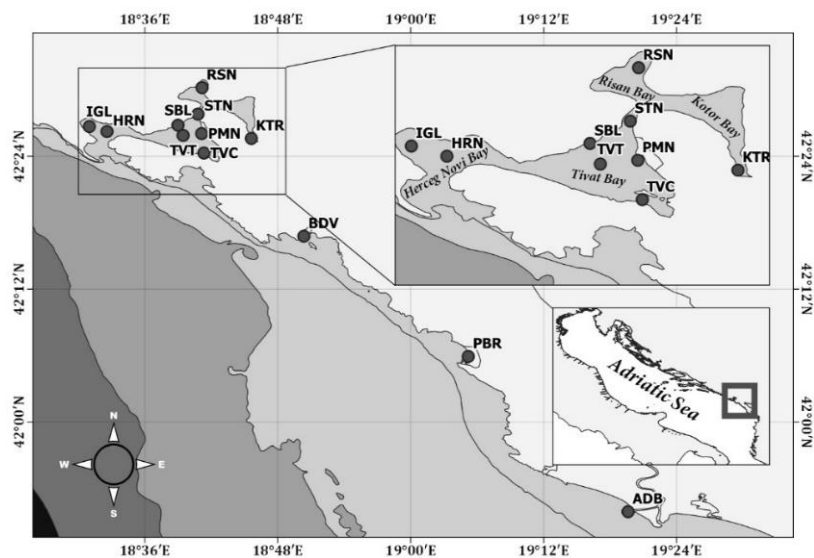
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868   **Figure 1.** A map of Boka Kotorska Bay with marked sampling locations.

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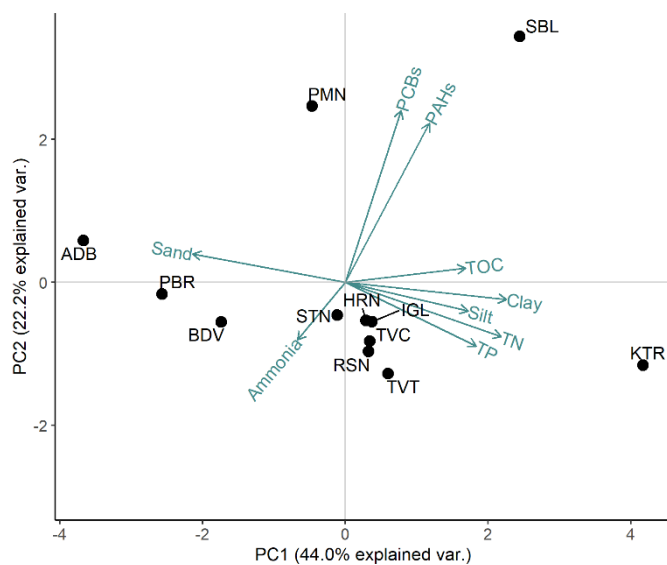
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871   **Figure 2.** Principal component analysis (PCA) of chemical parameters and granulometric
872   composition of sediments.

873
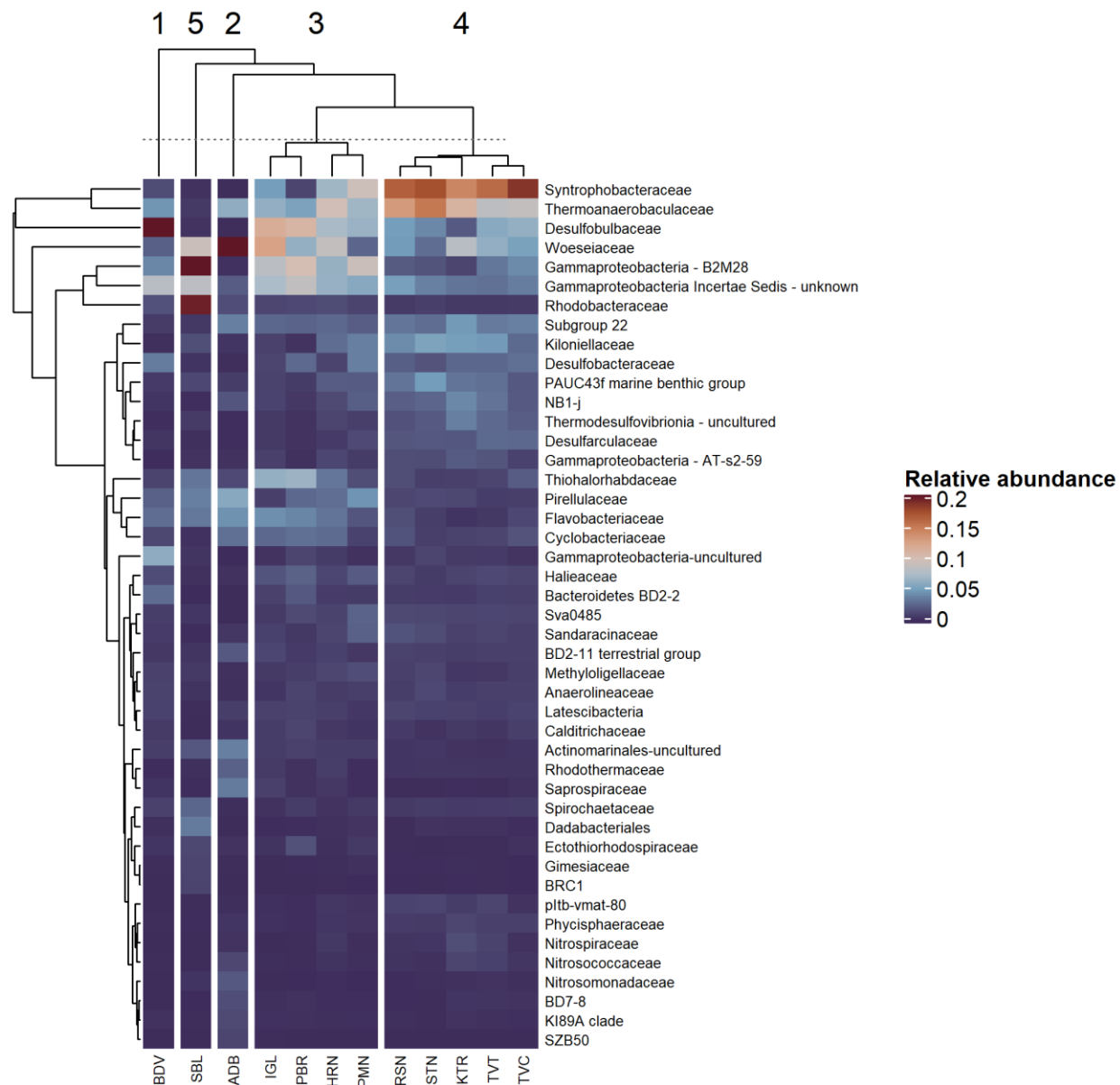
Figure 3. Family-level hierarchical clustering of bacterial distribution from twelve samples based on Bray-Curtis dissimilarity. Rows represent the relative abundance of abundant bacterial family (≥0.01) and column represents different sample. The relative abundance for each family is indicated by color key legend.
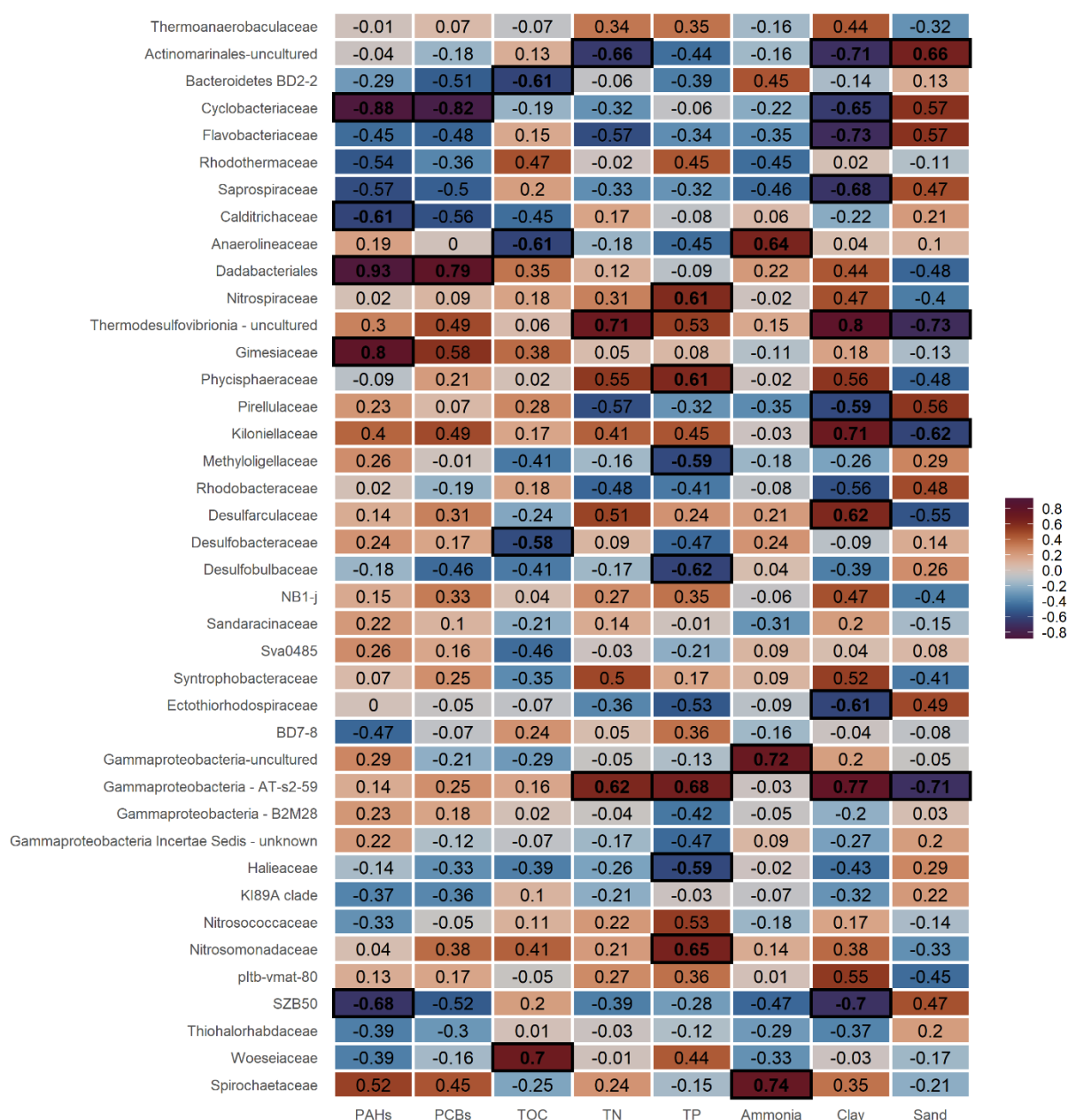
Figure 4. Spearman's rank correlation analysis at the family level with chemical and granulometric parameters. Color indicates whether the correlation is positive (red) or negative (blue). Bold values showed significant correlation of bacterial family and parameter (p≤0.05).
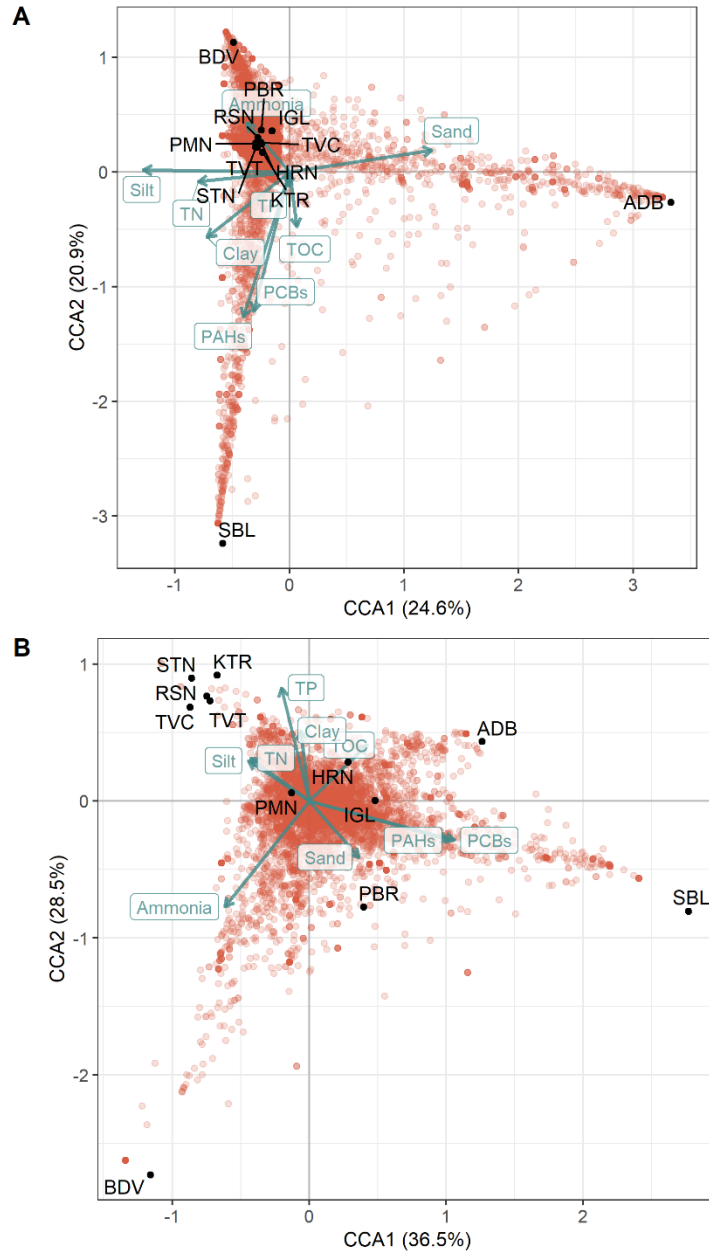
Figure 5. Canonical correspondence analyses (CCAs) ordination diagram. Relationship between chemical and granulometric parameters presented as vectors (PAH, PCB, TOC, TN, TP, Ammonia, Clay, Silt, Sand) and the taxonomic composition at the OTU level (A) and the KO level (B). Black dots represent sampling sites; red dots represent OTUs (A) and KOs (B). The vectors are represented as arrows. According to ANOVA permutation test statistically significant (p≤0.05) correlation was found on the OTU level (A) with PAHs, TOC and Silt.

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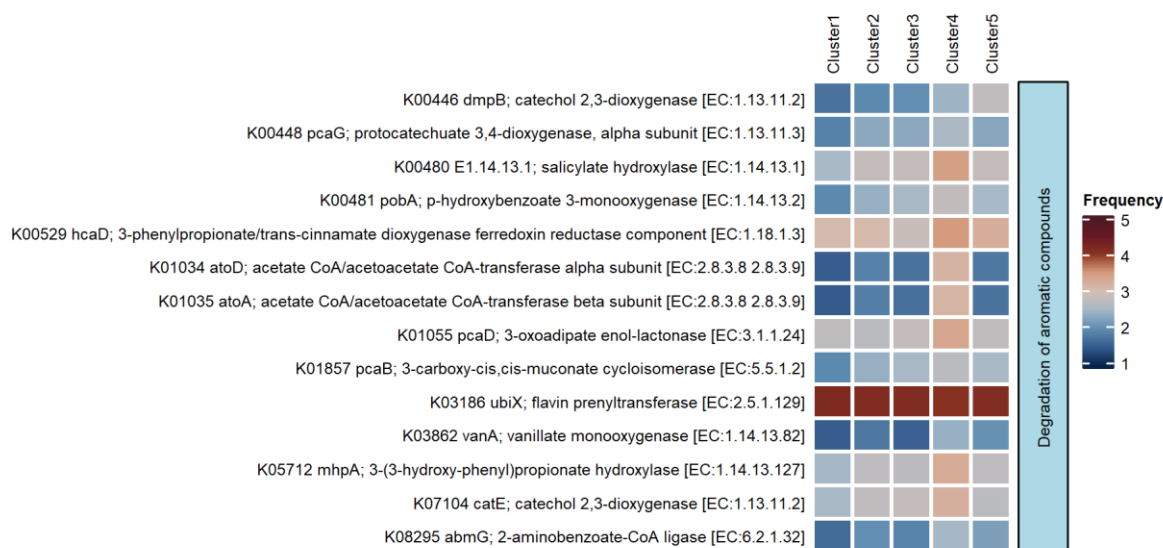
Figure 6. Frequency heatmap of the significantly different KOs related to the degradation of aromatic compounds between the clustered samples ($p \leq 0.05$). The frequency data has been transformed in log10 for better visualization of the differences between the frequencies. Clusters represent the average 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
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0
-2

896 **Figure 7.** Frequency heatmap of the significantly different KOs related to ABC transporter
897 proteins between the clustered samples (p ≤ 0.05). The frequency data has been transformed in
898 log10 for better visualization of the differences between the frequencies. Clusters represent the
899 average of gene frequencies: Cluster 1 (TVT, KTR, RSN, STN, TVC), Cluster 2 (IGL, HRN,
900 PMN), Cluster 3 (BDV, PBR), Cluster 4 (SBL) and Cluster 5 (ADB).
901

902 **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
$\sum$LPAH/$\sum$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

903

904

905 **Table 2.** Alpha diversity indices estimated using Richness, Shannon, Simpson, ACE, Chao1 and
906 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

907

908

909 **Supplementary Material**

910
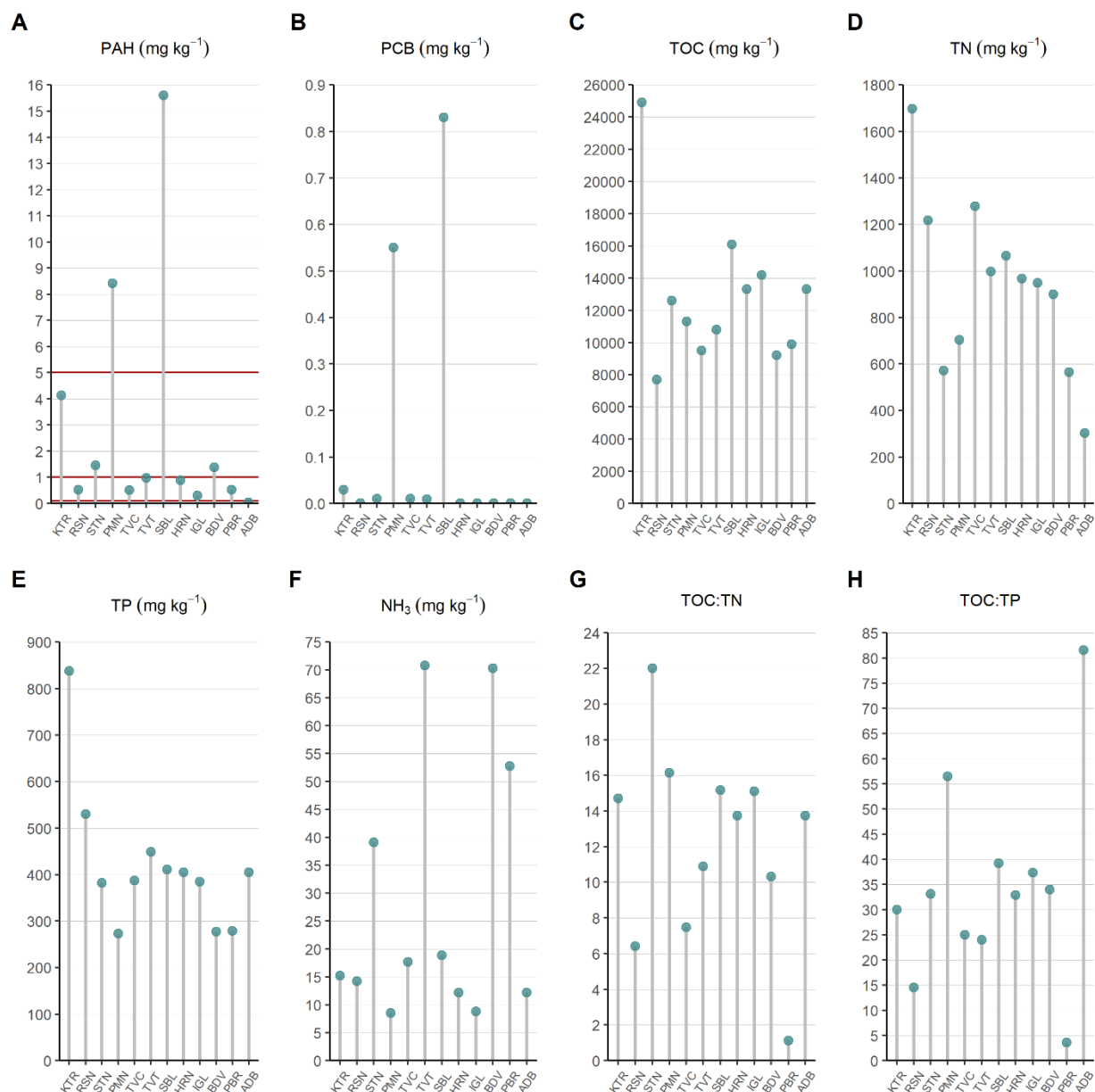
Figure S1. The concentration values and ratios of chemical parameters: (A) PAH (mg kg^{-1}), (B) PCB (mg kg^{-1}), (C) TOC (mg kg^{-1}), (D) TN (mg kg^{-1}), (E) TP (mg kg^{-1}), (F) NH$_3$ (mg kg^{-1}), (G) TOC/TN and (H) TOC/TP. In (A) the red lines indicate PAH pollution levels: low <0.1 mg kg^{-1}, moderate 0.1-1 mg kg^{-1}, high 1-5 mg kg^{-1} and very high >5 mg kg^{-1}.
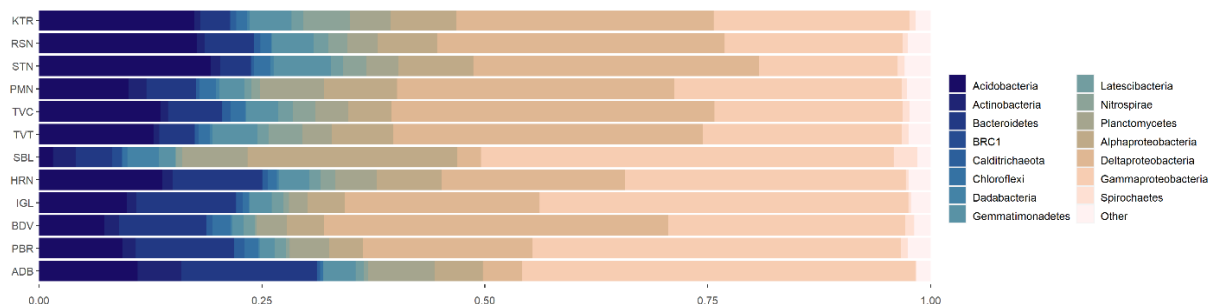
915

916 **Figure S2.** Relative abundance of the most abundant phylum or classes (for Proteobacteria)

917 (≥0.01) based on the classified OTUs in investigated samples. Phyla with relative abundance <0.01

918 were aggregated into the group reported as "Other".
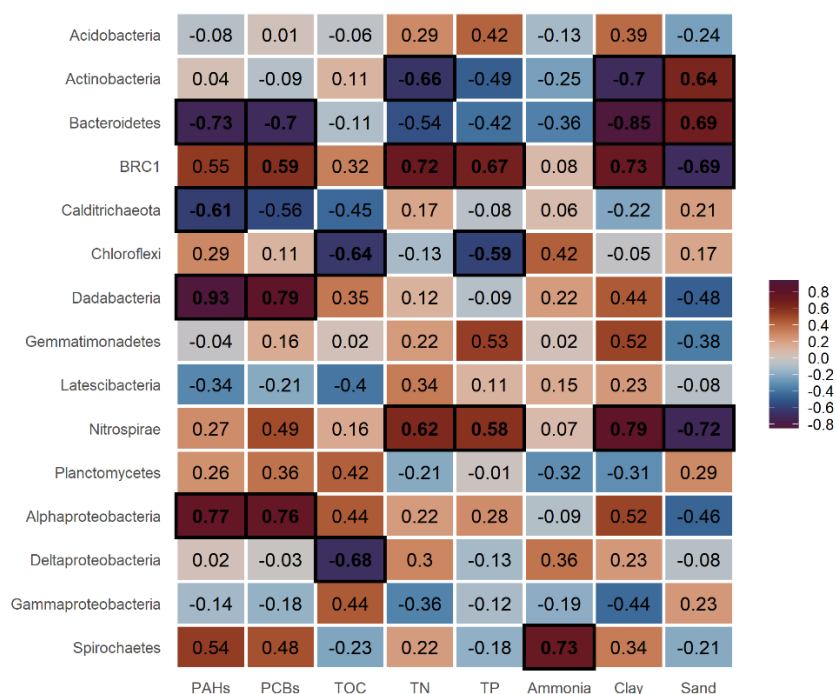
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920

921

922 **Figure S3.** Spearman's rank correlation analysis at the phylum and class level (for Proteobacteria)

923 with chemical and granulometric parameters. Color indicates whether the correlation is positive

924 (red) or negative (blue). Bold values showed significant correlation of bacterial phylum (class) and
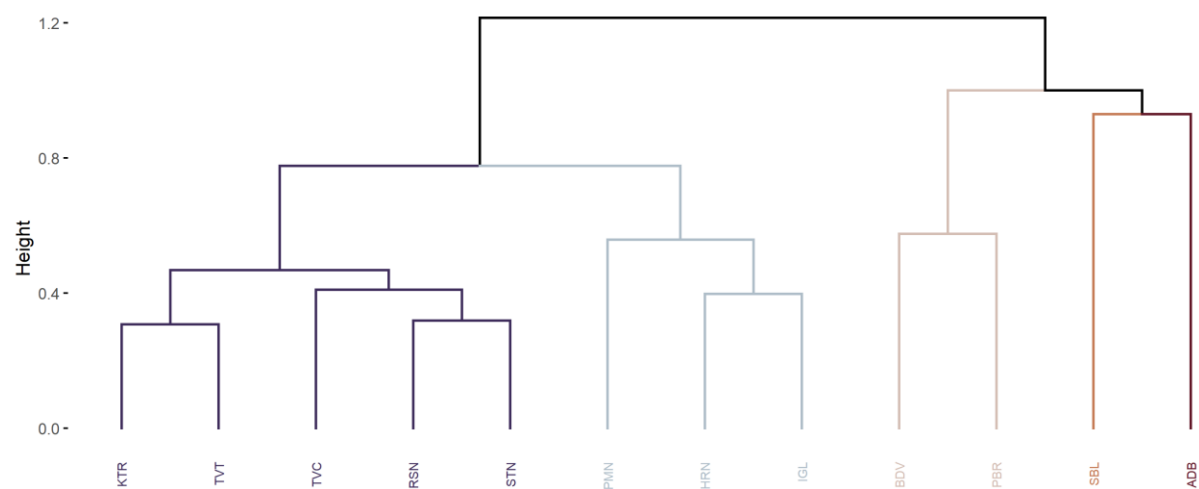
925 parameter (p≤0.05).

926

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.

929

930

931 **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.

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943 **Table S2.** Assessment criteria for ascertained components of PAHs and PCBs in sediment. Bold

944 shaded cells indicate components with values higher than Effects Range Low (ERL).

945 Concentrations above ERL values could be considered to have bad environmental status (Webster

946 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

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948 **Table S3.** Granulometric composition of the sediment samples. Sediments were classified

949 according to Shepard's classification scheme (1954).

	KTR	RSN	STN	PMN	TVC	TVT	SBL	HRN	IGL	BDV	PBR	ADB
Clay (%)	31.5	15.1	19.6	7.7	12.7	27.5	26.1	11.4	11.5	8.6	5.5	1.1
Silt (%)	66.1	68.5	72.1	65.7	78.3	68.2	68.2	79	81.7	54.7	44.7	6.6
Sand (%)	2.4	16.5	8.3	26.7	9	4.3	5.7	9.6	6.8	36.8	49.8	92.3
Shepard's classification	Clayey silt	Sandy silt	Clayey silt	Sandy silt	Silt	Clayey silt	Clayey silt	Silt	Silt	Sandy silt	Silty sand	Sand

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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:

CRediT author statement

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