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## Keystone microbial taxa organize micropollutant-related modules shaping the microbial community structure in estuarine sediments

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1                   **Keystone microbial taxa organize micropollutant-**  
2                   **related modules shaping the microbial community structure**  
3                   **in estuarine sediments**

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16

## 17 **Abstract**

18 The fluctuation of environmental conditions drives the structure of microbial communities in  
19 estuaries, highly dynamic ecosystems. Microorganisms inhabiting estuarine sediments play a key  
20 role in ecosystem functioning. They are well adapted to the changing conditions, also threatened  
21 by the presence of pollutants. In order to determine the environmental characteristics driving the  
22 organization of the microbial assemblages, we conducted a seasonal survey along the Adour  
23 Estuary (Bay of Biscay, France) using 16S rRNA gene Illumina sequencing. Microbial diversity  
24 data were combined with a set of chemical analyses targeting metals and pharmaceuticals.  
25 Microbial communities were largely dominated by *Proteobacteria* (41%) and *Bacteroidota*  
26 (32%), showing a strong organization according to season, with an important shift in winter. The  
27 composition of microbial communities showed spatial distribution according to three main areas  
28 (upstream, middle, and downstream estuary) revealing the influence of the Adour River. Further  
29 analyses indicated that the microbial community was influenced by biogeochemical parameters  
30 ( $C_{org}/N_{org}$  and  $\delta^{13}C$ ) and micropollutants, including metals (As, Cu, Mn, Sn, Ti, and Zn) and  
31 pharmaceuticals (norfloxacin, oxolinic acid and trimethoprim). Network analysis revealed  
32 specific modules, organized around keystone taxa, linked to a pollutant type, providing  
33 information of paramount importance to understand the microbial ecology in estuarine  
34 ecosystems.

## 35 **Environmental implication**

36 The study aims to decipher the impact of anthropogenic multi-contamination on microbial  
37 communities in the Adour Estuary (France) sediments in order to identify the main environmental  
38 drivers as well as the keystone microbial taxa organizing the microbial communities.

39 The environmental conditions (hydrological and geochemical parameters; metals and  
40 pharmaceuticals content) combined with 16S rRNA gene metabarcoding data, revealed the effect  
41 of antibiotics and metals contamination on microbial community structures. We propose that  
42 *Nitrospira*, identified as keystone microbial taxa establishing microbial relationships under  
43 various contamination levels, corresponds to an ideal partner that should be considered when  
44 implementing strategies for environmental management.

45 **keywords:** multi-contamination; sequencing; co-occurrence network; microbial ecology;  
46 antibiotics

47

## 48 **1 Introduction**

49 Estuaries are ecotones, transitional waters at the interface of freshwater and marine habitats. They  
50 are described as highly productive areas providing ecosystem services such as nurseries for many  
51 bird and fish species, regulation of nutrient flow and cycling, and recreational activities (Kennish,  
52 2002; Basset et al., 2013). They are highly sensitive environments showing important fluctuations  
53 of environmental parameters, including salinity, temperature, and nutrient concentrations.  
54 Estuaries have been considered as naturally stressed environments (Elliott and Quintino, 2007),  
55 further threatened by various contaminants released by human activities. Among the  
56 contaminants, metals and pharmaceuticals are of primary concern because they are biologically  
57 active compounds threatening aquatic biota, ecosystems and even human health (Fabbri and  
58 Franzellitti, 2016). These compounds are ubiquitous, being detected in various estuarine  
59 compartments, including surface water (Aminot et al., 2016; Obimakinde et al., 2017), effluent  
60 from wastewater treatment plants (Li et al., 2016), sediments (Shi et al., 2014), and aquatic  
61 organisms (He et al., 2019). Microorganisms play a crucial role in numerous environmental  
62 processes and functions, being involved in carbon, nitrogen, phosphorus cycles (Bauer et al.,  
63 2013; Damashek and Francis, 2018; Watson et al., 2018). In estuarine sediments, microorganisms  
64 are highly diverse with complex organization, representing the main biotic compartment  
65 (Lozupone and Knight, 2007; Yi et al., 2020). The microbial communities are sensitive to  
66 environmental variations such as temperature and salinity, and the presence of micropollutants  
67 (Lozupone and Knight, 2007; Sun et al., 2012; Jeanbille et al., 2016). Hence, in ecosystems as  
68 much fluctuating than estuaries, the shift in microbial community structuration are likely to be  
69 observed in response to the input of metals and pharmaceuticals. The presence of pollutants, both  
70 organic and inorganic, has been shown to influence the organization of microbial communities in  
71 aquatic environments (Bordenave et al., 2004; Vercraene-Eairmal et al., 2010; Cravo-Laureau et  
72 al., 2011; Duran et al., 2015). It is thus of paramount importance to understand how  
73 micropollutants in sediment modify the microbial community structure in order to identify key  
74 microorganisms around which microbial assemblages are organized in response to the presence  
75 of a pollutant. Network analysis allows to determine the interactions between microorganisms  
76 and other organisms and their habitat (Fuhrman and Steele, 2008; Williams et al., 2014),

77 revealing ‘specialists’ microorganisms specifically associated with a pollutant type and its biotic  
78 interactions (Lladó et al., 2015; Duran et al., 2015).

79 The Adour Estuary (France), located in the Bay of Biscay (Atlantic Ocean), features contrasted  
80 areas that are under the influence of different human activities, exhibiting different types and  
81 levels of pollution (Stoichev et al., 2004). The upstream of the watershed is mainly agricultural  
82 with livestock activities, while downstream is highly urbanized, with industrial, port and urban  
83 activities.

84 In this work, we hypothesize that the environmental factors influence the organization of  
85 microbial assemblage in sediments. Particularly, the presence of pollutants promotes the selection  
86 of specific taxa that represent potential biomarkers of pollution, although estuarine environments  
87 constitute highly mixing zones. The main objective of the study was thus to characterize the  
88 relationships between the microbial communities, hydrological and geochemical characteristics,  
89 and pollutants occurrence and content (metals and pharmaceuticals), in estuarine sediments along  
90 ten sampling sites located in the Adour Estuary at different seasons, in order to estimate the  
91 significant parameters driving the estuarine microbial communities.

## 92 **2 Material and Methods**

### 93 **2.1 Study site and sample collection**

94 The Adour Estuary is located at the southwestern of France, extends along 308 km from the  
95 Pyrenees to the Bay of Biscay (Atlantic North-East). This mesotidal estuary presents a highly  
96 heterogenous content river discharge, with  $290 \text{ m}^3 \cdot \text{s}^{-1}$  in annual mean and varying between 50  
97 and  $3,200 \text{ m}^3 \cdot \text{s}^{-1}$ . Three sampling campaigns were performed: May 2017 (spring), September  
98 2017 (summer), and from January to February 2018 (winter). Samples were collected at low tide  
99 slackwater, the tidal coefficient ranged between 78 and 109. Estuarine sediments were sampled in  
100 ten sites from maximum salinity intrusion in the Adour River (site 1) and in the Nive River (site  
101 2) to the downstream estuary (site 10) (Fig. 1). Sites were selected in order to sample both  
102 upstream preserved sites and downstream contaminated sites according to area structuration and  
103 previous observations (Stoichev et al., 2004; Point, 2004; Cavalheiro et al., 2017). Triplicate of  
104 surface sediment samples (0 - 1 cm) were collected in sealable plastics bags. In total, the analyses

105 were performed on 90 samples (10 sites \* 3 replicates \* 3 seasons). Samples were transported in  
106 coolers at approximately 4°C and in the dark to the laboratory, within 2h. Samples were then  
107 stored at -20°C until analysis. During sample collection, an aliquot of each replicate was stored  
108 specifically in 2 mL Eppendorf tubes directly shock-frozen in liquid nitrogen carriers and stored  
109 at -80°C prior microbial DNA extraction.

## 110 **2.2 Hydrological and geochemical characteristics**

111 Sediments were freeze-dried in a freeze-dryer (VaCo2 - Zirbus, Germany). Dry sediments were  
112 visually homogenized, plants, stone and other debris were manually removed. An aliquot of 1 g  
113 was collected for grain size distribution characterization by laser diffraction (Mastersizer 2000,  
114 Malvern). Sediments were then grinded in a planetary ball mill (PM 100, Retsch) and stored at -  
115 20°C. Sediments water contents (%WC) were obtained by measuring the weight loss before and  
116 after drying at 60°C for 48h. Total carbon and particulate organic carbon (TC and POC) were  
117 analyzed by infrared spectroscopy via high temperature combustion on a Shimadzu TOC-  
118 LCSH/CSN/SSM-5000A analyzer. POC was measured after removal of carbonates with 1.2 N  
119 HCl from 200 mg of powdered sample. Sediments for carbon and nitrogen isotopic compositions,  
120 particulate organic nitrogen (PON) and C/N ratios were weighed into silver cups and  
121 decarbonated using 1.2 N HCl, and then analyzed using an elemental analyzer (Flash 2000,  
122 Thermo Fisher Scientific) coupled to an isotope ratio mass spectrometer (IRMS; Isoprime, GV  
123 Instruments). Chlorophyll-*a* was measured in sediments by spectrophotometry according to  
124 Lorenzen (1967) and Aminot and K  rouel (2004) using a Shimadzu UV-1800 spectrophotometer.  
125 All concentrations were expressed in dry weight. River discharge values were obtained from  
126 HYDRO database (<http://hydro.eaufrance.fr>).

## 127 **2.3 Micropollutants characterization: Metals, trace elements and** 128 **pharmaceuticals analyses**

129 Trace metals and metalloids (Ag, As, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sb, Se, Sn, Th, Ti, U, V,  
130 Zn) except Hg were measured in 0.05 to 1 g sample of dry sediments after an acid digestion and  
131 by inductively coupled plasma-mass spectrometry (ICP-MS; 7500 series, Agilent). Briefly, 3 mL  
132 of HNO<sub>3</sub> (70%, Instra Analysed, J.T. Baker) and 1 mL of HCl (37%, Ultrex, Fisher Scientific)

133 were added to dried sediment samples in acid-cleaned and uncolored PP tubes (DigiTUBE, SCP  
134 Science). Sediments solutions were heated on a hot plate at 85°C for 4h (DigiPREP, SCP  
135 Science). Then, 1 mL of hydrogen peroxide (30-32%, Optima, Fisher Chemical) was added drop  
136 by drop and solutions were heated again at 85°C for 2h (DigiPREP, SCP Science). Final volumes  
137 were adjusted to 15 mL with ultrapure water (MilliQ, Millipore). Solutions were centrifugated  
138 and the supernatants were filtered with 0.22 µm PTFE (Millipore) syringe filters. Filtrates were  
139 spiked with internal standards (Bismuth, Bi). Samples were analyzed by ICP-MS using the  
140 external calibration method with internal standard correction (Bi) and limits of detection are  
141 detailed in Table S1.

142 Quality of analyses was controlled with Standard Reference Materials (SRM 1944, National  
143 Institut of Standards & Technology), with mean recoveries ranging, with a few exceptions (2 out  
144 of 16), from 42 to 128%. Total mercury (Hg) content was measured by atomic absorption  
145 spectrophotometry (AAS) (Costley et al., 2000) using Advanced Mercury Analyzer (AMA-254,  
146 LECO). To avoid any grain size effect, trace metal concentrations were normalized with thorium  
147 (Th) concentrations (Lanceleur et al., 2011). For further analyses and statistics, triplicate samples  
148 were averaged per site.

149 A total of 43 pharmaceuticals was measured in surface sediment by liquid chromatography  
150 coupled to tandem mass spectrometry (LC- MS/MS), including 7 non-steroidal anti-inflammatory  
151 drugs (acetaminophen, aspirin, niflumic acid, diclofenac, ibuprofen, ketoprofen and phenazone),  
152 22 antibiotics (ampicillin, azithromycin, ciprofloxacin, clarithromycin, doxycycline,  
153 erythromycin, flumequine, josamycin, metronidazole, norfloxacin, ofloxacin, oxolinic acid,  
154 piperacillin, rifampicin, roxithromycin, spiramycin, sulfadiazine, sulfamethazine,  
155 sulfamethoxazole, tetracycline, trimethoprim and tylosin), 3 anxiolytics (lorazepam, nordazepam  
156 and oxazepam), 3 cardiovascular drugs (atenolol, losartan and metoprolol), as well as an  
157 antiarrhythmic agent (amiodarone), an anticancer drug (cyclophosphamide), one hormone  
158 (norethindrone), a diuretic (hydrochlorothiazide), a compound from fibrate group of medications  
159 (gemfibrozil), a diuretic and carbonic anhydrase inhibitor (acetazolamide), a neuroleptic  
160 (carbamazepine) and caffeine. The latter can be considered as a stimulant and can be used as a  
161 domestic sewage tracer since it is one of the most consumed substances worldwide (Quadra et al.,  
162 2020). Internal standard solutions were composed of carbamazepine-d10, atenolol-d7,



163 ciprofloxacin-d8, norfloxacin-d5, ofloxacin-d3, diazepam-d5, nordazepam-d5 and ibuprofen-d3.  
164 All internal standards were purchased from Sigma-Aldrich. Extraction of pharmaceuticals in  
165 surface sediment was performed following the micro-QuEChERS (Quick, Easy, Cheap,  
166 Effective, Rugged and Safe) method coupling solid extraction protocol (Ben Salem et al. 2016;  
167 Siedlewicz et al. 2016; Azaroff, et al. 2020) and liquid SPE pre-concentration protocol (Gros et  
168 al. 2017). As previously described, the sample volume was adapted to reduce the matrix effect  
169 (Maillet et al. 2017; Guironnet et al. 2022) optimizing the recovery of important sensitive  
170 pharmaceuticals (Peysson and Vulliet, 2013). Briefly, extraction was processed by adding to 200  
171 mg of dry sediment spiked with 40  $\mu\text{L}$  of internal standard solutions (at 50  $\text{mg}\cdot\text{L}^{-1}$  in methanol), a  
172 mixture of 1 mL methanol (MeOH), 1 mL supersaturated aqueous ammonium chloride solution  
173 and 200  $\mu\text{L}$   $\text{Na}_2\text{EDTA}$  at 0.1  $\text{mol}\cdot\text{L}^{-1}$  (Sigma Alrich). Samples were sonicated for 8 min and  
174 centrifuged 5 min at 4,500 rpm. Supernatant were collected and transferred into 50 mL  
175 polypropylene tubes. Sample volume was adjusted to 50 mL with ultrapure water (MilliQ,  
176 Millipore). Solutions were loaded in Oasis HLB cartridges (60 mg, 3 cc) (Waters) previously  
177 conditioned with 3 mL MeOH followed by 3 mL MeOH at 1  $\text{mL}\cdot\text{min}^{-1}$ . After loading samples,  
178 cartridges were rinsed with 5 mL ultrapure water and dried at room temperature for 20 min.  
179 Elution solvent was 5 mL MeOH at flow rate 1  $\text{mL}\cdot\text{min}^{-1}$ . Samples were evaporated under a  
180 gentle air stream (TurboVap LV, Biotage). Dried extracts were then reconstituted in 1 mL  
181 MeOH:H<sub>2</sub>O (MilliQ) (5: 95, v:v) and stored at -20°C prior to analysis by LC-MS/MS using an  
182 Acquity UPLC system connected to a Xevo TQ MS (Triple quadru- pole) with an electrospray  
183 (ESI) interface (Waters). An internal calibration with deuterated analogs was used to quantify  
184 concentrations of target compounds. Two matrix-matched calibration curves were prepared using  
185 200 mg of sediments with different granulometry (sandy and silty) spiked with increasing  
186 concentration of target pharmaceuticals ranging from 0 to 500  $\text{ng}\cdot\text{g}^{-1}$ . Solvent blanks  
187 (MeOH:H<sub>2</sub>O, 5: 95, v:v) and procedural blanks were prepared to evaluate system performances  
188 and detection limits (Table S1). Recoveries were measured from sediment samples adding a  
189 mixture of selected pharmaceuticals at the concentration of 200  $\text{ng}\cdot\text{g}^{-1}$ . With a few exceptions (6  
190 out of 44 compounds), recoveries ranged between 80 and 110%.

## 191 **2.4 Microbial community composition**

192 Total DNA was extracted from environmental samples (~500 mg of wet sediment) using  
193 PowerSoil DNeasy (Qiagen) according to the manufacturer's instructions. The microbial  
194 universal primers 515F-Y 5'-GTGYCAGCMGCCGCGGTAA-3' (Parada et al., 2016) and 926R  
195 5'-CCGYCAATTYMTTTRAGTTT-3' (Quince et al., 2011) were used to amplified V4-V5  
196 hypervariable 16S rRNA gene regions. Triplicate polymerase chain reaction (PCR) reaction  
197 mixtures containing 1  $\mu\text{L}$  of DNA at  $10 \text{ ng} \cdot \mu\text{L}^{-1}$ , 2X AmpliTaq Gold DNA Polymerases (Thermo  
198 Fisher Scientific), 0.5  $\mu\text{M}$  forward primer, 0.5  $\mu\text{M}$  indexed reverse primer in a final reaction  
199 volume of 25  $\mu\text{L}$ . PCR amplification conditions were an initial denaturing step of 10 min at  $95^\circ\text{C}$   
200 followed by 35 cycles of  $95^\circ\text{C}$  for 30 s,  $60^\circ\text{C}$  for 30 s and  $72^\circ\text{C}$  for 45 s and then a final extantion  
201 of 7 min at  $72^\circ\text{C}$ . Per sample, a pool of triplicate PCR reaction was sent to Genotoul (INRA  
202 Toulouse, France) for sequencing process. Amplicon were sequenced using MiSeq Illumina 2x  
203 250 bp. Sequences were processed in R software (version 4.0.2) (R Core Team, 2020). R package  
204 dada2 (version 1.16.0) was used to analyze and cleaned 16S rRNA gene sequence reads and  
205 identified amplicon sequence variants (ASVs) (Callahan et al., 2016a) according to the default  
206 settings. The ASV identification was done using pseudo-pooling sample strategy. The taxonomic  
207 assignment of ASVs was made using SILVA SSU (small subunit) database (version 138) (Quast  
208 et al., 2013) and IDTAXA (Murali et al., 2018). The treatment of ASV and taxonomic table were  
209 processed with R package phyloseq (version 1.32.0) following instruction from Callahan et al.  
210 (2016b) but also including a rarefaction step (calculated on observed richness, R). After diversity  
211 analysis, within ASV and taxonomic table, replicates were merged by mean by site.

## 212 **2.5 Statistical analysis**

213 All graphics and statistical analyses were processed using R software (R Core Team, 2020). All  
214 analyses were performed on thorium-normalized data and average triplicates for chemical data.  
215 For sequencing data, the mean of triplicate for each sample was used to build the ASV table.

216 Principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) were  
217 performed using vegan package (Oksanen et al., 2020) based on Bray-Curtis dissimilarity.  
218 Analysis of similarity (ANOSIM) were performed based on 999 permutation using vegan  
219 package. Permutational multivariate analysis of variance (PERMANOVA) based on 1,000

220 permutations using Bray-Curtis matrices and vegan R package were used to estimate the relative  
221 contribution of environmental variables. Canonical-correlation analysis (CCA) with 9,999 Monte  
222 Carlo permutation tests were conducted, with variables that present significant contribution on  
223 the PERMANOVA and ASV table at the genus taxonomic level, using vegan package.

## 224 **2.6 Co-occurrence networks construction**

225 Co-occurrence network was built from overall ASV profil (all campaign, all site) merge by site,  
226 under Molecular Ecology Network Analyses pipeline (MENAp) using a random matrix theory  
227 (RMT) based method (Zhou et al., 2011; Deng et al., 2012). Based on the relative proportion of  
228 ASVs abundance and ASVs present in a minimum of 37% of all samples (all campaigns  
229 included), networks were constructed using Pearson correlation matrix (Zhou et al., 2011).  
230 Within networks, modules, groups of nodes showing higher densities connections within each  
231 group than between (Newman, 2004), were identified by fast greedy modularity optimization  
232 (Newman, 2004; Clauset et al., 2004). Modules composed of a minimum of 5 nodes were  
233 conserved in further analysis. Gephi 0.9.2 was used to visualize resulting networks and modules  
234 (Bastian et al., 2009).

## 235 **3 Results and discussion**

### 236 **3.1 Geochemical parameters and contamination level**

237 Although the sites on the Adour Estuary were located in different areas, i) downstream and  
238 middle-stream estuarine area under urban contributions and ii) upstream area under agricultural  
239 and fluvial influence (Fig. 1), surface sediments exhibited homogenous geochemical patterns  
240 (Table S2). According to the isotopic signatures and C/N ratios, sediments were dominated by  
241 river derived organic matter with a slight proportion of organic matter from sewage treatment  
242 plants for urban sites (Savoie et al., 2012; Kubo and Kanda, 2017). These geochemical  
243 parameters did not show significant seasonal variations, but the chlorophyll-a and POC contents  
244 were higher in spring and summer, especially in downstream sites, suggesting that a major  
245 amount of sediment organic matter in the Adour Estuary came from autochthonous primary  
246 production (Kubo and Kanda, 2017).

247 The PCA, explaining 46% of the variance on the first two axes, showed that the samples were  
248 distinguished mainly by hotspots values (Fig. 2). The third axis of the PCA, explaining additional  
249 11% of the variance, was also driven by the same hotspot values (Fig. S1). Nevertheless, the  
250 separation of samples according to location can be mainly observed along the axis 2, while the  
251 axes 1 and 3 discerned the samples mainly according to the season (Fig. 2, Fig. S1). The PCA  
252 showed the main distribution of samples as follows: i) the middle-stream sites were linked to  
253 metoprolol and abiotic parameters (water content and fraction < 50  $\mu\text{m}$ ), and Mn in winter and  
254 summer, ii) the upstream sites were linked to norfloxacin in summer and in winter, and Mo in  
255 spring, and iii) the downstream sites were associated to norethindrone, As and Sb in summer, to  
256 oxolinic acid in winter, and to Mo and Zn in spring (Fig. 2). However, the seasonal variability of  
257 both hydrological and geochemical parameters was not significant contrary to that observed in  
258 previous studies on the Adour Estuary (Stoichev et al., 2004), and even other estuaries (Shi et al.,  
259 2014; Aminot et al., 2016). The highest concentration of antibiotics was found in winter (Table  
260 S3), which corresponds to the highest Adour River discharge season contrarily to that observed in  
261 other estuaries (Liu and Wong, 2013; Aminot et al., 2016). The contamination profile of the  
262 Adour Estuary was characterized by higher contaminant concentrations in the middle (mainly site  
263 7) or downstream estuarine Urban area (sites 8, 9 and 10), than upstream area (Fig. 2; Tables S3  
264 to S6). Since it has been shown that the presence of contaminants plays a crucial role in shaping  
265 the structure of microbial communities (Misson et al., 2016; Jacquiod et al., 2018; Wang et al.,  
266 2021), the microbial communities were characterized in order to decipher the environmental  
267 parameters controlling the microbial assemblages.

268

## 269 **3.2 Microbial community diversity and composition**

270 The composition of microbial communities, determined by 16S rRNA sequencing, provided  
271 16,288 ASVs (Amplicon Sequences Variants) coming from 1,629,171 reads (after filtration and  
272 chimera removal). A mean of 1,819 ASVs  $\pm$  494 (richness, R) per sample was obtained, which  
273 was consistent with previous reports showing similar diversity in estuarine sediments (Yan et al.,  
274 2018). Observed richness rarefaction curves showed a plateau (Fig. S2), suggesting that the  
275 sequencing effort was sufficient to collect most of the diversity. The microbial  $\alpha$ -diversity

276 (Simpson evenness) was homogenous among samples (Table S7), indicating that the microbial  
277 diversity level was not affected by the presence of contaminants. It was surprising to observe  
278 such similar diversity indexes in sediments presenting different contaminant types and contents  
279 since previous studies showed that the presence of pollutants affects (increase or decrease) the  
280 microbial diversity (Johnston and Roberts, 2009; Duran et al., 2015; Jacquiod et al., 2018; Li et  
281 al., 2020). However, similar observations have been reported when comparing contaminated and  
282 non-contaminated sediments (Paissé et al., 2008; Ben Salem et al., 2019). The legacy of  
283 microbial communities exposed to fluctuating presence of contaminants combined with the  
284 coalescence of microbial communities, mixing microbial communities along tidal estuarine  
285 continuum, may explain the homogenous microbial diversity observed in the Adour Estuary. This  
286 was also previously proposed in river sediments (Yin et al., 2015).

287 After rarefaction step, in total 42 phyla were observed in this study. More than 80% of the  
288 relative abundance corresponded to two phyla: *Proteobacteria* ( $41 \pm 3\%$ ) and *Bacteroidota* ( $32 \pm$   
289  $6\%$ ) (mean  $\pm$  SD of all samples) (Fig. 3). The dominance of these two phyla is consistent with the  
290 major groups found in estuarine environments (Fortunato and Crump, 2015; Guo et al., 2018;  
291 Vidal-Durà et al., 2018), where they play a critical role in biogeochemical cycles (Xia et al.,  
292 2013). *Proteobacteria* were dominated by *Gammaproteobacteria* ( $23 \pm 9\%$ ) and  
293 *Alphaproteobacteria* ( $9 \pm 3\%$ ). The *Burkholderiales* order, formerly affiliated to  
294 *Betaproteobacteria* class now affiliated to *Gammaproteobacteria*, dominated the  
295 *Gammaproteobacteria* ( $20 \pm 6\%$ ). Interestingly, the abundances of *Alphaproteobacteria* and  
296 *Burkholderiales* showed significant seasonal variability (pairwise wilcox test, p-value  $< 0.05$ )  
297 with opposite trends. From summer/spring to winter, corresponding to the intrusion of freshwater  
298 in the estuary due to higher Adour River discharge in winter, the abundance of  
299 *Alphaproteobacteria* decreased (from 11 to 7%) while that of *Burkholderiales* increased (from 16  
300 to 25%). It is likely that the variation of the abundance of these taxa was a consequence of the  
301 variation of the Adour River discharge (maximum in winter) and the intrusion of freshwater in  
302 the estuary. Accordingly, previous reports showed that *Burkholderiales* occur commonly in  
303 estuarine and freshwater ecosystems (Aylagas et al., 2017; Roberto et al., 2018) while  
304 *Alphaproteobacteria* have been identified as a major group in marine microbial communities  
305 (Pommier et al., 2007; Zinger et al., 2011). The *Bacteroidetes* phylum, which members have been  
306 identified to play a crucial role in the degradation of biopolymers (e.g. chitin, cellulose)

307 (Fernández-Gómez et al., 2013; Kirchman, 2002), was dominated by the *Bacteroidia* class ( $30 \pm$   
308  $6\%$ ), especially by the *Flavobacteriales* order (Table S8). In accordance with previous report  
309 showing the critical role of the *Flavobacterium* genus in the microbial loop (detrital food web)  
310 (Kisand et al., 2002), the dominance of the *Flavobacterium* genus suggested that the organic  
311 matter played a key role in the organization of microbial communities in the Adour Estuary.  
312 Besides, several phyla were present in lower abundance in the bacterial community, including  
313 *Deltaproteobacteria* (*Desulfobacterota*; Waite et al., 2020), ( $5.5 \pm 2.7\%$ ), *Acidobacteriota* ( $4.2 \pm$   
314  $2.2\%$ ), *Verrucomicrobiota* ( $3.1 \pm 2.4\%$ ), *Actinobacteriota* ( $1.7 \pm 0.9\%$ ), *Planctomycetota* ( $1.7 \pm$   
315  $0.8\%$ ), *Chloroflexi* ( $1.6 \pm 0.8\%$ ), and *Firmicutes* ( $1.2 \pm 0.9\%$ ). Members of these phyla are  
316 commonly found in estuarine or freshwater environments (Feng et al., 2009; Guo et al., 2018;  
317 Vidal-Durà et al., 2018). The *Archaea* were found at low abundance ( $< 1\%$ ) showing also  
318 seasonal variation, particularly in the estuarine Urban area, being not detected in spring while  
319 showing the highest abundance ( $1\%$ ) in winter. Despite such low abundance, it is worth to take  
320 into account *Archaea* since they are known to play an important role in biogeochemical cycles,  
321 especially the nitrogen cycle, methanogenesis and even metal resistance (Webster et al., 2015).  
322 The low abundance of *Archaea* in our study was in accordance with previous studies in similar  
323 ecosystems (Kaci et al., 2016; Vidal-Durà et al., 2018), although methodological bias  
324 underestimating *Archaea*, such as high variability of 16S rRNA gene for multiples *Archaea*  
325 lineages and a lack of specificity of universal primers for *Archaea* (Bahram et al., 2019), cannot  
326 be excluded. *Crenarchaeota*, *Halobacterota* and *Euryarchaeota*, were the main phyla,  
327 *Crenarchaeota* representing the more abundant archaeal phylum ( $> 0.2\%$ ; Fig. 3). These archaeal  
328 phyla are usually observed in estuarine sediment (Abreu et al., 2001; Kaci et al., 2016).

### 329 **3.3 Spatial and seasonal variation in microbial community** 330 **structure**

331 ANOSIM showed that the microbial communities in the Adour sediment were grouped according  
332 to season ( $R = 0.78$ ,  $p\text{-value} = 0.001$ ), further confirmed by the hierarchical clustering (Fig. 4A)  
333 revealing that the microbial communities in winter were separated from those of the other  
334 seasons (ANOSIM,  $R = 1$ ,  $p\text{-value} = 0.001$ ). These results suggested that the microbial  
335 communities were influenced by seasonal parameters such as temperature and Adour River  
336 discharge (Table S2). Nevertheless, differences between microbial communities were also

337 observed along the Adour Estuary (ANOSIM,  $R = 0.15$ ,  $p\text{-value} = 0.028$ ), the NMDS analysis  
338 showing three clear clusters (Fig. 4B): microbial communities of upstream sites (1 and 2) being  
339 separated from those of the middle sites (3, 4, 5, 6 and 7) and downstream sites (8, 9 and 10).  
340 Noteworthy, the seasonal effect can be seen within these clusters in which the spring and summer  
341 microbial communities are clearly separated (Fig. 4A). Such observation was in accordance with  
342 the level of contaminant concentrations observed along the Adour Estuary showing a gradient of  
343 increasing pollutant concentration from upstream to downstream (Fig. 2; Tables S5 and S6), but  
344 the clear separation of the most upstream sites (1 and 2) from the other sites confirmed that they  
345 are under freshwater influence. It is likely that despite the microbial communities exhibited  
346 seasonal variations, they were adapted to both hydrological/geochemical conditions and  
347 contaminant concentrations prevailing at each site, as observed in other estuaries (Wang, L. et al.,  
348 2013; She et al., 2016; Guo et al., 2019).

### 349 **3.4 Influence of micropollutants on microbial assemblages**

#### 350 **3.4.1 Major parameters shaping microbial assemblages**

351 Correlation and PERMANOVA analyses revealed that the microbial communities were  
352 influenced by 12 main environmental parameters ( $p\text{-value} < 0.05$ ), including 6 metal(loid)s (As,  
353 Cu, Mn, Sn, Ti and Zn), 3 pharmaceuticals (norfloxacin, oxolinic acid and trimethoprim),  
354  $C_{\text{org}}/N_{\text{org}}$ ,  $\delta^{13}\text{C}$ , a parameter linked to organic matter transformation processes and the season  
355 (Table 1). Additional collinear parameters V, Co, losartan and flumequine respectively associated  
356 to As, Mn, Zn and oxolinic acid were also revealed (Table 1). Interestingly, the correlation of Zn,  
357 proxy used as urban water marker (Pringault et al., 2012), with losartan, antihypertensive drug,  
358 supported the urban contamination. However, oxolinic acid and flumequine, two quinolone  
359 antibiotics, are used in aquaculture and farming (cattle, pig and poultry) as well (Delépée et al.,  
360 2004). The presence of losartan together with Zn probably modifies the effect of Zn on microbial  
361 communities that has been shown to affect microbial activities (Pringault et al., 2008, 2010,  
362 2012). The CCA also shows the link of Zn with pharmaceuticals, especially with the antibiotic  
363 norfloxacin (Fig. 5, Fig. S3). Interestingly, the CCA revealed that the abundance of 9 bacterial  
364 genera correlated with norfloxacin concentration (Fig. 5). Most of the genera are usually found in  
365 anaerobic digesters such as *Acetobacteroides*, *Acidaminobacter*, *Simplicispira*, *Leucobacter*,

366 *Williamwhitmania*, and *Comamonas* (Su et al., 2014; Wen et al., 2017; Szabó et al., 2017;  
367 Schumann and Pukall, 2017; Mei et al., 2020).

368 Additionally, the CCA showed the distribution of microbial communities according to their  
369 location in the estuary along the axis 2 (explaining 11% of the variation), while they were  
370 distributed according to the season mainly winter along the axis 1 (explaining 20% of the  
371 variation) and mainly spring along the axis 3 (explaining 8% of the variation; Fig. S3). The CCA  
372 showed that: i) the upstream sites were driven by trimethoprim in spring and summer, and  
373 oxolinic acid in winter, ii) the middle-stream sites were associated to the organic carbon/nitrogen  
374 ratio ( $C_{org}/N_{org}$ ) in spring and summer, and to Cu and Ti in winter, and iii) the downstream sites  
375 were driven by As and Sn in spring and summer, and by Cu and Ti in winter (Fig. 2. Fig. S3).  
376 Farming activities, such as cattle, sheep, pig and poultry breeding and aquaculture, on the  
377 watersheds could be related with the exposure of microbial communities to antibiotics  
378 (trimethoprim and oxolinic acid) probably on upstream sites. Several bacterial genera were found  
379 associated with trimethoprim, including genera belonging to *Plantomycetes* (*Gemmata* and  
380 *Fimbriiglobus*) known to exhibit wide antibiotic resistance (Godinho et al., 2019).

381 Similarly, twelve genera were associated to oxolinic acid. The majority exhibit potential  
382 antibiotic resistance, including two members of *Chitinophagaceae* (*Terrimonas* and  
383 *Ferruginibacter*) (Cui et al., 2021), *Haliscomenobacter* (Zhang et al., 2020), *Geobacter* (Kashefi  
384 et al., 2003), *Deefgea* (Chen et al., 2010), JGI 0001001-H03 belonging to *Blastocatellaceae*  
385 (Jauregi et al., 2021) or again *Haliangium*, a genus known to synthesize antimicrobial compound  
386 (Sun et al., 2016). Moreover, the CCA showed the association of As and Sn with specific  
387 bacterial genera, including among them *Desulfosarcina*, *Defluviitaleaceae* UCG-011,  
388 *Propionigenium*, *Ruminococcus*, *Malonomonas*, *Macellibacteroides* described as anaerobic  
389 bacteria (Schink and Pfennig, 1982; Dehning and Schink, 1989; Jabari et al., 2012), together with  
390 the cyanobacteria *Chroococciopsis*, *Calothrix* (Boone and Castenholz, 2001) and other aerobic  
391 bacteria such as *Craurococcus*, *Imperialibacter*, *Rubellimicrobium* (Saitoh et al., 1998; Wang, H.  
392 et al., 2013; Jiang et al., 2019).



### 393 3.4.2 Microbial co-occurrence network description

394 The interactions between various microbial functional groups are pivotal for ensuring ecosystem  
395 functioning, as shown for fluctuating polluted environments (McGenity et al., 2012; Duran et al.,  
396 2015). In order to deeper describe the microbial relationships and their interactions with  
397 contaminants (metals and pharmaceuticals), a co-occurrence network was constructed based on  
398 strong and significant Spearman correlations. The microbial network contained 347 nodes and  
399 404 edges, with topological features of complex systems such as scale-free, small-world, and  
400 modularity (M) (Table S9) indicating non-random network as previously proposed (Wan et al.,  
401 2020). The microbial network possessed high connectivity and modularity (Table S9) suggesting  
402 stability and resilience of the system as reported for microbial networks in paddy soil (Wan et al.,  
403 2020) and marine coastal sediment (Jeanbille et al., 2016). Following the 16S rRNA gene  
404 composition, the microbial network was dominated by *Proteobacteria* (40%), *Bacteroidetes*  
405 (25%), *Desulfobacterota* (7%), *Acidobacteriota* (6%), *Planctomycetota* (4%), and  
406 *Verrucomicrobiota* (4%) (Fig. 6A1).

407 Modularity analysis identified a total of 17 modules structuring the network, each composed of 9  
408 to 29 nodes. Noteworthy, the modules were mainly composed by ASVs occurring in summer  
409 and/or spring (Fig. S6), probably because the ASV associated to winter did not show the minimal  
410 occurrence required for network construction (Liu et al., 2015), in accordance with the seasonal  
411 variability observed by CCA (Fig. 5). The modules correspond to groups of strongly connected  
412 ASVs within the group but with very few connections outside the group. Modules showing  
413 significant correlations ( $p$ -value  $< 0.05$ ) with the most significant pollutants (PERMANOVA,  
414 Table 1) were detected (Fig. 6B).

415 Some modules showed negative correlations with environmental parameters (Fig. 6B) suggesting  
416 that these modules gathered microbial taxa either sensitive to metals (modules 4, 5, 6, 9, 12 and  
417 15) or to antibiotics (modules 8 and 11). Such sensitive microbial taxa might be useful to report  
418 ecosystem quality, as for example in the MicrogAmbi integrative microbial community index  
419 combining sensitive and tolerant taxa (Aylagas et al., 2017). However, further investigations are  
420 required to understand the role of such sensitive microbial taxa in the ecosystem functioning. The  
421 other modules showed positive correlations with either antibiotics (modules 1, 6, 9, and 15) or  
422 metals (modules 8, 10, 11, and 16) (Fig. 6B). It was surprising that none of the modules correlate

423 with both antibiotics and metals since several studies have shown co-selection of resistances to  
424 these two kinds of compounds (Baker-Austin et al., 2006; Liu et al., 2021).

### 425 **3.4.3 Antibiotics correlated modules**

426 Regarding the modules positively correlated to antibiotics, four were correlated to both oxolinic  
427 acid and trimethoprim (modules 1, 6, 9, and 15), while the module 7 was correlated only with  
428 oxolinic acid (Fig. 6B). Both antibiotics, used in human and animal therapies, have been detected  
429 together in effluents of wastewater treatment plants (Rodriguez-Mozaz et al., 2020). The modules  
430 correlated with both trimethoprim and oxolinic acid (modules 1, 6, 9, and 15) were structured  
431 around ASVs affiliated to *Hydrogenophaga*, *Ignavibacterium*, *Leptospira* genera, and  
432 *Comamonadaceae* family, which represented keystone ASVs by exhibiting the highest  
433 connectivity within the network (Fig. 6C). The *Comamonadaceae* family, harboring a large  
434 diversity of metabolisms (Willems, 2014), to which *Hydrogenophaga* genus belongs.  
435 *Ignavibacterium album*, the unique member of *Ignavibacteriaceae* family, was isolated from  
436 microbial mats developed in hot spring water streams (Iino et al., 2010). Some species of  
437 *Leptospira* sp. are pathogen, responsible of leptospirosis, a disease targeting humans and animals  
438 (Costa et al., 2015). These three genera have been reported to show resistance to trimethoprim  
439 and/or oxolinic acid, as well as to other antibiotic (Chakraborty et al., 2010; Iino et al., 2010;  
440 Gerzova et al., 2014). In addition, the modules included several less connected ASVs, which  
441 related genera were reported as trimethoprim resistant, such as: *Geobacter* (modules 1 and 6)  
442 (Kashefi et al., 2003), *Gemmata* (module 1) (Cayrou et al., 2010), and *Pseudomonas* (module 15)  
443 (Meng et al., 2020) that is also known as oxolinic acid resistant (Concha et al., 2021). It is also  
444 worth to note that other genera, including *Luteolibacter* (module 1) (Pascual et al., 2017),  
445 *Nitrospira* (modules 1 and 9) (Mehrani et al., 2020), or *Terrimonas*, *Hyphomicrobium*,  
446 *Ferruginibacter* (module 9) (Cui et al., 2021) have been described as antibiotic-resistant taxa. The  
447 presence of well-structured microbial modules around antibiotic-resistant taxa represents an  
448 important concern for human health that should be considered in monitoring aquatic  
449 environment.

#### 450 **3.4.4 Metal(loid)s correlated modules**

451 Regarding the modules positively correlated to metal(loid)s (Fig. 6B), one was correlated to Mn  
452 (module 10) and three to As (modules 8, 11, and 16). The concentration of these elements ranged  
453 between 176 and 1,100  $\mu\text{g}\cdot\text{g}^{-1}$  for Mn and 6.6 and 19  $\mu\text{g}\cdot\text{g}^{-1}$  for As (Tables S4 and S6). These  
454 concentrations are between the lowest effect level (LEL; 460 and 6  $\mu\text{g}\cdot\text{g}^{-1}$  for Mn and As  
455 respectively) and the severe effect level (SEL; 1100  $\mu\text{g}\cdot\text{g}^{-1}$  and 33  $\mu\text{g}\cdot\text{g}^{-1}$  for Mn and As  
456 respectively) for sediment (Persuad et al., 1993), indicating that Mn and As can reach a moderate  
457 contamination level in the Adour Estuary.

458 The module 10, significantly correlated to Mn, included keystone ASVs affiliated to *Lewinella*,  
459 uncultured IheB3-7 bacteria, and two ASVs corresponding to the families of *Comamonadaceae*  
460 and *Rhodobacteraceae* (Fig. 6C2). *Lewinella* and the uncultured IheB3-7 bacteria are marine  
461 bacteria, typically found in sediment and deep-sea systems respectively (Nakagawa et al., 2005).  
462 Interestingly, several members of *Comamonadaceae* family are recognized as manganese-  
463 oxidizing bacteria (Breda et al., 2017). Mn-oxides have been shown to be involved in the  
464 nitrogen cycle, Mn geochemistry being important for controlling redox processes in sediments  
465 (Anschutz et al., 2019). Beside these keystone ASVs, the module gathered several ASVs that  
466 have been described for their Mn removal capacity such as ASVs associated to the  
467 *Flavobacteriaceae* and *Pirellulaceae* families (Carmichael et al., 2013; Molari et al., 2020),  
468 *Nitrospira* and *Oscillatoria* genera (Gerasimenko et al., 2013; Palomo et al., 2016) as well as  
469 metal(loid)s resistant genera such as *Arenimonas* and *Roseomonas* (Li, F. et al., 2021; Wang, F.  
470 et al., 2021).

471 The modules correlated to As were organized around keystone ASVs affiliated to  
472 *Rhodobacteraceae* and *Holophagaceae* (module 8), *Ekhidna*, *Pir4* lineage and TRA3-20 family  
473 (module 11) and *Nitrospira* (module 16) (Fig. 6C3). Some of the genera have been described as  
474 As resistant taxa, including the genera of the *Rhodobacteraceae* (Crognale et al., 2019) and  
475 *Holophagaceae* (Islam et al., 2005; Stroud et al., 2014) families, and members of the *Nitrospira*  
476 genera (Palomo et al., 2018). Such observations supported their structuring role in As resistant  
477 modules. It was particularly interesting to found *Nitrospira*, a nitrite-oxidizing genera able to  
478 perform complete nitrification (comammox), which has been demonstrated to carry As resistance  
479 mechanisms (Palomo et al., 2018). *Nitrospira* might play a crucial role promoting the formation

480 of bacterial assemblages in association with other ASVs related to *Dechloromonas* (module 8)  
481 and *Aquabacterium* (module 16) As resistant genera (Suhadolnik et al., 2017). Additionally, the  
482 modules correlated with As included sulphate-reducing bacteria (SRB) *Desulfonatrobacter*,  
483 *Desulfuromonadia* (module 16), and *Desulfobulbus* (module 8), which have been shown involved  
484 in As methylation and demethylation processes (Chen et al., 2019). Such observation is in  
485 accordance with several studies reporting the presence of SRB and other sulphate-reducing  
486 microorganisms (SRM) in As rich environments (Dias et al., 2008; Bruneel et al., 2008;  
487 Giloteaux et al., 2013; Liu et al., 2018). SRM might play a crucial role in the organization of  
488 microbial networks in response to As.

489 The presence of ASVs related to *Nitrospira* in almost all modules significantly correlated with  
490 pollutants was the most striking observation, which suggested that the presence of *Nitrospira* is  
491 an essential link in the microbial networks. Such omnipresence of *Nitrospira* is probably  
492 explained by its involvement on the nitrogen cycle, particularly via comammox, combined with  
493 its capacity to face the presence of organic and inorganic contaminants (Palomo et al., 2018). The  
494 presence of *Nitrospira* has been reported in microbial networks in various ecosystems, including  
495 wastewater treatment plant (Petrovski et al., 2020), saline soil (Li, X. et al., 2021), alpine  
496 grassland (Qi et al., 2021), agricultural soil (Han et al., 2021), estuarine mudflat (Wang, X. et al.,  
497 2021). Such cosmopolitan behaviour suggests that *Nitrospira* is a “good friend”, an ideal partner  
498 for microorganisms for the colonization of a wide range of habitats. Such capability is  
499 particularly interesting for treatments of contaminated sediments, making *Nitrospira* a potential  
500 bio-augmentation agent.

## 501 **4 Conclusion**

502 Our study shows the stratification of microbial communities along an estuarine continuum  
503 highlighting the fragmentation according to habitat filtering despite the mixing zone that  
504 represents the conditions conducive to the coalescence of microbial communities. Specific  
505 microbial communities were observed, depending on the occurrence and level of contaminants,  
506 that allowed to detect specific and keystone taxa representing “specialists” particularly well  
507 adapted to the presence of contaminants. Such “specialist” taxa, like *Hydrogenophaga*,  
508 *Ignavibacterium*, *Dechloromonas* and *Oscillatoria* represent potential bio-indicators or

509 “sentinels” to be included in integrative indices for evaluating the ecological status of aquatic  
510 environments. The network analysis showed that the microbial communities were organized in  
511 specialized modules around these “specialists” keystone taxa. The modules were tailored to  
512 respond specifically to the presence of a contaminant type, providing useful information to  
513 understand how the environmental parameters shape the organization of microbial assemblages.  
514 Particularly, *Nitrospira* was found to be an ideal partner for establishing microbial relationships  
515 under various conditions, which should be considered in environmental management. The results  
516 pave the way for future studies, particularly at the functional level, for deciphering the metabolic  
517 networks involved in the ecosystem functioning in estuarine ecosystems.

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## 526 **Data Availability Statement**

527 The sequencing datasets generated for this study are available at NCBI Sequence Read Archive  
528 (SRA) database under accession number **SUB9572817**.

## 529 **Annex: Supplementary material**

530

531 **References**

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985 **Figure captions**

986 **Fig. 1. Sampling area and site location along the Adour Estuary.** Sites are identified by  
987 numbers and colors. Shapes indicate site position along the estuary, (■) upstream, (●) middle and  
988 (▲) downstream.

989 **Fig. 2. Comparison of Adour Estuary sites based on hydrological and geochemical**  
990 **parameters and pollutants content.** PCA for (A) sites discriminating by sampling season and  
991 site position, and (B) variables including metals (red), pharmaceuticals (purple), geochemical  
992 parameters (blue) concentrations and abiotic parameters (black).

993 **Fig. 3. Microbial community composition.** Relative abundance (average of three replicates) of  
994 dominant microbial groups (phylum, class or order) observed by site and season in Adour Estuary  
995 sediment. NA, not assigned sequences; Other, taxa with abundance below 0.2% for phyla and  
996 classes and 1 % for orders.

997 **Fig. 4. Comparison of microbial community composition.** A) Hierarchical clustering based on  
998 Bray-Curtis dissimilarity computed on ASVs composition using the Ward D2 linkage method.  
999 The analysis was performed on the average of three replicates. The four groups were determined  
1000 according to the explained inertia (83% with four groups). The seasons are identified by colors:  
1001 spring (green), summer (pink) and winter (yellow). B) NMDS showing k-means clusters, based  
1002 on the Bray-Curtis distances with samples pooled by site (without taking account to the season).

1003 **Fig. 5. Correlations between microbial community structures (genus level) with the**  
1004 **environmental chemical parameters by CCA.** The sites, the chemicals (arrows), and the ASVs  
1005 at the genus level (grey points) are plotted. The bacterial genera discussed in the text are shown  
1006 in red.

1007 **Fig. 6. Adour sediments microbial communities network analysis.** (A) Global network  
1008 representation where each node corresponds to an ASV, node size is proportional to the node  
1009 betweenness. Networks are first colored according to phylum (A1) where NA, designated not  
1010 assigned sequences and Other, phyla with abundance below 1%. Or, they are colored by (A2)  
1011 modules, where modules significantly positively correlated with environmental parameters

1012 selected are identified in colors and Others designed nodes that does not belong to these modules.  
1013 (B) Heatmap of correlation between modules and environmental variables indicating Spearman  
1014 correlation values (p-value). Only correlation with p-value higher or equal to 0.05 were  
1015 represented. (C) Adour sediments microbial communities modules representation. Visualization  
1016 of selected modules correlated to trimethoprim and oxolinic acid (C1), to Mn (C2) and to As (C3)  
1017 illustrating ASVs within each module and their interactions. Each node corresponds to an ASV,  
1018 node size is proportional to the node betweenness. Modules are identified by colors according to  
1019 the legend in (A2). For a better resolution version of the Fig., see Fig. S4 for (A1) and (A2) and  
1020 Fig. S5 for (C1), (C2) and (C3).

1021

1022 **Table 1** Contribution of environmental variables on the organization of microbial communities  
 1023 by PERMANOVA based on Bray-Curtis index. Monte Carlo's level of significance (p) below  
 1024 0.05 are indicated in bold. Only the most significant collinear variables ( $\text{coefficient}_{\text{pear}} \geq |0.85|$ )  
 1025 revealed by PERMANOVA are indicated.

| <b>Environmental variable</b>          | Pseudo-F ratio (F) | R <sup>2</sup> | p*           | Correlation between variables ( $\text{coefficient}_{\text{pear}}$ ) |
|--|--------------------|----------------|--------------|--|
| <b>As</b>                              | 4.47               | 0.061          | <b>0.007</b> | Vanadium (0.87)  |
| <b>Cr</b>                              | 2.09               | 0.028          | 0.065        |  |
| <b>Cu</b>                              | 4.25               | 0.058          | <b>0.001</b> |  |
| <b>Mn</b>                              | 3.39               | 0.046          | <b>0.019</b> | Cobalt (0.90)  |
| <b>Sn</b>                              | 5.91               | 0.081          | <b>0.001</b> |  |
| <b>Ti</b>                              | 6.95               | 0.095          | <b>0.002</b> |  |
| <b>Zn</b>                              | 4.96               | 0.068          | <b>0.002</b> | Losartan (0.85)  |
| <b>Acetaminophen</b>                   | 2.24               | 0.030          | 0.052        |  |
| <b>Caffeine</b>                        | 2.05               | 0.028          | 0.066        |  |
| <b>Lorazepam</b>                       | 1.81               | 0.025          | 0.126        |  |
| <b>Metoprolol</b>                      | 1.57               | 0.021          | 0.151        |  |
| <b>Norfloxacin</b>                     | 2.57               | 0.035          | <b>0.048</b> |  |
| <b>Ofloxacin</b>                       | 2.25               | 0.031          | 0.074        |  |
| <b>Oxolinic acid</b>                   | 2.61               | 0.036          | <b>0.043</b> | Flumequine (0.85)  |
| <b>Trimethoprim</b>                    | 3.77               | 0.051          | <b>0.011</b> |  |
| <b>C<sub>org</sub>/N<sub>org</sub></b> | 2.91               | 0.040          | <b>0.028</b> |  |
| <b>δ<sup>13</sup>C</b>                 | 4.22               | 0.058          | <b>0.008</b> |  |
| <b>Season</b>                          | 2.70               | 0.073          | <b>0.021</b> |  |

\*: Monte Carlo approximated level of significance