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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 HNO₃ (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 μL 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 μL Na_2EDTA 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:H₂O (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:H₂O, 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 μL of DNA at $10 \text{ ng} \cdot \mu\text{L}^{-1}$, 2X AmpliTaq Gold DNA Polymerases (Thermo
198 Fisher Scientific), 0.5 μM forward primer, 0.5 μM indexed reverse primer in a final reaction
199 volume of 25 μL . PCR amplification conditions were an initial denaturing step of 10 min at 95°C
200 followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 45 s and then a final extantion
201 of 7 min at 72°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 μm), 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 α -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 -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 -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_{org}/N_{org} , $\delta^{13}C$, 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

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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.

Environmental variable	Pseudo-F ratio (F)	R ²	p*	Correlation between variables ($\text{coefficient}_{\text{pear}}$)
As	4.47	0.061	0.007	Vanadium (0.87)
Cr	2.09	0.028	0.065	
Cu	4.25	0.058	0.001	
Mn	3.39	0.046	0.019	Cobalt (0.90)
Sn	5.91	0.081	0.001	
Ti	6.95	0.095	0.002	
Zn	4.96	0.068	0.002	Losartan (0.85)
Acetaminophen	2.24	0.030	0.052	
Caffeine	2.05	0.028	0.066	
Lorazepam	1.81	0.025	0.126	
Metoprolol	1.57	0.021	0.151	
Norfloxacin	2.57	0.035	0.048	
Ofloxacin	2.25	0.031	0.074	
Oxolinic acid	2.61	0.036	0.043	Flumequine (0.85)
Trimethoprim	3.77	0.051	0.011	
C_{org}/N_{org}	2.91	0.040	0.028	
δ¹³C	4.22	0.058	0.008	
Season	2.70	0.073	0.021	

*: Monte Carlo approximated level of significance