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Abstract

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

Environmental implication

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

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

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

1 Introduction

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

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

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

2 Material and Methods

2.1 Study site and sample collection

The Adour Estuary is located at the southwestern of France, extends along 308 km from the Pyrenees to the Bay of Biscay (Atlantic North-East). This mesotidal estuary presents a highly heterogenous content river discharge, with 290 $m^3.s^{-1}$ in annual mean and varying between 50 and 3,200 $m^3.s^{-1}$. Three sampling campaigns were performed: May 2017 (spring), September 2017 (summer), and from January to February 2018 (winter). Samples were collected at low tide slackwater, the tidal coefficient ranged between 78 and 109. Estuarine sediments were sampled in ten sites from maximum salinity intrusion in the Adour River (site 1) and in the Nive River (site 2) to the downstream estuary (site 10) (Fig. 1). Sites were selected in order to sample both upstream preserved sites and downstream contaminated sites according to area structuration and previous observations (Stoichev et al., 2004; Point, 2004; Cavalheiro et al., 2017). Triplicate of surface sediment samples (0 - 1 cm) were collected in sealable plastics bags. In total, the analyses were performed on 90 samples (10 sites * 3 replicates * 3 seasons). Samples were transported in coolers at approximately 4°C and in the dark to the laboratory, within 2h. Samples were then stored at -20°C until analysis. During sample collection, an aliquot of each replicate was stored specifically in 2 mL Eppendorf tubes directly shock-frozen in liquid nitrogen carriers and stored at −80°C prior microbial DNA extraction.

2.2 Hydrological and geochemical characteristics

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

2.3 Micropollutants characterization: Metals, trace elements and pharmaceuticals analyses

Trace metals and metalloids (Ag, As, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sb, Se, Sn, Th, Ti, U, V, Zn) except Hg were measured in 0.05 to 1 g sample of dry sediments after an acid digestion and by inductively coupled plasma-mass spectrometry (ICP-MS; 7500 series, Agilent). Briefly, 3 mL of HNO_3 (70%, Instra Analysed, J.T. Baker) and 1 mL of HCl (37%, Ultrex, Fisher Scientific) were added to dried sediment samples in acid-cleaned and uncolored PP tubes (DigiTUBE, SCP Science). Sediments solutions were heated on a hot plate at 85°C for 4h (DigiPREP, SCP Science). Then, 1 mL of hydrogen peroxide (30-32%, Optima, Fisher Chemical) was added drop by drop and solutions were heated again at 85°C for 2h (DigiPREP, SCP Science). Final volumes were adjusted to 15 mL with ultrapure water (MilliQ, Millipore). Solutions were centrifugated and the supernatants were filtered with 0.22 µm PTFE (Millipore) syringe filters. Filtrates were spiked with internal standards (Bismuth, Bi). Samples were analyzed by ICP-MS using the external calibration method with internal standard correction (Bi) and limits of detection are detailed in Table S1.

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

A total of 43 pharmaceuticals was measured in surface sediment by liquid chromatography coupled to tandem mass spectrometry (LC- MS/MS), including 7 non-steroidal anti-inflammatory drugs (acetaminophen, aspirin, niflumic acid, diclofenac, ibuprofen, ketoprofen and phenazone), 22 antibiotics (ampicillin, azithromycin, ciprofloxacin, clarithromycin, doxycycline, erythromycin, flumequine, josamycin, metronidazole, norfloxacin, ofloxacin, oxolinic acid, piperacillin, rifampicin, roxithromycin, spiramycin, sulfadiazine, sulfamethazine, sulfamethoxazole, tetracycline, trimethoprim and tylosin), 3 anxiolytics (lorazepam, nordazepam and oxazepam), 3 cardiovascular drugs (atenolol, losartan and metoprolol), as well as an antiarrhythmic agent (amiodarone), an anticancer drug (cyclophosphamide), one hormone (norethindrone), a diuretic (hydrochlorothiazide), a compound from fibrate group of medications (gemfibrozil), a diuretic and carbonic anhydrase inhibitor (acetazolamide), a neuroleptic (carbamazepine) and caffeine. The latter can be considered as a stimulant and can be used as a domestic sewage tracer since it is one of the most consumed substances worldwide (Quadra et al., 2020). Internal standard solutions were composed of carbamazepine-d10, atenolol-d7, ciprofloxacin-d8, norfloxacin-d5, ofloxacin-d3, diazepam-d5, nordazepam-d5 and ibuprofen-d3. All internal standards were purchased from Sigma-Aldrich. Extraction of pharmaceuticals in surface sediment was performed following the micro-QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method coupling solid extraction protocol (Ben Salem et al. 2016; Siedlewicz et al. 2016; Azaroff, et al. 2020) and liquid SPE pre-concentration protocol (Gros et al. 2017). As previously described, the sample volume was adapted to reduce the matrix effect (Maillet et al. 2017; Guironnet et al. 2022) optimizing the recovery of important sensitive pharmaceuticals (Peysson and Vulliet, 2013). Briefly, extraction was processed by adding to 200 mg of dry sediment spiked with 40 µL of internal standard solutions (at 50 $mg.L^{-1}$ in methanol), a mixture of 1 mL methanol (MeOH), 1 mL supersaturated aqueous ammonium chloride solution and 200 µL Na_2EDTA at 0.1 $mol.L^{-1}$ (Sigma Alrich). Samples were sonicated for 8 min and centrifuged 5 min at 4,500 rpm. Supernatant were collected and transferred into 50 mL polypropylene tubes. Sample volume was adjusted to 50 mL with ultrapure water (MilliQ, Millipore). Solutions were loaded in Oasis HLB cartridges (60 mg, 3 cc) (Waters) previously conditioned with 3 mL MeOH followed by 3 mL MeOH at 1 $mL.min^{-1}$. After loading samples, cartridges were rinced with 5 mL ultrapure water and dried at room temperature for 20 min. Elution solvent was 5 mL MeOH at flow rate 1 $mL.min^{-1}$. Samples were evaporated under a gentle air stream (TurboVap LV, Biotage). Dried extracts were then reconstituted in 1 mL MeOH:H_2O (MilliQ) (5: 95, v:v) and stored at -20°C prior to analysis by LC-MS/MS using an Acquity UPLC system connected to a Xevo TQ MS (Triple quadru- pole) with an electrospray (ESI) interface (Waters). An internal calibration with deuterated analogs was used to quantify concentrations of target compounds. Two matrix-matched calibration curves were prepared using 200 mg of sediments with different granulometry (sandy and silty) spiked with increasing concentration of target pharmaceuticals ranging from 0 to 500 $ng.g^{-1}$. Solvent blanks (MeOH:H_2O, 5: 95, v:v) and procedural blanks were prepared to evaluate system performances and detection limits (Table S1). Recoveries were measured from sediment samples adding a mixture of selected pharmaceuticals at the concentration of 200 $ng.g^{-1}$. With a few exceptions (6 out of 44 compounds), recoveries ranged between 80 and 110%.

2.4 Microbial community composition

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

2.5 Statistical analysis

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

Principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) were performed using vegan package (Oksanen et al., 2020) based on Bray-Curtis dissimilarity. Analysis of similarity (ANOSIM) were performed based on 999 permutation using vegan package. Permutational multivariate analysis of variance (PERMANOVA) based on 1,000 permutations using Bray-Curtis matrices and vegan R package were used to estimate the relative contribution of environmental variables. Canonical-correlation analysis (CCA) with 9,999 Monte Carlo permutation tests were conducted, with variables that present significant contribution on the PERMANOVA and ASV table at the genus taxonomic level, using vegan package.

2.6 Co-occurrence networks construction

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

3 Results and discussion

3.1 Geochemical parameters and contamination level

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

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

3.2 Microbial community diversity and composition

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

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

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

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

3.3 Spatial and seasonal variation in microbial community structure

ANOSIM showed that the microbial communities in the Adour sediment were grouped according to season (R = 0.78, p-value = 0.001), further confirmed by the hierarchical clustering (Fig. 4A) revealing that the microbial communities in winter were separated from those of the other seasons (ANOSIM, R = 1, p-value = 0.001). These results suggested that the microbial communities were influenced by seasonal parameters such as temperature and Adour River discharge (Table S2). Nevertheless, differences between microbial communities were also observed along the Adour Estuary (ANOSIM, R = 0.15, p-value = 0.028), the NMDS analysis showing three clear clusters (Fig. 4B): microbial communities of upstream sites (1 and 2) being separated from those of the middle sites (3, 4, 5, 6 and 7) and downstream sites (8, 9 and 10). Noteworthy, the seasonal effect can be seen within these clusters in which the spring and summer microbial communities are clearly separated (Fig. 4A). Such observation was in accordance with the level of contaminant concentrations observed along the Adour Estuary showing a gradient of increasing pollutant concentration from upstream to downstream (Fig. 2; Tables S5 and S6), but the clear separation of the most upstream sites (1 and 2) from the other sites confirmed that they are under freshwater influence. It is likely that despite the microbial communities exhibited seasonal variations, they were adapted to both hydrological/geochemical conditions and contaminant concentrations prevailing at each site, as observed in other estuaries (Wang, L. et al., 2013; She et al., 2016; Guo et al., 2019).

3.4 Influence of micropollutants on microbial assemblages

3.4.1 Major parameters shaping microbial assemblages

Correlation and PERMANOVA analyses revealed that the microbial communities were influenced by 12 main environmental parameters (p-value < 0.05), including 6 metal(loid)s (As, Cu, Mn, Sn, Ti and Zn), 3 pharmaceuticals (norfloxacin, oxolinic acid and trimethoprim), C_{org}/N_{org}, $\delta^{13}C$, a parameter linked to organic matter transformation processes and the season (Table 1). Additional collinear parameters V, Co, losartan and flumequine respectively associated to As, Mn, Zn and oxolinic acid were also revealed (Table 1). Interestingly, the correlation of Zn, proxy used as urban water marker (Pringault et al., 2012), with losartan, antihypertensive drug, supported the urban contamination. However, oxolinic acid and flumequine, two quinolone antibiotics, are used in aquaculture and farming (cattle, pig and poultry) as well (Delépée et al., 2004). The presence of losartan together with Zn probably modifies the effect of Zn on microbial communities that has been shown to affect microbial activities (Pringault et al., 2008, 2010, 2012). The CCA also shows the link of Zn with pharmaceuticals, especially with the antibiotic norfloxacin (Fig. 5, Fig. S3). Interestingly, the CCA revealed that the abundance of 9 bacterial genera correlated with norfloxacin concentration (Fig. 5). Most of the genera are usually found in anaerobic digesters such as *Acetobacteroides*, *Acidaminobacter*, *Simplicispira*, *Leucobacter*, *Williamwhitmania*, and *Comamonas* (Su et al., 2014; Wen et al., 2017; Szabó et al., 2017; Schumann and Pukall, 2017; Mei et al., 2020).

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

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

3.4.2 Microbial co-occurrence network description

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

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

Some modules showed negative correlations with environmental parameters (Fig. 6B) suggesting that these modules gathered microbial taxa either sensitive to metals (modules 4, 5, 6, 9, 12 and 15) or to antibiotics (modules 8 and 11). Such sensitive microbial taxa might be useful to report ecosystem quality, as for example in the MicrogAmbi integrative microbial community index combining sensitive and tolerant taxa (Aylagas et al., 2017). However, further investigations are required to understand the role of such sensitive microbial taxa in the ecosystem functioning. The other modules showed positive correlations with either antibiotics (modules 1, 6, 9, and 15) or metals (modules 8, 10, 11, and 16) (Fig. 6B). It was surprising that none of the modules correlate with both antibiotics and metals since several studies have shown co-selection of resistances to these two kinds of compounds (Baker-Austin et al., 2006; Liu et al., 2021).

3.4.3 Antibiotics correlated modules

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

3.4.4 Metal(loid)s correlated modules

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

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

The modules correlated to As were organized around keystone ASVs affiliated to *Rhodobacteraceae* and *Holophagaceae* (module 8), *Ekhidna*, *Pir4 lineage* and TRA3-20 family (module 11) and *Nitrospira* (module 16) (Fig. 6C3). Some of the genera have been described as As resistant taxa, including the genera of the *Rhodobacteraceae* (Crognale et al., 2019) and *Holophagaceae* (Islam et al., 2005; Stroud et al., 2014) families, and members of the *Nitrospira* genera (Palomo et al., 2018). Such observations supported their structuring role in As resistant modules. It was particularly interesting to found *Nitrospira*, a nitrite-oxidizing genera able to perform complete nitrification (comammox), which has been demonstrated to carry As resistance mechanisms (Palomo et al., 2018). *Nitrospira* might play a crucial role promoting the formation of bacterial assemblages in association with other ASVs related to *Dechloromonas* (module 8) and *Aquabacterium* (module 16) As resistant genera (Suhadolnik et al., 2017). Additionally, the modules correlated with As included sulphate-reducing bacteria (SRB) *Desulfonatronobacter*, *Desulfuromonadia* (module 16), and *Desulfobulbus* (module 8), which have been shown involved in As methylation and demethylation processes (Chen et al., 2019). Such observation is in accordance with several studies reporting the presence of SRB and other sulphate-reducing microorganisms (SRM) in As rich environments (Dias et al., 2008; Bruneel et al., 2008; Giloteaux et al., 2013; Liu et al., 2018). SRM might play a crucial role in the organization of microbial networks in response to As.

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

4 Conclusion

Our study shows the stratification of microbial communities along an estuarine continuum highlighting the fragmentation according to habitat filtering despite the mixing zone that represents the conditions conductive to the coalescence of microbial communities. Specific microbial communities were observed, depending on the occurrence and level of contaminants, that allowed to detect specific and keystone taxa representing “specialists” particularly well adapted to the presence of contaminants. Such “specialist” taxa, like *Hydrogenophaga*, *Ignavibacterium*, *Dechloromonas* and *Oscillatoria* represent potential bio-indicators or "sentinels" to be included in integrative indices for evaluating the ecological status of aquatic environments. The network analysis showed that the microbial communities were organized in specialized modules around these "specialists" keystone taxa. The modules were tailored to respond specifically to the presence of a contaminant type, providing useful information to understand how the environmental parameters shape the organization of microbial assemblages. Particularly, *Nitrospira* was found to be an ideal partner for establishing microbial relationships under various conditions, which should be considered in environmental management. The results pave the way for future studies, particularly at the functional level, for deciphering the metabolic networks involved in the ecosystem functioning in estuarine ecosystems.

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Data Availability Statement

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

Annex: Supplementary material

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Figure captions

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

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

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

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

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

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

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

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

Environmental variable	Pseudo-F ratio (F)	R^2	p*	Correlation between variables ($coefficient_{pear}$)
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**	
$\mathbf{C_{org}/N_{org}}$	2.91	0.040	**0.028**	
$\boldsymbol{\delta^{13}C}$	4.22	0.058	**0.008**	
Season	2.70	0.073	**0.021**	

*: Monte Carlo approximated level of significance