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### ► To cite this version:

Anthony Bertucci, Claire Hoede, Emilie Dassié, Pierre-Yves Gourves, Amandine Suin, et al.. Impact of environmental micropollutants and diet composition on the gut microbiota of wild european eels (*Anguilla anguilla*). *Environmental Pollution*, 2022, 314, pp.120207. 10.1016/j.envpol.2022.120207 . hal-03788269

**HAL Id: hal-03788269**

**<https://hal.inrae.fr/hal-03788269v1>**

Submitted on 11 Nov 2023

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## Impact of environmental micropollutants and diet composition on the gut microbiota of wild european eels (*Anguilla anguilla*)

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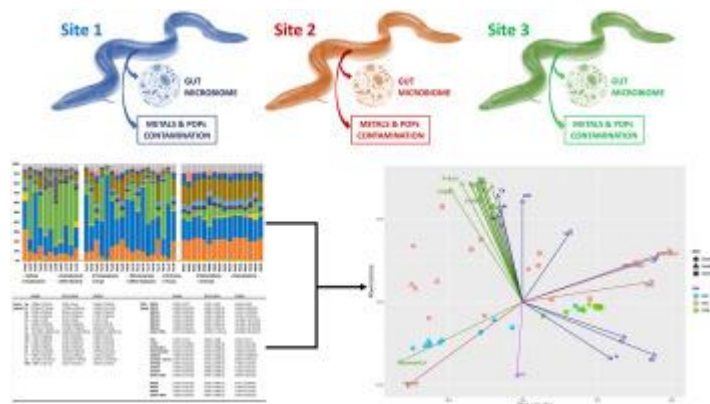
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### Abstract :

In fish, the gut microbiome plays a crucial role in homeostasis and health and is affected by several organic and inorganic environmental contaminants. Amphidromous fish are sentinel species, particularly exposed to these stressors. We used whole metagenome sequencing to characterize the gut microbiome of wild European eels (*Anguilla anguilla*) at a juvenile stage captured from three sites with contrasted pollution levels in term of heavy metals and persistent organic pollutants. The objectives were to identify what parameters could alter the gut microbiome of this catadromous fish and to explore the potential use of microbiota as bioindicators of environment quality.

We identified a total of 1079 microbial genera. Overall, gut microbiome was dominated by Proteobacteria, Firmicutes and Actinobacteria. Alpha and beta diversity were different amongst sites and could be explained by a reduced number of environmental and biological factors, specifically the relative abundance of fish preys in eels' diet, PCB101,  $\gamma$ HCH (lindane), transnonachlor and arsenic. Furthermore, we identified a series of indicator taxa with differential abundance between the three sites. Changes in the microbial communities in the gut caused by environmental pollutants were previously undocumented in European eels. Our results indicate that microbiota might represent another route by which pollutants affect the health of these aquatic sentinel organisms.

## Graphical abstract



## Highlights

► The gut microbial diversity of wild European eels differed amongst sites of catching. ► POPs and metals levels showed strong associations with gut microbiota composition. ► The relative abundance of fish in eels' diet also impacted gut microbiota. ► 323 microbial genera were differentially abundant between the catching sites.

**Keywords** : European eel, Gut microbiome, Heavy metals, Persistent organic pollutants, Diet

36 **INTRODUCTION:**

37 In fish, as in mammals, the collection of commensal, symbiotic and pathogenic microorganisms  
38 (eukaryotes, bacteria, archaea, and viruses) that occupy a particular environment, like the internal  
39 and external epidermal surfaces of metazoans, has the ability to fulfil key functions in nutritional  
40 provisioning, metabolic homeostasis, and immune defence. These microbial partners are referred to  
41 as “microbiota”, whereas the term “microbiome” often refers to the collection of genomes within  
42 this microbiota (Burokas et al., 2015). Owing to the ability of the microbiota to modulate the host  
43 physiology, an increasing number of studies have been carried out to characterize and determine the  
44 mechanisms of action of these microbes, particularly concerning the gut microbiota. This importance  
45 is highlighted by the fact that some authors consider gut microbiota as an “extra organ” (Feng et al.,  
46 2018) or a “second genome” (Grice and Segre, 2012). Though well behind mammalian models,  
47 teleost microbiome research is a quickly developing field (Llewellyn et al., 2014). Despite early works  
48 published in the 1920’s and 1930’s, this topic has known a recent expansion (for a review, see  
49 Egerton et al., 2018). More recently, next-generation sequencing techniques has represented a rapid  
50 and cost-effective method (i) to obtain data that provide more accurate information on the  
51 composition as well as the genetic and metabolic potential of a microbial community and (ii) to  
52 compare communities and test hypotheses. The microbiota associated with the fish intestine is  
53 supposedly dominated by Bacteria, mostly due to the caveat that they have been almost the only  
54 focus of research so far. The fish intestine may harbour  $10^7$  to  $10^{11}$  bacteria per gram of intestinal  
55 content. In the different fish species studied, *Proteobacteria*, *Bacteroidetes* and *Firmicutes*, represent  
56 90% of the intestinal microbiota (Ghanbari et al., 2015). This composition shows substantial inter-  
57 species and inter-individual variation depending on various factors such as life stage, trophic level,  
58 diet, season, habitat, sex and genetics of the host (Butt and Volkoff, 2019; Egerton et al., 2018;  
59 Tarnecki et al., 2017). Gut bacterial communities have been assessed in approximately 150 fish  
60 species covering a diverse range of physiology and ecology. However, most of the research effort is  
61 being carried out on economically important species such as salmonids, carp or tilapia (Perry et al.,  
62 2020), to meet the needs of the expanding aquaculture industry.

63 The European eel *Anguilla anguilla* is a catadromous fish with a complex life cycle shared between  
64 marine (reproduction, larval growth, and sexual maturation) and continental (growth) environments.  
65 European eels spawn in the Sargasso Sea before larvae drift towards the continental shelf of Europe  
66 and North Africa where they first metamorphose into unpigmented juvenile (glass eel) before  
67 reaching the growth phase (yellow eel) in continental water bodies where they remain up to 15-20  
68 years, often moving across different habitat types. The yellow eel stage ends with a second  
69 metamorphosis into maturing eels (silver eels) that will migrate back to the Sargasso Sea to  
70 reproduce and die. This life cycle makes *A. anguilla* particularly vulnerable to pollution (Feunteun,

71 2002; Guimaraes et al., 2009). The species has been considered as critically endangered since 2008  
72 and added to the IUCN red list (Jacoby and Gollock, 2014). Its catadromous migratory behaviour, long  
73 life, drastic habitat reduction, migration barriers, pollution, human-introduced diseases, overfishing,  
74 as well as climatic events may be amongst the causes of the catastrophic collapse of the European  
75 eel population observed over the past decades (Bevacqua et al., 2015; Knights, 2003; van den Thillart  
76 et al., 2009). Whereas the sensitivity of *A. anguilla* to metal and persistent organic pollutants is well  
77 documented (e.g. Baillon et al., 2015; Belpaire et al., 2008; Bertucci et al., 2019; Perrier et al., 2020;  
78 Pujolar et al., 2012), metagenomic studies in this species are scarce. Some were performed on the  
79 microbiome associated with skin mucosal surfaces (Carda-Dieguez et al., 2017; Carda-Diéguez et al.,  
80 2014). They showed the dominance of *Gammaproteobacteria*, especially the genus *Vibrio*, in eels  
81 from estuary and wetland while a mixture of genera predominated in lake and river eels. Which  
82 evidenced the role of salinity in shaping microbiota in fish. To our knowledge, community  
83 composition of intestinal microbiota of European eels was only assessed in cultivated individuals  
84 (Huang et al., 2018). The results showed the dominance *Proteobacteria* and *Fusobacteria*. However,  
85 more recently, gut microbiota was studied in other *Anguilla* species like the giant mottled eel *A.*  
86 *marmorata* (Lin et al., 2019), the Indonesian shortfin eel *A. bicolor* (Kusumawaty et al., 2020), and  
87 the Japanese eel *A. japonica* (Zhu et al., 2021).

88 The function of the gut microbiota and the subsequent physiological responses of the host  
89 depend on which microbes are present in the intestinal tractus and the interaction with the immune  
90 system. Then, the alteration of the microbiota composition and / or its metabolic functions might  
91 impact host's health and survival (Claus et al., 2016). This phenomenon is called dysbiosis and its  
92 links with environmental stressors suggest that the microbiome has the potential to provide  
93 innovative biomarkers (Boutin et al., 2013; Webster et al., 2019) and the identification of dysbiosis  
94 might be informative regarding adverse outcomes. Still, processes that drive microbial community in  
95 fish are poorly understood. The incorporation of the microbiome in studies investigating the effects  
96 of chemical compounds could then be a promising approach in ecotoxicology (Adamovsky et al.,  
97 2018; Duperron et al., 2020). In this study we investigated the impact of environmental pollutants,  
98 diet composition, and biometric parameters on the composition of intestinal microbiota of wild  
99 European eels sampled from three sites with contrasted anthropogenic pollution profiles. We  
100 measured a total of 15 metals in fish liver, and 28 persistent organic pollutants including  
101 polychlorinated biphenyls (PCBs), polybromodiphenylethers (PBDEs) and organochlorine pesticides in  
102 fish muscle. PCBs and PBDEs are widespread contaminants. PCBs are man-made chlorinated  
103 industrial chemicals that were used in electrical equipment hydraulic fluids, heat transfer fluids,  
104 lubricants, plasticizers, surface coatings and inks. PBDEs are used in plastics, electronics, building  
105 materials and textiles as flame retardants. Pesticides are used in agriculture and are rarely selective

106 enough to prevent effect on non-target organisms. Many studies have documented the accumulation  
107 of these xenobiotics in aquatic organisms and more recently their toxicity for gut microbiota (see  
108 Evariste et al., 2019). Diet composition was assessed through DNA sequencing of the intestinal  
109 content. Our aim was not only to characterize the microbiota of these migratory fish but also to  
110 determine what factors can drive the taxonomy of their microbial partners. The results of this study  
111 allowed the identification of a limited number of pollutants and diet component that could explain  
112 dysbiosis in European eels.

113

#### 114 **MATERIAL AND METHODS:**

115 **Sample collection** – Wild European eels (*Anguilla anguilla*) specimens were collected in Batejin  
116 (BAT: 44°55'53.87"N, 01°07'22.94"W; 12 individuals in July 2019), Pas-du-Bouc (PDB: 44°50'27.03"N,  
117 01°09'07.58"W; 20 individuals in July 2019) and Pauillac (PAU: 45°14'00.26"N, 00°44'01.80"W; 20  
118 individuals in September 2019). Immature yellow eels of a pre-defined size (20-40 cm) were targeted  
119 in order to have the same range for all sites. BAT and PDB samples were collected by electro-fishing  
120 according to the authorizations from the “Direction Départementale des Territoires et de la Mer de la  
121 Gironde” edicted on May 22, 2019 and June 7, 2019. PAU samples were trapped by a professional  
122 fisherman. Fish were kept in tanks with aerated water from the site for few minutes after collection  
123 until being sacrificed and dissected at the proximity of the sampling site. Individuals were measured  
124 (cm) and weighted (g). Liver, spleen, and kidney were collected and weighted for organo-somatic  
125 indices calculation and stored at -20°C. The intestinal section located shortly down the stomach and  
126 up the colon was dissected, transferred in RNA-later and placed at 4 °C overnight before storage  
127 at -20 °C until DNA extraction. Otoliths were collected for age determination. For all sites,  
128 temperature, salinity, and dissolved oxygen concentrations were measured using a multi-parameter  
129 portable device or by using data coming from the automated observation network of the Gironde  
130 (MAGEST network).

131 **Biometric indices** – Fulton index (K), hepato-somatic index (HSI) and spleno-somatic index (SSI)  
132 were calculated as  $K = (\text{total weight} / \text{length}^3) \times 100$ ;  $HSI = (\text{liver weight} / \text{total weight}) \times 100$ ; and  $SSI$   
133  $= (\text{spleen weight} / \text{total weight}) \times 100$ ; where weight is in grams and length is in centimetres.

134 **Age determination** – Age was determined according to the methods described in Annex 4 of the  
135 report of the ICES Workshop on Age Reading of European and American Eel (ICES, 2009). Briefly,  
136 sagittal otoliths were extracted, cleaned with distilled water, dried, and stored in plastic Eppendorf  
137 tubes at ambient room temperature until processing. Otoliths were embedded in epoxy resin;  
138 grinded on 1200-grit paper with water until the nucleus is reached and polished on 4000-grit paper;  
139 etched for 3 minutes with a 5% EDTA solution, washed with distilled water and stained with 0.01%

140 toluidine blue for 3 minutes. Otoliths were rinsed with distilled water and observed with a binocular  
141 microscope under transmitted light.

142 **Trace metal analyses** – Liver tissues were dried, weighted, and digested in polypropylene tubes  
143 using nitric acid (67%). Digesta were heated at 100 °C for three hours. After complete mineralization,  
144 samples were diluted with ultrapure water. In order to verify the accuracy of the method blank  
145 samples, Internationals Certified Reference Materials (CRM) for trace metals elements: DOLT-5 (fish  
146 liver), TORT-3 (lobster hepatopancreas), and IAEA-413 (algae) were prepared in similar conditions as  
147 the samples. A series of 15 elements were analysed simultaneously by Inductively Coupled Plasma  
148 Optical Emission Spectrometer (700 Series ICP-OES, Agilent): Silver (Ag), Aluminium (Al), Arsenic (As),  
149 Cadmium (Cd), Cobalt (Co), Chrome (Cr), Copper (Cu), Iron (Fe), Manganese (Mn), Molybdenum  
150 (Mo), Nickel (Ni), Lead (Pb), Selenium (Se), Vanadium (V), and Zinc (Zn). Metals concentrations in  
151 tissue samples were expressed in  $\mu\text{g}\cdot\text{mg}^{-1}$  dry weight (dw). Average recovery rates were between  
152 97% and 102%. When not detectable, metal concentrations were replaced by the maximum  
153 theoretical detection limit ( $DL_{\text{max}}$ ) to allow multivariate analysis.  $DL_{\text{max}}$  of a sample is calculated as  $(DL$   
154  $\times W) / V$ ; where DL is the detection limit for the metal in  $\mu\text{g}\cdot\text{L}^{-1}$ , W the mass of the sample in g, and V  
155 the volume of the sample in ml. DL values are given in supplementary file 1. The metal pollution  
156 index was calculated as in Khan et al. (2020).

157 **Organic pollutants analyses** – Analysis of the seven polychlorinated biphenyls (CB28; CB52;  
158 CB101; CB118; CB138; CB153 and CB180), thirteen organochlorine pesticides (hexachlorobenzene or  
159 HCB; lindane or  $\gamma$ -HCH; heptachlor; heptachlor epoxide; *cis*-chlordane; *trans*-nonachlor; mirex;  
160 2,4'DDE; 2,4'DDD; 4,4'DDE; 4,4'DDD; 2,4'DDT and 4,4'DDT), and 8 polybromodiphenylethers (BDE28,  
161 BDE47, BDE99, BDE100, BDE153, BDE154, BDE183 and BDE209) were performed on individual  
162 muscle samples by liquid / liquid extraction followed by purification by adsorption chromatography  
163 as described previously (Tartu et al., 2015). PCB and OCP were analysed by gas chromatography  
164 coupled to an electron capture detector (GC-ECD), while PBDEs were determined by GC hyphenated  
165 with mass spectrometry (GC-MS). Results were expressed in  $\text{ng}\cdot\text{g}^{-1}$ . When not detectable,  
166 concentrations were replaced by the quantification limit of the compound to allow multivariate  
167 analysis (supplementary file 1).

168 **DNA extraction, sequencing and filtering** – In order to capture the widest microbial diversity,  
169 three gut sections of approximately 5 millimetres each were cut out at the beginning, the middle  
170 and the end of the intestine sample dissected previously. These three pieces were sliced open and  
171 minced with a sterile blade, then mixed and processed with the QIAamp PowerFecal Pro DNA Kit  
172 (Qiagen) following the supplier's instructions.

173 DNAseq was performed at the GeT-PlaGe core facility, INRAE Toulouse. DNA-seq libraries were  
174 prepared according to Illumina's protocols using the Illumina TruSeq Nano DNA HT Library Prep Kit.

175 Briefly, DNA was fragmented by sonication, size selection was performed using SPB beads (kit beads)  
176 and adaptors were ligated to be sequenced. Library quality was assessed using an Advanced  
177 Analytical Fragment Analyzer and libraries were quantified by qPCR using the Kapa Library  
178 Quantification Kit. DNA-seq experiments have been performed on an Illumina NovaSeq6000 using a  
179 paired-end read length of 2x150 pb with the Illumina NovaSeq6000 Reagent Kits.

180 Each sample was sequenced twice on separate lanes of the same flowcell and the two paired files  
181 were merged. Raw data were cleaned as follows by using first step of metagWGS v1.1 available at:  
182 <https://forgemia.inra.fr/genotoul-bioinfo/metagwgs/-/tree/master> (Fourquet et al., 2020): (i) we  
183 checked quality of raw reads by fastQC v0.11.9, (ii) we remove adaptors with cutadapt v2.10, (iii)  
184 trimmed paired reads if nucleotide quality is lesser than 20 with sickle v1.33, (iv) we used bwa mem  
185 v0.7.17-r1188 to map trimmed reads against *Anguilla anguilla* genome (GCA\_013347855.1),  
186 samtools view v1.10 and bam2fastq from bedtools v2.29.2 were used to obtain cleaned paired reads  
187 fastq files without host reads, (v) finally we used fastQC again on cleaned reads to obtain quality  
188 metrics.

189 **Taxonomic profiling** – Because we used gut sections, we anticipated that bacteria and other  
190 microbial partners would represent a small portion of our libraries. For each sample, taxonomic  
191 classification of quality trimmed and filtered metagenomic paired-end reads was performed using  
192 Kaiju (v.1.7.4) (“greedy” heuristic approach, -e 3), with the NCBI nr database (release dated May  
193 25th, 2020; containing all proteins belonging to Archaea, Bacteria and Viruses and proteins from  
194 fungal and microbial eukaryotes) as reference. We used kaiju2table to build taxonomy table at each  
195 taxonomy levels (superkingdom, phylum, class, order, family, genus, species). To obtain one unique  
196 matrix for all samples we used the home-made script merge\_kaiju\_results.py available in metagWGS  
197 (<https://forgemia.inra.fr/genotoul-bioinfo/metagwgs/-/tree/master/bin>). Counts of sequences  
198 successfully assigned and taxonomic information were imported into RStudio (v. 1.2.5019), based on  
199 R (v. 3.6.1), for further processing with the phyloseq (v. 1.30.0) and vegan (v. 2.5.5) packages.  
200 Filtering of the most abundant Genera were based on a minimum abundance threshold of 100 cpm  
201 (counts per million reads). Taxa not exceeding these thresholds in at least half the samples of one  
202 site (50% prevalence) were removed. The core microbiota was determined with a 0.10% relative  
203 frequency threshold and a prevalence of at least 80%. This was performed using the core function in  
204 microbiome R package version 1.17.42 (Leo and Sudarshan, 2017). In order to assess the diet of the  
205 fish, taxonomic classification of quality trimmed and filtered metagenomic paired-end reads was  
206 performed using Kraken2 (v. 2.0.8-beta) with ‘--paired’ parameter against the entire NCBI nt  
207 database downloaded on January 24, 2022. Metazoan sequences were classified at the Class level  
208 and taxa not exceeding 1% of the reads in half of the samples from at least one site were removed.



209 The numbers of reads used in both kaiju and kraken analysis for each sample are presented in  
210 supplementary file 2.

211 **Statistics and data analysis** – Alpha diversity was calculated using the R package “vegan”  
212 (Oksanen et al., 2020) based on observed, Shannon, Chao1 and Pielou’s Evenness indices calculated  
213 from non-normalized genus-level read counts. Beta diversity between the sites were visualized using  
214 nonmetric multidimensional scaling (NMDS) and Principal Coordinates Analysis (PCoA) plots based on  
215 the Bray-Curtis dissimilarity. Beta diversity analysis were performed on cpm-transformed counts at  
216 the genus level to control for sequencing depth between libraries. Pairwise differences were tested  
217 using PERMANOVA (n=1000 permutations) based on Bray-Curtis dissimilarity and adjusted P values  
218 (FDR  $\leq$  0.05 were considered as statistically significant. Beta dispersion was assessed by an ANOVA-  
219 like permutation test (n=1000 permutations) on the Bray-Curtis distance matrix. A Linear  
220 discriminant analysis Effect Size (LEfSe) was used to identify bacterial taxa responsible for differences  
221 between sampling sites at the genus level (Segata et al., 2011) with a LDA score threshold of 2.0 and  
222 a FDR of 0.01.

223 The statistical analysis of morphometric data and pollutant levels were performed using RStudio  
224 (v.1.2.5019). Metal concentrations and morphologic variables were expressed as mean  $\pm$  SE. The  
225 comparison among sites was performed using a linear model after checking for normality and  
226 homoscedasticity of residuals. When necessary, post-hoc analysis was performed by the Tukey test.  
227 A p-value  $\leq$  0.05 was considered significant.

228 Covariates of gut microbiome  $\beta$ -diversity were identified by performing linear correlations to  
229 calculate the association between pollutant content, diet, morphometry and genus-level community  
230 coordinate with the envfit() function in the “vegan” R-package. Then, in order to identify non-  
231 redundant determinants of microbiota variation, covariates selected by envfit() function were sub-  
232 selected by forward stepwise redundancy analysis on genus-level community ordination calculated  
233 by Bray-Curtis distance with the ordiR2step() function in the “vegan” R-package.

234 **Data availability** – The molecular data set generated and analyzed for this study were deposited  
235 in NCBI repository under the BioProject accession number PRJNA825732.

236

## 237 **RESULTS:**

238 **Differences among sampling sites** – Three sites located along the Gironde estuarine ecosystem were  
239 selected. Batejin (BAT) and Pas-du-Bouc (PDB) were located on the ‘canal des étangs’ located  
240 between the Lacanau lake and the Arcachon lagoon, while Pauillac (PAU) was located in the Gironde  
241 estuary. The major difference was salinity, but variability also exists in term of dissolved oxygen level  
242 and temperature on the date of sampling (Table 1). According to one-way ANOVAs followed by  
243 Tukey’s HSD tests (p $\leq$ 0.05), differences were observed in eels’ morphologic features even though fish

244 were initially selected according to their size range (20-40 cm). PDB and PAU individuals were  
245 significantly longer and heavier than BAT individuals. PDB eels were older and exhibited a higher  
246 Fulton index. These individuals also had lower HSI, and higher SSI values compared to BAT and PAU.

247 **Heavy metal pollution in eels' liver** – Fifteen trace metal concentration were measured in hepatic  
248 tissue. Co was the only element for which no difference was observed between the 3 sites ( $p \leq 0.05$ ).  
249 BAT eels are characterized by the highest levels of Cr ( $0.426 \pm 0.094 \mu\text{g}\cdot\text{mg}^{-1}$ ) and the lowest levels of  
250 Cu, Mn and Zn compared to eels from the other sites ( $p \leq 0.05$ ). PDB eels are characterized by the  
251 highest level of Fe ( $2,936.914 \pm 298.146 \mu\text{g}\cdot\text{mg}^{-1}$ ), Mo ( $1.581 \pm 0.082 \mu\text{g}\cdot\text{mg}^{-1}$ ), Se ( $39.264 \pm 6.647$   
252  $\mu\text{g}\cdot\text{mg}^{-1}$ ) and V ( $2.01 \pm 0.198 \mu\text{g}\cdot\text{mg}^{-1}$ ) and the lowest levels of Pb ( $0.05 \pm 0.003 \mu\text{g}\cdot\text{mg}^{-1}$ ) in their livers  
253 ( $p \leq 0.05$ ). PAU individuals showed the highest levels of Ag ( $0.968 \pm 0.104 \mu\text{g}\cdot\text{mg}^{-1}$ ), As ( $4.444 \pm 0.438$   
254  $\mu\text{g}\cdot\text{mg}^{-1}$ ) and Cd ( $1.729 \pm 0.184 \mu\text{g}\cdot\text{mg}^{-1}$ ) and the lowest levels of Al ( $13.489 \pm 1.373 \mu\text{g}\cdot\text{mg}^{-1}$ ) and Ni  
255 ( $0.052 \pm 0.019 \mu\text{g}\cdot\text{mg}^{-1}$ ) compared to BAT and PDB ( $P \leq 0.05$ ). Moreover, PAU samples ( $3.014 \pm 0.178$   
256  $\mu\text{g}\cdot\text{mg}^{-1}$ ) showed a significantly higher MPI value than BAT ( $1.689 \pm 0.217 \mu\text{g}\cdot\text{mg}^{-1}$ ) and PDB ( $2.022 \pm$   
257  $0.193 \mu\text{g}\cdot\text{mg}^{-1}$ ), suggesting a higher total content of metals in their livers. Details are given in Table 2.

258 **Organic contaminants in eels' muscle** – Four OCPs (2,4'DDE; heptachlor; heptachlor epoxide and  
259 mirex) and five BDEs (BDE100; BDE153; BDE154; BDE183 and BDE209) were not detected in any  
260 sample and were then removed from the analysis. Except for  $\gamma$ -HCH ( $0.551 \pm 0.058 \text{ng}\cdot\text{g}^{-1}$ ) and BDE28  
261 ( $0.353 \pm 0.055 \text{ng}\cdot\text{g}^{-1}$ ) that are significantly more present in BAT eels, all organic pollutants showed  
262 the highest levels in PAU fish ( $p \leq 0.05$ ). With a mean  $\pm$  SE of  $678.480 \pm 93.419 \text{ng}\cdot\text{g}^{-1}$ ,  $\Sigma$ PCBs is 20- and  
263 25-times higher in PAU than in PDB and BAT, respectively;  $\Sigma$ OCPs in PAU ( $56.997 \pm 8.13 \text{ng}\cdot\text{g}^{-1}$ ) is 16-  
264 and 22-times higher than in PDB and BAT, respectively; and  $\Sigma$ BDEs in PAU ( $2.623 \pm 0.422 \text{ng}\cdot\text{g}^{-1}$ ) is 4-  
265 and 3-times higher than in PDB and BAT, respectively (Table 2).

266 A Principal Component Analysis (PCA) was carried out on the whole pollutants' levels (Figure 1).  
267 Eigenvalues in components one and two represent 51% and 10% of the variation in the data,  
268 respectively and the site of origin appears as the major explanatory variable. The first component is  
269 associated with POPs levels and separates the more contaminated estuarine samples (Pauillac) from  
270 the freshwater samples (Batejin and Pas-du-Bouc). The second component is rather associated with  
271 metals and discriminates Batejin samples and Pas-du-Bouc samples within the freshwater cluster.

272 **Assessment of eels' diet** – Following quality trimming, filtering, taxonomic classification with  
273 Kraken2 (v. 2.0.8), and removal of unclassified sequences we analysed a total of 18,182,766  
274 metazoan sequences from eels' intestine, more than 90% of which were classified within five Classes:  
275 *Insecta*, *Actinopteri* (referred to as Fish thereafter), *Mammalia*, *Aves* (referred to as Birds thereafter),  
276 *Bivalvia*, *Gastropoda*, and *Malacostraca*. Based on individual percentages, 3 groups were created  
277 through k-mean clustering (R command "kmeans", the optimal number of clusters was determined  
278 with the Elbow method, supplementary file 3). Group A contained only 3 individuals that were

279 characterized by a higher percentage of Malacostraca. Group B contained 15 individuals with higher  
280 percentages of insects, bivalves and gastropods. Group C contained 27 individuals whose intestines  
281 contained more fish, mammals, and birds as preys (Table 3).

282 **Differences in microbial community structure between sampling sites** – Following quality  
283 trimming, filtering, taxonomic classification with Kaiju (v.1.7.4), and removal of unclassified  
284 sequences we analysed a total of 7,416,386 microbial sequences. Library sizes ranged from 28,877 to  
285 726,035 with an average size of 151,354 (details are given in supplementary file 2). After removal of  
286 unassigned reads, rarefaction curves at the species-level indicated that the sequencing depth was  
287 sufficient to capture microbial complexity in each library (supplementary file 4). The gut microbial  
288 community was mainly composed of Bacteria, accounting for an average of 69% of the sequences,  
289 while the Eukaryota, Archaea and viruses accounted for 26%, 1.3% and 2.7% of the sequences,  
290 respectively (Figure 2A and Supplementary file 5).

291 A total of 1,079 taxa were identified at the genus level. Among which 645 bacterial genera from  
292 24 phyla were detected. The most dominant bacterial phyla in the eel gut samples, averaged for all  
293 samples, were *Proteobacteria* (36.97% of the Bacteria), *Firmicutes* (30%) and *Actinobacteria* (16.17%)  
294 followed by *Bacteroidetes*, *Fusobacteria*, and *Cyanobacteria*. The remaining 18 phyla combined  
295 represented only 10% of the relative abundance of Bacteria. Amongst microbial eukaryotic  
296 sequences, a total of 335 fungal genera from 8 phyla were detected. Fungi represented most of the  
297 microbial Eukaryotes (58%). The most abundant fungal phyla were *Ascomycota* (48%) and  
298 *Basidiomycota* (35%) followed by *Mucoromycota* (6%) and *Chytridiomycota* (5%). The remaining  
299 microbes belonged to viruses (2.75%) and Archaea (1%). The *A. anguilla* core microbiota is made of  
300 33 bacterial genera that were identified at an 80% prevalence threshold (Figure 2B and  
301 Supplementary file 5) from the Phyla *Actinobacteria* (10 genera), *Proteobacteria* (9 genera) and  
302 *Firmicutes* (6 genera). *Streptomyces* (*Actinobacteria*); *Clostridium* and *Bacillus* (*Firmicutes*); and *Vibrio*  
303 and *Pseudomonas* (*Proteobacteria*) were the only genera found in all the samples. This core  
304 microbiota was completed by *Fungi* from the phylum *Ascomycota*, other Eukaryotes and Viruses.

305 Differences in microbial community structures were observed between sites (Figure 2 and  
306 Supplementary file 5). BAT samples were dominated by *Firmicutes* (58%) with *Clostridiales* being the  
307 major Order (88.5% of *Firmicutes*). *Actinobacteria* were dominant in PDB samples (34%) with  
308 *Streptomyetales* being the major Order (36.8%) and *Proteobacteria* dominated PAU samples (52%)  
309 with a majority of *Vibrionales* (63.3%). We also observed large differences in Fungi abundance that  
310 represents an average of 6.5%, 12.4% and 24% of sequences in BAT, PAU and PDB, respectively.

311 We calculated four within-sample (alpha) diversity indices: number of observed OTUs, Shannon  
312 index, Chao1 and Pielou's Evenness (Figure 3). Significant differences at the genus level were  
313 observed among sites. BAT eels had significantly higher observed number of OTUs and Chao1 index,

314 while PDB eels had significantly higher Shannon index and Evenness values. This suggests that  
315 species richness was higher in BAT samples while relative taxonomic abundance was more  
316 homogeneous in PDB samples, with less dominant genera.

317 A Principal Coordinate Analysis (PCoA) was used to study the genus composition and the effect of  
318 the sampling site on the  $\beta$ -diversity of microbial communities (Figure 4). The site of origin of the fish  
319 accounted for a substantial variation in  $\beta$ -diversity of the gut microbiome at the genus level  
320 (PERMANOVA  $R^2 = 0.429$ ,  $p = 0.001$ ). This effect was detected at all taxonomic levels (Supplementary  
321 file 6 and Supplementary file 7). Moreover, the microbial composition of PDB appeared more  
322 homogeneous since these samples showed a significantly lower beta dispersion compared to BAT  
323 (permuted  $p$ -value = 0.009) and PAU (permuted  $p$ -value = 0.001). BAT and PAU showed a similar beta  
324 dispersion (permuted  $p$ -value = 0.175). Following site, diet also had an impact on microbial  $\beta$ -  
325 diversity (PERMANOVA  $R^2 = 0.277$ ,  $p = 0.001$ ) as the microbiome of fish harbouring Diet B  
326 significantly differed from those with Diet C ( $p = 0.003$ ). Salinity (*i.e.* BAT and PDB = freshwater and  
327 PAU = estuary) also had an effect, though smaller, on  $\beta$ -diversity (PERMANOVA  $R^2 = 0.150$ ,  $p = 0.001$ ).

328 **Identification of covariates impacting gut microbiota in European eels** – Site of origin (42.9%)  
329 and diet (27.7%) were identified to be the main factors impacting microbial diversity. A univariate  
330 analysis revealed that amongst all the covariates associated to these parameters (15 metals, MPI, 9  
331 biometric values and indices, 19 POPs, 3 sums of POPs, and proportion of 7 classes of preys), 32 were  
332 significantly associated with microbiome composition (FDR  $\leq 0.01$ ) (Figure 4). POPs levels (18  
333 covariates) showed strong associations with gut microbiota especially PCBs, followed by metals (9  
334 covariates). Four out of the seven classes of prey in eels' diet influenced microbiota. The Hepato-  
335 somatic index was the only morphometric variable to associate with gut microbiota of eels. Some  
336 variables showed overlapping directionalities for the ordination of gut microbiota composition  
337 suggesting a redundancy amongst covariates. Five non-redundant covariates with a cumulative effect  
338 size ( $r^2$ ) of 0.534 were identified by a forward stepwise redundancy analysis. The relative abundance  
339 of fish in eels' diet was the main non-redundant factor impacting gut microbiota ( $p$ -value = 0.002)  
340 with an effect size of 0.220, followed by the levels of PCB101 ( $r^2 = 0.154$ ,  $p$ -value = 0.002),  $\gamma$ HCH ( $r^2 =$   
341 0.090,  $p$ -value = 0.002), transnonachlor ( $r^2 = 0.044$ ,  $p$ -value = 0.004) and Arsenic ( $r^2 = 0.023$ ,  $p$ -value =  
342 0.014).

343 **Differential abundance of microbial genera between sites** – Here we focused on differences  
344 amongst sites since it appeared as the main factor explaining microbiome composition in eels'  
345 intestines. Within the 1079 genera we identified previously, a LEfSe analysis identified 726  
346 discriminant features ( $p$ -value  $\leq 0.01$ , LDA score  $> 2$ ), amongst which 323 at the Genus level (details  
347 in Supplementary file 8). As previously suggested by lower Pielou's evenness values (Figure 3), a  
348 limited number of dominant taxa were identified in BAT and PAU samples. Most of the 32

349 significantly enriched genera in BAT samples belonged to the Phylum *Firmicutes* (25 genera), class  
350 *Clostridia* (17 genera). Amongst the 8 significantly enriched genera in PAU samples, 7 were the class  
351 *Gammaproteobacteria* with the order *Vibrionales* accounting for most of them (5 out of 7). Dominant  
352 taxa were harder to identify in PDB samples which had 283 enriched genera. Most of those (132  
353 genera) are Fungi with 50.0% of *Ascomycota* and 33.3% of *Basidiomycota*. Bacteria enriched in PDB  
354 (135 genera) were in majority from the phyla *Actinobacteria* (42 genera) and *Proteobacteria* (63  
355 genera). These results are in agreement with what was previously suggested by the taxonomic  
356 composition in Figure 2 (and Supplementary file 5). No enrichment was observed in relation to the  
357 diet group. The most differentially abundant taxa (LDA > 4.0) enriched in each site are presented in  
358 Figure 5. A Spearman correlation analysis based on the most abundant genera in each site showed  
359 significant associations with pollution- and diet-related covariates (Figure 6).

360

## 361 **DISCUSSION:**

362 Gut microbiomes of fish are complex, dynamic communities influenced by a wide variety of  
363 extrinsic factors such as environmental parameters, and intrinsic factors such as physiology or  
364 genetic background of the host (Butt and Volkoff, 2019). The characterization of the microbial  
365 communities that are present in fish and the comprehension of what factors influence that  
366 composition are equally important and may contribute to the diagnostic of habitat health condition,  
367 especially in sentinel organisms. Micropollutants of the environment are now recognized as strong  
368 and pivotal factors shaping the gastrointestinal microbiome (Adamovsky et al., 2018; Claus et al.,  
369 2016; Duperron et al., 2020). Altogether, our results indicate that environmental parameters,  
370 particularly pollutants, may alter microbial community in the gut of eels and that microbial taxa may  
371 represent innovative biomarkers of environmental quality. Moreover, as gut microbiota may differ  
372 between wild and captured / cultured fish (Egerton et al., 2018; Uren Webster et al., 2018), the use  
373 of wild specimens increases the degree of ecological relevance. One should note that our work was  
374 based on observations of both the gut digesta and mucosa of fish and that both resident and  
375 transient microbial communities (also referred to as autochthonous and allochthonous, respectively)  
376 were studied simultaneously. Most of the current literature on teleost microbiomes was obtained  
377 through 16S rRNA barcoding (Llewellyn et al., 2014). In this study, we used whole metagenome  
378 sequencing (WMS) that has revealed an effective method for analysing both the feeding habits and  
379 gut microbiomes of fish (Pan et al., 2021). In addition to taxa information down to species and even  
380 strain levels that are not restricted to prokaryotes, the use of WMS can allow analysis of functional  
381 potentials of microbiomes. Unfortunately, the sequencing depth here was not sufficient to assemble  
382 a metagenome and we were able to annotate only a small proportion of kaiju reads (Supplementary  
383 files 9 and 10). Sample preparation and DNA extraction methods should be improved toward this

384 objective in the future. However, shallow whole-metagenome shotgun sequencing may still provide  
385 highly similar data to WMS and more accurate taxonomic assignments than rRNA targeted  
386 sequencing (Xu et al., 2021).

387 We identified 1,079 taxa at the genus level. Unsurprisingly, Bacteria were the main component of  
388 eels' intestinal microbiota. This result is similar to other fish species that received more interest from  
389 the microbiome research community in which the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*,  
390 *Bacteroidetes*, *Fusobacteria*, and *Tenericutes* are commonly reported to be amongst the most  
391 dominant members present in the gastrointestinal tracts of fish species (Ghanbari et al., 2015;  
392 Tarnecki et al., 2017). A small proportion of the microbiome was made of *Archaea* and viruses.  
393 Though they are common components of vertebrates' gut microbiota, information in fish is scarce,  
394 either in terms of composition or functionality. Finally, amongst eukaryotes, *Ascomycota* and  
395 *Basidiomycota* have been previously identified in several marine and freshwater fish. Various yeasts  
396 have been identified in the normal intestinal microbiota of fish in aquaculture. Their presence is  
397 generally considered as commensalism, even though opportunistic strains may reveal pathogenic in  
398 some case (Gatesoupe, 2007). In our results, Fungi were particularly abundant in eels collected from  
399 Pas-du-Bouc (Figures 2 and 5). This result is a first hint on the potential bioindicative role that some  
400 microbial taxa could play regarding the links between European eels' health and/or ecosystem  
401 quality.

402 Our results showed similarities with previous studies in European eel such as the dominance of  
403 *Proteobacteria* (Huang et al., 2018) in all the samples or the abundance of *Fusobacteria* and  
404 *Firmicutes* in larger individuals (Shi et al., 2020). However, we must remain careful before we  
405 extrapolate from previous literature since we used wild animals and a larger number of individuals  
406 compared to previous literature. In addition, this discrepancy may come from the much larger use of  
407 bacterial 16S rRNA gene amplicon targeted sequencing in fish literature to date (Talwar et al., 2018).  
408 PCR-based sequencing, unlike WMS, may indeed bias results toward certain bacterial phyla  
409 depending on the primer that were used (Hansen et al., 1998).

410 Numerous drivers of gut microbiome diversity have been reported, amongst which external  
411 environment and diet (Johny et al., 2021; Legrand et al., 2020; Sullam et al., 2012; Tarnecki et al.,  
412 2017). In our study, differences in alpha-diversity were observed between sites (Figure 3). This first  
413 result seems in accordance with Kim *et al.* (2021) who showed that the gut microbiota is primarily  
414 determined by host habitat, rather than by genetic factors. Fish from Batejin, that were less  
415 contaminated by micropollutants (Table 2 and Figure 1), exhibited a significantly higher microbial  
416 richness as indicated by higher numbers of observed OTUs and Chao1 values. As Chao1 evaluate the  
417 total richness, the fact that these two values were similar confirm that we were able to capture most  
418 of the microbial diversity in all our samples. This was also evidenced by saturation curves

419 (Supplementary file 4). Pas-du-Bouc samples exhibited a higher microbial diversity demonstrated by  
420 a higher value of Shannon index and more indicator taxa identified by LEfSE in this group of fish. The  
421 site of origin was also the main factor impacting beta diversity. We observed a larger dissimilarity in  
422 beta-diversity of PAU samples (Figure 4) that were characterized by higher pollution levels (*i.e.*  
423 significantly higher MPI,  $\Sigma$ PCBs,  $\Sigma$ OCPs, and  $\Sigma$ BDEs). This result might be an example of the Anna  
424 Karenina principle (AKP) (Zaneveld et al., 2017). The AKP predicts that stressors have stochastic  
425 rather than deterministic effects on community composition and that healthy microbiomes are  
426 similar whereas each dysbiotic microbiome is dysbiotic in its own way. To explain these differences,  
427 we performed linear correlations that identified a series of persistent organic pollutants, metals, as  
428 well as diet components that are drivers of this beta diversity (Figure 4). Amongst those, we  
429 identified five non-redundant determinants with a relatively high cumulative effect size on  
430 community variation (53.4%).

431 The proportion of fish preys in eels diet showed the largest effect (22.0%). Fish can employ a large  
432 variety of feeding strategies, and numerous studies have shown diet can influence intraspecies  
433 differences in fish gut microbiota (*e.g.* Desai et al., 2012). For instance, the guts of carnivorous fish  
434 was dominated by protease producing bacteria whereas herbivorous fish guts by cellulose degrading  
435 bacteria (Liu et al., 2016). In *A. anguilla* at the yellow eel stage, dietary differences may result in two  
436 morphotypes amongst individuals from the same population and of similar size. Eels feeding on hard  
437 food developed broader heads than soft feeders with narrow-heads (De Meyer et al., 2016; Provan  
438 and Reynolds, 2000), with consequences on growth rate (De Meyer et al., 2017). According to its  
439 physiological role, it would then be interesting to test whether these phenotypic differences could be  
440 linked with alterations of the microbiome. The intestinal microbial diversity usually increases  
441 sequentially from carnivores to herbivores and to omnivores (Kashinskaya et al., 2018). *A. anguilla* is  
442 a generalist predator. Though it feeds mainly on invertebrates and fish, some individuals exhibit  
443 scavenger behaviour feeding on dead animals (Sporta Caputi et al., 2020). Our results meet this  
444 assumption since eels from Pas-du-Bouc, which have a microbiome composition influenced by the  
445 proportion of fish, mammals and bird preys in their diet, also showed a lower microbial alpha-  
446 diversity (Observed and Chao1). However, cautions must be taken regarding the impact of diet on  
447 microbiota in wild individuals. First, most studies linking diet and microbiome composition were  
448 performed in culture or controlled conditions (Perry et al., 2020; Tarnecki et al., 2017). Then, in fish,  
449 gut microbiome composition and its predicted metabolic function may vary shortly after a feeding  
450 event and/or during the digestion cycle (Parris et al., 2019), and finally, the DNA detected in the  
451 dietary material can either come from the environment (eDNA), from the food eaten by the eels, or  
452 from remnant DNA contained in the diet of eels' prey. However, many authors reported a nocturnal  
453 feeding activity in *A. anguilla* (Tesch and Thorpe, 2003) suggesting that, since sampling was

454 performed during daytime, the differences in microbiota composition observed here unlikely  
455 resulted from a recent food intake.

456 If chemicals can alter microbial communities, the microbiota also has the ability to biotransform a  
457 wide variety of chemicals, hence influencing fundamental properties of toxicants (Claus et al., 2016).  
458 The comprehension of how chemicals may perturb the host-microbiome relationship is crucial to  
459 establish links between chemical exposures and adverse effects. This can have long-term implications  
460 for adaptation of organisms in highly contaminated environments (Adamovsky et al., 2018). In the  
461 present study, three POPs (PCB101,  $\gamma$ HCH, and Transnonachlor) and arsenic showed a cumulative  
462 effect size of 31.3%. Recent studies have investigated the impact of POPs exposure on the gut  
463 microbiota in mammals and the potential consequences on health and disease (Chiu et al., 2020; Tian  
464 et al., 2020). But in fish, even though POPs are known to impair endocrine functions, early  
465 development, growth / condition / energy reserves, reproduction, immunity, and behaviour (Johnson  
466 et al., 2013), links with the microbiome are still poorly investigated. The fact that, in the present  
467 study, the level of many POPs showed strong association with gut microbiota should encourage  
468 research toward this direction. Amongst all the environmental and biological parameters that we  
469 measured in each individual eel; our work allowed the identification of a reduced number of factors  
470 that drive microbial communities in this particular context. Those should be further investigated in  
471 controlled conditions to better characterize their mode of action and specific consequences on fish  
472 microbiome and health.

473 Migratory amphidromous fish experience extreme modifications of environmental conditions,  
474 especially salinity (Sullam et al., 2012), during transit from freshwater to seawater, along with  
475 physiological changes associated with metamorphosis that may impact gut microbiota (*e.g.* Dehler et  
476 al., 2017; Llewellyn et al., 2016; Zhao et al., 2020). This suggests that gut microbial community  
477 adaptations allow fish to cope with changing environmental conditions, even though differences may  
478 exist between species. In our study, PERMANOVA analysis revealed that salinity had a smaller effect  
479 on microbiota structure (10.5 %) than site of origin (42.9 %) and diet composition (27.7 %). At yellow  
480 stage, *A. anguilla* has the ability to move back and forth between seawater, estuaries and freshwater  
481 mostly for feeding purposes. Some individuals may even never live in freshwater (Daverat et al.,  
482 2006). This phenotypic plasticity could partly result from the limited impact of salinity on gut  
483 microbial composition that we observed here. The effects of salinity and metamorphosis on gut  
484 microbiota should certainly be further investigated in mature individuals (silver eels) prior or during  
485 their catadromous migration.

486 Owing to their involvement in crucial processes in all environmental compartments such as  
487 wastewaters, continental and coastal surface water, groundwater, and sediments, along with their  
488 sensitivity to alterations, their quick and early responses, microorganisms are now commonly used as



489 early-warning bioindicators in ecotoxicological studies (Ghiglione et al., 2016; Pesce et al., 2020). The  
490 recent coupling of host-associated microbiome research with ecotoxicology in multicellular  
491 organisms is a promising approach (Duperron et al., 2020). Here, 323 genera showed differential  
492 abundance amongst sites (Figure 5). Due to a larger variability in their beta-diversity (see above),  
493 *Vibrionales* was the only enriched taxon (LDA = 5.14, p-value  $\leq$  0.001) identified in the fish from the  
494 Gironde estuary (Pauillac). *Vibrionales* bacteria are abundant in marine fish (e.g. Egerton et al., 2018;  
495 Sullam et al., 2012) and contain beneficial and pathogenic representatives. In chinook salmon  
496 (*Oncorhynchus tshawytscha*), juveniles exposed to organic chemical pollutants in a contaminated  
497 urban estuary were immunosuppressed and more susceptible to pathogenic strains, such as *Vibrio*  
498 *anguillarum* (Arkoosh et al., 1998). In Teleosts, the spleen is the major organ of immunity and fish  
499 with smaller spleen-somatic index show lower immune response and disease resistance (Hadidi et  
500 al., 2008). SSI values were smaller ( $0.08 \pm 0.01$ ) in PAU eels (Table 1) and micropollutants, particularly  
501 POPs, had an important effect on the abundance of *Vibrionales* (e.g. Transnonachlor, Figure 6). Thus,  
502 we cannot exclude the hypothesis that these compounds may increase the risk of infection by  
503 pathogenic members of *Vibrionales* by impairing the immune system of European eels.

504 In Batejin, that could be considered the less contaminated of our three study sites, taxa of the  
505 class *Clostridia* (Phylum *Firmicutes*) were significantly more abundant. Within *Clostridia*, the order  
506 *Clostridiales* is associated with the enhanced production of the short chain fatty acids acetate,  
507 butyrate, and propionate and with the degradation and metabolism of sugars in animals. In the  
508 common carp (*Cyprinus carpio L.*), a greater proportion of *Clostridiales* was associated with higher  
509 food intake and growth rate (Li et al., 2013) suggesting the good health of eels there. This hypothesis  
510 was partly confirmed by the higher values of Hepatosomatic Index (HSI) in BAT eels. In fisheries  
511 science, this index is used as an indicator of energy reserves in the liver. Correlation studies showed  
512 that, in our eels, the abundance of *Clostridiales* was negatively impacted by heavy metals (Mo and  
513 Zn) and diet composition (Fish and Mammal preys). The physiological role of these bacteria and the  
514 impact of pollutants should be further investigated by functional studies.

515 *Basidiomycota* and *Ascomycota* phyla were enriched in our fish from Pas-du-Bouc (Figure 5 and  
516 Supplementary file 5). Fungi colonizing animal gut, also referred to as mycobiota, is an important, yet  
517 largely neglected component of animal microbiome (Lavrinenko et al., 2021), partly because in wild  
518 animals, resident gut mycobiota is often overwhelmed by the large proportion of food-borne *Fungi*.  
519 However, strong and consistent covariation between fungal and bacterial communities were  
520 identified across animal kingdom by Harrison et al. (2021) suggesting an important role in host  
521 physiology through metabolic interactions with *Bacteria*. For instance, In *Actinopterygii*, these  
522 authors found that *Basidiomycota* and *Ascomycota* showed positive co-occurrence with *Firmicutes*  
523 and *Proteobacteria*. In PDB fish, genus *Aspergillus* was the indicator taxon with the larger LDA score,

524 and was positively correlated to Mo, Zn and the proportion of fish in diet. *Aspergillus* sp. is a  
525 saprophyte fungus occurring on decaying vegetation that could have been more abundant in the PDB  
526 area facilitating ingestion by the fish. Likewise, the free-living soil bacteria *Streptomyces* sp. was  
527 significantly more present in the gut of PDB eels. The presence of this genus as symbiont in gut  
528 microbiota has been previously reported in animals (Seipke et al., 2012), including freshwater fish  
529 (Jami et al., 2015) where they could help in the digestion of insoluble organic polymers, like chitin or  
530 cellulose but their precise role remains unclear. The class *Actinobacteria* are known to produce many  
531 secondary metabolites, including anti-inflammatory cytokines. *Actinobacteria*, that were more  
532 abundant in PDB (LDA = 4.85, p-value  $\leq$  0.001), are usually found on the bottom mud of lakes and  
533 water streams. In bighead carp (*Aristichthys nobilis*), they were more abundant in the gut of  
534 omnivorous juvenile fish living in lower water layers than in adult filter feeders that live in middle to  
535 upper water layers (Lu et al., 2022). In European eels also, these modifications could hence reflect a  
536 difference in feeding habits.

537

#### 538 **CONCLUSION:**

539 Microbiome response to pollutants is crucial but remains an underestimated element to better  
540 understand the toxicity of environmental contaminants on aquatic organisms (Duperron et al., 2020;  
541 Evariste et al., 2019). The sensitivity of microbiomes to environmental stressors suggests that  
542 microbial communities could represent novel ecological indicators for biomonitoring of ecosystem or  
543 host health (Sehnal et al., 2021). In the present study, we were able to associate environmental  
544 contamination and biologic variables with modifications in taxonomic composition of intestinal gut  
545 microbiome of European eels from three contrasted sites. The comprehension of how this species  
546 responds to contamination is crucial for environmental risk assessment. We also identify microbial  
547 genera specifically enriched in each of these sites. That suggests that changes in the structure of  
548 microbial communities associated with the gut of European eels could be an underestimated route  
549 by which the health of this endangered species is threatened by pollutants present in the  
550 environment. To the best of our knowledge, this work constitutes the first example trying to link gut  
551 microbiome modifications with the ecotoxicological landscape in wild fish. Due to the conservation  
552 status of *A. anguilla*, the use of gut microbial modifications as novel environmental biomarkers of  
553 contamination shall be coupled to non invasive sampling methods.

554 In the future, it will be necessary to complement the description of microbiome changes with the  
555 measurement of other endpoints of toxicological relevance in the host such as markers of the  
556 immune status or histology, as well as with functional studies of host-microbiota interactions.

557

#### 558 **ACKNOWLEDGEMENTS:**

559 This study has been carried out with financial support from the French National Research Agency  
560 (ANR) in the frame of the Investments for the future Programme, within the Cluster of Excellence  
561 COTE (ANR-10-LABX-45). The GeT core facility was supported by France Génomique National  
562 infrastructure (ANR-10-INBS-09). We thank the analytical platforms “Majeurs et Métaux traceS”  
563 (M&Ms) and PLATINE at UMR 5805 EPOC (University of Bordeaux) for performing the quantification  
564 of trace elements and POPs in fish tissues, respectively. This work benefited from the French GDR  
565 “Aquatic Ecotoxicology” framework which aims at fostering stimulating scientific discussions and  
566 collaborations for more integrative approaches. The authors thank Romaric Le Barh and Gwilherm  
567 Jan as well as Jean-Christophe Huguet, and all the volunteers from UMR 5805 EPOC and  
568 FDAAPPMA33 for their field support in fishing and dissections.  
569

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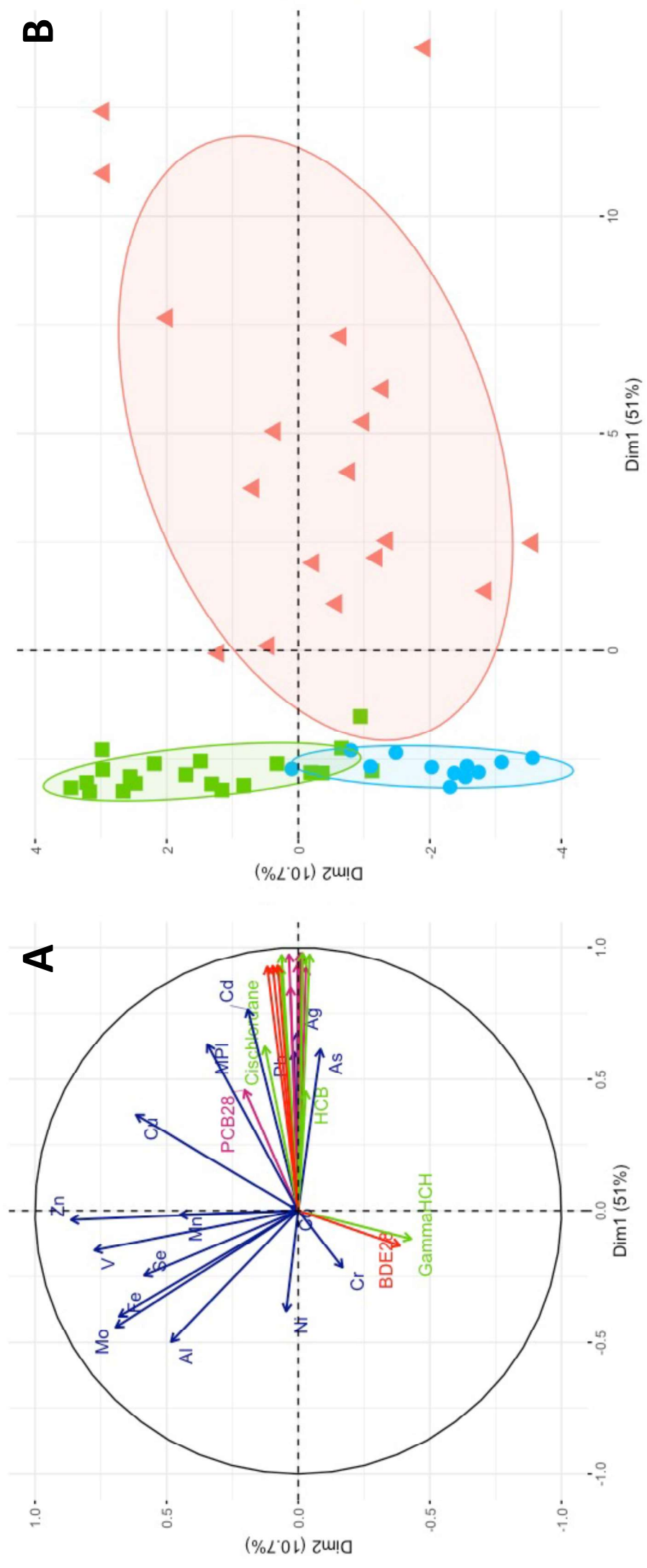
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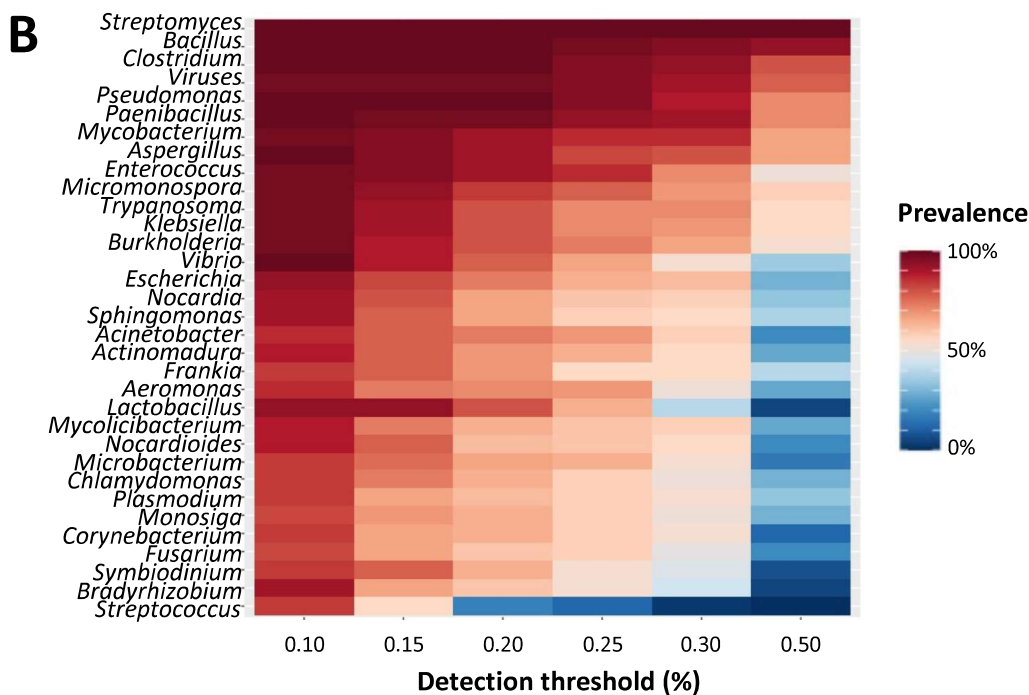
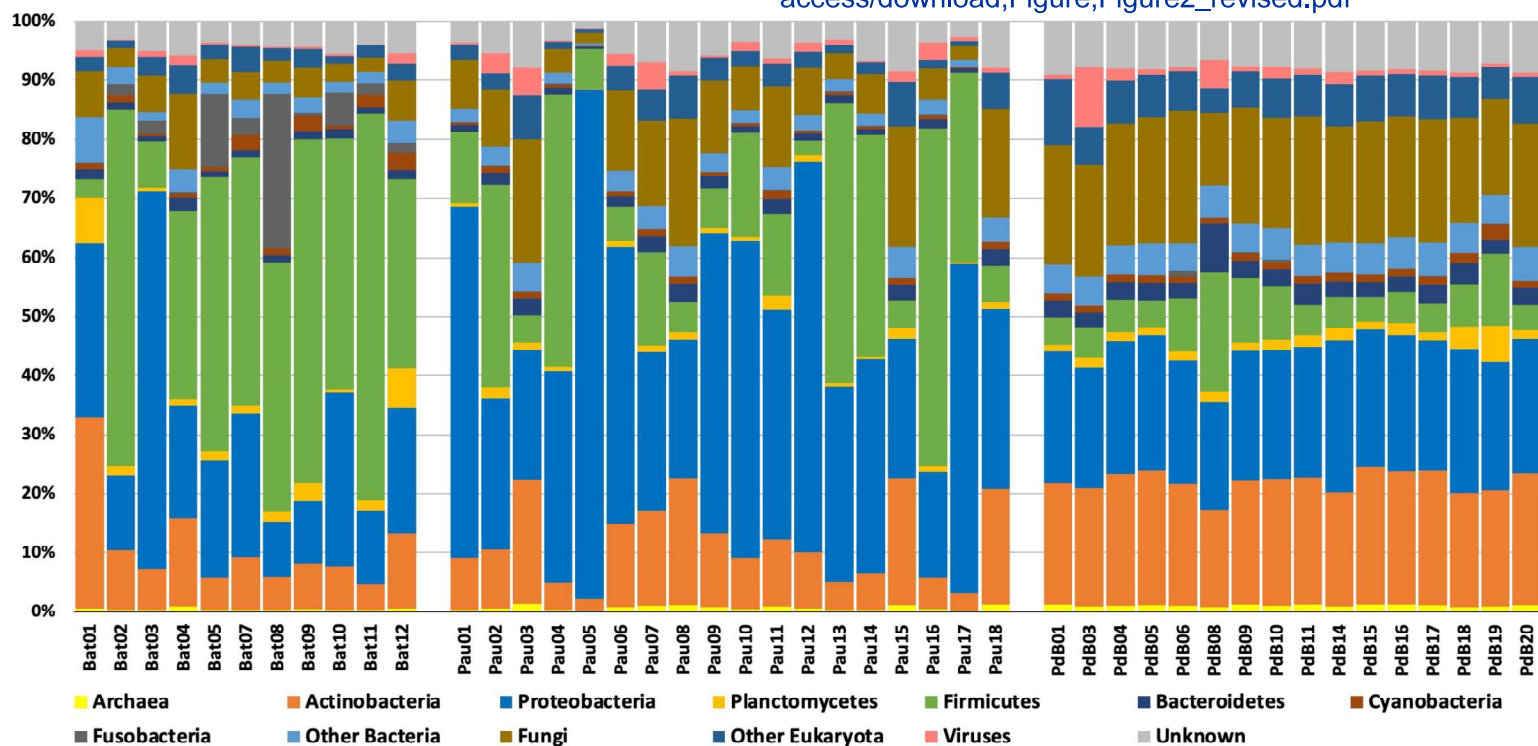
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Figure 1

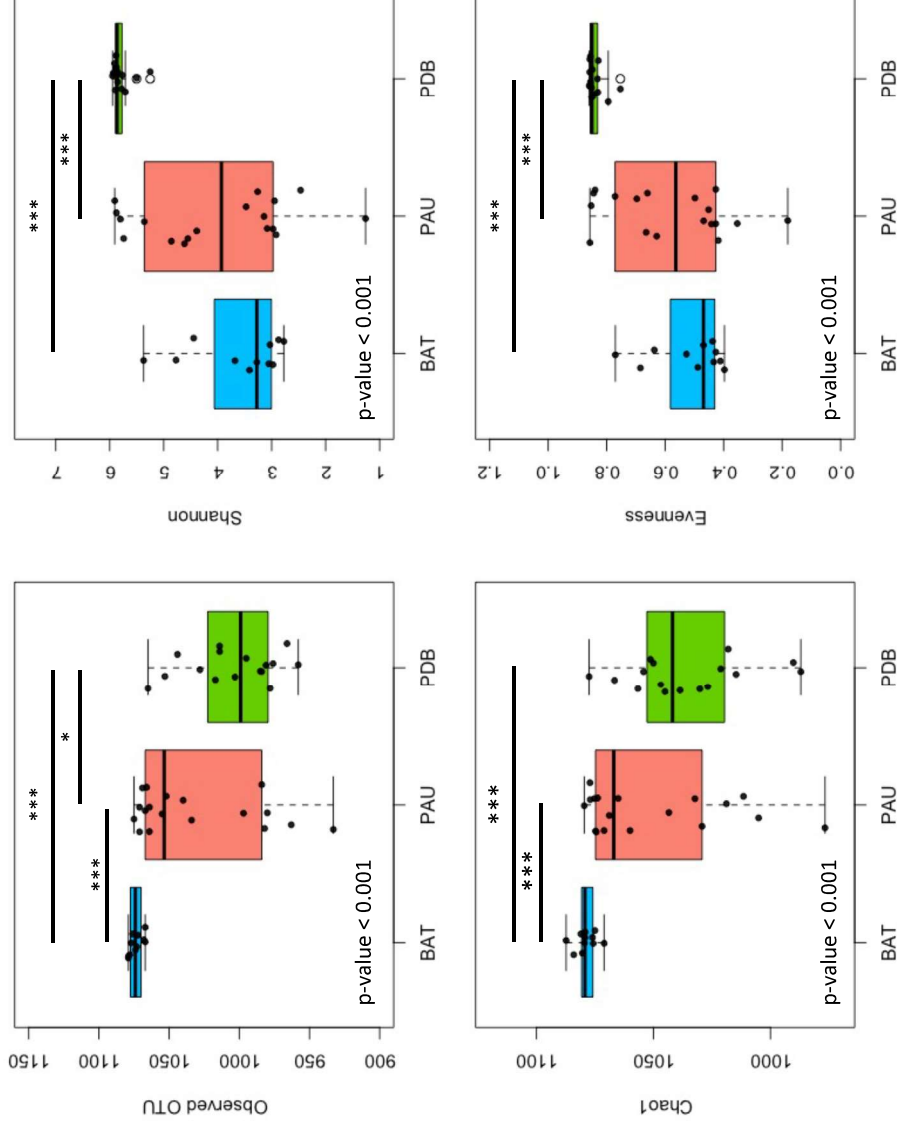
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**Figure 1:** Principal Component Analysis results for the pollutants levels. (A) Correlation circle for all variables. Blue, purple, green and red arrows represent metals, PCBs, OCPs and BDEs, respectively. (B) Distribution of individuals and grouping according to the site of origin. Blue dots = Batejin, green squares = Pas-du-Bouc, red triangles = Pauillac. Ellipses are represented with a 0.99 confidence level.



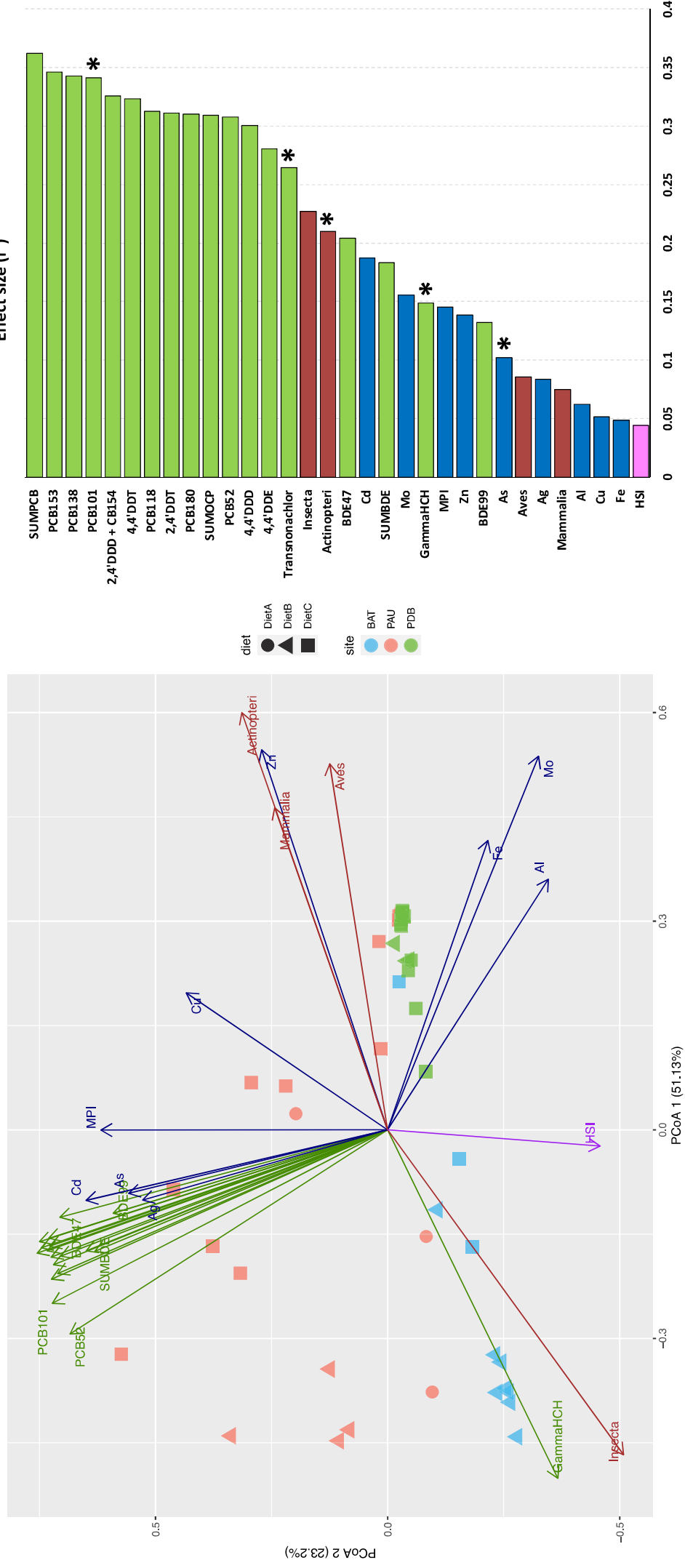
**Figure 2:** Taxonomic composition of the eels' intestine microbiome. **(A)** Relative abundance of metagenomic shotgun sequences classified at the domain (Archaea, Bacteria, Eukaryota and virus) and Phylum levels in all samples. Only the most abundant bacterial phyla are presented. **(B)** Core gut microbiota of *A. anguilla* at the Genus level. Selected taxa were above 0.1% or relative abundance in 80% of samples.



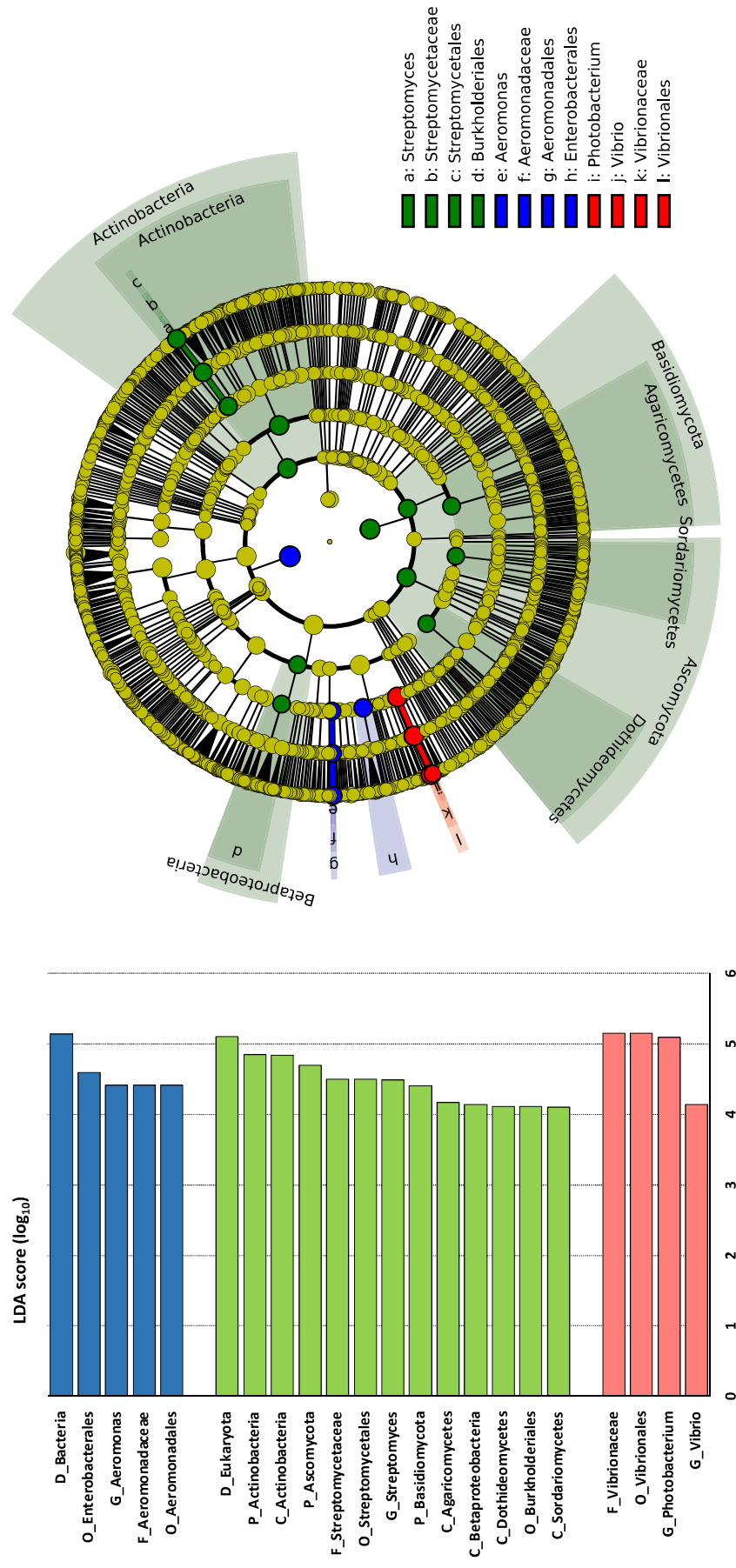
**Figure 3:** Within-sample microbial alpha diversity in eels' intestines at the genus level. Boxplots of Observed OTU, Shannon, Chao1 and Pielou's Evenness diversity indices calculated from non-normalized counts of reads. Individuals are represented by a point and grouped by sampling site. Statistical analysis was performed using Kruskal-Wallis tests (global p-value indicated on each panel) followed by pairwise Wilcoxon rank sum tests (\*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ).

Figure 4

[Click here to access/download;Figure;Figure4\\_revised.pdf](#)

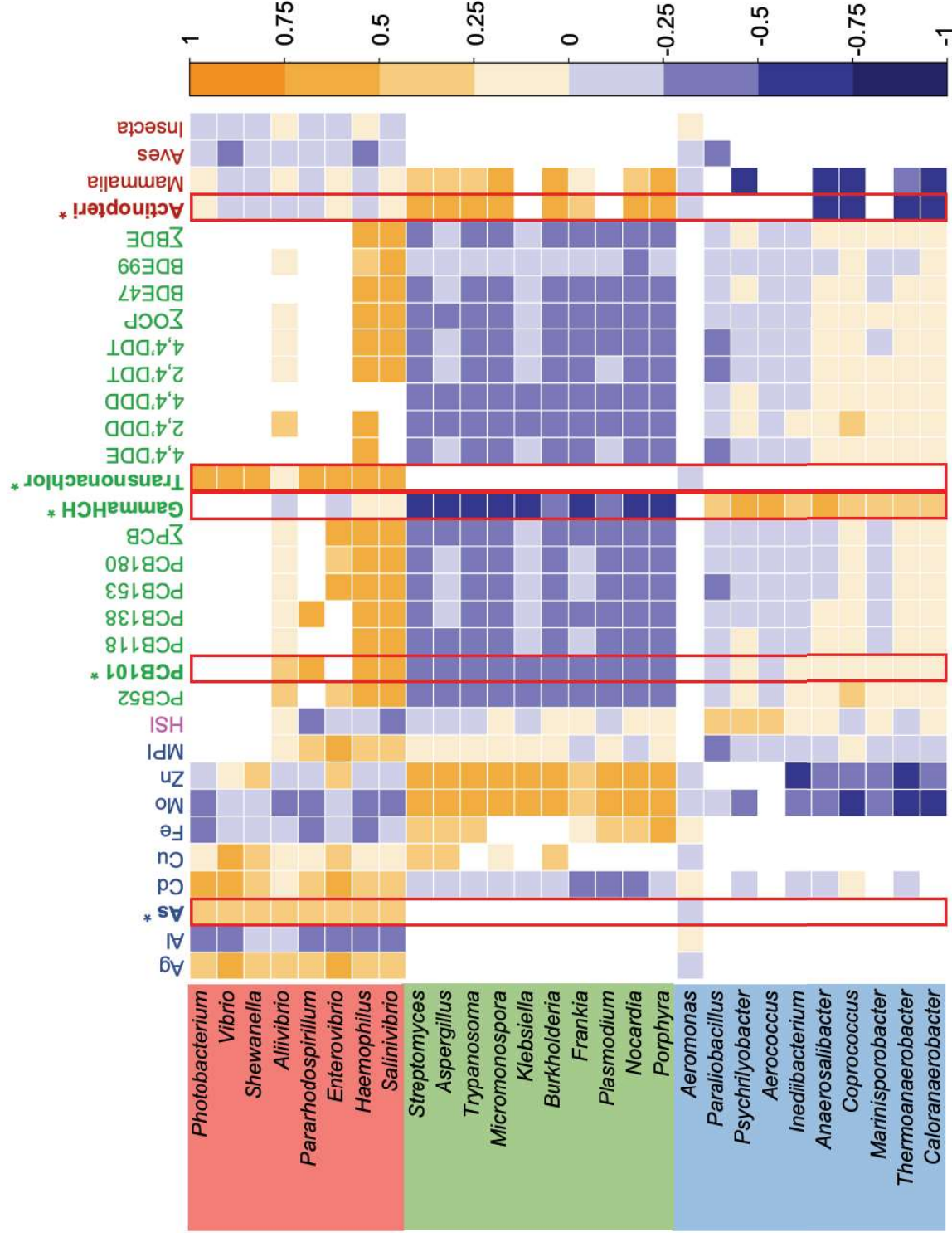


**Figure 4:** PCoA-based on Bray-Curtis distance (left panel) and effect size of microbiome covariates (right panel). Arrows show the ordination of 32 covariates for overall microbiome community variation in eels' gut. Factors are coloured based on their category (blue = metals, green = POPs, red = diet composition, and purple = biometry). Individuals are grouped by site (colour) or diet (shape). \* symbol indicates non-redundant covariates.



**Figure 5:** Linear discriminant analysis (LDA) effect size (LEfSe) results. Left panel: Indicator taxa with scores of 4 or greater in the gut microbiota of eels from Batejin (blue), Pas-du-Bouc (green) and Pauillac (red). D\_domain; P\_phylum; C\_class; O\_order; F\_family; G\_genus. Right panel: circular cladogram of the microbiome components classified from Phylum to genus. Yellow nodes represent genera with no significant differences. Blue, green, and red nodes represent the microbial groups that are significantly more present in Batejin, Pas-du-Bouc and Pauillac groups, respectively. Abbreviations of lower taxonomic levels are displayed in the bottom right legend.





**Figure 6:** Spearman correlation matrix heatmap between dominant (LDA > 3) indicator taxa at the genus level as identified by LEfSe in each group (blue: Batejin, green: Pas-du-Bouc, red: Pauillac) and covariate significantly associated with microbiome composition. Non-redundant covariates selected by stepwise redundancy analysis are indicated by the \* symbol. Only significant correlations ( $p \leq 0.05$ ) are indicated.



**Table 1:** Summary of environmental and morphologic measurements on each sampling site. Values are means  $\pm$  SE. Letters indicate significant differences between groups ( $p$ -value < 0.05).

		BAT	PDB	PAU
	<b>Location</b>	44°55'53.87"N	44°50'27.03"N	45°14'00.26"N
	<b>(GPS coordinates)</b>	01°07'22.94"W	01°09'07.58"W	00°44'01.80"W
	<b>Sampling date</b>	04-JUL-2019	22-JUL-2019	16-SEP-2019
<b>Environmental</b>	<b>Temperature (°C)</b>	28.00	23.80	22.36
	<b>Salinity (‰)</b>	0.12	0.12	10.36
	<b>pH</b>	8.19	6.42	nd
	<b>Dissolved O<sub>2</sub> (mg/l)</b>	8.24	7.67	5.84
<b>Eels characteristics</b>	<b>n samples</b>	11	16	18
	<b>Age</b>	4.75 $\pm$ 0.55 (a)	5.25 $\pm$ 0.29 (b)	3.89 $\pm$ 0.22 (a)
	<b>Length (cm)</b>	26.57 $\pm$ 1.92 (a)	29.44 $\pm$ 0.90 (b)	31.54 $\pm$ 0.83 (b)
	<b>Mass (g)</b>	31.16 $\pm$ 8.74 (a)	44.70 $\pm$ 4.20 (b)	46.89 $\pm$ 3.97 (b)
	<b>Fulton K</b>	0.16 $\pm$ 0.01 (ab)	0.16 $\pm$ 0.01 (b)	0.14 $\pm$ 0.01 (a)
	<b>HSI</b>	1.36 $\pm$ 0.08 (b)	0.93 $\pm$ 0.05 (a)	1.20 $\pm$ 0.07 (b)
	<b>SSI</b>	0.19 $\pm$ 0.04 (b)	0.25 $\pm$ 0.03 (c)	0.08 $\pm$ 0.01 (a)

**Table 2:** Concentrations of heavy metal in the liver (left panel) and organic pollutants in muscles (right panel) of European eels from the different sampling sites. Mean  $\pm$  SE, letters indicate significant differences between groups (p-value < 0.05).

	Batejin	Pas-Du-Bouc	Pauillac		Batejin	Pas-Du-Bouc	Pauillac
<b>Metals</b>				<b>POPs</b>			
<b>(<math>\mu\text{g}/\text{mg}</math>)</b>				<b>(ng/g)</b>			
<b>Ag</b>	0.008 $\pm$ 0.001 (a)	0.005 $\pm$ 0 (a)	0.968 $\pm$ 0.104 (b)	<b>PCB28</b>	0.823 $\pm$ 0.077	1.651 $\pm$ 0.567	6.784 $\pm$ 4.649
<b>Al</b>	27.684 $\pm$ 3.768 (a)	35.782 $\pm$ 2.553 (a)	13.489 $\pm$ 1.373 (b)	<b>PCB52</b>	4.838 $\pm$ 0.634 (a)	4.296 $\pm$ 0.519 (a)	13.864 $\pm$ 2.601 (b)
<b>As</b>	0.036 $\pm$ 0.004 (a)	0.440 $\pm$ 0.156 (a)	4.444 $\pm$ 0.438 (b)	<b>PCB101</b>	5.583 $\pm$ 0.865 (a)	4.698 $\pm$ 0.633 (a)	31.132 $\pm$ 5.983 (b)
<b>Cd</b>	0.762 $\pm$ 0.168 (a)	0.667 $\pm$ 0.085 (a)	1.729 $\pm$ 0.184 (b)	<b>PCB118</b>	4.354 $\pm$ 0.569 (a)	5.290 $\pm$ 1.046 (a)	37.347 $\pm$ 5.291 (b)
<b>Co</b>	0.232 $\pm$ 0.059	0.149 $\pm$ 0.042	0.151 $\pm$ 0.020	<b>PCB138</b>	6.659 $\pm$ 0.682 (a)	8.249 $\pm$ 0.567 (a)	281.848 $\pm$ 34.575 (b)
<b>Cr</b>	0.426 $\pm$ 0.094 (a)	0.258 $\pm$ 0.043 (b)	0.221 $\pm$ 0.010 (b)	<b>PCB153</b>	3.990 $\pm$ 0.365 (a)	8.651 $\pm$ 3.651 (a)	165.551 $\pm$ 24.128 (b)
<b>Cu</b>	26.472 $\pm$ 1.769 (a)	49.738 $\pm$ 4.568 (b)	60.049 $\pm$ 4.740 (b)	<b>PCB180</b>	0.966 $\pm$ 0.094 (a)	1.236 $\pm$ 0.114 (a)	141.954 $\pm$ 22.759 (b)
<b>Fe</b>	1721.904 $\pm$ 204.857 (a)	2936.914 $\pm$ 298.146 (b)	1128.601 $\pm$ 128.825 (a)	<b>SUM of PCBs</b>	27.214 $\pm$ 2.980 (a)	34.071 $\pm$ 6.711 (a)	678.480 $\pm$ 93.419 (b)
<b>Mn</b>	7.013 $\pm$ 0.468 (a)	8.793 $\pm$ 0.320 (b)	8.578 $\pm$ 0.410 (b)	<b>HCb</b>	0.446 $\pm$ 0.074	0.482 $\pm$ 0.104	0.630 $\pm$ 0.111
<b>Mo</b>	1.154 $\pm$ 0.048 (a)	1.581 $\pm$ 0.082 (b)	1.043 $\pm$ 0.073 (a)	<b><math>\gamma</math>-HCH</b>	0.551 $\pm$ 0.058 (b)	0.223 $\pm$ 0.047 (a)	0.310 $\pm$ 0.031 (a)
<b>Ni</b>	0.355 $\pm$ 0.033 (a)	0.268 $\pm$ 0.067 (a)	0.052 $\pm$ 0.019 (b)	<b>Cis-chlordane</b>	0.016 $\pm$ 0.005 (a)	0.037 $\pm$ 0.008 (a)	0.221 $\pm$ 0.051 (b)
<b>Pb</b>	0.570 $\pm$ 0.240 (a)	0.050 $\pm$ 0.003 (b)	0.906 $\pm$ 0.113 (a)	<b>Trans-nonachlor</b>	0.126 $\pm$ 0.015 (a)	0.279 $\pm$ 0.070 (a)	1.006 $\pm$ 0.142 (b)
<b>Se</b>	15.000 $\pm$ 1.238 (a)	39.264 $\pm$ 6.647 (b)	17.448 $\pm$ 1.012 (a)	<b>4,4'DDE</b>	0.789 $\pm$ 0.067 (a)	1.492 $\pm$ 0.162 (a)	31.581 $\pm$ 4.889 (b)
<b>V</b>	0.887 $\pm$ 0.183 (a)	2.010 $\pm$ 0.198 (b)	1.116 $\pm$ 0.158 (a)	<b>2,4'DDD + CB154</b>	0.197 $\pm$ 0.034 (a)	0.259 $\pm$ 0.024 (a)	8.830 $\pm$ 0.941 (b)
<b>Zn</b>	113.280 $\pm$ 5.176 (a)	163.304 $\pm$ 5.796 (b)	143.791 $\pm$ 9.135 (b)	<b>4,4'DDD</b>	0.095 $\pm$ 0.013 (a)	0.231 $\pm$ 0.079 (a)	7.796 $\pm$ 1.424 (b)
<b>MPI</b>	1.689 $\pm$ 0.217 (a)	2.022 $\pm$ 0.193 (a)	3.014 $\pm$ 0.178 (b)	<b>2,4'DDT</b>	0.138 $\pm$ 0.016 (a)	0.189 $\pm$ 0.036 (a)	4.701 $\pm$ 0.501 (b)
				<b>4,4'DDT</b>	0.147 $\pm$ 0.044 (a)	0.284 $\pm$ 0.028 (a)	1.922 $\pm$ 0.302 (b)
				<b>SUM of OCPs</b>	2.504 $\pm$ 0.176 (a)	3.477 $\pm$ 0.369 (a)	56.997 $\pm$ 8.130 (b)
				<b>BDE28</b>	0.353 $\pm$ 0.055 (b)	0.263 $\pm$ 0.001 (a)	0.271 $\pm$ 0.008 (a)
				<b>BDE47</b>	0.214 $\pm$ 0.001 (a)	0.214 $\pm$ 0.001 (a)	1.648 $\pm$ 0.314 (b)
				<b>BDE99</b>	0.192 $\pm$ 0.001 (a)	0.192 $\pm$ 0.001 (a)	0.703 $\pm$ 0.114 (b)
				<b>SUM of BDEs</b>	0.759 $\pm$ 0.055 (a)	0.670 $\pm$ 0.001 (a)	2.623 $\pm$ 0.422 (b)

**Table 3:** Mean percentage ( $\pm$  SD) of metazoan sequences in the 5 classes used to assess diet for each group of eels created by k-mean clustering.

Diet group	N	Actinopteri	Mammalia	Aves	Insecta	Malacostraca	Bivalvia	Gastropoda
<b>A</b>	3	22.54 $\pm$ 1.93	11.26 $\pm$ 0.59	3.74 $\pm$ 0.25	39.80 $\pm$ 1.40	13.76 $\pm$ 3.47	1.36 $\pm$ 0.06	1.34 $\pm$ 0.01
<b>B</b>	15	26.76 $\pm$ 2.34	12.53 $\pm$ 0.45	4.26 $\pm$ 0.18	45.24 $\pm$ 2.91	1.70 $\pm$ 1.85	1.44 $\pm$ 0.05	1.39 $\pm$ 0.06
<b>C</b>	27	31.25 $\pm$ 0.71	13.29 $\pm$ 0.29	4.55 $\pm$ 0.13	40.39 $\pm$ 0.81	1.16 $\pm$ 0.65	1.41 $\pm$ 0.04	1.33 $\pm$ 0.04