Insights on the particle-attached riverine archaeal community shifts linked to seasons and to multipollution during a Mediterranean extreme storm event

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Insights on the particle-attached riverine archaeome at different seasons and in response to multipollution during a Mediterranean extreme storm event

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Abstract

Even if Archaea deliver important ecosystem services and are major players in global biogeochemical cycles, they remain poorly understood in freshwater ecosystems. To our knowledge, no studies specifically address the direct impact of xenobiotics on the riverine archaeome. Using environmental DNA metabarcoding of the 16S ribosomal gene, we previously demonstrated bacteriome significant responses to pollutant mixtures during an extreme flood in a typical Mediterranean coastal watercourse. Here, using the same methodology, we sought to determine whether archaeal community shifts were also driven by environmental stressors during the same flood. Further, we wanted to determine how archaea taxa compared at different seasons. In contrast to the bacteriome, the archaeome showed a specific community in summer compared to winter and autumn. We also identified a significant relationship between *in situ* archaeome shifts and changes in physicochemical parameters along the flood, but a less marked response to river hydrodynamics than bacteria. New urban-specific archaeal taxa, which were significantly related to multiple stressors, were identified. Through statistical modeling of both domains, our results demonstrate that Archaea, seldom considered as bioindicators of water quality, could provide a rapid assessment of microbial pollution risk and thus have the potential to improve monitoring methods of watersheds.

Keywords: microbial ecotoxicology; water quality; extreme storm event; coastal Mediterranean rivers; sewer overflow; environmental DNA; metabarcoding.

1. Introduction

In the 1970s, Woese and collaborators highlighted a new domain of life, the Archaea, distinct from Bacteria and Eukaryota domains (Woese and Fox 1977; Woese et al. 1978). With the advent of Next Generation Sequencing (NGS), archaeal research and knowledge are expanding (Adam et al. 2017; Bang and Schmitz 2018). Archaea are recognized as major players in the global biogeochemical cycles of carbon, nitrogen, hydrogen and sulfur (Casamayor and Borrego 2009; Offre et al. 2013; Castelle et al. 2015) and there are at least two metabolisms, essential for nutrient cycling, which are carried out exclusively by archaea: methanogenesis and anaerobic methane oxidation (Joye 2012). Despite their importance for ecosystem functioning and their ubiquitous presence (Chaban et al. 2006; Casamayor and Borrego 2009; Herfort et al. 2009; Auguet et al. 2010), the environmental archaeome has been overlooked for decades. Most studies have concentrated on marine archaea (Zinger et al. 2012; Zeglin 2015), and the diversity and importance of archaea in other environments have been largely disregarded (Casamayor and Borrego 2009; Zinger et al. 2012; Zeglin 2015; Adam et al. 2017). Only recently, continental freshwater habitats have emerged as one of the largest reservoirs of archaeal diversity (Auguet et al. 2010; Zinger et al. 2012). Some studies have linked archaeal communities to water physicochemical properties such as pH, temperature and nutrients (Herfort et al. 2009; Wang et al. 2018; Lei et al. 2020). *Methanobrevibacter smithii,* for example, was proposed as a potential indicator of human-specific sewage pollution (Johnston et al. 2010; McLellan and Eren 2014). To assess water quality, several studies have focused on freshwater archaea (Cannon et al. 2017; Dila et al. 2018), notably for their potential remediation role in heavily contaminated urban rivers (Samson et al. 2019; Lei et al. 2020). Cannon *et al.* (2017) found that a rain event induced changes in the structure of microbial communities, including archaea, from environmental DNA (eDNA) and stressed the importance of considering hydrological conditions when studying riverine microbiomes. Both chemical and microbial pollutants reach surface waters via point sources (such as urban sewage) or diffuse sources (linked to runoff) of pollution. It is well known that during rainfall events, particles from terrestrial soils and river basin sediments remobilize, and affect water quality (Garcia-Esteves et al. 2007; Dumas et al. 2015; Faure et al. 2015) because soils and sediments store pathogens, nutrients and pollutants. Thus, suspended particles have a crucial role in the transfer of contaminants to surface waters through runoff and in the resuspension of river sediments during storms (Turner and Millward 2002; Amalfitano et al. 2017). Moreover, some studies have reported that riverine archaea tend to be associated with particles and derive only from

allochthonous inputs (Crump and Baross 2000; Casamayor and Borrego 2009; Hu et al. 2018). This is all the more important in Mediterranean regions, where extreme hydrological events are expected to become more intense and frequent due to climate change (Cowling et al. 2005; Blanchet et al. 2016). Furthermore, first-flush events during rainfalls lead to Combined Sewer Overflows (CSOs), which carry large loads of contaminant mixtures over surface waters through in-sewer solids resuspension (Ashley et al. 1992; Osorio et al. 2012; Oursel et al. 2014; Reoyo-Prats et al. 2017, 2018). We previously demonstrated that an extreme Mediterranean flood produced shifts in the particle-attached bacterial compartment from eDNA, severely affecting resident riverine communities (Noyer et al. 2020). In this study, we went a step further by using metabarcoding of the 16S ribosomal RNA gene (rDNA) to explore how the diversity of the particle-attached archaeome changed within the same environmental samples, and to compare the responses between the bacteriome and the archaeome. Next, we modeled shifts in both communities using physical parameters as well as several families of chemical parameters as environmental forces, which is a first in environmental microbiology. This study addressed the following questions: how did the fluviatile particle-attached archaeal community change between seasons and how did it evolve over the course of a heavy rain event? How our findings compare to other studies of archaeal alpha and beta diversity in lotic ecosystems? Did environmental parameters drive structural shifts in the archaeome as they did in the bacteriome? Was there a strong relationship between key taxa and environmental dynamics? How did the seasonal and temporal succession of bacterial and archaeal communities compare? The answer to these questions could, in general, provide insights on the use of microorganisms in water quality assessment and, in particular, help on the rapid risk assessment of multiple pollutants in aquatic ecosystems.

2. Materials and Methods

2.1. Study site, sampling information

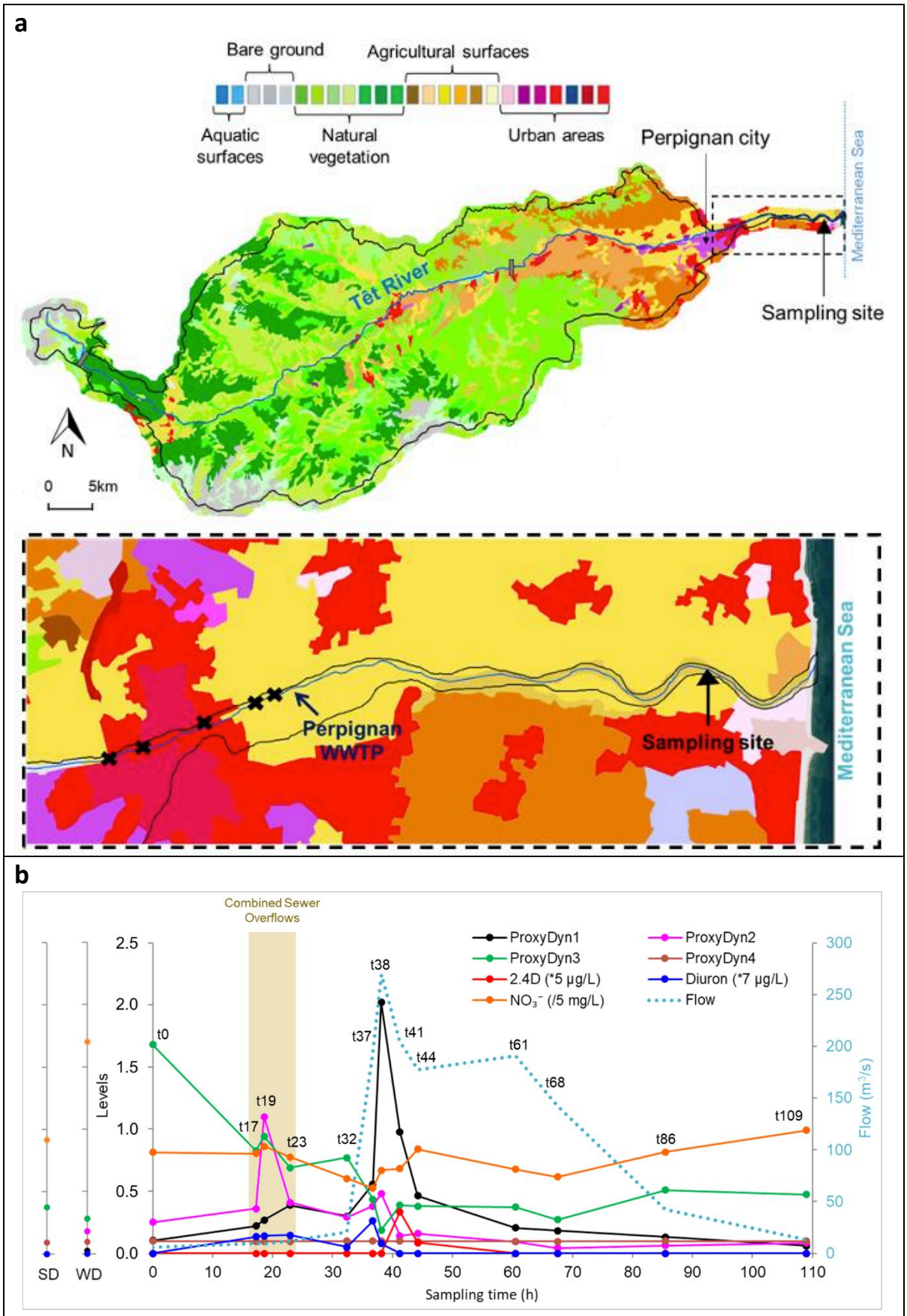
Sampling took place in the Têt River, a watercourse representative of Mediterranean coastal watercourses with a torrential regime that discharges into the Gulf of Lion (Southeast of France) (Dumas et al. 2015; Reoyo-Prats et al. 2017) downstream from the Perpignan city wastewater treatment plant, the main threat to the water quality of this river (Fig. 1a, Conseil Général des Pyrénées Orientales 2009, 2012; Reoyo-Prats et al. 2017). The Vinça dam, situated 40 km upstream from the sampling station, controls the downstream river flow, particularly

109 during floods. For sampling methodology and sampling site see Reoyo-Prats *et al.* (2017). In
110 short, ten liters of river water were collected in summer, winter, and autumn, before, during
111 and after a 5-year flood that we followed 24h/24h during more than 4 days (see Fig. 1b for
112 further details).
113 First flushes during the flood brought the highest levels of *E. coli* and *Enterococci* ever detected
114 in this river (Reoyo-Prats et al. 2017) as well as relative higher abundance of other typical
115 sewer bacteria (Noyer et al. 2020) and higher levels of dissolved pharmaceuticals (Reoyo-Prats
116 et al. 2018), which indicated the moment at which sewer overflows occurred (Fig. 1b).
117 Environmental DNA sampling was previously described by Noyer *et al.* (2020). In short, a liter
118 of mixed-water sample was entirely (or until clogged) filtered through cellulose acetate MF-
119 Millipore membrane filters with 3 µm porosity (Merck Darmstadt, Germany) and repeated
120 three times to obtain three replicates per sample.

121 **2.2. Nucleic acid extraction, 16S rRNA gene sequencing, and sequence analyses to**
122 **obtain Operational Taxonomic Units (OTUs) contingency table**

123 Nucleic acid extraction and bacterial sequencing from eDNA were described in a
124 previous study (Noyer et al. 2020) and were performed in triplicate per sample. For archaea
125 sequencing, a single set of DNA replicates from each sample was sent to the Research and
126 Testing Laboratory (RTL, Texas, USA). Two more replicates were later sent to the Genome
127 Quebec laboratory (GQ, Montreal, Canada), except for samples t61 and t68 which were only
128 sequenced once again for technical reasons. DNA from samples t19 and t23 from the first
129 replicate, which was already sequenced at RTL, was also sent to GQ to be sequenced again for
130 comparative purposes. Sequencing targeted V3 and V4 hypervariable regions of the 16S rDNA
131 by using 519wF (5'-CAGCMGCCGCGGTAA-3') and 1017R (5'-
132 GGCCATGCACCWCCTCTC-3') universal archaeal primers (Borrego et al. 2020) and was
133 performed on an Illumina MiSeq sequencer using a 2x300bp paired-end protocol. Libraries
134 were generated by pooling equimolar ratios of amplicons before sequencing. In contrast to RTL
135 sequences, those provided by GQ contained primers. In order to pool sequences together, we
136 eliminated primers from GQ sequences using Cutadapt version 1.8.3 for Unix (Martin, 2011).
137 Default options were used with the exception of sequences treatment as paired with -g and -G
138 options for forward and reverse primers respectively, taking wildcards into account, discarding
139 untrimmed sequences and setting an overlap of 14 bp, a quality-base of 33 and an error-rate of
140 0.1. Harmonized sequences were pooled together and archived before being uploaded to the

141 Galaxy platform (Afgan et al., 2016): http://sigenae-workbench.toulouse.inra.fr. FROGS
142 pipeline (v. r3.0-3.0, Escudié et al. 2018) was used to process sequences to form OTUs and to
143 taxonomically affiliate them as described in Noyer *et al.* (2020) except that VSEARCH (v2.6.2,
144 Rognes et al. 2016) was used as a read pair assembler, which allows a higher number of
145 sequences to be conserved when amplicon sizes are highly variable, as was the case for our
146 dataset. Read length was set to 300pb and the amplicon size was set to 450pb for the minimum
147 and optimized to 545pb for the maximum. We assigned each OTU up to the species taxonomic
148 level based on blast alignment using the affiliation tool (v. r3.0-2.0) and the Silva 138.1
149 database with a pintail score of 100, which allowed for the most accurate affiliation possible.
150 No archaea hits were removed hereafter. To resolve the taxonomic ambiguity of OTUs that
151 were multi-affiliated within the FROGS pipeline when using blast against the curated pintail
152 100 score silva database, we blasted these OTUs against the NCBI 16S nucleotide collection
153 database using the megablast algorithm.



Summer drought	Winter drought	Autumn drought	Autumn flood
SD	WD	t0	12 sampling times: t17 → t109

Fig. 1 Têt River archaeome sampling sites and environmental parameters measured in the same samples. (a) Watershed of the river with sampling site (black arrow), located after wastewater treatment plan (WWTP) of the city of Perpignan, combined sewers (black crosses) and water reservoirs indicated as grey rectangles (adapted from Reoyo-Prats et al. 2017). (b) Environmental parameter dynamics in the Têt River at different seasons and along an autumn flood. For sample names, see table below figure. Autumn sample names are followed by a number that indicates the sampling time in hours after t0, which was sampled at autumn basal level water discharges. Sampling took part at crucial moments of the flood that occurred thereafter: at first flushes (t17-t19-t23), before the flow peak (t32-t37), during the flow peak (t38-t41), following the release of water from the upstream Vinça reservoir (t44-t61) and during the return to basal level (t68-t86-t109). ProxyDyn1 corresponds to the dynamics of particulate organic carbon (/20 mg/l), which represented the dynamics of water flow, also represented in figure, total suspended solids, total organic carbon, total nitrogen, and terbuthylazine parameters. ProxyDyn2 corresponds to aminomethyl phosphonic acid (AMPA, µg/l), which represented glyphosate, phosphate, copper, temperature, *E. coli*, enterococci, diclofenac, sulfamethoxazole and carbamazepine parameters. ProxyDyn3 corresponds to lead (/150 µg/g) in the representation of the dynamics of cadmium, zinc, and conductivity parameters. ProxyDyn4 corresponds to pH (/70), which represented cobalt, nickel, and chrome parameters. Three parameters, Diuron, 2.4D and NO^{3-}, had a unique dynamic. For further details on statistical analyses for environmental parameters, see Noyer *et al.* (2020).

2.3. Archaeal diversity analyses

Diversity analyses were performed using the output OTUs contingency table, tree and dissimilarity matrices calculated using FROGS as input for *Phyloseq* within R package 1.24.2 (McMurdie and Holmes 2013) and a collection of additional R functions (https://github.com/mahendra-mariadassou/phyloseq-extended). Trimming rare OTUs affects alpha-diversity measurements sensitive to rare OTUs such as Chao1, Observed richness and Shannon indices, while rarefaction is controverted as well when concerning some alpha diversity indices (McMurdie and Holmes 2014; Cameron et al. 2021). Alpha diversity was therefore calculated using non-filtered and non-normalized replicates from the GQ laboratory only, because the lower sequence depth of RTL sequenced replicates precluded comparison. Fisher, Simpson, Shannon, and Pielou alpha diversity indices were used, together with the nonparametric Chao1 species richness estimator. These indices provide complementary information regarding evenness and richness aspects of alpha diversity that are interesting to take into account (see, for instance, Walters and Martiny 2020). Kruskal-Wallis test (KW) followed by a post hoc Dunn test with R software (v. 3.5.1, R Core Team 2018) were applied to evaluate diversity changes through time. Beta diversity was assessed on all replicates, independently of platform origin, because sequencing depth is not relevant in this case. Singletons were filtered out and then abundance was normalized to the sample with the lowest number of sequence reads. Using this dataset, relative abundances by phylum and class were plotted. To detect potential outliers in the dataset we proceeded by (i) checking the number of sequences of each replicate, (ii) checking OTU abundance distribution among replicates of the same sample and (iii) calculating qualitative Jaccard and quantitative Bray-Curtis dissimilarities from replicates separately and using Principal Coordinates Analysis (PCoA) to

194 visualize replicate dissimilarities. Once outliers checked, beta dissimilarities were recalculated
195 on the averaged OTU abundances. To this end, the number of OTUs decreased to less than
196 10,000 after the dataset was normalized, thus allowing qualitative Unifrac and quantitative
197 Weighted-Unifrac (W-U) dissimilarities, which also consider phylogeny of OTUs, to be
198 included within FROGS. PCoA and hierarchical clustering Ward.D2 methods were used to
199 visualize archaeal community dissimilarities among samples. A one-way analysis of similarity
200 (ANOSIM, Clarke 1993) was performed to test significant differences between sample groups
201 resulting from hierarchical clustering. To further check for significant differences in archaeal
202 community shifts at the class level, the Mann-Whitney test (MW) was implemented with R
203 software.

204 **2.4. Statistical analyses for inference**

205 2.4.1. Constrained (canonical) ordination analyses by environmental parameters dynamics

206 Physicochemical environmental parameters were previously measured (Reoyo-Prats et al.
207 2017) in the same samples in which nucleic acid extractions were performed. Measured
208 parameters included pH, temperature, conductivity, flow, total suspended solids, particulate
209 organic carbon, total organic carbon, nitrogen and 250 pesticide molecules, 90
210 pharmaceutically active compounds, polycyclic aromatic hydrocarbons and polychlorinated
211 biphenyls, nutrients, trace metals in the particulate fraction and fecal indicators (load in
212 *Escherichia coli* and Enterococci). Collinearity issues resolution and the seven major
213 environmental dynamics retained for further analyses are described in Noyer *et al.* (2020). For
214 clarity, the retained variables that will be used for further analyses as proxies of correlated
215 environmental variables are summarized in Fig. 1b. Constraint-based ordination analyses were
216 then used to evaluate the relationships between the normalized OTU contingency table and
217 retained environmental parameter dynamics using *vegan* R package version 2.5-3 (Oksanen et
218 al. 2018). Detrended correspondence analysis (DCA) performed on the OTU dataset rendered
219 a first axis gradient length of 3.7, so both canonical correspondence (CCA) and redundancy
220 analyses (RDA) were performed (ter Braak 1988). We also performed a Hellinger transformed-
221 based RDA (tbRDA) (Legendre and Gallagher 2001) and a distance-based RDA (dbRDA)
222 using all beta dissimilarities from the previous section. Permutation analyses of variance were
223 used to evaluate the significance of constraint-based models, axes, and variables. Variables
224 were tested by adding each of them independently and the number of permutations was set to
225 10,000. To further determine which OTUs best responded to environmental variables, we
226 reduced the OTU matrix to a percentage of abundance so that CCA/RDA modeling became

227 significant. Instead of 0.005%, as reported by Bokulich *et al.* (2012) and Noyer *et al.* (2020)
228 for bacteria, we found a percentage of 0.05% for the archaeal dataset. RDA modeling of OTUs
229 with ≥ 0.05% of the total read number (i.e. keeping 2,688,328 reads), allowed for the OTU
230 goodness of fit (GOF) to be calculated. As for bacteria, OTUs retained for further analyses had
231 a GOF ≥ $GOF_{average}$, which is considered a conservative approach to OTU selection.

232 #### 2.4.2. Network construction via module eigengene analysis

233 We used the Molecular Ecological Network Analyses Pipeline (MENAP:
234 http://ieg4.rccc.ou.edu/MENA/) to build the relationships among OTUs following the
235 developer's recommendations (Deng et al. 2012; Tao et al. 2018) but sample-specific OTUs
236 were kept for network construction by changing "OTUs present at least in one sample". An
237 automatically generated similarity threshold value (0.32) was obtained with Random Matrix
238 Theory (RMT)-based method, which allowed network construction ensuring that the
239 connections between microorganisms were non-random (R^2 of power-law = 0.28). The network
240 was separated into modules via the short random walk method (Pons and Latapy 2005), which
241 had the highest modularity (0.078) (Newman 2004). Module higher-order organization was
242 then performed via eigengene analysis (Langfelder and Horvath 2007) using default parameters
243 to obtain the correlation significance between modules and environmental parameters
244 dynamics. Cytoscape software (v. 3.7.1, Shannon et al. 2003) was used to visualize this
245 constraint-based network. We also used MENAP to explore the relationship between OTUs
246 from Bacteria (from the previous study by Noyer et al., 2020) and Archaea domains and flood
247 environmental dynamics. The network was constructed with the same parameters as before
248 except zero counts were replaced "by 0.01" instead of "on paired values", to avoid losing an
249 important number of OTUs when using the matrix of joined OTUs from both domains. The
250 highest modularity was obtained via the leading eigenvector method (0.520). The RMT
251 threshold was 0.940 and the R^2 of power law = 0.863.

252 ## 3. Results

253 ### 3.1. Changes in particle-attached archaeal diversity along a storm event in the Têt
254 River

255 To study changes in an urban archaeome along a storm event, a high-resolution environmental
256 DNA sampling from river water was performed during a major flood that occurred in autumn
257 2013. Two other seasons were also sampled during the summer and winter drought periods.

All reported sequence data passed standard quality controls of certified sequencing companies. A total of 1,804,302 reads corresponding to 350,106 operational taxonomic units (OTUs) were identified. Most OTUs (98%) were assigned to the archaeal domain, the rest were eliminated from further analysis.

Alpha diversity statistical analyses showed significant differences among samples for Shannon (KW = 0.03, Fig. 2) and Pielou (KW = 0.03, Table S1) indices only. These two indices changed similarly along seasons and during the flood, with equivalent significant differences between samples except for the Pielou index for the summer drought, which was significantly higher than the flow peak (t38-t41) indicating a greater evenness in OTU abundances in this sample. In general, both indices decreased significantly at t41, i.e. right after the flood peak, with respect to t17 and t19. Three hours later, a significant increase in diversity was noticed (t44). Even if the observed OTU number, Chao1 and Fisher indices did not show significant differences (Table S1), they had the same change pattern as the Shannon index (Fig. 2), except for a much lower value in the summer drought sample. This result contrast with the Pielou and Simpson indices (Table S1), which emphasize the evenness component of diversity, as opposed to Fisher and Shannon indices, which appear more related here to the richness component of diversity (Magurran 2004).

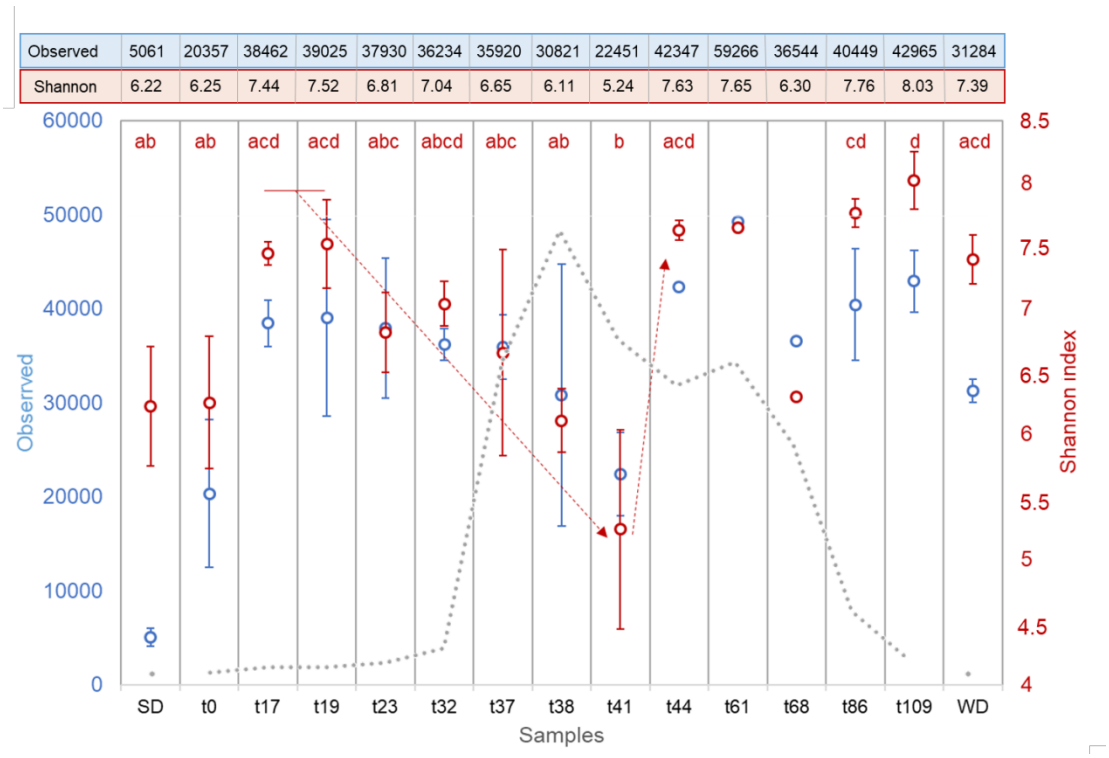


Fig. 2 Alpha diversity of the archaeome of the Têt river along time. Changes in observed OTU number (blue) and Shannon index (red) along the flood (tX) and at summer and winter droughts (SD and WD respectively). For the

Shannon index, different letters indicate a significant difference between samples (dunn.test<0.025) and red arrows show major significant differences. Observed OTU number was not significantly different (KW=0.14). The dotted profile is the flow level at each sampling point (see Fig. 1, also for sample names). Even though the absence of replicates for t61 and t68 samples impeded statistical testing, they are represented through time for comparison.

After normalization of OTU matrix once singletons were excluded, a total of 1,377 OTUs (3,777 sequences/sample) were retained for further analyses. We noticed a great difference in the taxonomic composition of the summer drought (SD) with respect to all other samples. SD had a significantly lower amount of Nitrososphaeria and Thermoplasmata classes when compared to all samples (Fig. 3, MW p=0.012 and p=0.021 respectively) and a higher amount of Nanoarchaeia (Nanoarchaeota phylum, Fig. 3) and Bathyarchaeia (Crenarchaeota phylum) classes when compared to most samples. These large differences in the summer sample community were confirmed with the beta diversity analyses (data not shown), as all samples significantly separated from the SD sample with all beta dissimilarity indices, contrarily to WD, which always clustered with t17, t19, and t32. Additionally, the taxonomic composition of the WD sample was very similar to t17, t19, and t32 samples, mainly composed of Nitrososphaeria class, followed by Nanoarchaeia, Thermoplasmata, and then three classes, Methanobacteria (Euryarchaeota phylum), Methanomicrobia (Halobacterota phylum) and Bathyarchaeia at variable smaller abundances (Fig. 3).

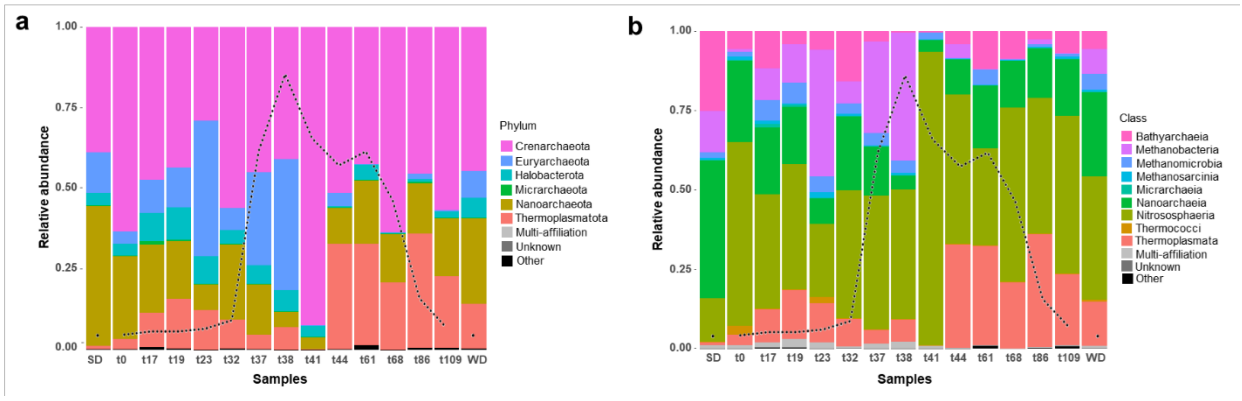


Fig. 3 Composition of archaeal communities averaged across replicates. Histogram of relative abundances (a) of the six major phyla and (b) of the ten major classes. Samples are organized according to sampling time from left to right: summer drought (SD), autumn flood (sample names are followed by a number that indicates the sampling time in hours after the beginning of the flood at t0), and winter drought (WD). The dotted profile is the flow level at each sampling point (see Fig. 1 for further details).

304 Given these results, only flood samples were used to further explore structural diversity
305 changes using beta dissimilarities. These samples comprised a total of 1,739 OTUs (19,355
306 sequences/sample) after eliminating singletons and normalization. Using different beta
307 dissimilarities allowed for a better assessment of which differences are responsible for the
308 community structure (either presence/absence or abundance and/or phylogeny of OTUs).
309 Using only OTU presence/absence with Jaccard qualitative dissimilarity, three communities
310 were significantly differentiated (Fig. S2.1a). The first community group included samples
311 collected at t0, t17, t19 and t32, the second included samples t23, t37, and t38, and the third
312 included the rest of the samples (t41-t109), which was the most differentiated cluster. But when
313 phylogenetic relationships were considered using Unifrac qualitative dissimilarity, t41 sample
314 grouped with t23, t37 and t38 instead (Fig. S2.1b). With Unifrac, this last cluster was the most
315 differentiated. When Bray-Curtis quantitative dissimilarity, which considers OTU abundance,
316 was used, three community groups of samples were distinguished (Fig. S2.1c). The first
317 included from t17 to t38 samples, the second included t0, t41, and t68 samples, and the third
318 t44, t61, t86, and t109 samples. When considering phylogenetic relationships using W-U,
319 observed community groups coincided with those of Bray-Curtis dissimilarity (Fig. 4, bold
320 black line), but the most significantly differentiated communities were those from the group of
321 t23, t37 and t38 (axis 1) and then t41 and t0 samples (axis 2). Even though the first taxonomic
322 changes occurred from the first flood sample at t17, samples t23, t37 and t38 had a particular
323 significant increase in Methanobacteria (Euryarchaeota phylum) (Fig. 3, MW, p=0.001).
324 Sample t0 differentiated significatively from samples from the end of the flood (from t44 to
325 t109) only when considering OTUs presence/absence but not when abundance alone or with
326 phylogeny were considered. This sample had a significantly lower abundance of taxa from the
327 Thermoplasmata class with respect to that group of samples (Fig. 3b, MW p=0.003). Regarding
328 t41 sample community, it had a particular taxonomy, significantly dominated by
329 Nitrososphaeria class (MW p=0.002) and W-U dissimilarity significantly separated t41 from
330 the rest of the samples (Fig. 4). With regard to the group of samples at the end of the flood
331 event, from t44 to t109, we could notice a significant increase of Thermoplasmata class with
332 respect to all other flood samples (Thermoplasmatota phylum, MW p=0.002, Fig. 3b).
333

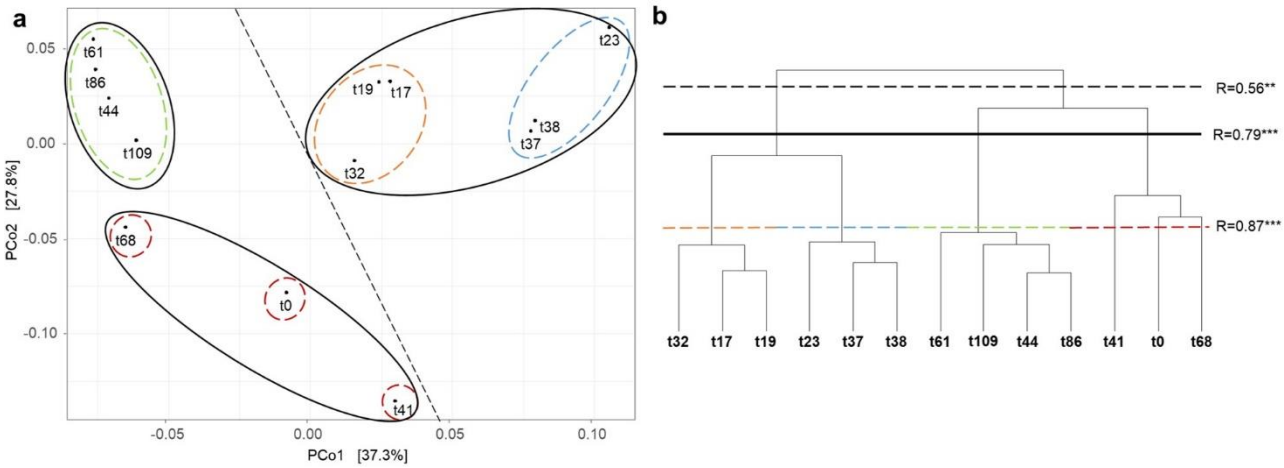


Fig. 4 Structure of archaeal communities averaged across replicates. (a) Principal Coordinate Analysis (PCoA) and (b) hierarchical clustering with Ward D2 linkage method using Weighted-Unifrac dissimilarity computed on OTU average abundance. Lines indicate ANOSIM significant groups. Sample names are followed by a number that indicates the sampling time in hours after t0 (see Fig. 1 for details). Significant codes ** and *** indicate p-value < 0.01 and < 0.001, respectively.

Table 1 Summary of constraint-based multivariate statistical models on archaea OTU matrix averaged over replicates and without singletons. (a) Permanova significance of the five models tested and the percentage of biological variance that is explained by each model using permutation test with anova.cca function. (b) Axes and modeled variables significance after permanova using anova.cca of significant models in (a). Axes not shown were not significant. p-values significance codes: (*** < 0.001 < ** < 0.01 < * < 0.05).

a	**OTUs matrix transformation**	**Model significance**	**Variance (%)**
dbRDA	**Jaccard**	0.003**	63.38%
	Unifrac	0.008**	66.35%
	Bray-Curtis	0.326	60.04%
	Weighted-Unifrac	0.004**	76.58%
CCA		0.765	56.44%
RDA	**Hellinger**	0.134	61.4%

b	**OTUs matrix transformation**	**Axes significance and variance explained (%)**	**Statistical significance of modeled variables**						
		CAP1	**ProxyDyn1**	**ProxyDyn2**	**ProxyDyn3**	**ProxyDyn4**	**2.4D**	**Diuron**	**NO_3^-**
dbRDA	**Jaccard**	0.002** (22.74)	0.001***	0.002**	0.001***	0.105	0.095	0.001***	0.096
	Unifrac	0.040* (30.24)	0.001***	0.021*	0.046*	0.458	0.383	0.036*	0.014*
	Weighted-Unifrac	0.006** (42.89)	0.006**	0.009**	0.039*	0.050*	0.004**	0.002**	0.166

346 **3.2. Modeling archaeome diversity according to multiple contaminant dynamics**

347 Constrained multivariate analyses were performed to determine if the retained environmental
348 dynamics (Fig. 1b) could statistically explain the observed community structure and diversity
349 shifts through time. Significance and percentage of variance explained by all models tested
350 were summarized in Table 1a. Jaccard, Unifrac and Weighted-Unifrac dbRDAs supported
351 significantly (p-value < 0.01) a link between environmental parameters included in the model
352 and our community data (Fig. S2.2). Models using Jaccard and Unifrac dissimilarities
353 explained between 63 and 66% of the total variance, respectively. Only the first canonical axis
354 was significant in both models, with a higher proportion of the variance in the dissimilarity
355 matrix explained when phylogeny was considered using Unifrac (33% vs 23% for Jaccard, Fig.
356 S2.2a-b). In both models, the same four environmental dynamics were significant, ProxyDyn1,
357 ProxyDyn2, ProxyDyn3, and Diuron (Table 1b). W-U dbRDA model performed best,
358 explaining 78% of variance in the OTU matrix. The first axis was significant and explained
359 44% of the total variance. Four environmental dynamics were significant according to this
360 model, ProxyDyn1, ProxyDyn2, 2.4-Dichlorophenoxyacetic acid (2.4D), and Diuron (Table
361 1b, Fig. S2.2c). Finally, only when the raw matrix was reduced to OTUs ≥ 0.05% of total read
362 number (see section 2.4.1 for details) tbRDA became significant (p-value = 0.046, DCA first
363 axis length < 3), with one significant axis and three significant dynamics, ProxyDyn1 (p-value
364 = 0.032), ProxyDyn2 (p-value = 0.007) and Diuron (p-value = 0.001) (Fig. 5). This matrix
365 included 53 OTUs, and 284,506 reads, i.e. 3% of total OTUs, representing 69% of the total
366 reads) which were conserved for further analyses. This model explained 66.32% of the total
367 variance, and the first axis was significant and explained 37.6% of the variance. Samples well
368 projected to ProxyDyn2 and Diuron were t23 and t37, followed by t17 and t19, and to a lesser
369 extent t38 (see Fig. 5), which was well projected to ProxyDyn1. Notice however that axis 2
370 was not significant.

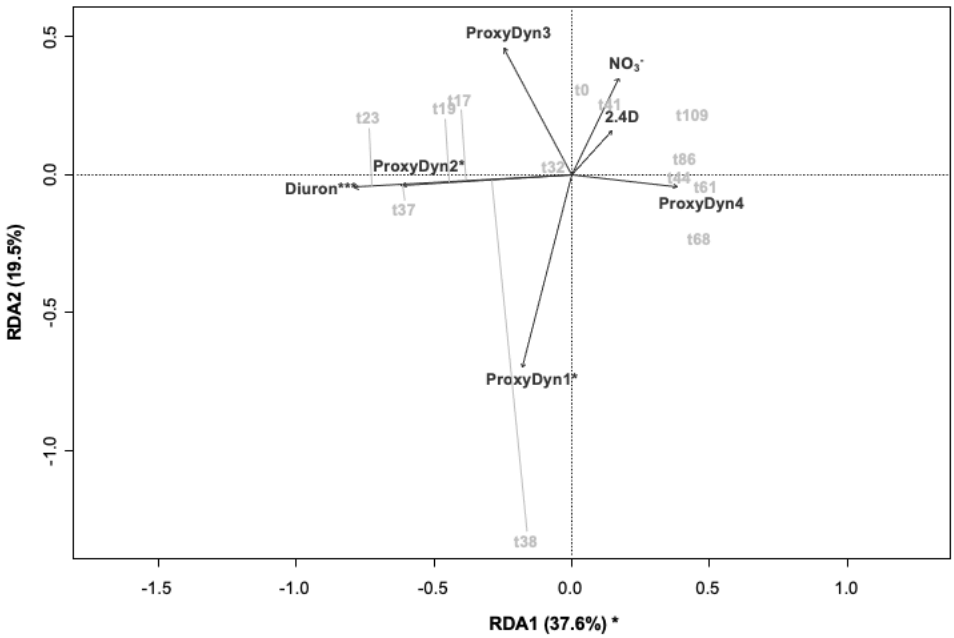


Fig. 5 Redundancy analysis (RDA) biplot with scaling by sites on the normalized matrix of OTUs with an abundance ≥ 0.05%. The model explained 66.32% of the variance ($p<0.05$). Significance for axes and environmental dynamics after permanova analyses are indicated, p-value significance codes: ***<0.001<**<0.01<*<0.05. Sample names are followed by a number that indicates the sampling time in hours after the beginning of the flood at t0. For further details on sample names and retained environmental variable dynamics, see Fig. 1b. Perpendicular grey lines represent the projection of the corresponding samples onto the corresponding dynamics and approximate the value of that sample along the variable (Legendre and Legendre 2012).

3.3. Constrained molecular ecological network analyses by environmental parameters dynamics

3.3.1. Archaeal network

Network analysis using MENAP resulted in four modules containing all OTUs and 774 links. Module eigengene analysis allowed correlation of modules with environmental variables. Only one module was significantly correlated to the environmental dynamics of the flood, particularly with ProxyDyn2 and Diuron (Fig. 1b). This module included 28 OTUs of which 17 had a significant module membership (Fig. 6a), comprised of 11 with positive (52,149 reads) and six with negative (18,573 reads) correlations (Table S3.1). Worth noting is that OTUs with positive module membership (Fig. 6b) had positive interactions with each other (Fig. 6a) and negative interactions with OTUs with negative module membership (Fig. 6c), and OTUs with negative module membership had positive interactions with each other and negative interactions with OTUs with positive module membership. All positively correlated OTUs were abundant in samples t23 (20,246 reads, which represent 39% of reads along the flood

from these OTUs, Table S3.1), t37 (20%), t19 (16%) and t17 (13%). A total of 60% of reads from these OTUs belonged to the Methanobacteria class (Euryarchaeota phylum). Two OTUs were particularly abundant, OTU3, matching *Methanobrevibacter (Mbr.) smithii* at 99% similarity after blastn search against NCBI database, and OTU11 matching *Methanobacterium (Mba.) palustre* (100%). The next most abundant OTUs also matched methane-related taxa: OTU41, which matched *Methanosaeta (Msa.) concilii*, 100%, from Halobacterota phylum), OTU19 (*Methanogranum* sp. 98.04%. from Thermoplamatota phylum), OTU26 (*Mbr. acidurans*, 99%, from Euryarchaeota phylum) and OTU16 (Methanomethylophilacea, 99.61%, from Thermoplamatota). The other positively correlated OTUs (not highlighted in bold in Table S3.1) did not match any further than the family level after a blast search of the NCBI database. Finally, negatively correlated OTUs were abundant in samples from the end of the flood (t44-t109) and belonged to Nitrososphaeria (Crenarchaeota phylum, representing 85% of the sequences of these OTUs) and Thermoplasmata (15%) classes (Thermoplasmatota phylum).

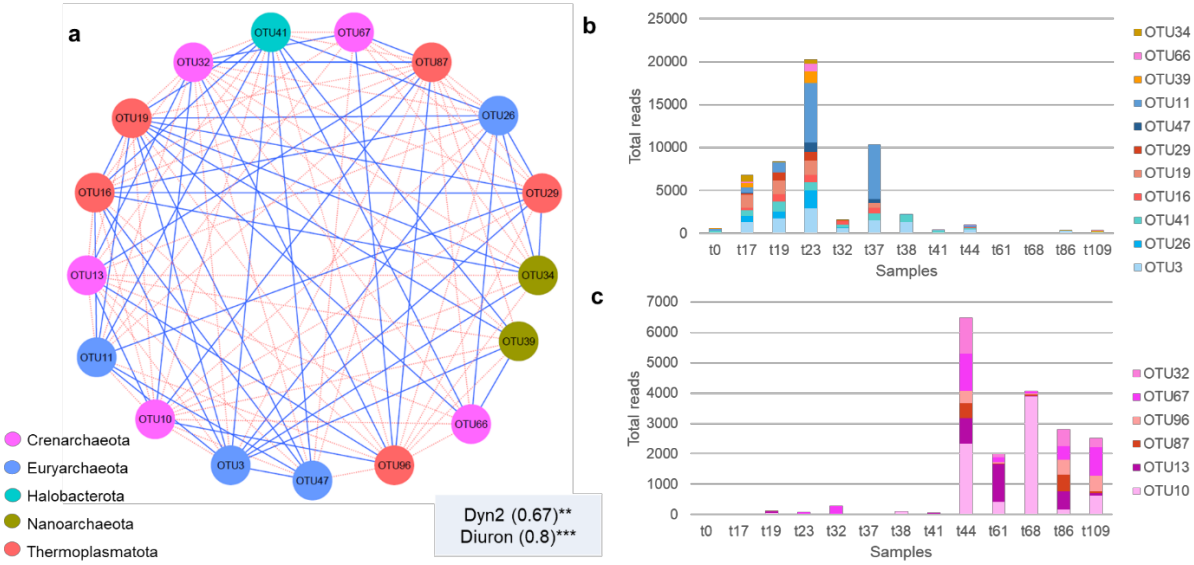


Fig. 6 (a) Molecular ecological network of the unique module significantly positively correlated with environmental dynamics, particularly ProxyDyn2 and Diuron (see Fig. 1b for details and sample names). (b) Histogram of total reads of OTUs with positive module membership in function of samples along the flood. (c) Same for OTUs with negative module membership. Environmental dynamics are followed by module correlation value between parenthesis and the p-value significant code as follows: ***<0.001<**<0.01<*<0.05. OTUs are colored according to their phylum. The positive and negative connectivities between OTUs are indicated by blue and red lines, respectively. Only OTUs with a significantly correlated abundance profile with module are represented in this figure.

418 3.3.2. Archaeal and bacterial joining network

419 To further explore the microbial relationships with environmental parameters, we performed a
420 second eigengene analyses by first constructing a network using the 53 retained archaeal OTUs
421 from this study, and the 260 retained bacterial OTUs from the previous study using the same
422 samples and methodology (Noyer et al. 2020). The network we obtained before considering
423 environmental parameters consisted in 200 nodes (OTUs) and 967 links and was composed of
424 21 modules. When considering environmental parameters, the network was composed of 136
425 OTUs, 442 links and 13 modules (Fig. 7, Table S3.2). All retained environmental dynamics
426 (Fig. 1b) were positively or negatively correlated with one or more modules and there were
427 127 bacterial and 9 archaeal significant OTUs. More than half of the total reads (53%) were
428 represented in module 1 (Fig. 7). Archaeal OTUs were present in five modules (Fig. 7). Six
429 archaeal OTUs represented 41% of the total reads among significant OTUs in the network and
430 were linked to ProxyDyn2 and/or Diuron. Notably, OTU3 and OTU41 in module 1, OTU16,
431 OTU26, and OTU61 (*Mba. acidurans*, 98.42%) in module 2, and OTU47 (*Mba. formicicum,*
432 100%) in module 5 (Table S3.2). They were mainly present in t17, t19, t23, and t37 samples.
433 Two archaeal OTUs, 94 and 44, in module 4 correlated to NO_3^- (4% of the total reads, Fig. 7),
434 and belonged to Nitrosotaleaceae family (Crenarchaeota phylum) and were mainly present
435 towards the end of the flood event. Of the 127 bacterial OTUs present in this joined network,
436 49 were present in the bacterial only network from the previous study (Noyer et al. 2020, in
437 purple in Table S3.2) and were significantly linked to the same environmental dynamics. Worth
438 noting is that the bacteria in modules which were positively correlated with ProxyDyn2 and
439 Diuron were also the most relatively abundant in t17, t19, t23, and t37 samples. The most
440 abundant of these bacterial OTUs was OTU5, which matched *Arcobacter cryaerophilus* and
441 represented 20% of the total reads included in the network.
442

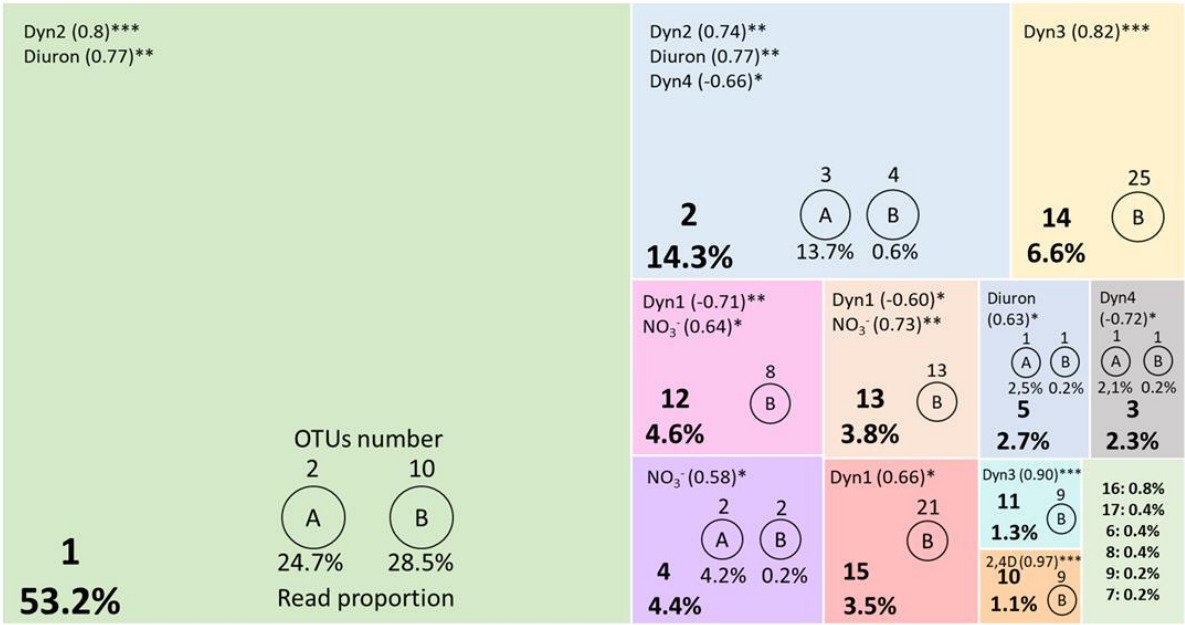


Fig. 7 Summary of joined bacterial and archaeal network analysis. Each colored rectangle represents a module whose number appears at the bottom left corner of each rectangle together with the percentage of reads in the module out of the total number of reads analyzed within the network. The number of OTUs and the proportion of reads within each module for each domain: archaea (A) and bacteria (B) are also indicated in each rectangle. At the top left of each rectangle the environmental dynamics are indicated with module eigengene correlation value between parentheses and the p-value significant code as follows: ***<0.001<**<0.01<*<0.05. The rectangle on the bottom right corner represents the six modules whose percentage of reads is less than 1% of the total number of reads analyzed in the network.

4. Discussion

4.1. Particle-attached archaeome diversity at three different seasons was higher and more even than in all other lotic ecosystems studied so far

Archaea have been studied in only a few river environments (Herfort et al. 2009; Hu et al. 2016, 2018; Cannon et al. 2017; Samson et al. 2019; Lei et al. 2020; Cao et al. 2020; Pinto et al. 2020; Shen et al. 2021) and to the best of our knowledge, no study has characterized their diversity over different seasons in lotic ecosystems. Here, eDNA was extracted from riverine water samples from summer, autumn, and winter and characterized using high-throughput Illumina sequencing of the archaeal rDNA in a coastal Mediterranean watercourse, the Têt River (Southeast France). Shannon alpha diversity (Fig. 2) from summer, winter and autumn drought samples were not significantly different (SD = 6.22; t0 = 6.25 and WD = 7.39). This was also the case for all alpha diversity indices (Table S1). Lei *et al.* (2020) obtained lower values of Shannon index (ranging 4.07-5.72) through Illumina sequencing of a highly polluted

465 river in China. Along a river in India sampled during summer and sequenced by metagenomic
466 analyses, Samson *et al.* (2019) found a Shannon diversity varying from 3.12 to 4.79, depending
467 on the sampling site. Hu *et al.* (2018) studied microbial diversity in a high-elevation river in
468 China using universal primers and found even lower Shannon values for archaea along the river
469 (2-2.7) and a Pielou evenness index very high (0.77-0.82) compared to ours (ranging 0.44-0.51
470 in Têt drought samples along seasons). These authors used 97% identity to define OTUs, while
471 the other two studies did not specify how they defined the OTUs in their studies. Nevertheless,
472 given the lower number of OTUs retained in these studies, the differences in alpha diversity
473 with respect to our study could be due to less throughput sequencing. Borrego *et al.* (2020)
474 studied particle-attached archaea in a Mediterranean water reservoir in summer with the same
475 specific primers as in the present study, and they also found a lower Shannon alpha diversity
476 than ours (4.9 ± 0.15). Gobet *et al.* (2014), in a comparative study of different alpha diversity
477 indices between ARISA versus pyrosequencing methodologies, determined significant
478 correlation results among methods, particularly with Shannon index, without a need for
479 correction for sequencing depth. In summary, although the Têt River archaeal diversity was
480 higher and less even (lower Pielou index) when compared to other riverine environments
481 studied so far, additional studies with higher throughput and standardized sequencing pipeline
482 analyses are needed to better understand the ecology of fluviatile archaea.
483 Studies on lotic ecosystems have revealed the presence of three major phyla, Crenarchaeota,
484 Euryarchaeota and Thaumarchaeota using the previous silva database (Hu et al. 2016, 2018;
485 Samson et al. 2019; Lei et al. 2020). When considering archaeal taxonomy changes since the
486 last silva database release, the major phyla observed in the Têt river corresponded to those
487 same phyla, but we also found the Nanoarchaeota phylum (Fig. 3a). In a recent study, which
488 used the same primers as those used in our study, Woesarchaeia class (now called
489 Nanoarchaeia) was found in a small water reservoir sampled in summer (Borrego et al. 2020).
490 In our study, the summer drought community showed, through beta diversity analyses, the
491 greatest difference with respect to communities present in autumn and winter and was also
492 dominated by this class of the Nanoarchaeota phylum, followed by Bathyarchaeia class
493 (Crenarchaeota phylum). Primer bias could thus be responsible for the absence of this class in
494 previous riverine studies. Nevertheless, winter drought and dry autumn weather (t0) samples
495 had a smaller proportion of these classes at the expense of Nitrososphaeria class
496 (Crenarachaeota phylum, Fig. 3). On the other hand, Herfort *et al.* (2009) sequenced five
497 DGGE fragments amplified from surface waters of the Têt River in June 2005 that matched
498 two classes, a marine benthic group Crenarchaeota and LDS/RCV from Halobacteriales order

499 (currently at Halobacteria class from Halobacterota phylum). In this study, we also found taxa
500 from the genus *Methanomicrobia*, which were present in all samples independently of season,
501 and are classified within Halobacterota and thus closely related to Halobacteria. While we also
502 found taxa from the marine benthic group A from Crenarchaeota phylum (not shown), they
503 were absent in the summer drought. Given the technical limitations of the DGGE fingerprinting
504 method, a biased result cannot be discarded.

505 4.2. Particle-attached archaeome shifts gave evidence of allochthonous inputs into 506 the Têt River along a Mediterranean extreme flood

507 In this manuscript, we characterized the temporal shift of the particle-attached riverine archaeal
508 community throughout an autumn storm event that included a 5-year flood and led to an
509 instantaneous peak discharge of 270 m^3/s. Cannon *et al.* (2017) is the only study that
510 characterized river archaeal composition in water samples from a contaminated urban river
511 before and after transient rainfall using Illumina sequencing of the rDNA. They found a weak
512 resolution in archaeal sequences of samples from before and after the rain event, which they
513 concluded might be due to a primer bias. In this study, we demonstrated major changes in
514 archaeal taxonomy, and therefore community shifts observed through beta diversity, occurred
515 at specific moments along the flood event and not when comparing samples from before and
516 after the flow peak. It is therefore possible that the absence of differences in Cannon *et al.*
517 (2017) was due to the resilience of archaeal communities at the end of the rainfall as was the
518 case at the Têt River. Communities from the end of the flood, from t44 to t109 were indeed
519 more similar to the t0 sample (sampled before the rainfall started, see Fig. 4). A first shift in
520 the structural diversity occurred after t17, particularly in samples t23 and t37. These samples
521 corresponded to moments of multipollution peaks that occurred at storm first flushes CSOs and
522 inputs from in-sewer sediment resuspension (Fig. 1b, see also Reoyo-Prats et al. 2017).
523 Methane-related classes such as Methanobacteria, Methanomicrobia and Methanosarcinia
524 became significantly abundant at these moments, and our results support these classes as major
525 components of riverine communities related to pollutants, organic matter inputs and/or hypoxic
526 sediments, as reported by Casamayor and Borrego (2009) with respect to the Euryarchaeaota
527 phylum. All these classes belonged indeed to the Euryarchaeaota before the last silva database
528 release. Lei *et al.* (2020) also found a predominance of different Euryarchaeota methanogens
529 in water samples from a black odor, highly polluted Chinese river and more particularly the
530 large presence of *Methanobacterium* genus (Methanobacteria class) in water as well as in hotter

531 and lower-oxygen, downstream sediments, what corroborates our hypothesis on the origin of
532 this river community shift. A second major community shift during the storm event occurred
533 at t41, just after the maximum water discharges (Fig. 3). Compared to Lei *et al.* (2020), who
534 did not observe a significant variation in alpha diversity along vertical and horizontal river
535 samples, we noticed a significant decrease in the archaea alpha diversity at t41 (Table S1). This
536 can be explained by the presence of three OTUs representing 90% of all reads in this sample
537 (not shown), which belonged to Nitrosopumilaceae and Nitrososphaeraceae families
538 (Nitrososphaeria class). Nitrososphaeria is the only known ammonia-oxidizing archaea (AOA)
539 class which accomplishes nitrification in all kinds of environments (Pinto et al. 2020) but has
540 been specifically found attached to terrestrial soil and freshwater sediments (Sonthiphand et al.
541 2013; Li et al. 2018). The AOA predominated assemblage at t41 is therefore derived from soil
542 runoff or sediments from upstream environments or from the resuspension of deep river
543 sediments, as they became predominant over polluted in-sewer sediments resuspension and
544 urban runoff-related assemblages that predominated until t38. The last community shift
545 included a significant enrichment of Thermoplamata class in samples t44 to t109, that
546 coincided with the start of the second peak of flow, which occurred due to the release of waters
547 from the upstream Vinça reservoir (Reoyo-Prats et al. 2017). This class has been associated
548 with anoxic, sulphide-rich lentic sediments (Fillol et al. 2015; Compte-Port et al. 2017). The
549 release of water from this reservoir during regular floods is performed via a valve situated at
550 the bottom of the dam. As the bottom reservoir waters are anoxic (Fovet et al. 2020) and flow
551 increase at the bottom level of the dam could potentially lead to sediment release as well, both
552 are potential causes for the increase of Themoplasmata when dam discharges became
553 predominant at t44.

554 **4.3. Major environmental forces were linked to particle-attached archaeome shifts**
555 **during an extreme event through comprehensive modelling analyses**

556 Among the few papers in the literature that have linked archaeal diversity to environmental
557 parameters, all addressed the effect of one family of parameters on changes in archaeal
558 communities, such as nutrients (Herfort et al. 2009; Hu et al. 2016, 2018; Lei et al. 2020; Cao
559 et al. 2020) or metals (Mahamoud Ahmed et al. 2020; Shen et al. 2021). This is, therefore, the
560 first study on the Archaea domain where both diversity and a large panoply of physicochemical
561 parameters (notably nutrients, trace metals, pharmaceuticals, and pesticides) have been
562 analyzed for the same samples. To analyze such complex datasets, we used a comprehensive

563 analysis as such performed on bacteria (Noyer et al. 2020). On one side, Bokulich *et al.* (2012)
564 suggested quality-filtering strategies to eliminate artifacts before interpretation of results and
565 recommended a conservative OTU threshold of 0.005% for bacteria. On the other side, it has
566 been suggested to use multivariate cut-offs instead of arbitrary thresholds to delineate abundant
567 versus rare OTUs, as the latest are largely dependent on sequence coverage (Jia et al. 2018).
568 Furthermore, multivariate cut-offs can be set considering environmental parameters when
569 available (Gobet et al. 2010). Based on these studies, we designed a strategy with the purpose
570 to define this cut-off at the point where the constraint-based model which considers OTU
571 relative abundance (tbRDA model) became significant. The environmental response of the
572 archaeal community was obtained with a tenfold higher threshold (0.05%) than bacteria
573 (0.005%, Noyer et al. 2020). This result indicated that archaeal taxa that responded to
574 environmental pollutants were ten times more abundant than bacteria. Less abundant archaeal
575 taxa were therefore less crucial than those same bacterial taxa in the response of their respective
576 communities to environmental parameters. We believe that was the reason why the constraint-
577 based model obtained when using Bray-Curtis dissimilarity i.e., that considers OTU abundance
578 only, turned out non-significant for the archaeome (Table 1a) in contrast to the bacteriome. The
579 other constrained multivariate analyses models that were significant, as well as the constrained
580 network analyses, gave evidence of two dynamics, ProxyDyn2 and Diuron, as responsible for
581 major structural diversity shifts observed on riverine particle-attached archaea during the
582 extreme flood event (Fig. 5 and 6b). These specific dynamics were discharged in the watershed
583 by CSOs as well as by urban runoff (Reoyo-Prats et al. 2017) from t17 to t38 and represented
584 several environmental pollutants including pesticides, as well as copper and dissolved
585 pharmaceutical products and a contaminant, phosphate (see Fig. 1b for further details). These
586 parameters have been found to affect bacterial communities in freshwater ecosystems, and
587 metals and nutrients also affect archaeal communities in freshwaters (Hu et al. 2016; Lei et al.
588 2020; Shen et al. 2021). Nevertheless, to our knowledge, the effect of xenobiotics such as
589 pesticides or pharmaceutical products on archaeal communities from freshwater ecosystems
590 has not yet been specifically reported. But, the pesticide glyphosate, for instance, interferes
591 with the aromatic-acids pathway in microorganisms, including archaea~~,~~ and alters microbial
592 communities (van Bruggen et al. 2021). On the other hand, eutrophication by phosphate has
593 been found to decrease glyphosate degradation by biofilms and increase AMPA accumulation
594 in surface waters (Carles et al. 2019), what could therefore indirectly affect microbial
595 communities. The third significant dynamics according to the tbRDA, ProxyDyn1, projected
596 on axis 2, was mainly linked to the flow peak sample at t38, but axis 2 turned out not significant

597 (Fig. 5). ProxyDyn1 was neither correlated to the significant module of network analyses (Fig.
598 6).

599 **4.4. Key players in the response of the riverine archaeome to multiple stressors**

600 One of the major objectives of this study was to identify OTUs which could play a key role in
601 the archaeal community response to environmental changes derived from the delivery of
602 xenobiotics into the river by different pollutant sources. OTUs with positive membership to
603 the significant module from constraint-based network analysis were affiliated with
604 Euryarchaeota, Halobacterota and Thermoplasmatota (Fig. 6a, Table S3.1). These major OTUs
605 played a significant role in the response of the archaeome to multiple stressors derived from
606 point sources of pollution, as they were most abundant in samples t17-t23, t37, and t38 (Fig.
607 6b, Table S3.1) and were linked to ProxyDyn2 and Diuron but not to ProxyDyn1, which is a
608 proxy for diffuse sources of pollution. The predominant OTU11, affiliated with *Mba. palustre,*
609 is a species isolated for the first time in peat bogs that has the ability to use secondary alcohols
610 to produce methane (Zellner et al. 1988; Chaban et al. 2006). *Mbt. smithii* (OTU3) was the
611 second most abundant key player, which is known to be derived from the human
612 gastrointestinal tract (Miller et al. 1982; Oliveira et al. 2016) and identified as a potential
613 indicator of sewage (Johnston et al. 2010; McLellan and Eren 2014). The third most abundant
614 OTU41 was affiliated with *Msa. concilii*, which is involved in methane production from acetate
615 (Zwain et al. 2017) and is abundant in-sewer biofilms (Sun et al. 2014). What was striking in
616 the present study was that other OTUs, which had not yet been linked to CSOs and/or pollutant
617 inputs in the literature, acted as major OTUs in the response of the archaeome to pollutant
618 mixtures (Fig. 6a, Table S3.1). Three of them matched Methanobacteria (OTU26) or
619 Thermoplasmata (OTUs 16 and 19) classes and were also related to methane; and the other
620 three major OTUs were affiliated to Nanoarchaeia and Bathyarchaeia classes (OTUs 39, 66
621 and 34). Lastly, several OTUs affiliated to Thermoplasmata and Nitrososphaeria classes, had
622 a significant negative membership to the module of network analysis linked to ProxyDyn2 and
623 Diuron (Fig. 6c). These key players were instead linked to samples from the end of the flood
624 event, from t44 to t109. As evidenced above, they were taxa from allochthonous origins. All
625 these urban taxa, which were identified as either positively or negatively correlated with major
626 stressors, could be used as alternative bioindicators for rapid risk assessment of the impact of
627 multiple stressors on aquatic ecosystems, as proposed for bacteria (McLellan and Eren 2014;
628 Dila et al. 2018; Noyer et al. 2020).

629 **4.5. Comparison between particle-attached archaeal and bacterial diversity at**
630 **seasons and along the flood event**

631 Few studies so far have compared archaeal and bacterial structural diversity in lotic ecosystems
632 (Cannon et al. 2017; Hu et al. 2018) and, to the best of our knowledge, the differences in the
633 responses of both domains to seasons have not yet been studied. Fluviatile archaeal response
634 to environmental dynamics differed from the bacterial response in three ways. First, the
635 archaeome showed a specific community in summer compared to winter and autumn samples,
636 while bacterial communities from these three seasons clustered together when compared to
637 communities along the flood (Noyer et al. 2020). Second, environmental forces structured the
638 archaeome diversity differently depending on beta diversity distance-based statistical models,
639 particularly when considering qualitative vs. quantitative dissimilarities. These models were
640 not always significant (Table 1), while for bacteria all models were significant. Third, while
641 the bacterial community structured differently at the two multipollution events of this extreme
642 flood, which were derived from CSOs and the flow peak (Reoyo-Prats et al. 2017), archaeal
643 structural shifts could only be interpreted after further statistical modeling of OTU abundances
644 and extreme event dynamics. On the one hand, the archaeal riverine resident communities
645 shifted significantly according to two environmental dynamics only, ProxyDyn2 and Diuron
646 (Fig. 5 and 6). These dynamics were derived from point sources of pollution, such as CSOs,
647 in-sewer sediments resuspension and urban runoff conducted through CSOs. On the other hand,
648 bacteria also responded significantly to other environmental parameters linked to diffuse
649 sources of pollution. Notably, at the highest water discharge at t38 and t41, bacteria showed a
650 specific community that was correlated to ProxyDyn1 and was thus attributed to allochthonous
651 inputs from runoff (Reoyo-Prats et al. 2017). The archaeome shifted at t41 into an anoxic
652 community, most likely related to the resuspension of deep sediments, and was not
653 significantly related to ProxyDyn1 (Fig. 3 and 5) and therefore the flow peak. In conclusion,
654 archaea can help to better understand the origin of watershed sediments and can thus be of
655 interest for quality assessments of suspended matter.
656 To further understand the different responses of archaea and bacteria domains of life to river
657 contaminants and hydrodynamics, we used a network analysis combined with a module
658 eigengene analysis of bacterial and archaeal taxa best fitted to significant beta diversity models.
659 One remarkable finding was the joint presence of archaea *Mbr. smithii* and *Msa. concilii* with
660 bacteria *Arcobacter cryaerophilus, Bacteroides graminisolvens, Cloacibacterium normanense*
661 and *Macellibacteroides fermentans* in Module 1, which was significantly correlated with

662 ProxyDyn2 and Diuron. All of these species have already been identified as potential indicators
663 of human-specific sewage pollution (Dick and Field 2004; Johnston et al. 2010; McLellan and
664 Eren 2014; McLellan and Roguet 2019) and *A. cryaerophilus* is a known human pathogen
665 (Collado et al. 2010). While no archaea has yet shown pathogenic effects on humans
666 (Cavicchioli et al. 2003; Bang and Schmitz 2018), archaea and bacteria can share genes through
667 horizontal gene transfer, which is particularly enhanced in anaerobic environments (Fuchsman
668 et al. 2017). Biofilms that develop in urban pipes are anaerobic (Guisasola et al. 2008), and the
669 co-habitation of archaea with bacterial pathogens in urban systems can increase the risk of
670 antibiotic resistance gene transfer. Sewers have indeed been identified as reservoirs of
671 antibiotic-resistance bacteria carried by human pathogens (Millar and Raghavan 2017; Auguet
672 et al. 2017). Similarly, resistance to pollutants could be enhanced between both domains
673 through the same mechanisms. Furthermore, the presence of both domains in the same habitat
674 pointed out to potentially common as well as complementary metabolic and physiological
675 functions. Another interesting finding that emerged from this analysis was the presence of
676 archaea together with bacteria OTUs in module 4, which significantly correlated with NO_3^-
677 (Fig. 7, Table S3.2). Notably, two Nitrososphaeria class OTUs, known to be dominant in
678 particle- attached ammonia-oxidizing archaeal communities (Cai et al. 2019; Pinto et al. 2020)
679 were linked to module 4. Wang *et al.* (2018) also demonstrated a significant influence of
680 dissolved inorganic nitrogen on the composition of bacterial and archaeal communities along
681 an urban river. In the present study, the addition of bacterial OTUs in the network analysis
682 strengthened the importance of nitrates to drive shifts in archaeal assemblages, otherwise
683 mitigated when archaea were modeled alone, which enhance the interest of studying different
684 domains of life to better understand environmental drivers of community structure in natural
685 ecosystems.

686 **5. Conclusion**

687 Our study provides the first overview of archaeal community shifts along an extreme storm
688 event that led to multiple pollutions in a typical coastal Mediterranean watercourse. Shifts were
689 also compared with changes in archaeal diversity during three seasons. Archaea from the Têt
690 river showed a specific community in summer compared to winter and autumn samples, and a
691 higher alpha diversity and lower evenness could be observed in this river compared to other,
692 yet less-thorough studies on riverine archaeal communities. Further studies on the spatio-
693 temporal shifts in archaeal assemblages in these ecosystems are therefore urgently needed to

694 better understand seasonal shifts as well as their ecological diversity. For the first time, a
695 comparison of the response of archaea alone and together with bacteria in a fluviatile ecosystem
696 has shed light on the similarities and differences in their responses to seasons and when facing
697 multiple stressors derived from an extreme event. The fluviatile bacteriome and archaeome did
698 not respond in the same way to environmental forces. Extreme events were stronger at
699 structuring bacterial communities than seasons, while the opposite was observed in archaeal
700 communities. In contrast to bacteria, which responded quickly and significantly to both sewage
701 overflow and river hydrodynamics and associated environmental parameter changes, the
702 archaeal community shifted in response to multipollution derived from point sources and from
703 the resuspension of deep anoxic sediments but not so clearly at the river flow peak. Archaeal
704 taxa already known as urban-specific, as well as new archaea, mainly methane-related and
705 never identified as urban-specific taxa, predominated assemblages during multiple stress
706 events and were confirmed through statistical modeling of archaeal alone and together with
707 bacteria. These taxa could be used as bioindicators of point sources of pollution. Our results
708 highlight fluviatile archaea, seldom considered as bioindicators of water quality, could provide
709 a rapid risk assessment of multiple pollutants in aquatic ecosystems, as is the case for bacteria.
710 Furthermore, a better understanding of parallel shifts in assemblages from both domains of life
711 when confronted with multiple stressors could help to improve how urban watersheds are
712 monitored and would thus be extremely helpful in the management of pollution risk.
713
714 **Data Availability**
715 Sequencing data are deposited on NCBI under BioProject ID PRJNA602803.
716
717 **Supplementary Information**
718 Additional Supporting Information may be found in the online version of this article at the
719 publisher's website.
720
721 **Supplementary Information S1**
722 **Table S1.** Alpha diversity indices at the Têt river with Kruskal-Wallis test results and graphical
723 representation for each index.
724
725 **Supplementary Information S2**
726 **Fig. S2.1.** Beta diversity based on additional dissimilarities.
727 **Fig. S2.2.** Jaccard, Unifrac and Weighted-Unifrac distance-based RDA triplot.

Supplementary Information S3

Table S3.1. Key player significant archaeal OTUs in module eigengene analysis significantly related to environmental dynamics and graphical representation of their total reads along time.

Table S3.2. Key player significant bacterial or archaea OTUs in modules derived from module eigengene analysis significantly related to environmental dynamics.

Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Contributions

Megane Noyer and Carmen Palacios contributed to conception and design of the study, organized the database, performed the statistical analyses and wrote the first draft of the manuscript. Maria Bernard helped with metabarcoding analyses. All authors contributed to manuscript revision, read and approved the submitted version.

Ethical Approval

Not applicable.

Consent to Participate

All authors are informed and agree to the study.

Consent to Publish

The authors declare no competing interests.

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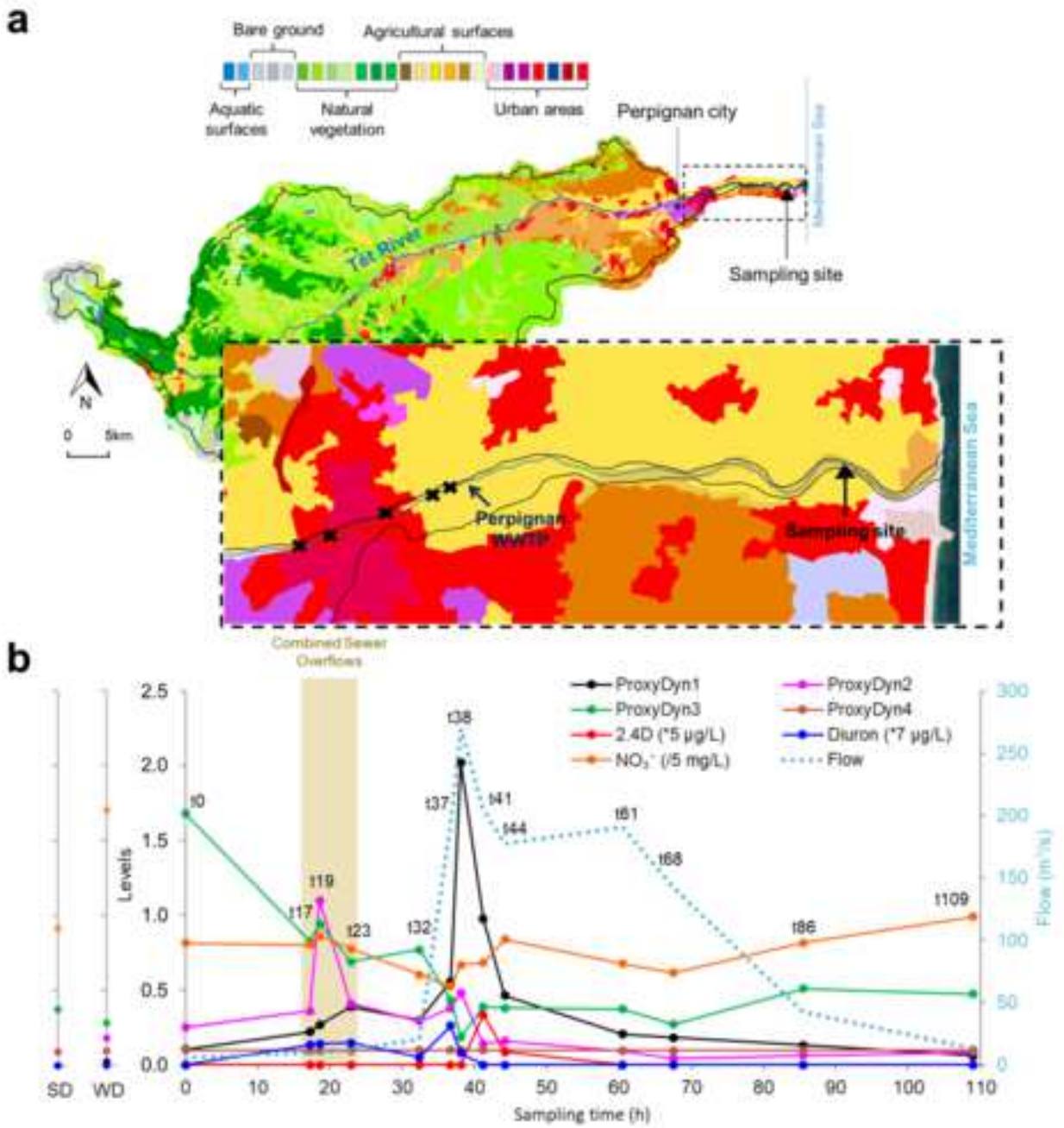
1011



Summer drought	Winter drought	Autumn drought	Autumn flood
SD	WD	t0	12 sampling times: t17 → t109

Figure2

Click here to access/download;Figure;Fig.2.jpg

	SD	t0	t17	t19	t23	t32	t37	t38	t41	t44	t61	t68	t86	t109	WD
Observed	5061	20357	38462	39025	37930	36234	35920	30821	22451	42347	59266	36544	40449	42965	31284
Shannon	6.22	6.25	7.44	7.52	6.81	7.04	6.65	6.11	5.24	7.63	7.65	6.30	7.76	8.03	7.39

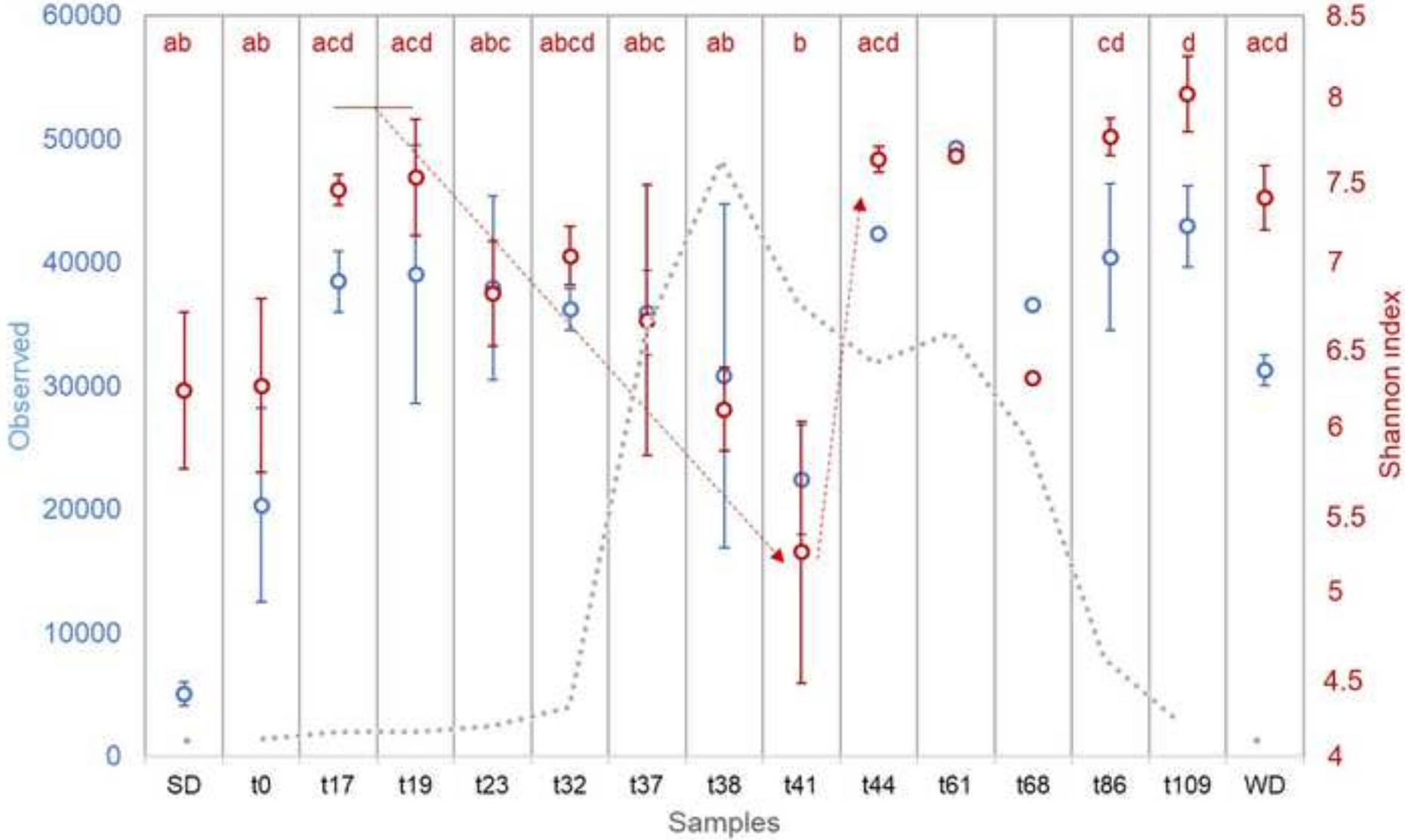


Figure3

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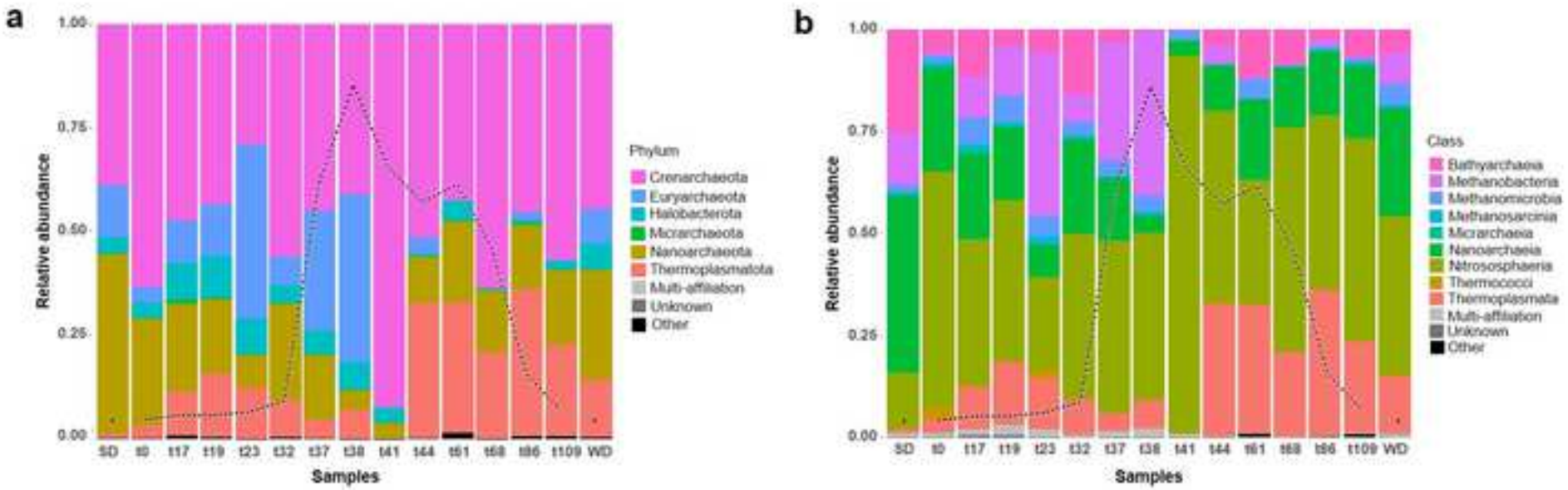

Figure4

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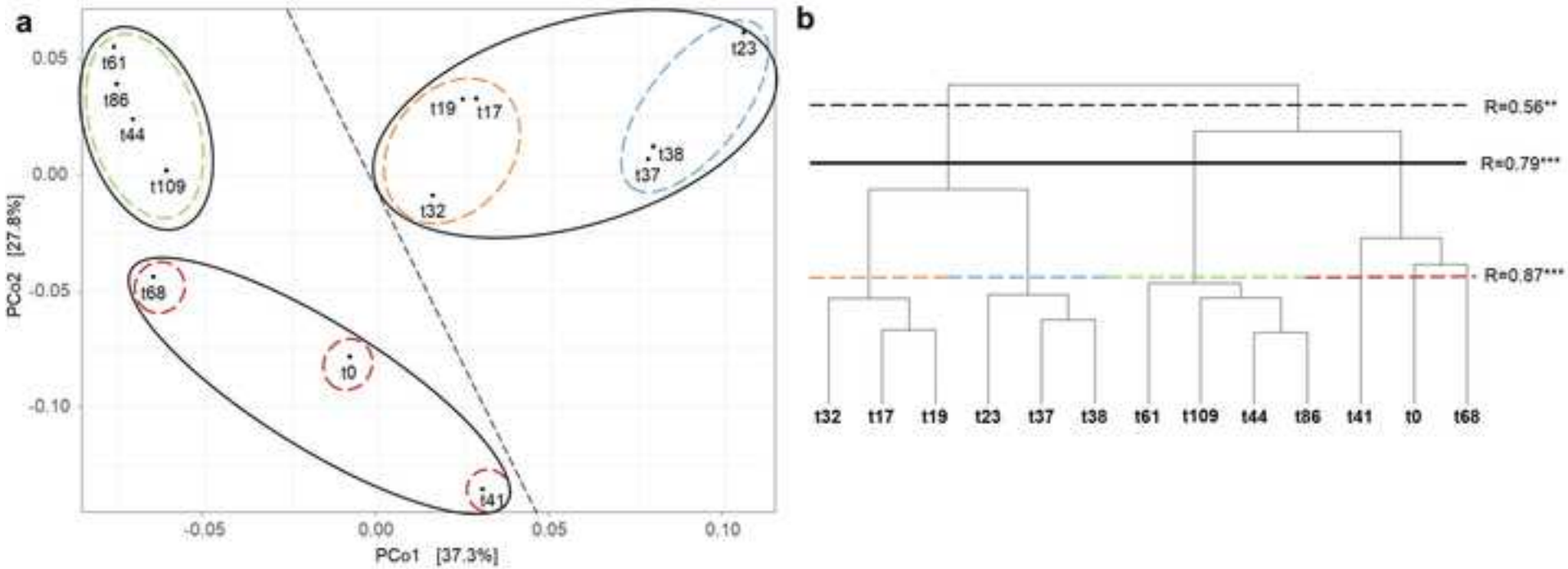

Figure5

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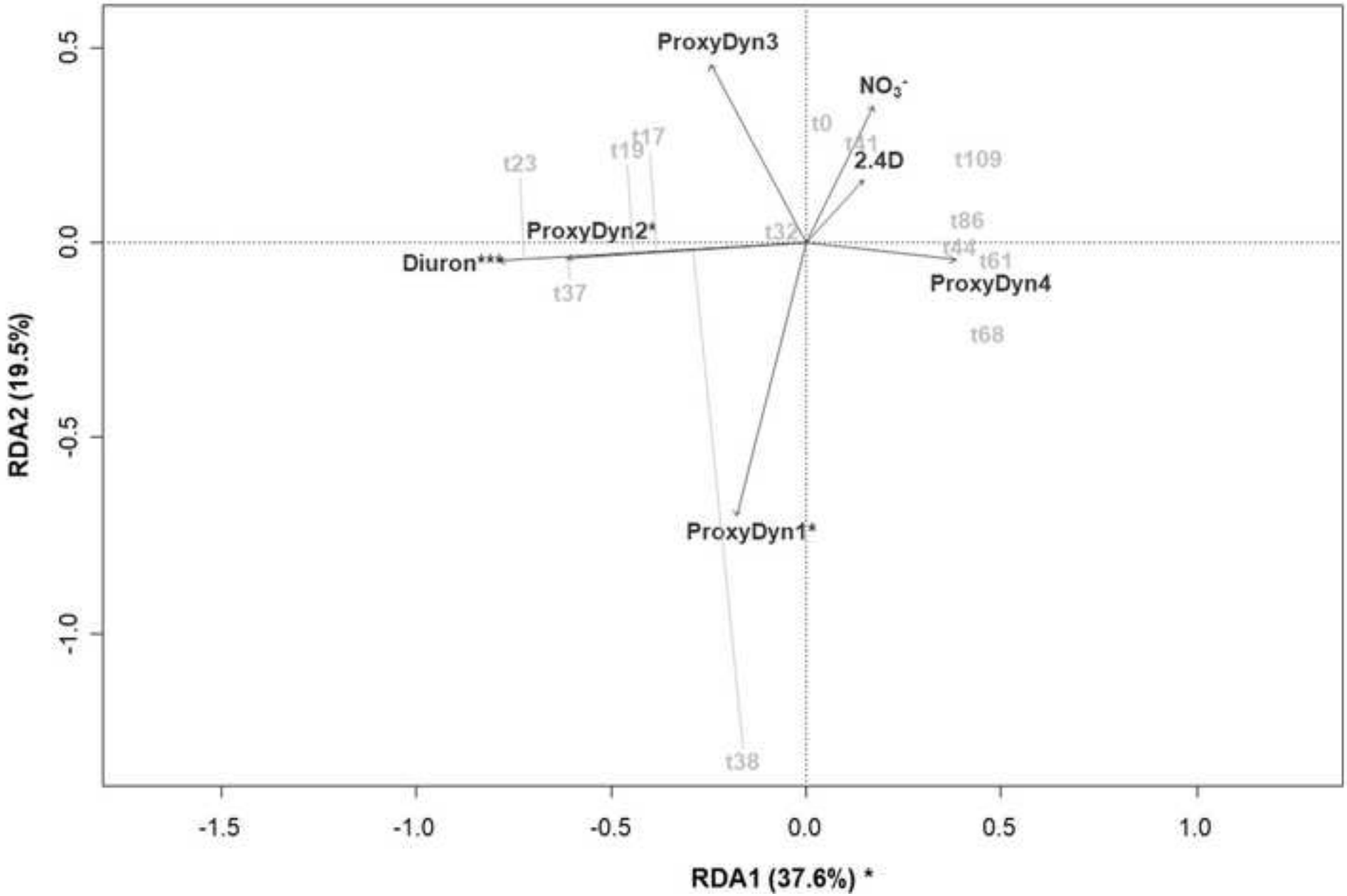

Figure6

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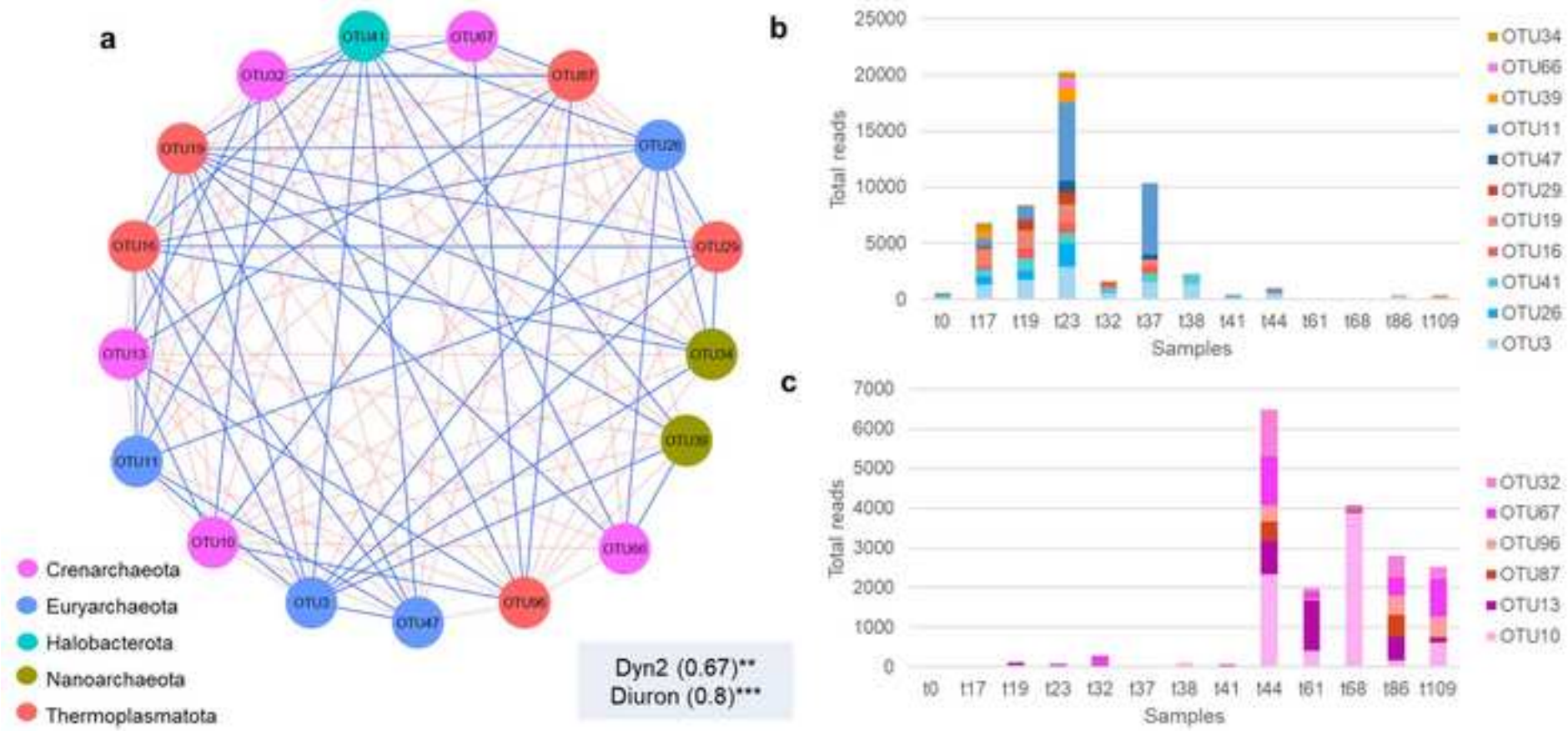

Figure7

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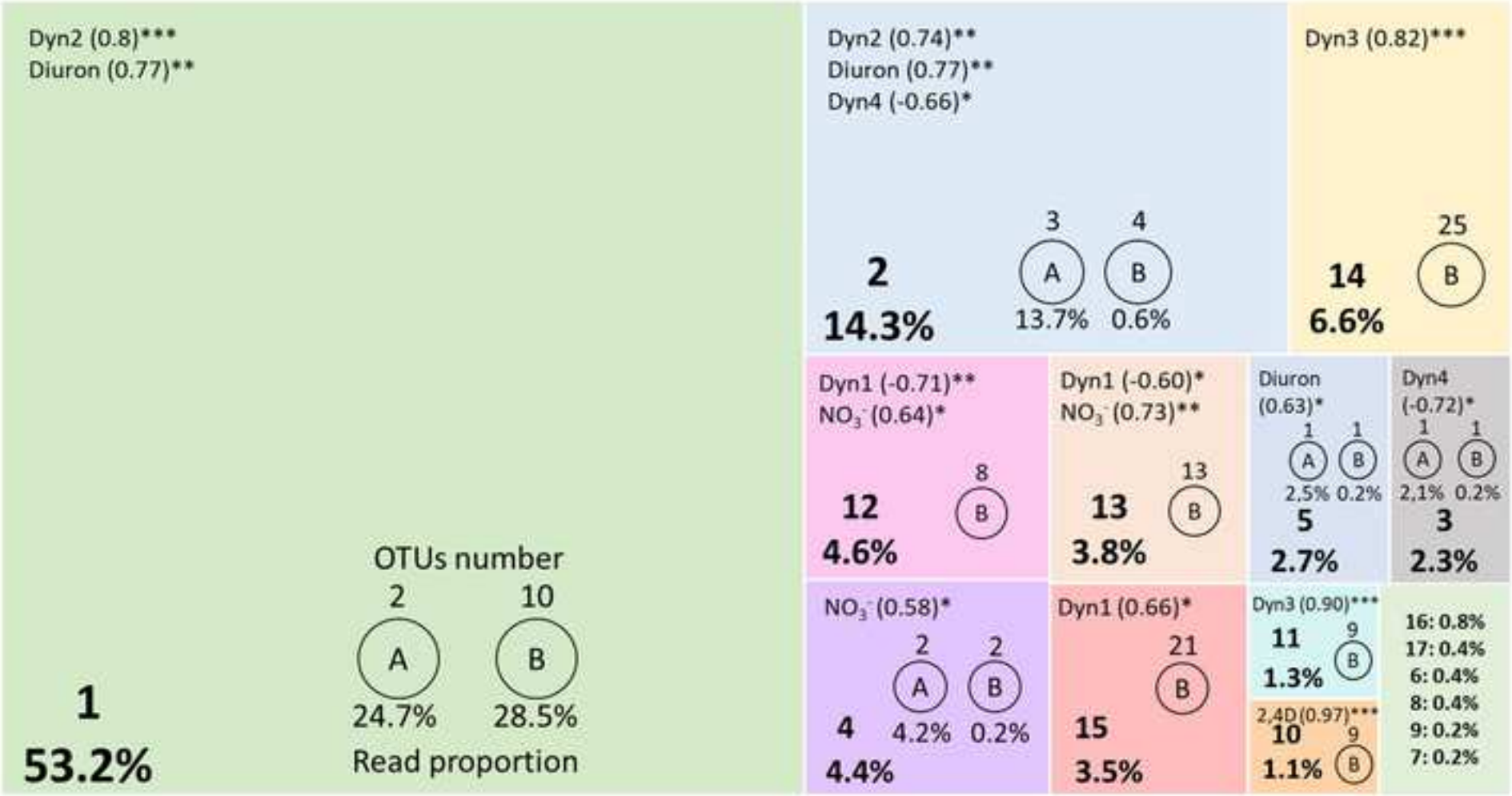

Fig. 1 Têt River archaeome sampling sites and environmental parameters measured in the same samples. (a) Watershed of the river with sampling site (black arrow), located after wastewater treatment plan (WWTP) of the city of Perpignan, combined sewers (black crosses) and water reservoirs indicated as grey rectangles (adapted from Reoyo-Prats et al. 2017). (b) Environmental parameter dynamics in the Têt River at different seasons and along an autumn flood. For sample names, see table below figure. Autumn sample names are followed by a number that indicates the sampling time in hours after t0, which was sampled at autumn basal level water discharges. Sampling took part at crucial moments of the flood that occurred thereafter: at first flushes (t17-t19-t23), before the flow peak (t32-t37), during the flow peak (t38-t41), following the release of water from the upstream Vinça reservoir (t44-t61) and during the return to basal level (t68-t86-t109). ProxyDyn1 corresponds to the dynamics of particulate organic carbon (/20 mg/l), which represented the dynamics of water flow, also represented in figure, total suspended solids, total organic carbon, total nitrogen, and terbuthylazine parameters. ProxyDyn2 corresponds to aminomethyl phosphonic acid (AMPA, µg/l), which represented glyphosate, phosphate, copper, temperature, E. coli, enterococci, diclofenac, sulfamethoxazole and carbamazepine parameters. ProxyDyn3 corresponds to lead (/150 µg/g) in the representation of the dynamics of cadmium, zinc, and conductivity parameters. ProxyDyn4 corresponds to pH (/70), which represented cobalt, nickel, and chrome parameters. Three parameters, Diuron, 2.4D and NO3-, had a unique dynamic. For further details on statistical analyses for environmental parameters, see Noyer *et al.* (2020).

Fig. 2 Alpha diversity of the archaeome of the Têt river along time. Changes in observed OTU number (blue) and Shannon index (red) along the flood (tX) and at summer and winter droughts (SD and WD respectively). For the Shannon index, different letters indicate a significant difference between samples (dunn.test<0.025) and red arrows show major significant differences. Observed OTU number was not significantly different (KW=0.14). The dotted profile is the flow level at each sampling point (see Fig. 1, also for sample names). Even though the absence of replicates for t61 and t68 samples impeded statistical testing, they are represented through time for comparison.

Fig. 3 Composition of archaeal communities averaged across replicates. Histogram of relative abundances (a) of the six major phyla and (b) of the ten major classes. Samples are organized according to sampling time from left to right: summer drought (SD), autumn flood (sample names are followed by a number that indicates the sampling time in hours after the beginning of the flood at t0), and winter drought (WD). The dotted profile is the flow level at each sampling point (see Fig. 1 for further details).

Fig. 4 Structure of archaeal communities averaged across replicates. (a) Principal Coordinate Analysis (PCoA) and (b) hierarchical clustering with Ward D2 linkage method using Weighted-Unifrac dissimilarity computed on OTU average abundance. Lines indicate ANOSIM significant groups. Sample names are followed by a number that indicates the sampling time in hours after t0 (see Fig. 1 for details). Significant codes ** and *** indicate p-value < 0.01 and < 0.001, respectively.

Fig. 5 Redundancy analysis (RDA) biplot with scaling by sites on the normalized matrix of OTUs with an abundance ≥ 0.05%. The model explained 66.32% of the variance ($p<0.05$). Significance for axes and environmental dynamics after permanova analyses are indicated, p-value significance codes: ***<0.001<**<0.01<*<0.05. Sample names are followed by a number that indicates the sampling time in hours after the beginning of the flood at t0. For further details on sample names and retained environmental variable dynamics, see Fig. 1b. Perpendicular grey lines represent the projection of the corresponding samples onto the corresponding dynamics and approximate the value of that sample along the variable (Legendre and Legendre 2012).

Fig. 6 (a) Molecular ecological network of the unique module significantly positively correlated with environmental dynamics, particularly ProxyDyn2 and Diuron (see Fig. 1b for details and sample names). (b) Histogram of total reads of OTUs with positive module membership in function of samples along the flood. (c) Same for OTUs with negative module membership. Environmental dynamics are followed by module correlation value between parenthesis and the p-value significant code as follows: ***<0.001<**<0.01<*<0.05. OTUs are colored according to their phylum. The positive and negative connectivities between OTUs are indicated by blue and red lines, respectively. Only OTUs with a significantly correlated abundance profile with module are represented in this figure.

Fig. 7 Summary of joined bacterial and archaeal network analysis. Each colored rectangle represents a module whose number appears at the bottom left corner of each rectangle together with the percentage of reads in the module out of the total number of reads analyzed within the network. The number of OTUs and the proportion of

reads within each module for each domain: archaea (A) and bacteria (B) are also indicated in each rectangle. At the top left of each rectangle the environmental dynamics are indicated with module eigengene correlation value between parentheses and the p-value significant code as follows: ***<0.001<**<0.01<*<0.05. The rectangle on the bottom right corner represents the six modules whose percentage of reads is less than 1% of the total number of reads analyzed in the network.

Table1

Table 1 Summary of constraint-based multivariate statistical models on archaea OTU matrix averaged over replicates and without singletons. (a) Permanova significance of the five models tested and the percentage of biological variance that is explained by each model using permutation test with anova.cca function. (b) Axes and modeled variables significance after permanova using anova.cca of significant models in (a). p-values significance codes: (*** < 0.001 < ** < 0.01 < * < 0.05).

a	**OTUs matrix transformation**	**Model significance**	**Variance (%)**
dbRDA	**Jaccard**	0.003**	63.38%
	Unifrac	0.008**	66.35%
	Bray-Curtis	0.326	60.04%
	Weighted-Unifrac	0.004**	76.58%
CCA		0.765	56.44%
RDA	**Hellinger**	0.134	61.4%

b	**OTUs matrix transformation**	**Axes significance and variance explained (%)**	**Statistical significance of modeled variables**						
		CAP1	**ProxyDyn1**	**ProxyDyn2**	**ProxyDyn3**	**ProxyDyn4**	**2.4D**	**Diuron**	**NO_3^-**
dbRDA	**Jaccard**	0.002** (22.74)	0.001***	0.002**	0.001***	0.105	0.095	0.001***	0.096
	Unifrac	0.040* (30.24)	0.001***	0.021*	0.046*	0.458	0.383	0.036*	0.014*
	Weighted-Unifrac	0.006** (42.89)	0.006**	0.009**	0.039*	0.050*	0.004**	0.002**	0.166