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Particle-attached riverine bacteriome shifts in a pollutant-resistant and pathogenic community during a Mediterranean extreme storm event

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Abstract

Rivers are representative of the overall contamination found in their catchment area. Contaminant concentrations in watercourses depend on numerous factors including land use and rainfall events. Globally, in Mediterranean regions, rainstorms are at the origin of fluvial multipollution phenomena as a result of Combined Sewer Overflows (CSOs) and floods. Large loads of urban-associated microorganisms, including faecal bacteria, are released from CSOs which place public health – as well as ecosystems – at risk. The impacts of freshwater contamination on river ecosystems have not yet been adequately addressed, as is the case for the release of pollutant mixtures linked to extreme weather events. In this context, microbial communities provide critical ecosystem services as they are the only biological compartment capable of degrading or transforming pollutants. Through the use of 16S rRNA gene metabarcoding of environmental DNA at different seasons and during a flood event in a typical Mediterranean coastal river, we show that the impacts of multipollution phenomena on structural shifts in the particle-attached riverine bacteriome were greater than those of seasonality. Key players were identified via multivariate statistical modelling combined with network module eigengene analysis. These included species highly resistant to pollutants as well as pathogens. Their rapid response to contaminant mixtures makes them ideal candidates as potential early biosignatures of multipollution stress. Multiple resistance gene transfer is likely enhanced with drastic consequences for the environment and human-health, particularly in a scenario of intensification of extreme hydrological events.

Keywords

microbial ecotoxicology; water quality; multipollution phenomena; coastal Mediterranean rivers; sewer overflow; multiple stressors.

1. Introduction

Rivers are representative of the overall pollution in their catchment area (Giri and Qiu, 2016). Freshwaters near urban areas are particularly impacted by contaminants, from river sediments derived from leakage and runoff as well as from in-sewer solids resuspension when the sewerage system overflows during heavy rainfalls or during snow melting periods (Madoux-Humery et al., 2013; Osorio et al., 2012; Oursel et al., 2014; Taghavi et al., 2011). Globally, in Mediterranean climate regions, urban system overflows often occur because of the recurrence of intense rainfalls (Cowling et al., 2005; Gasith and Resh, 1999; Reoyo-Prats et al., 2017), while combined sewers, which simultaneously carry wastewater and stormwater, are also common around the world (Commissariat Général au Développement Durable, 2011; U.S. Environmental Protection Agency, 2016). When CSOs occur, large loads of contaminant mixtures are discharged into surface waters not only from runoff but from private residences as well (Llopart-Mascaró et al., 2014; Pailler et al., 2009; Phillips and Chalmers, 2009; Reoyo-Prats et al., 2017; Weyrauch et al., 2010).

Mixtures of contaminants are recognised as a new major threat for freshwater ecosystems (Sabater et al., 2019), but only heterotrophic microorganisms contribute to the degradation or transformation of these organic or inorganic xenobiotics (Carles et al., 2019; Díaz, 2004). As such, microbial communities provide critical ecosystem services. Nevertheless, most environmental microbial ecotoxicology studies analyse a single substance or contaminant family type, through a functional approach (Lambert et al., 2012; Pesce et al., 2010a; Widenfalk et al., 2008) or through structural changes in microbial communities together with contaminant concentrations (Amaral-Zettler et al., 2010; Lambert et al., 2012; Palacios et al., 2008; Pesce et al., 2010b). Only few studies have examined the *in situ* effect of pollutant mixtures with a focus on periphyton biofilms or sediment microbes (Dorigo et al., 2010; Pesce et al., 2008). This highlights the need to assess shifts in the river water bacteriome in response to multiple contaminants (Staley et al., 2014).

The impact of urban water discharges during storm and flood events on faecal bacterial contamination (Baudart et al., 2000; Chu et al., 2011; Masters et al., 2016), nitrogen

or carbon cycles (Eyre and Ferguson, 2006; Fouilland et al., 2012), as well as on temporal succession changes in microbial communities through microscopic enumeration (Fisher et al., 1982; Muylaert and Vyverman, 2006), has been widely studied and has made clear the negative impact of rainstorms on water quality and thus on human health (Ahern et al., 2005). Recent studies have focused on tracking sources of urban-associated bacteria released through sewer discharges or storm water (Besemer et al., 2005; Wu et al., 2010; Newton et al., 2013; Fisher et al., 2015; Marti et al., 2017a, 2017b) but only two studies have described bacterial community dynamics throughout the course of a rainstorm event (Kan, 2018; Ulrich et al., 2016). Although contaminant mixtures released during CSOs occur mainly at first flushes compared to other moments of the event (Ashley et al., 1992; Reoyo-Prats et al., 2017), bacterial community changes at first flushes have yet to be addressed.

In the transfer of chemical substances during storm events, suspended particles play a fundamental role (Turner and Millward, 2002) as sediments and in-sewer solids act as both sources and sinks for nutrients and contaminants (see above and also Amalfitano et al., 2017). Particulate matter thus offers a broader niche differentiation to which microbes can respond, resulting in a more diverse compartment of the fluvial microbiome (Savio et al., 2015). Particle-associated bacteria, which refers to bacteria that form biofilms around particles and large aggregates (Bartram and Ballance, 1996), are more reactive to changes in water composition in both marine and limnetic environments (Besemer et al., 2005; Crump et al., 1999; Savio et al., 2015). For these reasons and given the main purpose of our study, rather than describing the whole microbial community, we concentrated on particle-attached bacteria.

Our main objective was to finely assess shifts in the fluvial bacteriome evolution along seasons and during a typical intense Mediterranean rainstorm event in comparison to the dynamics of physicochemical parameters, including a large variety of contaminants. The following questions were addressed: how is the diversity of the particle-attached bacterial community impacted by the flood event when compared to seasonal diversity? Are structural patterns of diversity driven by physicochemical dynamics along the extreme event? Do

contaminant mixtures impact the watercourse bacteriome differently at the beginning of a storm in comparison to the flow peak? Are there specific ecotypes significantly related to physicochemical dynamics?

2. Material and Methods

2.1. Study site and sampling campaigns

Campaigns took place at the Têt River, a typical Mediterranean coastal watercourse with a torrential regime that discharges into the Gulf of Lion (Southeast of France) (Dumas et al., 2015; Reoyo-Prats et al., 2017), at a site downstream from the Perpignan city wastewater treatment plant, the main threat to the water quality of this river (Conseil Général des Pyrénées Orientales (CG66), 2012, 2009; Reoyo-Prats et al., 2018, 2017). For a review on the hydrology, biogeochemistry and anthropogenic impacts on the Têt River, as well as for further details on sampling methodology, see Reoyo-Prats *et al.* (2017). Today, this river is still predominantly impacted by agriculture and urbanisation void of any major industries or farms within its catchment area. Samples were named according to sampling time (Fig. 1 for details). Ten litres water samples for both chemicals and microbiology were collected during periods of drought, in summer on the 17th September 2013 (SD sample) and in winter, on the 13th of February 2014 (WD sample). Then, in autumn, from 16th-21st November 2013, using the same sampling protocol, we sampled a 5-year flood event 13 times over the course of 109 hours, with a high frequency sampling (1 h minimum intervals) at strategic moments: before the event (t0 sample), at first flushes (t17-t19-t23), before the flow peak (t32-t37), during the flow peak (t38-t41) and during the return to basal level water discharges (from t44 to t109).

2.2. Field water samples processing and nucleic acids extraction

Field samples were processed less than 2 hours after sampling. One litre mixed-water sample from 10 L tank was entirely (or until clogged) filtered through 3 µm and 0.22 µm porosity cellulose acetate MF-Millipore membrane filters (Merck Darmstadt, Germany)

using a vacuum pump and a polysulfone filtration column (previously cleaned with HCl and thoroughly rinsed with Milli-Q Water) placed in a bleach-cleaned bench of a laminar flow hood. This operation was carried out three times to obtain three replicates per sample. Filters were stored at -80 °C until nucleic acid extraction. Environmental nucleic acid extraction was performed by resuspending filters in 425 µl lysis buffer (0.04 M EDTA, 0.05 M Tris, 0.75 M sucrose). Filters were then subject to three 65°C liquid nitrogen cycles to mechanically break cells. Lysozyme (Sigma-Aldrich, Merk) solution (1 mg/ml) was then added and placed in a rotary mixer at 37°C (45 min). Next, Proteinase K (Sigma-Aldrich, Merk) was added (0.2 mg/ml) and filters were incubated at 55°C (1 h) with agitation every 10 minutes. AllPrep DNA/RNA extraction kit (Qiagen, Hilden Germany) was finally used following manufacture recommendations. DNA quantification was performed using a Nanodrop 2000 (Thermo Fisher Scientific Wilmington, USA). A bacteria-targeted 16S rDNA PCR was performed to verify DNA amplification of water samples and DNAs were stored at -80°C before sending samples for sequencing.

2.3. Metabarcoding of environmental DNA using the 16S rRNA gene and sequence processing to form Operational Taxonomic Units (OTUs)

DNA extractions were paired-end sequenced using Next Generation Sequencing (NGS) Illumina MiSeq method (PE250). A pilot study with a single set of replicates from all samples was first sent to Research and Testing Laboratories (RTL, Texas, USA). Two more replicates were later sent to Genome Quebec laboratory (GQ, Montreal, Canada). Three sample replicates already sequenced at RTL were also sent to GQ to be sequenced again. These three double-sequenced replicates aimed to identify if differences in sequencing methods between laboratories would yield different sequencing results in the same replicates, which would impede a conjoint analysis. Sequencing targeted V3 and V4 hypervariable regions of the 16S rDNA by using 357wF (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3') universal bacterial primers (primer assay list provided by RTL). To process sequences to form OTUs, we used FROGS (Find Rapidly

OTU pipeline v. r3.0-3.0, Escudié et al., 2018, see Supplementary A1). When relevant, multi-affiliated OTUs were blasted using *blastn* on the NCBI nucleotide collection database optimised for the highly similar sequences program.

2.4. Bacterial diversity analyses

Diversity analyses were performed using the output OTUs matrix, tree and dissimilarity matrices calculated using FROGS as input for *Phyloseq* R package 1.24.2 (McMurdie and Holmes, 2013) and a collection of additional R functions (<https://github.com/mahendra-mariadassou/phyloseq-extended>). Sequencing depth largely influences alpha diversity, therefore we first checked reads number and rarefaction curves for all replicates. While saturation was achieved in all of them, sequence depth was lower for RTL sequenced replicates. Therefore, we analysed alpha diversity using non-filtered, non-normalised replicates from GQ laboratory only. Alpha diversity was estimated using Fisher, Simpson, Shannon and Pielou diversity indices and the non-parametric Chao1 species richness estimator. The evolution of the diversity through time was statistically tested using Kruskal-Wallis test (KW) followed by a post-hoc Dunn test on R software (v. 3.5.1, R Core Team, 2018). Beta diversity was assessed after filtering out singletons/doubletons OTUs and abundance normalisation to the sample with the lowest number of reads. Qualitative (Jaccard and Unifrac) and quantitative (Bray-Curtis, Morisita and Weighted-Unifrac) indices were first calculated with replicates separately. Outliers were neither observed for identical replicates sent to both sequencing laboratories nor were they observed for all replicates of the same sample, independent of sequencing laboratory origin. Thus, OTU abundances were averaged over replicates. Sample dissimilarities were visualized using Principal Coordinates Analysis (PCoA) and hierarchical clustering Ward.D2 method. A one-way analysis of similarity (ANOSIM, Clarke, 1993) was performed to test for significant differences between sample groups. The average abundances on filtered and normalised counts were used for further analyses.

2.5. Statistical analyses for inference

2.5.1. Constrained (canonical) ordination analyses

Bacterial community diversity was assessed using the same samples as those used to assess physicochemical environmental parameters in the 2013 storm event described elsewhere (Reoyo-Prats et al., 2018, 2017). Analyses were processed with *vegan* R package 2.5-3 (Oksanen et al., 2018). To infer the relationship between environmental variables and bacterial OTU abundances, we first checked collinearity through a statistical analysis of environmental parameters only. The first step in constrained ordination was to resolve collinear issues with environmental data. To do this, we performed a Principal Components Multivariate Analysis (PCA) to explore the relationship among samples and physicochemical parameters. We then performed Spearman correlations due to the non-normality and non-homoscedasticity of the data. Finally, we tested for collinearity using variance inflation factor (VIF) when performing constrained analyses. Variables with $VIF > 10$ were partialled out (O'brien, 2007). The matrix of retained variables was standardised for further analyses.

Constrained ordination methods are robust multivariate analyses that facilitate both pattern recognition in noisy data and statistical testing of the relationships between organisms and the environment. Using detrended correspondence analysis, (DCA) the gradient length of OTUs dataset was 3.58, so both canonical correspondence (CCA) and redundancy analyses (RDA) were performed (ter Braak, 1987). For RDA, a Hellinger transformation (Legendre and Gallagher, 2001) was performed. Also, a distance-based redundancy analysis (dbRDA) was performed with dissimilarity indices from the previous section. We then used permutation analyses of variance to evaluate significance of full constrained models, constrained axis and terms. Variables were tested by adding each variable independently. Triplots were then obtained with scaling focused on inter-species distances. We further determined what OTUs best responded to environmental variables by dividing the OTU matrix into $\geq 0.005\%$ and $< 0.005\%$ of total read number (1 017 326 reads) following Bokulich *et al.* (2012) and repeating CCA/RDA modelling (DCA rendered a gradient length of 3.42), to finally calculate, over most performant model, each OTU goodness of fit

(GOF). OTUs retained for further analyses had a $GOF \geq GOF_{average}$, which constitutes a conservative approach to OTU selection.

2.5.2. Network and module eigengene analyses

In order to group OTUs with same abundance profiles, network analysis was conducted on OTUs with $GOF \geq GOF_{average}$ (see 2.5.1 section), using the Molecular Ecological Network Analyses Pipeline (MENAP: <http://ieg4.rccc.ou.edu/MENA/>, Deng et al., 2012) with default parameters with two exceptions. First, zero counts were replaced by 0.01. Second, as we used an already curated dataset according to environmental parameters, OTUs present in at least one sample were retained to do not loose OTUs specific to one sample. Random matrix theory (RMT)-based method was used to construct the network using the automatically generated similarity threshold value (0.89). For network modularity properties, greedy modularity optimization (Newman, 2006) rendered the highest modularity value (0.46). To reveal a higher order of organisation among modules through an analysis of their relationship with environmental parameters, a module eigengene analysis (Langfelder and Horvath, 2007) was performed on MENAP using default parameters. This constrained network was visualised in Cytoscape software (v. 3.7.1, Shannon et al., 2003).

3. Results

3.1. Particle-attached bacterial diversity changes in the Têt River

To study urban bacteriome dynamics, a high-resolution sampling of river water was performed during a heavy rain event in autumn 2013. A pilot study demonstrated that particle-attached bacteria were more diverse and heterogeneous than free-living bacteria (Reoyo-Prats, 2014), which supported our decision to focus on this part of the community only. Summer and winter drought periods were also sampled for comparison. A total of 392 443 operational taxonomic units (OTUs) corresponding to 1 084 527 reads were identified of which 92% were singletons and 3% were doubletons. The vast majority of OTUs (99.8%) was assigned to the bacterial domain. Chloroplasts, which represented 64 928 reads, were

removed. Alpha diversity statistical analyses showed significant differences among samples for all indices (Table B1, $KW < 0.05$) except Fisher ($p = 0.068$, not shown). Fisher had the same evolution pattern along time as the observed OTU number (Fig. B1a), which significantly decreased just before the flow peak (t37) with respect to samples t17-t23. Estimated OTU number (Chao1 index) significantly increased at the flow peak (Fig. B1b) and significantly decreased only at the end of the flood. Shannon, Simpson and Pielou indices (Fig. B1c-e respectively) revealed a significantly low diversity and evenness at t19 compared to t32. While the Simpson index was significantly higher from t32 onward, Shannon and Pielou indices decreased later at the flow peak and then increased significantly at t44. Alpha diversity at drought periods did not show any major differences from t0.

For beta diversity, 9 211 OTUs (8 479 sequences/sample) were obtained after filtering and normalisation. Jaccard and Unifrac qualitative dissimilarities differentiated two communities, one included summer and winter drought samples (SD, WD) and a second included samples collected at the beginning of the autumn storm event (t0-t37), from the rest of the autumn samples (Fig. A1a-b). Flow peak samples (t38-t41), clustered together only when phylogeny with Unifrac was considered. When OTU abundances were applied using Morisita and Bray Curtis dissimilarities (Fig. A1c-d), autumn t0 sample clustered with summer and winter droughts while the other relationships among samples remained largely unchanged except that t38 clustered closer to post-flood samples with Morisita only. When phylogenetic relationships using Weighted-Unifrac (Fig. 2a-b) were also considered: i) t0, as well as summer and winter drought samples, clustered together; ii) t38 and t41 formed another group; iii) t32 and t37 clustered with samples at the end of the flood (t44-t109); and iv) t17 to t23 samples formed a new a more divergent group when compared with results obtained with other dissimilarity measures. Analysis of similarity (ANOSIM) confirmed all four (i to iv, above) community groups (Fig. 2) were significantly different among one another ($p < 0.001$, $R = 0.78$). Phyla relative abundances and heatmap plot confirmed structuration in four groups (Fig. 2c-d). Although the summer drought sample was slightly different from t0 and winter drought samples as a result of a larger amount of Actinobacteria, the first major

structural change occurred at t17 and lasted until t23, with a gain in Epsilonbacteraeota and Firmicutes at the expense of Proteobacteria and, to a lesser extent, Bacterioidetes. These last two phyla remained proportionally high at t32 and t37 to decrease only at the flow peak. Proteobacteria abundance increased at these two intermediate samples, but really exploded at t38 and t41, thus representing the second major temporal bacteriome change, reflected also by the increase of Planctomycetes and the decrease of all other major phyla. Then, we observed an inverse in the pattern, where Proteobacteria decreased and most other major phyla increased.

3.2. *Linking the bacteriome to multiple contaminants dynamics through constrained multivariate analyses*

During the same flood event of autumn 2013, Reoyo-Prats *et al.* (2018, 2017) studied the dynamics of all known contaminant families. A statistical analysis of these parameters (Supplementary A2 for details) allowed seven major environmental dynamics to be retained (Fig. 1). To determine if different environmental dynamics could statistically explain observed community shifts in diversity through time, we used several constrained multivariate analyses. Significance and percentage of variance explained by all models tested are summarised in Table 1a. Although all models were significant with the exception of CCA, Morisita and Weighted-Unifrac dbRDA models performed best ($p < 0.001$). A strong relationship between the two significant canonical axes and the environmental parameters could be observed, with up to six dynamics being significant, Dyn1 to 3, 2,4-Dichlorophenoxyacetic acid (2,4D), Diuron and NO_3^- (Table 1b, Fig. A3).

3.3. *Revealing OTUs significantly related to environmental parameters*

To further explore the relationship between the fluvial bacteriome and the environmental parameters, we split the normalised abundance OTU matrix into OTUs $\geq 0.005\%$ of total abundance and those $< 0.005\%$. While no-significant models were obtained with the second dataset, RDA and CCA models, which allow for the calculation of OTUs

292 goodness of fit (GOF), gained resolution with the $\geq 0.005\%$ dataset (602 OTUs) (Table A1).
 293 The RDA model best explained our data (Fig. 3). A total of 260 OTUs best fitted to this model
 294 ($GOF \geq 0.26$) and were submitted to network analysis in order to determine biological
 295 interactions. The resulting network (R^2 of the power law=0.874) was composed of 17
 296 modules with 167 nodes and 725 links (Fig. A4). Module membership and eigengene
 297 correlations obtained from module eigengene analysis identified OTUs that were significantly
 298 represented in each module (Table C1). Only the 13 modules that were significantly
 299 correlated to environmental dynamics were retained for further analyses. Module 1 was
 300 correlated to Dyn2, Dyn3 and Diuron and included 38 OTUs of which 13 were significant
 301 (Fig. 4) comprising a total of 17 851 out of 24 625 reads from retained OTUs (72.5%, Table
 302 C1). Most OTUs in this module were in samples t17, t19 and t23 and belonged to
 303 Epsilonbacteraeota phylum. Bacteroidetes, Firmicutes and Patescibacteria phyla were also
 304 abundant (Fig. C1a). OTU5, assigned to *Arcobacter cryaerophilus* (Epsilonbacteraeota), was
 305 particularly abundant (75.3% of reads in Module 1 and 55.6% of retained OTU abundances,
 306 Table C1). Three out of six Bacteroidetes OTUs were the most abundant, OTU41,
 307 *Bacteroides graminisolvans*, OTU126, *Macellibacteroides fermentans* and OTU95,
 308 *Cloacibacterium normanense*. Module 2 was related to Dyn 1. It included 33 OTUs from
 309 which 24 were significant (Fig. 4) comprising a total of 3 585 reads (15% of retained OTU
 310 abundances, Table C1). OTUs from this module were present in the summer drought
 311 sample, at the beginning of the flood, and even more so at the flow peak (t38, Fig. C1b).
 312 Proteobacteria was by far the most abundant phylum, particularly Rhodobacteraceae and
 313 Sphingomonadaceae families within class Alphaproteobacteria. Two OTUs were highly
 314 present from these families, respectively, OTU146 affiliated to *Trabizicola* sp. and OTU90
 315 *Porphyrobacter donghaensis*, while OTU145, *Cloacibacillus* sp. was unique from the second
 316 most abundant phylum, Synergistetes. Modules 3, 5, 7, 8, 9 and 11 comprised 6.4% of
 317 retained OTU abundances (1 571 reads, Table C1) and were related to 2.4D dynamic (Fig.
 318 4). OTUs in these modules peaked mainly at t41 (Fig. C1c). The most abundant were
 319 OTU1184 belonging to Rickettsiales order and OTU790 belonging to Gemmataceae family,

with no clear phylogenetic affiliations. The rest of the modules, overall represented less than 7% of retained OTU abundances (Table C1, Fig. C1d-g).

4. Discussion

4.1. *Particle-attached riverine bacterial community shifts are driven by water discharges and sewer overflows during Mediterranean rainstorms*

Studies that follow the ecological succession of microbial communities throughout rainstorms are very scarce in the literature (Kan, 2018; Ulrich et al., 2016). Earlier studies that described temporal changes throughout the duration of flood events using microscopic enumeration found bacteria invariant along the succession (Fisher et al., 1982; Muylaert and Vyverman, 2006). The impact of stormwater and floods on faecal bacteria loads are a major sanitary concern and have earned sustained attention from researchers for decades (Ahern et al., 2005). Comparatively, the non-faecal counterpart has received little attention, yet they can be used as biosignatures to track sources of faecal pollution (Fisher et al., 2015; Newton et al., 2013) perhaps to a better extent than the current faecal indicators used as sentinels of human pathogens (McLellan et al., 2015; McLellan and Eren, 2014; Staley et al., 2014; Wu et al., 2010). In this study, we sought to evaluate temporal succession in particle-attached riverine bacteria using high-throughput sequencing of water samples from summer, winter and autumn as well as during an autumn storm event that included a 5-year flood which led to a peak instantaneous discharge of more than 250 m³/s. A thorough knowledge of the coastal Mediterranean Têt River hydrodynamics (Dumas et al., 2015) allowed for fine-scale sampling at crucial moments of the rainstorm and we observed that major changes in the community diversity coincided with major changes in river flow. The base level autumn community captured at t0 (where starting sampling time actually began before the storm event), was more similar to bacterial assemblages associated with winter and summer droughts than to those which emerged during the storm event ($p < 0.001$, Fig. 2). Throughout the storm, a first major community shift occurred at first flushes (t17-t23, Fig. 2), coinciding with CSOs (Reoyo-Prats et al., 2017). At this point in time we noticed the highest proportion

of Epsilonbacteraeota and Firmicutes, commonly detected in sewage and sewer biofilms (McLellan and Roguet, 2019). During this phase, observed diversity and Fisher index were the highest, while Shannon, Pielou and Simpson indices were significantly lower indicating a community skewness (Fig. B1a-e). We hypothesize that CSOs brought new microorganisms into waters derived not only from runoff and wastewater, but also from the washout of in-sewer deposits, destabilizing the fluvial bacterial consortium (Fig. B1). This hypothesis is reinforced by *Escherichia coli* counts, which were highest at this same moment (Fig. A2). Ulrich *et al.* (2016) performed a microbial study on a Pennsylvania stream (USA) during a 100-year river flood of 200 m³/s, where *E. coli* abundance peaked at the highest flow level, which according to authors originated from previous sewer overflows. In our study, *E. coli* also peaked at the highest river discharge but counts were much lower than those during first flushes (Reoyo-Prats *et al.*, 2017), which thus far, have not been studied in-depth. Ulrich *et al.* (2016) concluded that hydrodynamic changes structured bacterial communities as a result of differences in composition and diversity after the flow peak, with an increase of Firmicutes observed in some flow peak samples. In our study, a second major community shift occurred at the highest water discharge (t38-t41), where we observed an increase of Proteobacteria and Planctomycetes. As in the Pennsylvania stream, we also noticed the highest presence of Firmicutes before and after this specific moment (t37, t44-t61, Fig. 2c). In contrast, t38 and t41 had the lowest observed and Shannon diversities but the highest Chao1 index (Fig. B1a-b), in other words, the Chao1 considered that not all the diversity had been sampled although saturation of rarefaction curves was reached. One hypothesis which may explain these contradictory results between parametric and non-parametric diversity indices, is that high quantities of attached-bacteria are washed-out from particles at the flow peak. This hypothesis is supported by the low equitability of these samples (Fig. B1e) in spite of having the lowest number of OTU counts among all samples (Fig. B1a). In the Pennsylvania storm event, both the highest evenness, Chao1 and observed diversities were obtained at the flow peak (Ulrich *et al.*, 2016). Similarly, Kan (2018), who studied two small floods, obtained inversed patterns for Simpson and Shannon indices compared to ours. These results

reinforce our hypothesis because they studied the whole community and not only attached bacteria. However, as we used replicates, statistical analyses were possible. Shannon, Simpson and Pielou indices at the flow peak samples were not significantly different to most of the other samples. After t38 and t41 samples, alpha diversity increased significantly, demonstrating the contribution of a more diverse upstream bacteriome (Savio et al., 2015) following runoff and rapid downflow. Finally, bacterial composition at the end of our sampling time, 4.5 days after t0 i.e. sample t109, was similar to t32 and t37, pointing towards community resilience (Fig. 2). Additionally, when summer and winter drought samples were excluded and only autumn samples were analysed (not shown), t0 (the flow base level community) grouped together with t32 and t37 samples, further supporting the evidence of the evolution towards a recovery of the fluvial community after the rainstorm. Apart from Ulrich *et al.* (2016), who also noticed community resilience five days after the storm, fluvial bacteriome recovery upon perturbation is hardly addressed in the literature and needs further attention.

4.2. Tracking microbial structural community changes according to environmental parameters: what is new?

Along with the study of riverine bacteriome shifts during a typical Mediterranean heavy rain event, we analysed a large selection of physicochemical parameters including pollutants such as pesticides, trace metals or pharmaceuticals. Studies with such a detailed follow-up are rare (Staley et al., 2014). Thanks to advances in NGS methods, research in environmental microbiology has entered into a new era where the driving forces exerted by environmental parameters can now be deeper explored than never envisaged before. Although the explanatory power of constrained multivariate gradient analysis for inference on how environmental parameters shape microbial community structures had already been demonstrated (Besemer et al., 2005; Ghiglione et al., 2008), how riverine communities are finely shaped by chemical inputs remained poorly studied prior to the NGS era, with few exceptions in extreme habitats with low diversity (Amaral-Zettler et al., 2010; Palacios et al.,

2008). As predicted by Ramette (2007), the power of high-throughput sequencing to capture most diversity opened new possibilities for inference (Newton et al., 2013; Savio et al., 2015; Staley et al., 2014). Most studies suggested that only very few taxa were influenced by physicochemical parameters, while biotic interactions were the primary drivers of variation in bacteriome structure, at least at a local scale (Fortunato and Crump, 2011; Staley et al., 2014). In a comprehensive literature review of the influence of environmental factors on stream microbiota, Zeglin (2015) raised the importance of pollutants such as metals compared to nutrient load in altering these communities. Our results demonstrate that six abiotic environmental dynamics linked to multicontamination phenomena derived from CSOs and runoff (Fig. 1) acted as environmental driving forces of the particle-attached bacteriome during a storm event (Fig. A4). Constrained analyses models based on beta diversity dissimilarities matrix (dbRDA) were used to achieve these results (Table 1). Instead, models CCA and RDA based on OTU abundance matrix were effective only when background noise was reduced keeping OTUs $\geq 0.005\%$ of total reads (Fig. 3 and next section). Using qualitative Jaccard dissimilarity index, 2.4D and Diuron dynamics significantly shaped bacterial presence/absence differences among samples, but dynamics Dyn2 and Dyn3 became significant only when considering phylogeny with Unifrac dissimilarity (Table 1). Quantitative Bray-Curtis, Morisita and Weighted-Unifrac indices also consider OTU abundances, revealing which parameters affect bacterial growth. Globally, all parameters except Dyn4, became significant in structuring samples. Surprisingly, Morisita rendered similar results to Weighted-Unifrac, both captured nearly the same variance of the diversity matrix (Table 1, 77% vs 73%). Thus, when phylogeny is not available, the Morisita dissimilarity index is a very interesting alternative (Palacios et al., 2008).

4.3. *Key players involved in the response of particle-attached riverine bacteria to environmental perturbations*

One of the greatest difficulties in the analysis of large high-throughput sequencing datasets resides in deciding how to extract, analyse and synthesise the data to identify taxa

which play a key role in the studied processes. Here, we sought to identify ecotypes that could act as key players in the riverine bacteriome response to rainstorm events. As such, we first used an aggregation distance of 1 for clustering sequences into OTUs instead of the classic 97% similarity. This method had a double purpose: 1) overcome the difficulty of using a similarity cut-off criterion for defining significant units in microbial ecology (Cohan, 2006); and 2) use an appropriate intraspecific level to find ecologically significant units (Acinas et al., 2004; Palacios et al., 2008). We then reduced the dataset to OTUs $\geq 0.005\%$ of total reads following Bokulich *et al.* (2012) and reconducted statistical analyses to retain OTUs best adjusted to the most significant multivariate model. We finally used network analysis to determine OTU associations according to their abundance profiles, that we then constrained by module eigengene analysis (Deng et al., 2016; Zhou et al., 2011) to reveal key OTU players driven by major environmental processes. This analysis allows for the calculation of the significant correlation between module consensus abundance profile and environmental constraints. Thus, it enabled us to go a step further, invoking identical driving forces which underpin OTUs with strong memberships to the same module. This has been widely assumed in functional genomics network analyses (Wolfe et al., 2005; Zhou et al., 2011).

According to our findings, shifts in particle-attached bacterial communities during typical rainstorms in coastal Mediterranean rivers are driven by: 1) runoff and in-sewer solids resuspension from CSOs at first flushes, and 2) runoff and riverbed sediment resuspension at the flow peak (Fig. 5). The most notable key player driven by CSOs was *Arcobacter cryaerophilus*, with significant membership to Module 1. *Arcobacter* genus is known to represent a sewer signature (McLellan and Roguet, 2019; Newton et al., 2013) and *A. cryaerophilus* is a human pathogen (Collado et al., 2010) with 25 antibiotic resistance categories (Millar and Raghavan, 2017) and high potential capacity for horizontal gene transfer to distant phylogenetic organisms (Jacquiod et al., 2017). Furthermore, Modules 1 and 4, correlated to Diuron, and/or Dyn2-3 released during CSOs (Fig. 5), contained several Bacteroidetes which is commonly used as faecal indicator due to its high abundance in warm-blooded animal faeces (Dick and Field, 2004). *Bacteroides graminisolvens*,

460 *Cloacibacterium normanense* and *Macellibacteroides fermentans* were particularly abundant.
461 These are relatively resistant species that are capable of surviving in extreme environments.
462 *C. normanense* removes heavy metals via production of extracellular polymeric substances
463 (Nouha et al., 2016) and *M. fermentans* degrades isosaccharinic acids linked to radioactive
464 wastes (Rout et al., 2017). Other key players during CSOs belonged to Firmicutes and
465 Fusobacteria, including OTUs from Ruminococcaceae a faecal-associated bacterial family
466 (McLellan et al., 2010; Newton et al., 2013) and Leptotrichiaceae, an underexplored family
467 common in gastrointestinal or urogenital tracts and oral cavities of humans and animals
468 (Eisenberg et al., 2016) (Fig. 5). The most remarkable finding from Module 2 was the
469 majority of Sphingomonadaceae and Rhodobacteraceae, which peaked at the flow peak,
470 suggesting these Alphaproteobacteria originated from terrestrial environments (Fig. 5).
471 Synergistaceae family was particularly abundant at the flow peak and during CSOs only,
472 concordant with Synergistetes frequent detection in wastewater (Jumas-Bilak and
473 Marchandin, 2014). In Modules linked to 2.4D, Gemmataceae, which are known for their
474 radiotolerance and complex cellular architectures (Mahajan, 2019), were highly abundant.
475 For instance, *Gemmata massiliana* (identity 87%) is a species isolated from hospital water
476 and resistant to beta-lactam antibiotics (Aghnatos et al., 2015). Rickettsiales, also a major
477 order in these modules, is known to include mammalian pathogens such as *Orientia*
478 *tsutsugamushi* (identity 91%) causing scrub typhus (Darby et al., 2007). Finally, we also
479 found genera *Novosphingobium* and *Flavobacterium*, which are known to degrade 2.4D (Dai
480 et al., 2015; Silva et al., 2007). *Flavobacterium* sp. is also linked to the degradation of
481 organophosphate compounds such as pesticides (glyphosate, AMPA...) (Singh and Walker,
482 2006) (Fig. 5). In summary, key bacterial players from suspended solids emerging at crucial
483 moments of the rainstorm event consisted of highly pathogenic and/or resistant to pollutants
484 taxa. Marti et al. (2017a) also noted in sediments accumulated in a detention basin, a
485 bacteriome evolution towards a specific community better adapted to a disturbed
486 environment. In a scenario such as the one described in the present study (Fig. 5), where
487 multipollution phenomena associated with CSOs and floods disturb resident particle-

associated communities, cross-resistance and co-resistance – leading to multiple resistance bacteria – (SCENIHR, 2009), are enhanced because horizontal gene transfer is i) induced by stressors such as antibiotics, metals or biocides (Bengtsson-Palme et al., 2018), and ii) preferentially facilitated in particle-attached bacteria (Stewart, 2013; Amalfitano et al., 2017). Our results therefore reveal that CSOs and floods pose a non-negligible threat to the capacity of resident fluvial communities to prevent the spread of resistances (Jørgensen et al., 2018), with significant consequences to both ecosystems and public-health. Further research on community changes in association with extreme events will help to confirm key players identified in this study as indicators of multipollution, a major challenge in combined stressors research (Sabater et al., 2019).

5. Conclusions

This study is the first to identify a significant relationship between the *in situ* fluvial bacteriome, river hydrodynamics and the major changes of physicochemical parameters, including several families of contaminants (faecal indicators, trace metals, pesticides and pharmaceuticals) and nutrients during a typical Mediterranean storm event. The combination of tools used in this work was crucial to the success of the results: a finely designed fieldwork, a focus on the particle-attached bacteriome compartment through 16S rRNA gene metabarcoding sequencing, together with an inference analysis using constrained multivariate and network module eigengene statistical tools. The riverine bacteriome reacted stronger to changes in pollutant dynamics during the storm event than to environmental changes according to seasons. We highlight major community shifts linked to multipollution phenomena at two critical moments of the storm event: the flow peak and the CSOs. Particle-attached resident bacterial communities became more specialised towards pollutant-resistant bacteria, some of which were pathogenic and/or capable of transforming chemical molecules. Our results clearly illustrate how urban wastewater management practices can trigger shifts from resident riverine communities to perturbed microbial assemblages, a situation which is of particular concern with respect to human-health. Even though resilience

was achieved, ecosystems and human-health are likely at risk because co-resistance and cross-resistance are typically enhanced in a scenario of continuous disturbance of resident bacterial communities subjected to multipollution phenomena from CSOs and floods. Furthermore, given the current trend towards the intensification of extreme hydrological events as a consequence of climate change and the increase in anthropogenic impacts such as urbanisation, deforestation and agriculture, not only in Mediterranean regions but also elsewhere in the world, the conclusions drawn from our study are of the utmost importance for urban management practises. For instance, as CSOs happen frequently in coastal rivers of Mediterranean regions around the world due to their particular torrential regimes under this climate, our study's findings highlight the need to transition from combined towards separate sewers as a priority for conservation action at least in these regions. The rapid and significant response of riverine particle-attached bacteria to environmental perturbations enhance the potential use of these microorganisms for rapid assessment of environmental risk in aquatic ecosystems.

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Declaration of interest

All authors declare that they have no conflict of interest.

Data availability

Sequencing data are deposited on NCBI under BioProject ID PRJNA602803.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Appendix_A

Supplementary A1 Processing of sequence data to form OTUs.

Supplementary A2 Results for statistical analyses of physicochemical environmental variables.

Fig. A1 Beta diversity based on additional dissimilarities.

Fig. A2 Principal Component Analysis biplot of environmental parameters and samples.

Fig. A3 Weighted Unifrac distance-based RDA triplot.

Fig. A4 Resulting network from best-fitted OTUs to the RDA canonical model.

570 **Table A1** Summary of constrained multivariate statistical analyses based on OTUs \geq
571 0.005%.

572 **Table A2** Probability values from Spearman correlation test on environmental parameters.

573

574 ***Appendix_B***

575 **Table B1** Alpha diversity indices at the Têt River and general Kruskal-Wallis test results.

576 **Fig. B1a-e** Evolution of alpha diversity indices along time.

577

578 ***Appendix_C***

579 **Table C1** Significant key player OTUs.

580 **Fig. C1a-g** Taxonomy evolution along time in significant modules.

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916

Table caption

Table 1 Summary of constrained multivariate statistical analyses. (a) Permanova significance of the five models tested and the percentage of biological variance that is explained by each model. (b) Axes and modelled variables significance after permanova analyses from significant models in (a). p-values significance codes: (***) <0.001 (**) <0.01 (*) <0.05).

Figure captions

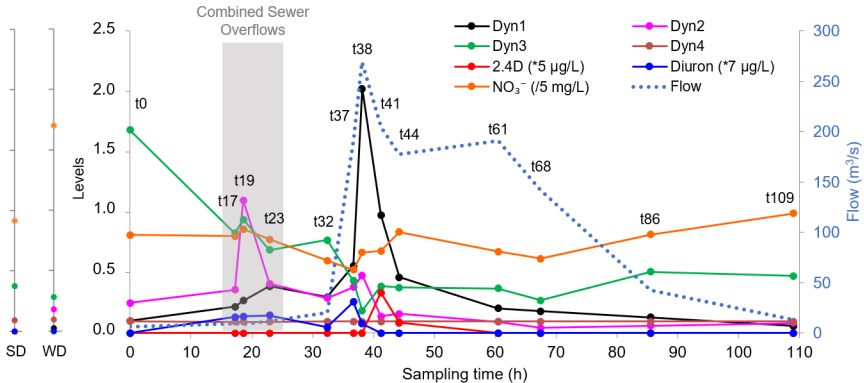
Fig. 1 Environmental parameter dynamics in the Têt River. Seven representative dynamics of environmental parameters along the flood. Samples were named as follows: tX, where X is the number of hours after starting sampling time at t₀, for the flood; and, on the left, SD and WD for summer and winter drought samples, respectively. Dyn1 corresponds to parameters with a dynamic similar to particulate organic carbon (/20 mg/l), Dyn2 to AMPA (µg/l), Dyn3 to Pb (/150 µg/g), Dyn4 to pH (/70). Three parameters, Diuron, 2.4D and NO₃⁻, had a unique dynamic (Supplementary A2 for details, modified from Reoyo-Prats et al., 2017).

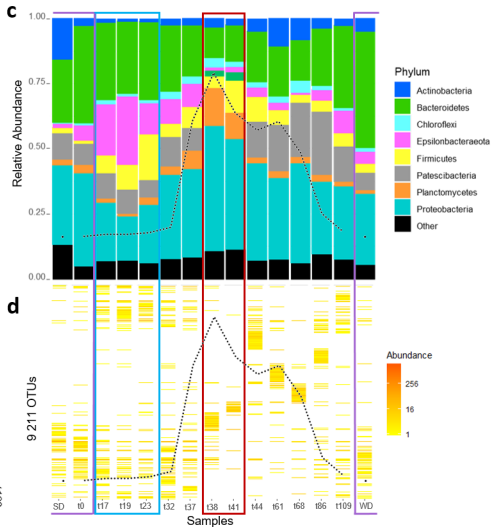
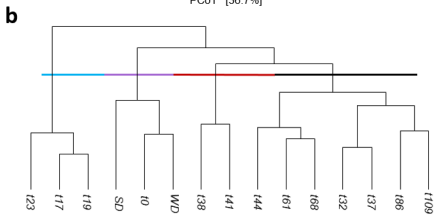
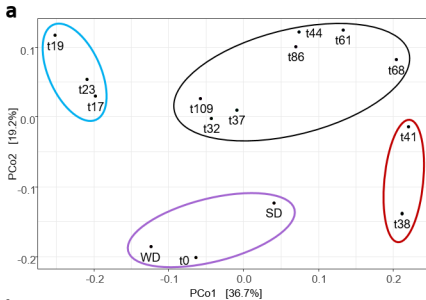
Fig. 2 Composition and structure of microbial communities averaged across replicates. (a) Principal Coordinate Analyses (PCoA) and (b) hierarchical clustering with Ward D2 linkage method using Weighted-Unifrac dissimilarity computed on OTU average abundances. Coloured lines indicate ANOSIM significant groups (R=0.78, p<0.001). (c) Histogram of relative abundances of seven major phyla and (d) heatmap based on PCoA. For (c) and (d) samples are organised according to sampling time: from summer drought (SD) to autumn flood samples (tX where X is the number of hours after t₀) and concluding with the winter drought (WD) sample. The dotted profile is the flow level of the flood (see Fig. 1).

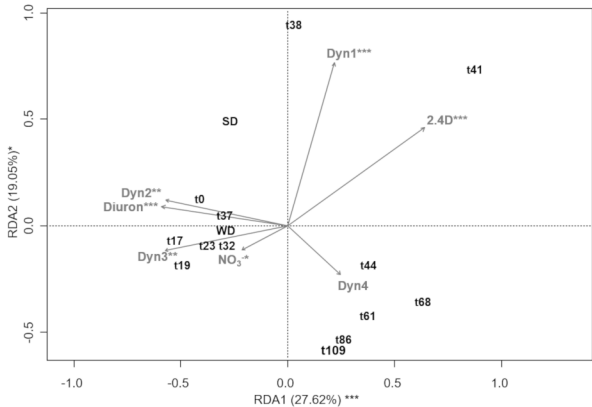
Fig. 3 Redundancy analysis (RDA) biplot with scaling by species on normalised matrix of OTUs with an abundance higher than 0.005%. The model explained 66.2% of the variance under constraints ($p < 0.001$). Environmental variables dynamics retained are plotted in Fig. 1. Significance for axes and environmental dynamics after permanova analyses are indicated, p-values significance codes: *** < 0.001 ** < 0.01 * < 0.05 . For sample and environmental parameter names, see Fig. 1.

Fig. 4 Module eigengene analysis. Eigengene network of thirteen modules (ModX, where X=module number) significantly positively correlated to environmental dynamics from Fig. 1. Each environmental dynamics is followed by module eigengene correlation value and the p-value significance code as follows: *** < 0.001 ** < 0.01 * < 0.05 . OTUs are represented by coloured fill according to phylum. Ellipse or round rectangle shape, respectively, represents significantly and non-significantly correlated OTU abundance profiles to module eigengene. The connectivity between OTUs is indicated by lines and their length is arbitrary.

Fig. 5 Illustration of the impact of combined sewer overflows and floods on particle-attached riverine bacterial communities in coastal Mediterranean rivers. Network modules contain only significantly correlated OTUs to environmental dynamics represented as black rectangles (see Fig. 4 for details). For each dynamic, all retrieved parameters are listed. Treemaps display phyla linked to relevant taxa and their relative abundance within each module or group of modules.







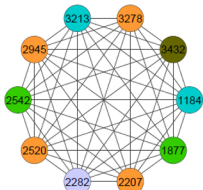
Mod1

Dyn2: 0.63**
 Dyn3: 0.55*
 Diuron: 0.64**

Mod2

Dyn1: 0.71**

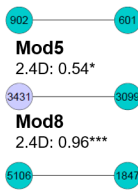
- Non-significant OTU
- Significant OTU
- Acidobacteria
- Armatimonadetes
- Bacteroidetes
- Chloroflexi
- Cyanobacteria
- Epsilonbacteraeota
- Firmicutes
- Fusobacteria
- Gemmatimonadetes
- Nitrospirae
- Patescibacteria
- Planctomycetes
- Proteobacteria
- Synergistetes
- WPS-2

**Mod3**

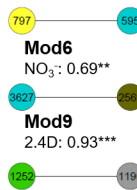
2.4D: 0.97***

**Mod4**

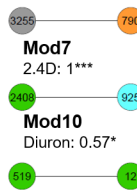
Dyn2: 0.66**
 Diuron: 0.9***

**Mod5**

2.4D: 0.54*

**Mod6**

NO₃⁻: 0.69**

**Mod7**

2.4D: 1***

**Mod9**

2.4D: 0.93***

**Mod8**

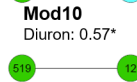
2.4D: 0.96***

**Mod11**

2.4D: 0.78***

**Mod12**

Dyn2: 0.53*

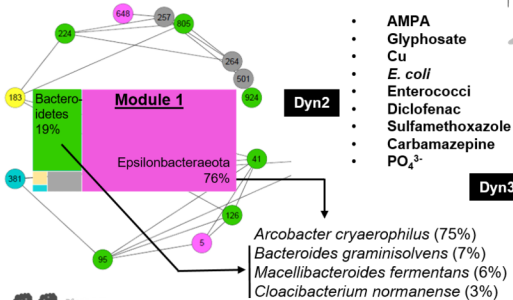
**Mod10**

Diuron: 0.57*

**Mod13**

NO₃⁻: 0.55*

Combined Sewer Overflows



Runoff



Families

Leptotrichiaceae (31%)

Ruminococcaceae (30%)

In-sewer solids resuspension



Riverbed sediments resuspension

2.4D

Diuron

Flow Peak

Dyn1

- Particulate Organic Carbon
- Flow
- Total Suspended Solids
- Total Organic Carbon
- Total Nitrogen NKJ
- Terbutylazine

Runoff

Order

Rickettsiales (13%)

Family

Gemmataceae (18%)

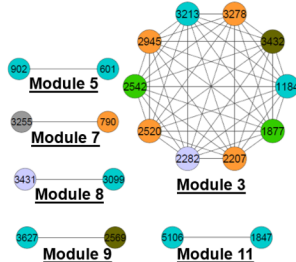
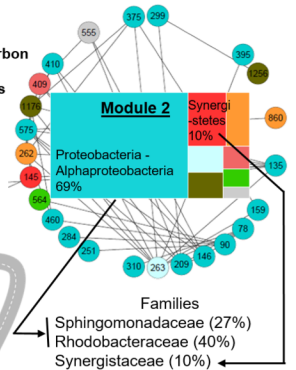
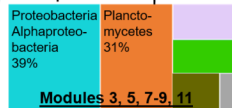


Table 1 Summary of constrained multivariate statistical analyses.

(a) Permanova significance of the five models tested and the percentage of biological variance that is explained by each model.

(b) Axes and modelled variables significance after permanova analyses from significant models in (a).

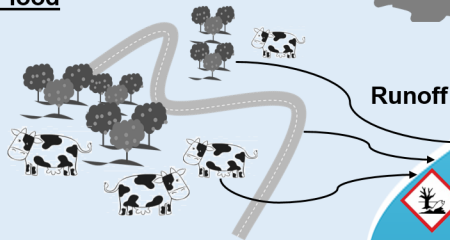
p-values significance codes: (*** < 0.001 < ** < 0.01 < * < 0.05).

a	OTUs matrice transformation	Model significance	Variance (%)
dbRDA	Jaccard	0.0120*	55.19
	Unifrac	0.003**	58.92
	Bray-Curtis	0.007**	59.70
	Morisita	0.001***	72.84
	Weighted Unifrac	0.001***	76.72
CCA		0.212	
RDA	Hellinger	0.018*	58.66

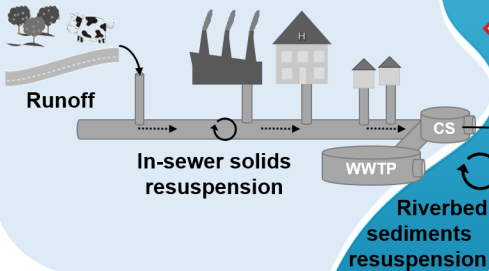
b	OTUs matrice transformation	Axes significance and variance explained (%)		Modelled variables significance						
		CAP1	CAP2	Dyn1	Dyn2	Dyn3	Dyn4	2.4D	Diuron	NO ₃ ⁻
dbRDA	Jaccard	0.006** (25.98)	0.203 (16.53)	0.090	0.065	0.050	0.428	0.002**	0.011*	0.284
	Unifrac	0.004** (30.62)	0.107 (17.89)	0.056	0.034*	0.035*	0.204	0.001***	0.010*	0.146
	Bray-Curtis	0.001** (28.85)	0.147 (17.65)	0.013*	0.012*	0.034*	0.269	0.001***	0.004**	0.142
	Morisita	0.001** (39.65)	0.031* (24.10)	0.001***	0.001***	0.003**	0.085	0.001***	0.001***	0.011***
	Weighted Unifrac	0.001** (43.04)	0.003* (21.87)	0.001***	0.001***	0.003**	0.104	0.002**	0.002**	0.001***
		Axes significance and variance explained (%)		Modelled variables significance						
		RDA1	RDA2	Dyn1	Dyn2	Dyn3	Dyn4	2.4D	Diuron	NO ₃ ⁻
RDA	Hellinger	0.008** (29.12)	0.160 (17.99)	0.034*	0.068	0.080	0.441	0.002**	0.007**	0.179

Storm Event

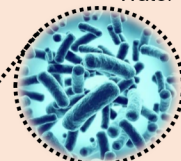
- Flood



- Combined Sewer Overflows



Water Samples



**Pollutant-resistant
and Pathogenic
Taxa**

**Risk on Human and
Ecosystem Health**

**Key
Players**

Potential **biosignatures** of
combined stressors