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Responses of resident (DNA) and active (RNA) microbial communities in fluvial biofilms under different polluted scenarios

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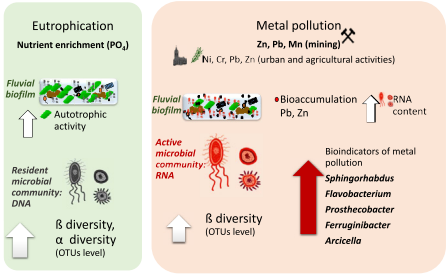
HIGHLIGHTS

- Effects of nutrients and heavy metals on microbial communities from fluvial biofilms were studied.
- Extraction of nucleic acids (DNA and RNA) provides complementary information of dynamics of microbial communities.
- Nutrients and conductivity change the diversity and composition of resident microbial community.
- Effects of mining metals are shown in the active microbial community at OTUs level.
- Five genus of bacteria are proposed as potential bioindicators of low and chronic metal pollution.

GRAPHICAL ABSTRACT



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ABSTRACT

Pollution from human activities is a major threat to the ecological integrity of fluvial ecosystems. Microbial communities are the most abundant organisms in biofilms, and are key indicators of various pollutants. We investigated the effects some human stressors (nutrients and heavy metals) have on the structure and activity of microbial communities in seven sampling sites located in the Ter River basin (NE Spain). Water and biofilm samples were collected in order to characterize physicochemical and biofilm parameters. The 16S rRNA gene was analysed out from DNA and RNA extracts to obtain α and β diversity. Principal coordinates analyses (PCoA) of the operational taxonomic units (OTUs) in the resident microbial community revealed that nutrients and conductivity were the main driving forces behind the diversity and composition. The effects of mining have had mainly seen on the taxonomic composition of the active microbial community, but also at the OTUs level. Remarkably, metal-impacted communities were very

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active, which would indicate a close link with the stress faced, that is probably related to the stimulation of detoxification.

1. Introduction

Rivers are influenced by the landscapes through which they flow. Consequently, the global transition from undisturbed landscapes to human-dominated ones with ever increasing agricultural, urban, forestry, mining and recreation land uses will impact habitat, water quality and biota, and become a principal threat to the ecological integrity of river ecosystems (Allan, 2004). Overall, these activities generate multiple pollutants such as metals (Beasley and Kneale, 2004; Brunzel et al., 2018; Mance, 1987) and nutrients (Drury et al., 2013; Smith et al., 1999; Withers and Lord, 2002) entering the river.

Nitrogen and phosphorous concentrations are of concern because they cause eutrophication that threatens the ecological status of the aquatic ecosystem (Lemm and Feld, 2017). Nutrients commonly generate an increase in algal biomass, which can result in increased diel swings in oxygen concentrations, thereby stressing some aquatic species (Correll, 1998). Occasionally, low levels of these perturbations enhance the productivity of a body of water if nutrients are limited under natural conditions. These favourable deflections are subsidiary responses (Odum et al., 1979).

Changing anthropogenic activities cause imbalances in N and P loading. P is the primary limiting nutrient in most aquatic ecosystems (McGarrigle, 1993; Water Framework Directive UK Tien and Chen, 2013), however, it is not the sole limiting nutrient in streams and rivers. Instead, this is N:P ratio that indicates which nutrient is likely to limit algal growth (Allan, 1996).

Metal pollution is still a great concern due to its high biotoxicity, perdurability and the bio-enrichment ability in food chain (Zhang et al., 2014) which causes adverse effects on biota and contributes to the deterioration of fluvial ecosystems' integrity (Corcoll et al., 2011). It is already known that the most common heavy metals found in all matrices at contaminated sites are, in order of abundance Pb, Cr, As, Zn, Cd, Cu, and Hg (Masindi and Muedi, 2018; PRC Environmental Management, 1997). The main sources of heavy metals are mines and industries, leading to high concentrations of Cd, Zn and Pb in water and sediment. For instance, Zang et al. (2017) studied remediation in a Dongdagou stream with sediments contaminated by Zn, Cd and Pb with concentrations of 1523.50, 24.90 and 857.75 $\mu g\, g^{-1}$, respectively, due to non-ferrous mining and smelting plants treated and untreated spills. Elevated concentrations of Pb in water (3 $mg\, L^{-1}$) were found near a Zn smelter plant in Brazil (Almeida et al., 2009) and in sediments from an industrial town in China (Zhu et al., 2013) with values of about 700 $\mu g\, g^{-1}$. There are other sources for heavy metals such as agriculture via fertilizers or the feed included in animal diets (Yu et al., 2016). For instance, Mendiguchía et al. (2007) associated the concentrations of dissolved Ni found in the Guadalquivir River (average of 2.31 $\mu g\, L^{-1}$) with agricultural activity. However, data for urban sources of metals are not as readily available and there are very few studies. Such studies that show values of 210 $\pm$ 30 $\mu g\, L^{-1}$ for Cr above the limits deemed permissible by the Environmental Protection Agency (EPA) (n.d) for wastewater (Khan et al., 2015). In another work, Rule et al. (2006) attribute domestic appliances as being sources of Cr and Ni.

Aquatic organisms living in fluvial systems reflect the historical and current effect the combined impact of chemical, physical, and biological stressors have. However, the interaction between natural stressors and toxicants is difficult to predict, thus complicating understanding the effects these multiple-stress scenarios have (Sabater et al., 2014). Biofilms, made up of prokaryotes, algae, fungi, and microfauna, located in close physical contact and embedded in a mucopolysaccharide matrix and which grow attached to any substrate submerged in water, are a ubiquitous component of fluvial systems. Because of their quick response to environmental changes, biofilms can be regarded as early warning systems that can be used to detect the effects toxicants are having on changes in aquatic systems (Sabater et al., 2007). Biofilms can accumulate heavy metals in high concentrations, Morin et al. (2008) described high Zn and Cd concentrations in biofilms (23,750 $\pm$ 2470 and 1809 $\pm$ 200 $\mu g\, g^{-1}$, respectively) in the Riou Mort (France) showing their effect in the diatom cell densities and taxonomic composition.

Biofilm microbial communities are very diverse and play a central role in the functioning of the ecosystem, as they interact with both biotic and abiotic components of the ecosystems. They are a key factor of specific functions such as biogeochemical cycling and the biodegradation of pollutants. Therefore, any perturbation in the community, for instance, could provoke a significant impact in fluvial ecosystems. Microbial ecotoxicology paves the way to assessing and evaluating the impact contaminants have on the taxonomic and functional microbial biodiversity which support ecosystem functions and ensure their stability and recovery (Ghiglione et al., 2016). In the case of fluvial biofilms, there remains a lack of knowledge concerning the structure of prokaryotic microbial communities (Zeglin, 2015). These communities can be described in terms of richness (e.g. number of taxonomic units) and composition (which taxonomic units are present). While important for biological understanding, metrics of richness are difficult to translate into diagnostics (Van Rossum et al., 2015). With the development of microbial community studies based on DNA and RNA sequencing, the effect of pollution on the ecosystem health can be better addressed.

A variety of methods have been developed to investigate microbial diversity and function, specifically amplicon sequencing allows to recover a high number of gene sequences since the sensitivity increases several orders of magnitude compared to previously used molecular techniques, for instance cloning or fingerprinting methods (Lear et al., 2013; Qu et al., 2017; Wang et al., 2011). Amplicon sequencing is carried out following PCR amplification and further sequencing of any of the target genes of interest such the 16S rDNA gene which codifies for the highly conserved 16S rRNA gene sequence from which it is possible to infer phylogenetic and taxonomic information (Rodicio & Mendoza, 2004). However, DNA-directed community analysis does not provide accurate information since DNA extracts contain DNA not present in actively growing cells. More precisely, extracellular DNA from deceased cells, DNA from dormant cells, DNA from non-growth active cells, i.e. microbial activities not linked to cell growth like cellular maintenance or motility (van Bodegom, 2007), or DNA from allochthonous microorganisms present in the environment due to passive migration driven by physical processes like water flow (Sobek et al., 2003). On the other hand, analysing the actual RNA pool, for which more than 90% consists of ribosomal RNA, may provide a better strategy for predicting the actual

performance of an ecosystem. RNA is only stable in active cells because it conducts metabolic processes, while potential extracellular RNA pools are rapidly degraded after cell death. Therefore, RNA makes a better indicator for extant microbial activity than DNA does although some limitations have been recently considered (Blazewicz et al., 2013). Overall, DNA (resident community) and RNA (active community) data provide complementary information of great value for understanding the complex dynamics of microbial communities and their response to human pressure. Most Occidental countries carry out water treatment and waste management programmes, and although this generates an important improvement in the water quality, pollution is still a problem. An example of this situation can be found in the Mediterranean rivers in Catalonia (NE Spain) because they are affected by low but chronic metal and nutrient pollution, as is reflected in the data obtained through the monitoring carried out by the Agència Catalana de l'Aigua (ACA) (n.d.). Gaining a better understanding of the composition of fluvial biofilm prokaryotic communities, their major drivers and their response to anthropogenic pressures is of critical importance to obtain insights into ecosystem health and to preserve its biodiversity and function. Accordingly, in order to determine the effects of metal-pollution and/or eutrophication on fluvial biofilms, in the present study amplicon 16S rRNA gene sequencing analysis of the active (RNA fraction) and resident (DNA fraction) prokaryotic community was performed. Subsequently, we analysed 19 biofilm samples taken in the winter from 7 different sites and measured a large set of environmental and biofilm variables. The sites were chosen to represent a variety of human activities, thus, different types of metal pollution and nutrient enrichment of different magnitudes were expected. The analyses presented here aim to provide a foundational interpretation of the data that contributes to the understanding of the effect of water pollution on the prokaryotic microbial communities living in fluvial biofilms. This work will provide support for future developments in water quality monitoring based on the RNA and DNA sequence biomarkers indicative of eutrophication and chronic but low metal pollution, thus contributing to provide novel microbial bioindicators of pollution.

2. Material and methods

2.1. Study site

This study was carried out along the Ter (3), Osor (1) and Llémena (2) rivers which are located in the north-east of the Iberian Peninsula (Girona, Spain).

The source of the Ter River (3) is in the middle of the Catalan Pyrenees (at 2400 m) and flows into the Mediterranean Sea at the coastal town of L'Estartit. The Ter comprises 3010 km^2 of basin surface and is 208 km long. Its drainage area is mainly calcareous except for the head, which is siliceous (Céspedes et al., 2008). It receives the direct impact of metallurgic, pulp mill, textile and tannery industries (Céspedes et al., 2008) and forms the Sau-Susqueda-Pasteral dam system which has a total capacity of 375 hm^3 of water and supplies Barcelona city and its surroundings with raw water for drinking (Espadaler et al., 1997).

The Osor River (1) is a second-order stream that flows into the Ter River. It is 23.5 km-long and drains a catchment area (from the Guilleries Mountains) of 88.9 Km^2 (Corcoll et al., 2012). The stream's stone-bedded geological substratum is mainly siliceous with moderate mineralization (90.8 mg $CaCO_3$ L^{-1}, Agència Catalana de l'Aigua (ACA) (n.d.)) Urban pressures are low, and it receives small amounts of residual sewage from Osor village (354 inhabitants) and from a wastewater treatment plant located upstream (St. Hilari Sacalm, 5064 inhabitants). The stream is also affected by effluents and runoff from a former mine that extracted sphalerite ((Zn,Fe)S) and galena (PbS). Although the mining activity finished in 1980, no environmental rehabilitation has been carried out and the stream is still receiving a continuous input of a mine effluent (Bonet et al., 2014)

The Llémena River (2) is also a tributary of the Ter River. It is a small calcareous stream that is 32 km-long. Although the upper part of the stream is very well preserved (Bonnineau et al., 2010; Corcoll et al., 2015) human activity increases downstream mainly through agriculture, livestock, and water diversion for irrigation, as well as urban activity.

Seven sampling points along these rivers were selected (Fig. 1). Three sampling points were located in the Osor River, the first (1a) upstream from the mine, the second (1b) downstream from the mine effluent and the third (1c) 12 km downstream from 1b. Another sampling point was selected in the lower part of the Llémena River (Sant Gregori) (2a), which is expected to be moderately polluted as a result of urbanization, agricultural and farming activities. The Ter River was sampled first (3a) before the confluence of the Osor River (Cellera del Ter) and considered as a reference site, the (3b), after the confluence of the Osor River but before the confluence with the Llémena River, thus potentially impacted by the Osor River and finally (3c) in Celrà, downstream from the city of Girona (98,255 inhabitants) and below a wastewater treatment plant with a tertiary treatment with a removal efficiency of 5-day Biochemical Oxygen Demand (BOD_5) (95%), Chemical Oxygen Demand (DQO) (92%), N (75%) and P (97%) with a flow nearly 45,000 (m^3 day^{-1}) (Agència Catalana de l'Aigua (ACA), 2017).

2.2. Experimental design and sample collection

A passive biomonitoring experiment with biofilm was conducted from late-February to mid-April for two consecutive years (2016 and 2017). Artificial substrata were used for biofilm growth. Colonization lasted for almost seven weeks (49 days for the first year and 46 days for the second). The artificial substrata consisted of different sized pieces of sand-blasted glass: the smaller ones being 1.2 × 1.2 cm and the larger 7 × 7 cm. These were glued onto pieces of cement cobbles (75 × 27 × 10 cm) with silicone sealant. Two of the cement cobbles were placed on the streambeds at a depth of 20–30 cm to guarantee similar light and current conditions at each sampling site.

In both years, biofilm samples for *Chl-a* fluorescence measurements (small glass substrata) were taken 7–8 times to monitor algal growth. For the rest of the analyses, biofilm was sampled once at the end, but in 2017 the biofilm samples had two replicates (one from each cement cobble) in order to collect more data and to be able to validate the objective. Water samples were taken three times in 2016, whereas a more intense sampling was performed in 2017.

Three to six small glass substrata were placed at the bottom of methacrylate boxes and were covered for 15–20 min with a small quantity of water from the sampling point. *Chl-a* fluorescence measurements were performed "in situ" with a portable amplitude modulated fluorimeter (Mini-PAM fluorometer Walz, Effeltrich, Germany). Biofilm from large sandblasted glass pieces was scrapped with brushes and cell scrapers. Such material was collected with Pasteur pipettes and used for metal bioaccumulation and organic matter (OM) analysis after having been transported to the laboratory and stored at −20 °C. Also, biofilm from large sandblasted glass pieces was scrapped using sterilized sampling material (cell scrapers, Pasteur pipettes and gloves) and samples were

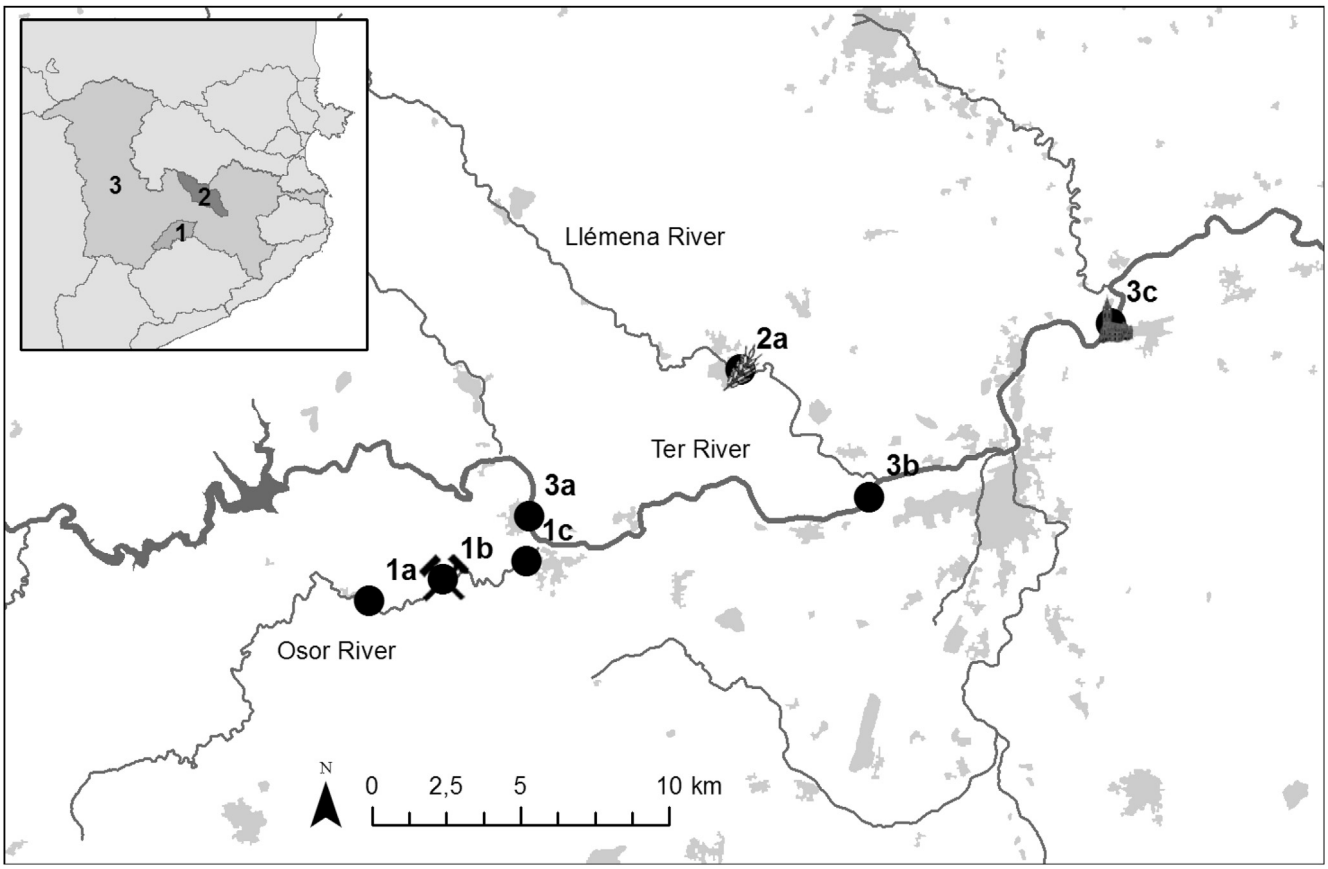

Fig. 1. Localization of the sampling sites: Osor upstream (1a), Osor mine (1b), Osor downstream (1c), Ter upstream (3a), Ter middle (3b), Llémena (2a), and Ter downstream (3c).

immediately flash frozen and stored at −80 °C for later microbial community analysis. At each sampling site and time, temperature, pH, dissolved oxygen and conductivity were measured "in situ" with a multi-parametric probe (WTW Meters, Weilheim, Germany). Water samples were filtered through 0.7 μm pore-diameter glass fiber filters GFF (Whatman), while water samples for metal analysis were filtered through 0.2 μm pore-diameter nylon membrane filters (Whatman) and acidified with 1% HNO_3 (65% suprapure, Merck). These samples were also stored at −20 °C.

2.3. Laboratory methods and sample analysis

2.3.1. Water samples

Monthly rainfall data (February, March and April) were obtained from two observatories located near the study sites: Sant Gregori ("Estació meteorològica Sant Gregori (Gironès)," n.d.) and Sant Hilari ("Meteoguilleries," n.d.).

Phosphate and ammonium were analysed as previously described by Murphy and Riley (1962) and Reardon et al. (1966), respectively, while nitrite and nitrate were analysed by ion chromatograph DIONEX ICS-5000.

Suspended solids were analysed according to standard methods (APHA) (Elosegui and Butturini, 2009). Analyses of total dissolved metal concentration were done by inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies, Inc., Wilmington, DE). Detection limits were 0.90 μg Zn L^{-1}, 1.96 μg Mn L^{-1}, 2.14 μg Fe L^{-1}, 2.02 μg Cd L^{-1}, 0.91 μg Pb L^{-1}, 1.47 μg Cr L^{-1}, and 1.23 μg Ni L^{-1} in the first experiment, and 0.28 μg Zn L^{-1}, 0.49 μg Mn L^{-1}, 0.41 μg Fe L^{-1}, 1.09 μg Cd L^{-1}, 0.32 μg Pb L^{-1}, 1.36 μg Cr L^{-1}, and 0.11 μg Ni L^{-1} in the second experiment. When the value was below the detection limit half of the detection limit, was used for data treatment (Helsel, 1990).

2.3.2. Biofilm samples

The samples for OM analysis were filtered through 0.7 μm pore-diameter glass fiber filters GF/F (Whatman), dried for 48 h at 50 °C in order to calculate dry matter. Afterwards, the samples were combusted in an oven at 450 °C (Obersal MOD MF12-124, Spain) for 4 h and then weighted again to calculate the mineral content. Samples for metal bioaccumulation in the biofilms were lyophilized, weighed and digested with 4 mL of HNO_3 (65% suprapure, Merck) and 1 mL of H_2O_2 (30% suprapure, Merck) in a high performance microwave digestion unit (Milestone, Ethos Sel) using the following method: 85 °C for 2 min, 145 °C for 5 min, 210 °C for 7 min and, finally, 210 °C for 10 min, and thereafter were diluted to 15 mL with Milli-Q water. After digestion, liquid samples were also measured with ICP-MS following the same procedure as for the total dissolved metals.

2.3.3. Molecular analysis of the microbial community

2.3.3.1. Nucleic acids extraction and RNA retrotranscription to cDNA. Biofilm samples were thawed, homogenized and weighted. RNA was extracted with the AllPrep DNA/RNA Mini kit (Qiagen) which included a step of mechanical cell disruptions (3 cycles at 5.5 power intensity for 30 s) with FastPrep®-24 Instrument. Each extract of RNA samples was treated with DNases using TURBO DNA-free Kit (Ambion, Inc). Afterwards, a negative PCR control was made to ensure that the samples were DNA-free, with universal eubacterial

primers for the 16S rRNA gene 357 F and 907 R (Weisburg et al., 1991) using a PCR Core Kit (Qiagen). PCR amplifications were performed in a GeneAmp PCR System 9700 (Applied Biosystems) following these conditions: 94 °C for 4 min, 10 cycles of 94 °C for 30 s, 61 °C for 45 s, 72 °C for 1 min; 30 cycles of 94 °C 30 s, 56 °C for 45 s and 72 °C for 1 min, finally 72 °C for 10 min. PCR products were checked by an agarose gel (1.5%, w/v) electrophoresis with a loading buffer using the Marker GeneRuler 1000 bp to check for the PCR product specificity. The gel was stained in an ethidium bromide solution (0.2 $\mu g\,mL^{-1}$) for the DNA visualization on a trans-illuminator Herolab UVT-20 M. The same samples were used for DNA extraction in accordance with the Soil DNA isolation plus kit (Norgen Biotek, Ontario, Canada), including the same mechanical cell disruption procedure described above. DNA and RNA extracts were quantified with a Qubit® RNA Assay Kit (Thermo Fisher Scientific, EEUU) and the quality was determined with a Nanodrop ND-1000 (NanoDrop Technologies Inc, New York, USA).

Retrotranscription was carried out with SuperScript® III First-Strand Synthesis System for RT-PCR KIT (Invitrogen, EEUU), using random hexamers to synthesize first-strand cDNA from purified total RNA. A PCR was used to verify that the samples contained cDNA template suitable for PCR amplification.

2.3.3.2. Bioinformatics analysis of microbial community. The analysis of 16S rRNA gene was carried out from the DNA and RNA extracts. Sequencing was performed at MSU Genomics Core (Michigan, USA) using a 2 × 250 bp paired-end Illumina MiSeq platform (Mardis, 2008). The V4 region of the 16S rRNA gene of the prokaryotes was amplified using the 515 F/806 R primer pair (Caporaso et al., 2011). Raw sequence data from this study was deposited via the Biosample Submission Portal (National Center for Biotechnology Information, n.d.) under the accession number PRJNA523926.The quality of raw reads was initially checked using the FastQC application (Babraham Bioinformatics, 2018). Raw sequences were demultiplexed, joined paired reads, quality-filtered, chimera checked and clustered into operational taxonomic units (OTUs) (97% cut-off) using MOTHUR version 1.39.5 (Kozich et al., 2013). Paired-end sequences were aligned, chimeras removed and sequences classified using the SILVA release 132 reference alignment and taxonomy database. To analyse the microbial community, alpha-diversity indicators of richness (Observed OTUs (Sobs) and Chao1) and diversity ((Shannon index (H') and Inverse Simpson index (D)) were calculated in Mothur after the normalization of the number of sequences in each sample by randomly selecting a subset corresponding to the lowest amount of sequences found in a sample (54,753 sequences per sample). Moreover, a matrix of the dissimilarity of the data from total number of OTUs was calculated for the β-diversity analysis by Yue & Clayton measure of dissimilarity (ThetaYC calculator) by MOTHUR. This matrix was used to perform by MOTHUR a Principal coordinate analysis (PCoA) to ordinate sampling sites and the axis were thereafter related with environmental variables with Pearson correlation. A PERMANOVA was also performed to test the differences of the community by site, year and the interaction (site*year) of biomonitoring and Mantel test to check if the resident and active microbial communities were correlated. These analyses were performed by PRIMER 6 and R (x64) 3.5.2 respectively. Moreover, a nonmetric multidimensional scaling (NMDS) was generated with relative abundance at order taxonomic level by the software PRIMER 6. A selection of no shared OTUs between polluted sites and non-polluted sites from the most abundant OTUs (20) was conducted. This selection was based in the PCoA information from active community by the package phyloseq of Rx64 3.5.2. software. This OTUs were selected to identify potential bioindicators of metal pollution.

2.4. Statistical analyses

A two-way ANOVA was used assuming independence between sampling sites to evaluate physicochemical differences, monthly rainfalls, F_0 and Y_{max} (biofilm parameters): among sites, between years and the interaction of annual temporality in each sampling site (site*year). Data was transformed when was required to accomplish with the assumptions of the model by napierian logarithm and square root. A post-hoc Bonferroni test was performed when significant differences ($p < 0.05$) were found between the sites. Moreover, a two-way ANOVA was performed with α-diversity indexes of microbial communities, among years and type of nucleic acid. In addition, a one-way ANOVA was performed with the rest of biofilm attributes to test differences among years of the experiment with a previous data transformation by napierian logarithm. Pearson correlations were used to explore the relationship between environmental variables and microbial diversity. ANOVA and correlation analyses were done by SPSS v.25 and Rx64 3.5.2. software. P-values of correlation analysis appear without correction.

3. Results

3.1. Physicochemical characterization of sampled sites

Monthly rainfalls in years 2016 and 2017 did not show significant differences in this area (Sant Gregori and Sant Hilari) ($p > 0.3$).

Physical and chemical water samples results are summarized in Table 1. Two-way ANOVA results show differences at specific sites, between the two sampling times (2016 and 2017) and for the interaction between sampling time and site.

Regarding temporal variability, pH was higher in 2017 ($p < 0.001$), with the exception of Osor upstream (1a). Conductivity was higher in 2017, mainly in the Ter River ($p < 0.001$). PO_4^{3-} and NH_4^+ concentrations were slightly higher in 2016 compared to 2017 ($p < 0.05$, $p > 0.05$, respectively).

Concerning site-specific differences, pH showed statistical differences among the sampling sites ($p < 0.001$) and with the interaction between the sampling site and year also significant ($p < 0.05$). The Ter and Osor downstream (3c and 1c) had lower pH values in relation to the Llémena, Osor upstream and the Osor mine ($p < 0.01$). O_2 concentration presented differences among sites ($p < 0.05$), which was statistically lower in the Ter downstream (3c) compared to Ter upstream (3a), Ter middle (3b) and Llémena (2a) ($p < 0.05$). Conductivity was two times higher in the Ter and Llémena rivers compared to Osor ($p < 0.001$) and an interaction between sampling site and year ($p < 0.001$) was found. PO_4^{3-} showed differences among sites ($p < 0.001$). The Osor River had the highest PO_4^{3-} concentration, especially upstream (1a) going over the standard concentrations for a good ecological status (28–70 $\mu g\,L^{-1}$) (Water Framework Directive UK TAG, 2013), whereas Llémena (2a) had the lowest values of all the sampled sites ($p < 0.001$). An average of three times higher N/P ratio was found in the Ter compared to the Osor River. Moreover, the Llémena had the highest values of N/P ratio (285.06 in average).

Metal dissolved in water showed temporal and site-specific variability (Table 1). Statistical differences in Zn concentrations were found among sites ($p < 0.05$) but not between years. The highest values were always found in the Osor past the mine site (1b), while the Llémena had the lowest Zn concentration, which was statistically different from 1b ($p < 0.05$). The Zn concentration found in the Osor mine exceeded the 1995 threshold set up by the US Environmental Agency (Environmental Protection Agency (EPA), n.d.) and which is determined to be maximum concentration of 120 $\mu g\,L^{-1}$ for acute and chronic exposure. Pb was only detected in

Table 1
Average and standard deviation of physicochemical parameters at each sampling site n = 21. A two-way ANOVA was performed to detect significant differences between year and sampling sites. Significance was set to $p < 0.05$. Metal concentrations above the toxicity threshold according US EPA (120 $\mu g\,L^{-1}$) and UK WFD, and phosphate concentrations above good ecological status levels established (Water Framework Directive UK Tien and Chen, 2013) (0.25 $mg\,L^{-1}$) are set in bold and underlined. bdl: below detection limit.

Sample code	1a		1b		1c		3a		3b		3c		2a		ANOVA		
Sample	Osor upstream		Osor mine		Osor		Ter upstream		Ter middle		Ter downstream		Llémena		Site	Year	Site*Year
Year	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017	P value	P value	P value
T (°C)	10.29 ± 3.15	9.73 ± 1.63	11.02 ± 2.61	11.7 ± 2.73	11.19 ± 2.65	11.05 ± 2.22	11.6 ± 1.68	11.33 ± 1.42	12 ± 1.47	13.35 ± 1.37	13.13 ± 1.63	14.7 ± 1.65	11.48 ± 2.66	13.58 ± 2.12	ns	ns	ns
pH	8.93 ± 0.38	8.6 ± 0.06	8.73 ± 0.31	8.87 ± 0.15	8.02 ± 0.27	8.46 ± 0.12	8.51 ± 0.16	8.65 ± 0.36	8.25 ± 0.09	8.71 ± 0.31	7.95 ± 0.14	8.56 ± 0.09	8.45 ± 0.34	8.96 ± 0.13	<0.001	<0.001	0.012
O_2 ($mg\,L^{-1}$)	11.51 ± 1.1	10.08 ± 0.47	11.24 ± 0.91	9.88 ± 0.66	11.11 ± 0.93	10.35 ± 0.3	11.95 ± 0.75	10.56 ± 1.03	10.81 ± 0.77	10.75 ± 1.3	9.04 ± 0.77	9.23 ± 0.41	10.18 ± 1.93	11.35 ± 1.74	0.029	ns	ns
% O_2	105.52 ± 6.50	92.85 ± 0.97	104.17 ± 3.49	92.98 ± 0.94	104.57 ± 1.54	95.83 ± 2.48	111.99 ± 2.57	98.33 ± 10.01	100.99 ± 3.81	105.65 ± 8.71	86.46 ± 4.88	91.5 ± 2.54	93.72 ± 12.22	111.28 ± 20.27	0.016	ns	ns
Cond ($\mu S\,cm^{-1}$)	233.49 ± 17.44	227.28 ± 35.47	254.4 ± 22.63	240.25 ± 33.08	248.31 ± 20.9	239.63 ± 30.39	431.33 ± 36.5	518.25 ± 16.92	416.11 ± 4.29	485.25 ± 13.33	483 ± 13	554.25 ± 44.13	500.17 ± 25.45	499.5 ± 62.69			
SS ($g\,L^{-1}$)	0.08 ± 0.13	0.01 ± 0.02	0.08 ± 0.13	0 ± 0	0.08 ± 0.12	0 ± 0	0.1 ± 0.17	0.15 ± 0.23	0.12 ± 0.21	0.02 ± 0.02	0.07 ± 0.11	0.01 ± 0.01	0.10 ± 0.11	0.03 ± 0.01			
NH_4^+ ($mg\,L^{-1}$)	0.08 ± 0.08	0.06 ± 0.04	0.04 ± 0.02	0.02 ± 0.02	0.04 ± 0.04	0.04 ± 0.04	0.06 ± 0.03	0.07 ± 0.11	0.05 ± 0.03	0.03 ± 0.01	0.38 ± 0.27	0.07 ± 0.04	0.13 ± 0.11	0.0 ± 0.01	ns	0.006	ns
PO_4^{3-} ($mg\,L^{-1}$)	**0.21 ± 0.03**	**0.15 ± 0.07**	**0.13 ± 0.01**	0.06 ± 0.01	**0.16 ± 0.05**	**0.09 ± 0.01**	0.03 ± 0.01	0.03 ± 0.02	0.07 ± 0.03	0.03 ± 0.01	**0.08 ± 0.04**	**0.1 ± 0.01**	0.02 ± 0.00	0.01 ± 0.01	<0.001	0.001	ns
NO_3^- ($mg\,L^{-1}$)	1.51 ± 0.25	1.3 ± 0.38	0.97 ± 0.19	0.44 ± 0.12	1.01 ± 0.28	0.99 ± 0.25	0.91 ± 0.04	0.99 ± 0.09	1.08 ± 0.13	1.12 ± 0.09	1.25 ± 0.08	1.31 ± 0.13	1.55 ± 0.07	1.27 ± 0.38	<0.001	0.042	0.048
N/P	17.29 ± 4.25	1.24 ± 0.38	17.32 ± 1.27	0.82 ± 0.52	16.22 ± 7.77	1.21 ± 1.16	68.87 ± 17.89	11.96 ± 22.04	36.93 ± 10.47	2.63 ± 1.47	52.77 ± 36.76	2.14 ± 1.98	184.44 ± 51.73	9.65 ± 7.51	0.001	<0.001	ns
Zn ($\mu g\,L^{-1}$)	69.06 ± 41.23	45.15 ± 27.45	**139.84 ± 45.76**	**292.08 ± 252.34**	96.46 ± 9.71	109.32 ± 109.08	26.25 ± 9.71	100.78 ± 49.12	89 ± 34.16	43.62 ± 17.15	35.91 ± 15.41	117.42 ± 128.17	22.17 ± 4.13	99.47 ± 65.98	0.016	ns	ns
Fe ($\mu g\,L^{-1}$)	121.35 ± 91.04	49.86 ± 32.05	75.39 ± 24.55	24.36 ± 14.59	86.93 ± 37.72	37.13 ± 24.93	47.96 ± 34.28	31.1 ± 17.22	109.61 ± 77.85	24.18 ± 13.01	77.72 ± 9.35	38.03 ± 18.15	56.65 ± 12.69	59.79 ± 52.72	ns	<0.001	ns
Pb ($\mu g\,L^{-1}$)	**8.10 ± 4.25**	bdl	**11.52 ± 5.48**	bdl	**2.77 ± 0.29**	bdl	**3.19 ± 1.09**	bdl	**9.22 ± 11.49**	bdl	**4.97 ± 5.07**	bdl	**3.33 ± 3.67**	bdl	ns	<0.001	ns
Mn ($\mu g\,L^{-1}$)	15 ± 5.42	7.1 ± 2.26	32.57 ± 0.73	98.36 ± 7.34	24.17 ± 7.42	16.23 ± 8.2	9.21 ± 3.79	9.95 ± 2.7	8.56 ± 3.48	7.42 ± 0.99	15.01 ± 2.17	26.5 ± 3.37	5.07 ± 2.62	12.79 ± 8.41			
Ni ($\mu g\,L^{-1}$)	bdl	0.81 ± 0.45	bdl	2.69 ± 1.94	bdl	1.15 ± 0.9	bdl	1.69 ± 1.07	bdl	1.28 ± 0.44	bdl	1.39 ± 0.26	bdl	1.85 ± 1.36			

2016 with its highest concentration in 1b. All sampling points in 2016 showed a Pb concentration above the 2.5 $\mu g\,L^{-1}$ concentration limit for chronic exposure in freshwater systems (Environmental Protection Agency (EPA), n.d.). Cr and Cd were below the detection limit for all sampling points in both years.

3.2. Biofilm characterization

Biofilm measurements are shown in Table 2. The biofilms did not present significant differences between their principal attributes in relation to the year of evaluation ($p > 0.06$). Although, F_0 did not show significantly differences between sampling sites ($p > 0.1$), photosynthetic efficiency (Y_{max}) was significantly higher in Osor and Ter upstream (1a and 3a, respectively) and Osor downstream (1c) sampling points ($p < 0.01$). Although differences among sites could not be demonstrated statistically for the rest of attributes due to the lack of replication, it was possible to point out some patterns. Regarding RNA, the results obtained in Osor mine (1b) stand out remarkably because of high values of almost ten times more than in the other sampling sites. The Llémena and the Ter rivers showed a notably temporal difference with higher values in 2016 mainly in 3a and 3c (up and downstream).

Neither were significant differences found between the different years in bioaccumulated metals ($p > 0.08$) but some differences between sampling sites were detected. Cd bioaccumulation had always been mainly below the detection limit, except in the Osor mine site (1b), where it was detected in both years. In fact, sampling site 1b differs from the rest of the sites principally by its bioaccumulated metals (Zn, Mn, Pb and Cd) provided by the mine (Table 2). In addition, Zn exceeded the threshold (150 $\mu g\,g^{-1}$) proposed by Corcoll, (2012).

3.3. Microbial community response

Microbial communities were studied based on the 16S rRNA gene sequences. A total of 4,488,081 sequences passed quality filtering. On average, 118,107 sequences with a length of 246 bp were obtained per sample. This sampling effort was enough to capture most of the bacterial diversity as indicated by the rarefaction plots (data not shown).

Clustering of sequences into operational taxonomic units (OTUs) at a 97% taxonomic cut-off ranged from 484 to 6134 OTUs per sample. The prokaryote richness (Sobs and Chao1) and diversity (Shannon and Inverse Simpson) were significantly higher in the RNA fraction (active community) than in the DNA fraction (resident community) with $p < 0.005$ (means 3729–2147), $p < 0.0001$ (means 6291–3075) for richness and $p < 0.05$ (means 5.840–5.250), $p < 0.1$ (means 96.07–73.06) for diversity, respectively (Table 3), but no significant differences were observed between years ($p > 0.1$).

Differences between sites could not be tested, as in the biofilm characterization. However, it is worth highlighting that in the Osor River, 1a and 1c had a high richness and diversity mainly in the active fraction (Table 3), while in 1b richness and diversity were lower, principally in the RNA fraction but also in the DNA fraction. The Ter upstream was very similar to 1a and 1c sampling points in

Table 2
Average and standard deviation of biofilm parameters for both experimental years in the different sampling sites n = 7 in 2016 and n = 14 in 2017. Values above the toxicity threshold according to structural and functional changes in biofilms (Corcoll, 2012) (150 μgZn g-1) are showed in bold and underlined. mv:missing value.

Sample code	1a		1b		1c		3a		3b		3c		2a	
Sample	Osor upstream		Osor mine		Osor downstream		Ter upstream		Ter middle		Ter downstream		Llémena	
Year	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017
Zn (μg g^{-1})	63.57	115.19	**2438.3**	**2677.81 ± 840.21**	**1139.39**	**538.27 ± 167.88**	30.77	50.27 ± 0.76	97.47	**177.99 ± 68.54**	98.11	**283.14**	38.12	29.76 ± 0.23
Fe (μg g^{-1})	15,234.0	18,344.55	17,215.49	14,984.18 ± 9679.06	15,586.3	17,353.56 ± 10,329.69	3784.31	9193.00 ± 2292.64	69.14	10,088.49 ±3940.60	6934.77	20,800.16	13,451.6	10,395.47 ± 1200.51
Pb (μg g^{-1})	19.72	23.72	225.04	214.81 ± 99.97	145.17	109.98 ± 51.03	4.71	17.48 ± 11.32	14.04	13.71 ± 12.42	24.16	64.96	3.36	5.56 ± 0.19
Mn (μg g^{-1})	835.69	539.87	2772.74	1039.71 ± 706.21	1618.62	657.19 ± 298.49	387.31	699.12 ± 212.39	856.38	632.81 ± 77.10	577.87	1143.83	361.59	461.97 ± 65.18
Cr (μg g^{-1})	7.5	7.86	10.97	7.62 ± 4.18	9.3	10.31 ± 5.82	3.94	9.79 ± 1.46	5.15	10.35 ± 3.92	11.28	25.3	8.16	15.45 ± 5.67
Ni (μg g^{-1})	3.77	4.5	9.94	12.11 ± 7.70	6.53	5.78 ± 2.35	5	10.37 ± 1.44	6.26	6.64 ± 1.20	9.02	21.6	28.23	29.31 ± 3.55
Cd (μg g^{-1})	bdl	bdl	3.03	1.98 ± 0.90	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Fo final	222.83	386.35	14.91	476.08	452.16	139.1	643.5	221.3	28.33	338.85	82.83	121.25	9.5	348.9
Ymax	0.533	0.541	mv	0.43	0.573	0.559	0.609	0.47	mv	0.471	mv	0.449	mv	0.459
OM (mg cm $^{-2}$)	2.61	1.18	0.67	0.81	2.44	1.59	0.6	1.62	0.7	0.62	1.66	0.52	mv	0.53
DNA (ng mg^{-1})	4.7	5.06	6.39	9.25	6.73	4.92	5.64	8.93	1.3	4.67	9.31	6.61	3.5	1.98
RNA (ng mg^{-1})	13.01	14.16	177.36	188.02	17.19	65.54	117.57	22.41	86.11	61.02	89.5	16.25	59.7	18.92

Table 3
Average and standard deviation of α-diversity results with estimated coverage of DNA (above) and RNA (below) fractions of the sampling sites and different years' n = 7 in 2016 and n = 14 in 2017.

Sample code	1a		1b		1c		3a		3b		3c		2a	
Sample	Osor upstream		Osor mine		Osor downstream		Ter upstream		Ter middle		Ter downstream		Llémena	
Year	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017
Parameters DNA														
coverage	0.99	0.98	0.99	0.99 ± 0.01	0.98	0.96 ± 0.00	0.98	0.99 ± 0.00	1.00	1.00 ± 0.00	1.00	0.99	0.98	1.00 ± 0.00
Sobs	2649	3641	1501	1488 ± 522	2941	5324 ± 386	3374	2153 ± 619	484	598 ± 88	776	2414	2623	633 ± 223
Chao 1	3322.98	5061.05	1894.17	2291.3 ± 863.23	4154.43	8649.69 ± 200.87	4463.50	2791.00 ± 527.55	722.07	825.84 ± 18.48	1102.41	3025.60	4399.61	791.98 ± 282.60
Inverse Simpson	162.18	86.03	61.54	15.38 ± 6.15	126.17	202.74 ± 113.64	86.40	85.77 ± 74.87	36.85	28.53 ± 7.96	13.60	64.80	28.62	28.54 ± 8.26
Shannon	6.21	6.01	5.37	4.11 ± 0.30	5.95	6.61 ± 0.46	6.02	5.59 ± 1.03	4.50	4.63 ± 0.32	3.71	5.83	5.03	4.62 ± 0.36
Parameters RNA														
Coverage	0.96	0.95	0.98	0.98 ± 0.00	0.97	0.96 ± 0.01	0.97	0.96 ± 0.01	0.99	0.97 ± 0.01	0.98	0.95	0.99	0.97 ± 0.01
Sobs	4267	5326	2872	2563 ± 58	3356	5226 ± 1604	4040	4437 ± 1126	1770	3805 ± 792	1881	6134	1718	3706 ± 635
Chao 1	7019.10	9277.20	4993.26	4592.18 ± 118.22	5561.45	8415.18 ± 2118.54	6022.93	7361.83 ± 2127.93	2980.81	6761.03 ± 1244.86	2998.83	10,744.80	2207.18	6732.47 ± 1035.10
Inverse Simpson	175.91	146.47	52.07	38.93 ± 3.20	87.52	147.05 ± 26.88	159.05	153.40 ± 52.22	36.45	63.14 ± 24.75	28.32	139.67	87.67	53.57 ± 9.42
Shannon	6.44	6.53	5.45	4.88 ±0.03	5.91	6.52 ± 0.57	6.47	6.37 ± 0.52	4.84	5.68 ± 0.57	4.66	6.82	5.81	5.56 ± 0.33

terms of the richness and diversity values. On the other hand, the Ter middle and the Llémena had the lowest values of richness in DNA and RNA fractions, respectively. Ter downstream was very different between years with the lowest values for diversity found in 2016 in both resident and active communities.

To determine the effect that relevant environmental (physico-chemical and biofilm parameters) variables have on α diversity, Pearson correlation analyses were performed. α diversity indices of the resident community had more correlations with temperature and conductivity (negative), however, the richness and diversity of the active community was more correlated with metals and Y_{max} positively, while negatively with activity (RNA concentration) (Supplementary Table 1).

Most of the sequences identified in the samples corresponded to the Bacterial domain (4,703,891 sequences) and only 1377 belonged to Archaeal domain. Overall, all the samples were dominated by the phylum *Proteobacteria* (38.42%–88.88%) mainly *Alphaproteobacteria* in the DNA fraction (22.48%–52.46%), followed by *Bacteroidetes* (5.15%–37.53%) more abundant in the RNA fraction (12,52%–28.19%) (Supplementary Fig. 1).

The differences in the structure of both resident and active microbial communities were analysed using a PCoA (Figs. 2 and 3). The PCoA based on the relative OTUs abundance of the microbial communities corresponding to the resident community explained 48.8% of the observed variation. The first axis explained 30.87% of the variance and was positively correlated with water temperature

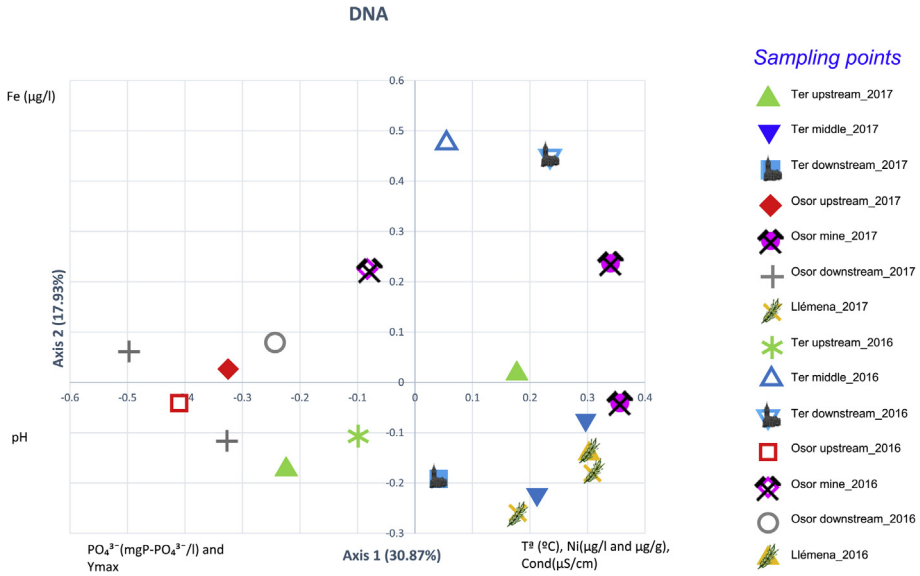

Fig. 2. PCoA of the dissimilarities between samples positions respect to the taxonomical structures (total OTUs) of resident communities using thetaYC distances and correlations with the environmental and biofilm parameters.

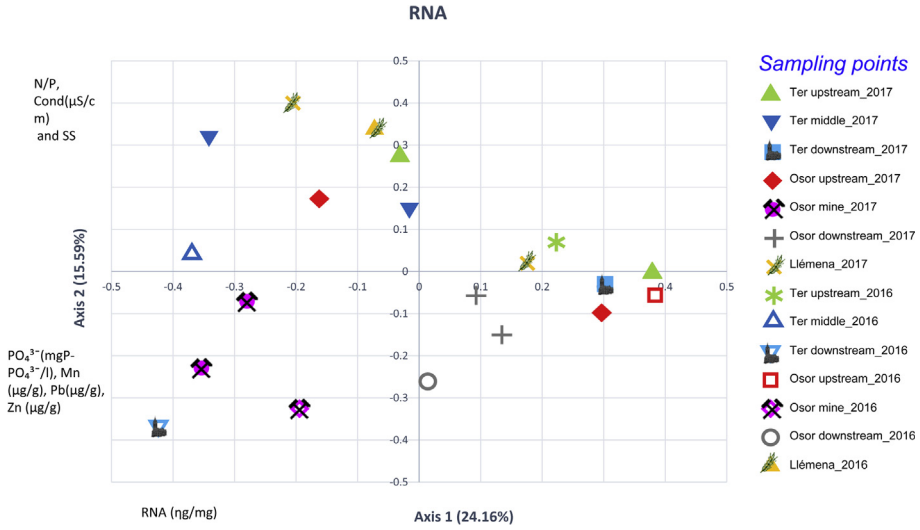

Fig. 3. PCoA of the dissimilarities between samples positions respect to the taxonomical structures (total OTUs) of active communities using thetaYC distances and correlations with the environmental and biofilm parameters.

(T^a) ($r = 0.614$, $p < 0.05$), Ni in water (µg L^{-1}) and Ni in biofilm (µg g^{-1}) ($r = 0.482$, $p < 0.05$ and $r = 0.516$, $p < 0.05$, respectively) and Cond (µS/cm) ($r = 0.528$, $p < 0.05$) and negatively correlated with PO_4^3 ($r = -0.668$, $p < 0.005$) and Y_{max} ($r = -0.609$, $p < 0.05$). The second axis explained 17.93% of the variance and it was positively correlated with Fe (µg L^{-1}) ($r = 0.458$, $p < 0.05$) and negatively to pH ($r = -0.536$, $p < 0.05$), clustering metal affected samples near the positive X axis and biofilm growth under high phosphate in the opposite site. On the contrary, the distribution of the sampling sites in the PCoA corresponding to the active community was different (Fig. 3). In this case, axis 1 explained 24.16% of the variance and was correlated with the RNA content ($r = -0.555$, $p < 0.05$) and axis 2 explained 15.59% of the variance and was correlated with N/P ($r = 0.553$, $p < 0.05$), Cond (µS/cm) ($r = 0.511$, $p > 0.05$), SS g L^{-1} and negatively with Mn (µg g^{-1}) ($r = -0.542$, $p < 0.05$), Pb (µg g^{-1}) ($r = -0.589$, $p < 0.005$), Zn (µg g^{-1}) ($r = -0.527$, $p < 0.05$) and PO_4^{3-} ($r = -0.603$, $p = 0.006$), showing a higher metal impact in the samples with the highest concentration of RNA. Some abundant and determinant OTUs from this active community ordination were found and are showed in Table 4 and in Supplementary Fig. 2 at order level. The most abundant genus found only in metal polluted sites were *Sphingorhabdus, Flavobacterium, Prosthecobacter, Ferruginibacter* and *Arcicella*.

β-diversity differences between resident and active community were checked out by PERMANOVA and Mantel 's test analysis. The resident community was different for site ($p = 0.005$), year ($p = 0.006$) and site * year ($p = 0.034$), while it was only different for site ($p = 0.026$) in the active community (Supplementary Table 2). Moreover, β-diversity of DNA and RNA fractions were not significantly correlated ($r = 0.06$, p-value = 0.292, permutations = 999).

Table 4
Specific Metal polluted and Nonmetal polluted OTUs of active microbial community (RNA fraction) from a taxonomic selection of 20 OTUs with more abundance. Metal polluted sites corresponds to Osor mine_2016, Osor mine_2017 and Ter downstream_2016) and Nonmetal polluted sites to (Osor upstream_2016, Osor_upstream_2017, Ter upstream_2016 and Ter upstream_2017).

Phylum	Metal polluted sites	Non-metal polluted sites
Acidobacteria		• **Acidobacteria GP3**
Bacteroidetes	• **Ferruginibacter** (Fam. Chitinophagaceae, Ord. Spingobacteriales). • **Flavobacterium** (Fam.Flavobacteriaceae, Ord. Flavobacteriales). • **Arcicella** (Fam. Cytophagaceae, Ord. Cytophagales).	• **Flavobacteriaceae** (Ord. Flavobacteriales)
Gemmatinomonadetes		• **Gemmatimonas** (Fam. Gemmatimonadaceae, Ord. Gemmatinomonadales).
Alphaproteobacteria	• **Sphingorhabdus** (Fam.Sphingomonadaceae, Ord. Sphingomonadales) • **Sphingomonadaceae** (Ord. Sphingomonadales)	• **Acetobacteraceae** (Ord.Rhodospirillaes)
Betaproteobacteria		• **Comamonadaceae** (Ord. Burkholderiales)
Deltaproteobacteria		• **Polyangiaceae** (Ord. Myxococcales)
Gammaproteobacteria		• **Pseudomonaceae** (Ord. Pseudomonadales) • **Haliea** (Fam. Alteromonaceae, Ord. Alteromonadales)
Verrucomicrobia	• **Prosthecobacter** (Fam. Verrucomicrobiaceae, Ord. Verrucomicrobiales)	

4. Discussion

4.1. Complementary approach of DNA and RNA microbial communities

In this study, the analysis of DNA and RNA to determine α and ß diversity of prokaryotic communities provided different and complementary information about the ecological integrity of the ecosystem as it was shown in Mantel's test and PERMANOVA (Supplementary Table 2).

The information provided by the DNA fraction, that represents the total community that is resident in the environment, including spores, dormant or non-growing active cells and dead cells (Blagodatskaya and Kuzyakov, 2013) was not very conclusive in terms of bioindication of pollution, since it was mainly attributed to ecological differences between river sites (i.e. upstream-downstream gradients of mineralization and nutrient contents). On the contrary, the RNA fraction which is based on the active community members was related to chronic but low metal pollution. However, there are some constraints in the interpretation of the RNA results as indicator of microbial activity that should be considered. For instance, growth rate of many prokaryotes is not always simply correlated to RNA content and can differ significantly among taxa (Worden and Binder, 2003). In addition, dormant cells can contain higher number of ribosomes than in the vegetative state (Sukenik et al., 2012). Overall, these considerations should be contemplated when RNA is used as a proxy of cell activity.

Based on the assumption that the RNA represents only a fraction of the resident community i.e. that only active cells (growing or non-growing) contain significantly amounts of RNA while all cells being active or not harbor genomic DNA, one would expect higher diversity and richness in the DNA fraction (Lennon et al., 2018). Contrarily, it is noteworthy to highlight that the richness and diversity were significantly higher in the active community than in the resident community in accordance to other studies (Baubin et al., 2019; Gill et al., 2017). In this study, we could also attribute this result to the greater activity of rare taxa that were undetectable in the DNA fraction. Although it cannot be excluded post-transcriptional processes effect. Alternative splicing can generate more diverse transcripts than their genomic templates (Gill et al., 2017).

Despite the limitations inherent in the RNA analysis, we strongly indicate that the complementarity of both RNA and DNA analyses provides a more complete and comprehensive characterization of complex environmental microbial communities and their response to different stressors.

4.2. Different response of microbial communities to different stressors

In this investigation, nutrient enrichment and metal pollution were used as a proxy to characterize water pollution in fluvial systems. This rather simple approach confirmed some of the alterations which were expected as a result of human activity (namely agriculture, urban, and mining effluents), in the different catchments and, which were, in most cases, consistent between years. The differences between two sampling periods (2016 and 2017) were better detected by physicochemical variables of water and resident community of prokaryotes, while the effects of the different metal polluted sites were found out mainly by biofilm parameter and active community of prokaryotes.

4.2.1. Response of biofilms to nutrient enrichment

Phosphate concentration followed the opposite pattern to that for water conductivity, (i.e., higher in the Osor and lower in the Llémena). The lowest conductivity and maximum phosphate concentrations were measured in the Osor (the siliceous stream). In this stream, phosphate concentration, mainly upstream, was

always high. This was attributed to the effluent from the wastewater treatment plant (WWTP) in Sant Hilari Sacalm and Osor, leading to concentrations 10–20 times those of background phosphate concentrations in undisturbed streams which are around 10 μg L^{-1} (Water Framework Directive UK TAG, 2013). Phosphate concentration was also high in the Ter downstream from the WWTP of Girona, but lower in the Ter upstream due to the reservoirs which act as nutrient purification tanks (Sabater et al., 2018). This concentration was minimum in the calcareous stream (Llémena) where chemical removal was expected due to co-precipitation of phosphate with carbonate (Otsuki and Wetzel, 1972). It is interesting to highlight that phosphate and conductivity were correlated with the first axis of the PCoA from the resident microbial community which would explain the 30.87% of variance in community composition (Fig. 2), and also phosphate with the second axis (which explains 15.59% of variance) of the PCoA of the active microbial community both performed at OTUs level (Fig. 3). This points to nutrient enrichment and mineralization as being the driving forces behind the resident microbial community composition, which has been already shown in other studies such as (Drury et al., 2013; Van Horn, Sinsabaugh, Takacs-Vesbach, Mitchell and Dahm, 2011; Wakelin et al., 2008), with a lower influence on the active community. It is also important to note that this pattern was not observed at phylum or order level, indicating that species differing in their preference concerning nutrient concentration and/or water conductivity may belong to the same order or phyla (Chodak et al., 2013).

The first axis of the PCoA of the resident microbial community was also correlated with Y_{max}, suggesting a causal relationship with nutrients that have a subsidiary effect on autotrophic organisms (Aristi et al., 2015; Gücker et al., 2006); this effect is shown mainly in the Osor, except in 1b where the inhibitory effect of metals on photosynthetic organisms is not counterbalanced by the availability of phosphate. As mentioned above, phosphate concentration followed the opposite pattern to conductivity, Ni in water and bioaccumulated and temperature. This co-occurrence does not allow the role that each factor plays on the variability observed to be discriminated.

The second axis of the PCoA of the active microbial community was correlated with phosphate but also with Mn, Pb and Zn accumulated in biofilm, with concentrations which were well above toxicity thresholds, indicating that metal pollution may have a major contribution determining the composition of the active microbial community - as discussed below.

α diversity was correlated negatively with conductivity and water temperature and positively with Y_{max}. On one hand, a higher number of species in the sites with lower water conductivity may also be related to the subsidiary response to nutrients in the Osor as predicted by the intermediate disturbance hypothesis (Odum et al., 1979). High Y_{max} could lead to increased α diversity of heterotrophic bacteria due to positive interaction between algae and bacteria biofilms (Battin et al., 2016; Rier and Stevenson, 2002). On the other hand, differences in species richness may also be attributed to mineralization and an upstream-downstream gradient that exerts a selection pressure towards a lower number of species. Overall, we can conclude that the differences between streams in this region have a great influence on the resident community as reported in Findlay and Sinsabaugh, (2006).

4.2.2. Metal pollution effects on biofilm

In addition to nutrients and conductivity, the sites differed in terms of the metals in water and accumulated in biofilm. The highest concentrations (Zn, Pb and Mn in water and Zn and Pb in biofilms) measured in the Osor downstream from the mine effluent (mainly in 1b but also in 1c). Ni, Cr, Pb and Zn were measured in biofilms in the Ter downstream from Girona (3c, mainly in 2017) and Ni and Cr in the biofilms of the Llémena (2a, also in 2017). While metal pollution in the Osor was attributed to mining, Ni and Cr are commonly associated with urban, industrial and agricultural activities (Tien and Chen, 2013; Victoria and Gómez, 2010). The lowest concentrations measured in the Osor upstream from the mine (1a) and in the Ter downstream from the reservoirs (3a), are within the range of background metal contents reported elsewhere (Bonet et al., 2014; Vishnivetskaya et al., 2011; Zhu et al., 2013).

As for the nutrients, the effects of metal pollution were mainly observed at OTUs levels. Ni (in water and bioaccumulated) was correlated with the first axis of the PCoA of the resident microbial community, whereas Mn, Pb and Zn bioaccumulated were correlated with the second axis of the PCoA of the active microbial community.

Focusing on the resident community, although Ni was correlated with the first axis of the PCoA, this may be due to a co-occurrence rather than a causa-effect relationship between Ni and the community composition since the values reported (maximum 29.31 μg Ni g^{-1}) were as mentioned earlier, moderate.

Regarding the active community, the results obtained support our hypothesis of the effects metal pollution on the composition of the community. Metals coming from the mine effluent (Pb and Zn accumulated in biofilm) are correlated with the PCoA of the active microbial community (Fig. 3). Likewise, the combination of metals in the biofilms explained the greater proportion of the variations observed in the bacterial communities (Ancion et al., 2013). The sampling sites most affected by mining metals were separated from the rest. These sites had values of Zn concentration above the 150 μg g^{-1} toxicity threshold in accordance with Corcoll, 2012. These values, detected in previous studies, inhibited antioxidant enzyme mechanisms such as glutathione-S-tranferase (Bonet et al., 2014), decreased photosynthetic efficiency, enhanced protection mechanisms through the xanthophyll cycle, changed the diatoms community (Corcoll et al., 2012) and exerted structural pressure by selecting the most metal-tolerant species (Tlili et al., 2011). Focusing on DNA values (Table 2), which is a proxy to the biomass of the whole microbial community (including autotrophic and heterotrophic organisms), the values measured in metal-polluted sites were relatively high, indicating that metal toxicity was mostly affecting the accrual of the biomass of the autotrophic component of the biofilm. Moreover, RNA content was even higher (in relative numbers), indicating the presence of a microbial community which was very active (Besaury et al., 2014). Since this community was active, one could envisage that prokaryotes respond to metal exposure by means of metallic rate reduction as previously shown for Zn, Cd and Pb (Almeida et al., 2009).

Patterns observed in α diversity did not follow the gradient of metal pollution unlike β diversity as in Yang et al (2013), although some differences were observed between sites located in the same stream. Microbial richness decreases downstream from the mine effluent (1b) with respect to the sampling sites on the Osor River. Several authors have pointed out that heavy metal pollution of aquatic, soil and biofilm ecosystems induces a decrease in the microbial diversity and richness (Almeida et al., 2009; Ancion et al., 2010; Kavamura and Esposito, 2010; Singh et al., 2014).

In addition, RNA content is correlated negatively with the richness and diversity of active microbial communities; therefore, bacteria were less diverse but more active indicating a shift towards a polluted-resistant community suggesting the presence and activity of detoxifying genes (Desai and Madamwar, 2007).

Once the effects of the heavy metals on the microbial communities had been detected, it was possible to identify 5 bacterial

genus proposed as bioindicators of the heavy metal contamination. *Sphingorhabdus* which has been described by Jogler et al., 2013 is known for their ability to cope with various metals because contain multiple genes associated with resistance to copper, cobalt, zinc, cadmium and mercury (Silva et al., 2018). In fact, the family *Sphingomonodaceae* harbors members with known ability to biodegrade pollutants and generate exopolysaccharides (Mahmoud et al., 2005). Concerning *Flavobacterium*, Maja, Menke, Höckner and Sommer, 2019 suggested this genus as a biomarker of heavy metals in soils and some authors found it in stream water or composting plants associate to metals (Najiah et al., 2009; Zhao et al., 2019). In addition, the genus *Prosthecobacter* is very common in freshwater (Bao et al., 2017) but also appeared as indicator of metal pollution in soil (Maja et al., 2019). *Ferruginibacter* which can reduce Fe (III) was found as an abundant genus in riparian soil adjacent to a mine drainage settling pond of Pb–Zn smelter (Fan et al., 2016) and although *Arcicella* is a non-dominant bacteria in freshwater. Londono, Donovan, Shi, Geisler and Liang, 2019 noticed that increased with the addition of metals such as Ti and Zn.

5. Conclusions

- Nutrients and conductivity were the main driving factors behind the diversity and composition of the community. These were very clear in terms of resident community, but also affected the active one. However, the effects of each factor could not be disentangled due to their co-occurrence in our specific study carried out in a human-impacted area with multiple environmental stressors.
- Metal pollution was found in many sites. Not only in streams affected by mining activities but also in zones with intense human activity, namely agriculture, industry and urbanization. Biofilms with elevated Zn and Pb content were characteristic of streams affected by mining pollution, whereas Cr and Ni increased in those presumably impacted by a myriad of pollution sources.
- The influence of mining as a determinant of the microbial community was very clear leading to the identification of 5 genus: *Sphingorhabdus, Flavobacterium, Prostheconbacter, Ferruginobacter and Arcicella* as bioindicators of low but chronic metal pollution.
- Such effect contrasted, with the contribution of other metals that could not be discerned from the effects of other environmental factors such as nutrients and mineralization and those that were not specifically addressed in this study like pharmaceuticals, pesticides, and so on.
- In contrast to nutrients and conductivity, the effects of mining were mainly seen on the active microbial community. This indicates that metal exposure may not affect the whole (resident) community but will selectively stimulate the activity of a set of species that respond to this specific type of stressor. Moreover, metal-impacted communities were very active, indicating a close link with the stress faced, probably related to the stimulation of detoxification processes.
- The differences between streams in this region have a great influence on the α and β-diversity of resident community. However, metal pollution has mainly influence on β-diversity of the active community. Both of these approaches provide complementary information needed to discriminate the human stressors studied.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.125108.

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