Universitat de Girona

MICROBIAL COMMUNITIES RESPONSES IN FLUVIAL BIOFILMS UNDER METAL STRESSED SCENARIOS

María Argudo Fernández

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DOCTORAL THESIS

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Dr. Frederic Gich Batlle of the Biology Department of the University of Girona and Dra Helena Guasch Padró of Continental Ecology Department of CEAB-CSIC,

DECLARE:

That the thesis entitled "**Microbial communities responses in fluvial biofilms under metal stressed scenarios"** presented by **MARÍA ARGUDO FERNÁNDEZ** to obtain a doctoral degree, has been conducted under our supervision.

For all intents and purposes, we hereby sign this document.

Dr Frederic Gich Batlle

Dra Helena Guasch Padró

Girona, December 2020

A MI PADRE

“The essence of the ocean cannot be seen in a drop of seawater”

Kurt Tucholsky, 1925

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LIST OF PUBLICATIONS

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- Guasch, H., **Argudo, M**., García, M., Gich F., Gutiérrez, M., & Romaní, A.M. Environmental drivers of microbial community structure in a high iron calcareous-spring. Prepared for submission.

LIST OF ABBREVIATIONS

AAPB: Aerobic Anoxygenic Phototrophic Bacteria

ACA: Agència Catalana de l'Aigua

AEA: Antioxidant Enzyme Activities

AFDW: Ash Free Dry Weight

ANCOVA: Analysis of Covariance

ANOVA: Analysis of Variance

AOA: Ammonia-Oxidizing Archaea

AOB: Ammonia-Oxidizing Bacteria

AOM: Anaerobic Oxidation of Methane

APHA: American Public Health Association

ASV: Amplicon Sequence Variant

BOD: Biochemical Oxygen Demand

bp: Base pair

CAP: Canonical Analysis of Principal coordinates

CCC: Criterion Continuous Concentration

cDNA: Complementary DNA

CEA: Animal experimentation Committee

Chao 1: Maximum richness index

Chl-a: Chlorophyll-a

COD: Chemical Oxygen Demand

Cond: Conductivity

D: Simpson index

DNA: Deoxyribonucleic acid

DOC: Dissolved Organic Carbon

DOM: Dissolved Organic Matter

EDTA: Ethylenediaminetetraacetic acid

EPS: Extracellular Polymeric Substances

F: Mesocosm with fish

F_0: Minimal fluorescence yield

Fam: Family

H': Shannon index

HDPE: High Density Polyethylene

HPLC: High Performance Liquid Chromatography

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometry

Mini-PAM: Portable Amplitude Modulated fluorometer

NE: North East

NF: Mesocosm without fish

NMDS: Non-metric Multidimensional Scaling

NOR: Normal Operating Range

Ord: Order

OTU: Operational Taxonomic Unit

PBS: Phosphate-Buffered Saline

PCA: Principal Component Analysis

PCoA: Principal Coordinate Analysis

PCR: Polymerase Chain Reaction

PERMANOVA: Permutational Multivariate Analysis of Variance

qPCR: Real-Time PCR

RNA: Ribonucleic acid

rRNA: Ribosomal ribonucleic acid

Sobs: Observed richness

SPSS: Statistical Package for Social Science (software)

SS: Suspended Solids

US EPA: United States Environmental Protection Agency

WWTP: Wastewater Treatment Plant

Y_{max}: Maximal or optimal quantum yield

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RESUM

Els ecosistemes fluvials són un dels ecosistemes més complexos i diversos del planeta. Les comunitats que hi viuen depenen de les interaccions entre els factors ambientals i biòtics que hi succeeixen. Actualment, els ecosistemes aquàtics estan sotmesos a condicions d'estrès múltiple que poden incloure factors d'estrès tant d'origen natural com antròpic. Un factor d'estrès que segueix sent motiu de preocupació és la contaminació per metalls, degut a la seva alta biotoxicitat, perdurabilitat i a la seva capacitat de bioacumulació en la cadena tròfica, que acaba causant efectes adversos en la biota i contribuint a la deterioració de la integritat dels ecosistemes fluvials. Un bon indicador d'estrès per metalls és la resposta del biofilm fluvial, sobretot la dels procariotes que hi viuen. Els procariotes responen ràpidament als canvis ambientals ja que la seva abundància, diversitat i taxa de creixement són elevades. Aquests porten a terme funcions ecosistèmiques importants assegurant l'estabilitat i recuperació dels ecosistemes fluvials, de manera que qualsevol factor d'estrès que afecti a aquests microorganismes pot comportar importants conseqüències pels ecosistemes. Per tant, l'ecotoxicologia microbiana, amb l'ajuda de la metagenòmica, proporciona una bona aproximació pel coneixement i avaluació de l'impacte que la contaminació per metalls té en l'estructura i funcionalitat de les comunitats microbianes. No obstant això, encara existeix una certa incertesa en l'avaluació i predicció dels efectes del metalls a gran escala. Els estudis de camp ajuden a aproximar-se a aquesta realitat ecològica per mitjà de l' enfoc holístic.

Aquesta tesi té l'objectiu d'investigar els efectes dels metalls d'origen natural i antròpic sobre la composició i funcionalitat de les comunitats de procariotes que viuen als biofilms epilítics fluvials a través d'anàlisis moleculars i estudis de camp. Aquest estudi inclou tres treballs de camp duts a terme en diferents escales temporals i espacials, per tal d'abordar la complexitat dels ecosistemes fluvials a múltiples escales. Primer, es va realitzar un estudi de monitorització passiva de biofilm en diferents zones dels rius Osor, Llémena i Ter per entendre els procediments i l'interès de realitzar anàlisis de seqüenciació de les fraccions ADN i ARN del gen 16SrRNA de les comunitats de procariotes que creixen en el biofilm dels ecosistemes eutròfics amb contaminació lleu de metalls. El segon estudi es va basar en un experiment amb mesocosmos que contenien còdols de riu colonitzats per biofilm al llarg del riu Osor, on es controlava la presència de peixos, per tal de determinar el seu impacte en l'estructura i funcionalitat de les comunitats microbianes en un ambient d'estrès múltiple. Finalment, el tercer va ser un estudi realitzat en una font de ferro procedent d'aigües subterrànies (Can Verdaguer) a la conca del riu Llémena, on es van

prendre mostres d'aigua, biofilm i fulles per reconèixer els factors ambientals i biòtics que determinen l'estructura i funcionalitat de la comunitat microbiana.

Els resultats d'aquesta tesi mostren que les anàlisis d'ADN i ARN per determinar la α i β-diversitat de les comunitats de procariotes proporcionen una informació diferent i complementària sobre la integritat ecològica de l'ecosistema. La fracció d'ADN (la comunitat resident) esdevingué un indicador pobre de la contaminació per metalls, però va detectar un canvi en la composició de les comunitats de bacteris al llarg d'un gradient de mineralització i segons el contingut de nutrients. En canvi, la fracció d'ARN (la comunitat activa) va mostrar la resposta de la comunitat a nivells lleus de contaminació de metalls amb comunitats bacterianes similars en els llocs afectats per metalls. A més, l'alt contingut en ARN d' aquestes mostres va evidenciar la presència d'una comunitat microbiana molt activa, suggerint una resposta específica a l'exposició de metalls, com podrien ser els processos de detoxificació.

Pel que fa a la presència o absència de peixos en un escenari d'estrès múltiple, els resultats obtinguts mostren que la combinació dels efectes de contaminació, manca d'aigua i la presència de peixos van tenir un fort impacte en l'estructura de les comunitats microbianes. La biomassa, el consum de nutrients per part del biofilm i la α-diversitat dels procariotes no van seguir el gradient de contaminació de metalls. En canvi, les diferències en la composició de la comunitat (β-diversitat) van ser molt més clares al llarg del gradient de contaminació de metalls, seleccionant famílies indicadores de cada lloc. És interessant ressaltar l'aparició de bacteris endosimbionts en els llocs més alterats, amb alta concentració de nutrients, contaminació de metalls i alteració hidrològica. A més, la bioturbació va reduir el contingut de clorofil·la i biomassa del biofilm i va incrementar la toxicitat dels metalls, confirmant la importància d'aquests macro consumidors en la seva arquitectura i en conseqüència, en el funcionament de l'ecosistema fluvial.

En relació a la font de ferro, els principals factors determinants de la composició de la comunitat microbiana van ser la química de l'aigua i la competència entre comunitats. Tal com s'ha vist en el cas de contaminació per metalls procedents de la mina, l'α-diversitat dels procariotes tampoc es va veure influïda per l'estrès químic en aquest ambient aquàtic. De fet, un gran nombre d'espècies va a traspassar el filtre ambiental d'estrès químic provocat pel ferro, apareixent una comunitat única i molt diversa caracteritzada per espècies quimiolitotròfiques. No obstant això, la β-diversitat dels procariotes, la producció primària i les taxes de descomposició de la fullaraca van seguir el gradient d'estrès químic. Aquestes condicions extremes van resultar molt nocives per a les algues i els organismes responsables de la descomposició de les fulles, portant a valors molt baixos de producció primària i de descomposició. Per

altra banda, a mesura que va disminuir la concentració de ferro aigües avall, la producció primària i la descomposició van incrementar generant una comunitat de procariotes molt diferent, però amb valors més baixos de diversitat i amb altres funcions atribuïts a l'exclusió competitiva.

En general, aquesta tesi mostra com els metalls d'origen natural i antròpic canvien de forma important la composició de les comunitats de procariotes, sobre tot dels bacteris. La β-diversitat és la variable més sensible als efectes dels metalls, a diferencia de la α-diversitat, que no es mostra gairebé afectada o fins i tot és beneficiada. En escenaris amb contaminació alta i crònica de metalls, la comunitat resident (fracció d'ADN) pateix canvis en la seva composició que poden ser detectats a nivell de fílum o classe. Si més no, en ecosistemes fluvials sotmesos a nivells més baixos de contaminació de metalls només la fracció d' ARN es veu afectada seleccionant els OTUs/ASVs més actius. El coneixement de la composició de la comunitat bacteriana i la identificació de taxons especialment sensibles, ajuden a trobar funcions potencials de resposta dels bacteris a situacions d'estrès causades per metalls.

RESUMEN

Los ecosistemas fluviales son uno de los más complejos y diversos del planeta. Las comunidades que viven en estos ecosistemas dependen de las interacciones entre los factores ambientales y bióticos que tienen lugar en ellos. Actualmente los ecosistemas acuáticos están sujetos a condiciones de estrés múltiple que pueden incluir estresores de origen natural o antrópico. Un estresor que sigue siendo motivo de preocupación es la contaminación por metales debido a su alta biotoxicidad, perdurabilidad y su capacidad de bioacumulación en la cadena trófica, lo cual causa efectos adversos en la biota y contribuye al deterioro de la integridad de los ecosistemas fluviales. Un buen indicador del estrés por metales es la respuesta del biofilm fluvial, sobre todo la de los procariotas que viven en él. Los procariotas responden rápidamente a los cambios ambientales debido a que su abundancia, diversidad y tasa de crecimiento son muy altas. Además, éstos llevan a cabo funciones ecosistémicas importantes asegurando la estabilidad y recuperación de los ecosistemas fluviales, con lo que cualquier estresor que afecte a estos microorganismos puede conllevar serias consecuencias para los ecosistemas. Por tanto, la ecotoxicología microbiana con la ayuda de la metagenómica, proporciona una buena aproximación al conocimiento y evaluación del impacto que la contaminación por metales tiene en la estructura y función de las comunidades microbianas. Sin embargo, todavía existe cierta incertidumbre en la evaluación y predicción de los efectos de los metales a gran escala (escala ecosistémica). Son los estudios de campo los que ayudan a aproximarse a esta realidad ecológica por medio de un enfoque holístico.

Esta tesis tiene el objetivo de investigar los efectos de los metales de origen natural y antrópico sobre la composición y función de las comunidades de procariotas que viven en los biofilms epilíticos fluviales, a través de análisis moleculares y estudios de campo. La tesis incluye tres estudios de campo llevados a cabo en diferentes escalas temporales y espaciales, como una forma de abordar la complejidad de los ecosistemas fluviales a múltiples escalas. Primero se realizó un estudio de monitorización pasiva de biofilm a lo largo de diferentes puntos de los ríos Osor, Llémena y Ter para comprender los procedimientos y el interés de realizar análisis de secuenciación de las fracciones ADN y ARN del gen 16S rRNA de las comunidades de procariotas que componen el biofilm, en ecosistemas eutróficos y con una contaminación leve de metales. El segundo estudio se basó en un experimento con mesocosmos que contenían códulos del río colonizados por biofilm a lo largo del río Osor, donde la presencia de peces estaba controlada, para determinar su impacto sobre la estructura y función de las comunidades microbianas en un ambiente de estrés múltiple. Finalmente, el tercero fue un estudio en una fuente de

hierro procedente de aguas subterráneas (Can Verdaguer) en la cuenca del río Llémena, donde se tomaron muestras de agua, biofilm y hojas para reconocer los factores ambientales y bióticos que determinan la estructura y función de la comunidad microbiana.

Los resultados de esta tesis muestran que el análisis de ADN y ARN para determinar la α y β-diversidad de las comunidades de procariotas proporciona una información diferente y complementaria sobre la integridad ecológica del ecosistema. La fracción de ADN (la comunidad residente) resultó un indicador pobre de la contaminación de metales, pero detectó un cambio en la composición de las comunidades de bacterias a lo largo de un gradiente de mineralización y en función el contenido de nutrientes. En cambio, la fracción de ARN (la comunidad activa) detectó respuestas de la comunidad ante una moderada contaminación de metales con comunidades bacterianas similares en los sitios afectadas por metales. Además, el alto contenido en ARN de estas muestras indicó la presencia de una comunidad microbiana muy activa, sugiriendo una respuesta específica a la exposición de metales, como podrían ser los procesos de detoxificación.

Respecto a la presencia o ausencia de peces en un escenario de estrés múltiple, los resultados obtenidos muestran que la combinación de los efectos de contaminación, falta de agua y peces tuvieron un efecto pronunciado en la estructura de las comunidades microbianas. La biomasa, la absorción de nutrientes por parte del biofilm y la α-diversidad de los procariotas no siguieron el gradiente de contaminación de metales. Sin embargo, las diferencias en la composición de procariotas (β-diversidad) fueron muy claras a lo largo del gradiente de contaminación de metales, seleccionando algunas familias indicadoras de cada sitio. Es interesante resaltar, la aparición de bacterias endosimbiontes en los sitios más afectados, con alta concentración de nutrientes, contaminación de metales y alteración hidrológica. Además, el efecto de la bioturbación de peces sobre el biofilm, redujo el contenido de clorofila y biomasa e incrementó la toxicidad de los metales, confirmando la importancia de estos macroconsumidores en la arquitectura del biofilm, y en consecuencia en el funcionamiento del ecosistema fluvial.

En relación a la fuente de hierro, los principales factores determinantes de la comunidad microbiana fueron la química del agua y la competición entre comunidades. Como ya ha sido citado para la contaminación por metales procedentes de la mina, la α-diversidad de los procariotas tampoco fue influenciada por el estrés químico en esta fuente. De hecho, un gran número de especies pasó el filtro ambiental provocado por una elevada concentración de hierro, creando una única y muy rica comunidad de procariotas sostenida por especies quimiolitotróficas. Sin embargo, la β-diversidad de los procariotas,

la producción primaria y las tasas de descomposición de las hojas siguieron el gradiente de estrés químico. Estas condiciones extremas fueron nocivas para las algas y los organismos responsables de la descomposición de las hojas, generando muy baja producción primaria y descomposición. Pero según se reducían las concentraciones de hierro aguas abajo, la producción primaria y la descomposición incrementaban, generando una comunidad de procariotas muy diferente, con baja diversidad y con otras funciones a causa de la exclusión competitiva.

En general, esta tesis muestra cómo los metales de origen natural y antrópico cambian de forma importante la composición de las comunidades de procariotas, sobre todo de las bacterias. La β-diversidad es la variable más sensible a los efectos de los metales, a diferencia de la α-diversidad, que no se muestra casi afectada o incluso es beneficiada. En escenarios con contaminación alta y crónica de metales, la comunidad residente (fracción de ADN) sufre cambios en su composición que pueden ser detectados a nivel de filo o clase. Sin embargo, en ecosistemas fluviales sometidos a niveles más bajos de contaminación de metales sólo la fracción de ARN se ve afectada selecionando los OTUs/ASVs más activos. El conocimiento de la composición de la comunidad bacteriana y por consiguiente la elección de algunos taxones como bioindicadores, ayuda a encontrar funciones potenciales de las bacterias que podrían ser importantes en las respuestas al estrés causado por metales.

SUMMARY

Fluvial ecosystems are one of the most complex and diverse systems on the planet. Biological communities that live in these ecosystems depend on environmental factors and biotic interactions. Nowadays, freshwater ecosystems are subjected to multiple stress conditions, which can include natural and anthropic stressors. A stressor, which is still under concern today, is metal pollution due to its high biotoxicity, perdurability and bioaccumulation across the food chain, which causes adverse effects on biota and contributes to the deterioration of fluvial ecosystem integrity. A good indicator of metal stress is the response of fluvial biofilm, especially prokaryotes living in it. Prokaryotes respond quickly to environmental changes due to their abundance, high diversity and fast growth rate. Moreover, they support important ecosystem functions and ensure the stability and recovery of fluvial ecosystems, so any stressor affecting microorganisms would cause serious consequences to the ecosystems. Consequently, microbial ecotoxicology, with the help of metagenomics, provides a good approach to understand and evaluate the impact of metal pollution on the structure and function of microbial communities. However, there is still some uncertainty in the assessment and prediction of the effects of metals on a large scale (ecosystem scale). Field studies help us get closer to this ecological reality over a holistic approach.

This thesis aims to investigate the effects of metals from natural and anthropogenic sources on the composition and function of the prokaryotic communities living in epilithic fluvial biofilms, based on molecular analyses and field studies. The thesis includes three field studies carried out at different temporal and spatial scales as a multi-scale way of approaching the complexity of fluvial ecosystems. First, a passive biomonitoring study with biofilms was conducted along different points of the Osor, Llémena and Ter Rivers to understand the procedures and interest of performing amplicon sequencing of the DNA and RNA fractions of the 16 rRNA gene analysis of the prokaryotic component of biofilms, in a eutrophic environment with low metal pollution. Second, an experiment with mesocosms filled with natural colonized cobbles was carried out over the Osor River, where the presence of fish was controlled, to determine their impact on the structure and function of biofilm microbial communities in a multiple stressed environment. Finally, the third study was performed in an iron (Fe) groundwater spring (Can Verdaguer) at the Llémena watershed, where water, biofilm and leaf samples were collected to examine the environmental and biotic drivers of the structure and function of microbial community.

The results obtained in this thesis show that the analysis of DNA and RNA to determine α and β-diversity of prokaryotic communities provided different and complementary information about the ecological integrity of the ecosystem. The DNA fraction (resident community) was a poor indicator of metal pollution, although it detected a change in the composition of the bacteria over upstream-downstream gradient of mineralization and nutrient contents. However, the RNA fraction (active community) detected community responses to low metal pollution with similar bacterial communities in sites affected by metals. In addition, the high content of RNA in the most polluted samples indicated the presence of a very active microbial community, which suggested a specific response to metal exposure, such as detoxification processes.

With respect to the presence or absence of fish in a multiple-stressed scenario, the results obtained show that the combined effects of pollution, water stress and fish had a pronounced effect on the structure of microbial communities. Biomass, nutrient uptake of biofilm and α-diversity of prokaryotes did not follow the gradient of metal pollution. However, the differences in the composition of prokaryotes (β-diversity) were very clear over the metal pollution gradient and therefore, some indicator families of each site could be classified. Interestingly, endosymbiotic bacteria appeared in the site most affected by nutrient enrichment, metal pollution and hydrological alteration. Moreover, the effects of the fish bioturbation on biofilm reduced Chl-a and AFDW and increased the toxicity of metals, thus confirming the importance of these macroconsumers in the biofilm architecture, and consequently, in the functioning of fluvial ecosystems.

In relation to the Fe spring, the main drivers of microbial community were water chemistry and biological competition. As reported for mining metals, α-diversity of prokaryotes was not affected by chemical stress in the Fe spring. In fact, a large number of species passed the extreme environmental filter of high Fe concentration creating a unique and very rich prokaryotic community sustained by chemiolitotrophic species. However, β-diversity of prokaryotes, primary production and leaf litter decomposition rates followed the chemical stress gradient. The extreme conditions were deleterious for algae and organisms responsible for leaf decomposition leading to a very low primary production and decomposition rates. As the concentration of Fe decreased downstream, the primary production and breakdown increased generating competitive exclusion, in such a way that a different prokaryotic community less diverse and with other functions was found.

Overall, this thesis shows how the metals of natural and anthropic origin change significantly the composition of the prokaryotic communities (mainly the composition of bacteria). The β-diversity is the most sensitive variable to the effects of metals, unlike α-diversity, which is hardly affected or even benefited. In scenarios with high and chronic metal pollution, the resident community (DNA fraction) suffers changes in its composition that can be detected at phylum or class taxonomic level. However, in fluvial ecosystems, subjected to lower levels of metal pollution, only the RNA fraction is affected by selecting more active OTUs/ASVs. In addition, the knowledge about the bacterial composition of communities and, consequently, the selection of specific taxa is useful to find some potential prokaryotes functions important in stress response caused by metals.

GENERAL INTRODUCTION

1. Ecological complexity of fluvial ecosystems

1.1. Fluvial ecosystems under study

Freshwater represents only 2.8% of all the water in the Earth. Ice caps and glaciers comprise most of it (2.24%), and groundwater (0.61%) is also a sizable percentage. Only 0.009% of the total of freshwater reserves are in lakes, about 0.001% in the atmosphere, and rivers contain 0.0001% (Allan & Castillo, 2007). Understanding the structure and function of these ecosystems is still a common goal for many aquatic ecologists.

Fluvial ecosystems are open, as well as hierarchical, dynamic and heterogeneous. They are submitted to physical, chemical and biological elements and processes across multiple spatial and temporal scales, which are interlinked. In particular, this thesis will focus on two important fluvial ecosystems: some rivers and a specific groundwater-fed spring, both located in Mediterranean climate regions.

Although **rivers** only represent a small percentage of the Earth's water, they have an important ecological relevance and they are among the most complex and diverse ecosystems on the planet. These ecosystems behave as a dynamic network of channels and floodplains intermittently connected by the action of the flow. Humphries, Keckeis & Finlayson (2014) put forward the river wave concept as an easy and familiar way to describe the river flow. This concept emphasizes the key processes that drive river ecosystem structure and function, such as the production, storage, transformation and transport of material and energy. The objective of the concept is to unite three hypothesis based on previously proposed models. These are the productivity model (Thorp, Delong, Greenwood & Casper, 1998), the river continuum concept (Vannote, Minshall, Cummins, Sedell & Cushing, 1980) and the flood pulse concept (Junk, Bayley, & Sparks, 1989). Following the predictions of the productivity model, the trough of a river wave equates to a low flow (baseflow) in which the local production of autochthonous and local inputs of allochthonous matter predominate. This fact contributes to the metabolism and the transformation of organic matter through decomposition and assimilation at various trophic levels. The ascending or descending limbs of river waves equate to rising and falling hydrographs, especially relevant in Mediterranean rivers, where upstream allochthonous inputs and longitudinal transport of material and energy are mainly found, according to the river continuum concept. Flood flows in rivers, referred in the model as the crest of the wave, are characterized by allochthonous inputs of material and energy from floodplain habitats due to lateral transport, autochthonous floodplain production and the storage and transformation of material. Also, upstream allochthonous production and transport continue to be substantial getting closer to the

predictions of the flood pulse concept (Figure 1). Moreover, these behaviors vary depending where the catchment is (upstream, middle and downstream), the climate, geology, geomorphology and human activity, which also influence the riverscape and its biota.

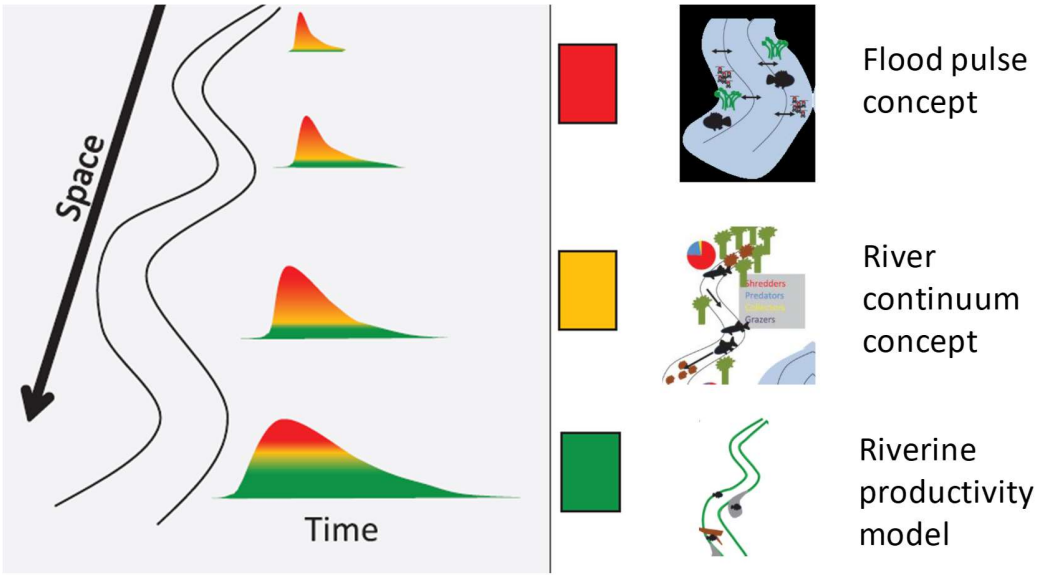


Figure 1. Theoretical examples of the variation in the time and space of the river waves through of productivity model (Thorp et al., 1998), the river continuum concept (Vannote et al., 1980) and the flood pulse concept (Junk et al., 1989). Modified from Humphries et al. (2014).

Other interesting and singular aquatic ecosystems are **groundwater-fed springs** arising from long transit time hydrogeological systems (eg., Beam et al., 2016; Chae, Yun, Kim & Mayer, 2006; Garrels & Mackenzie, 1967; Hurwitz, Hunt & Evans, 2012). In these systems strong hydrochemical changes often take place (Agnelli et al., 2015; Piqué, Grandia & Canals, 2010), creating steep physicochemical gradients, which most notably include a dramatic increase in dissolved oxygen and pH and the precipitation of solutes over short distances (< 100m). These springs are valued as extreme freshwater systems because of their unusual chemical composition, unique microbial assemblages and specific geological sources (Von Fumetti, Nagel & Baltes, 2007) allowing to show how the abiotic and biotic factors shape the distribution of species (Wellborn, Skelly & Werner, 1996).

Mediterranean climate is characterized by mild, wet winters and hot, dry summers (Lionello et al., 2006). Winter temperatures range from about 8° to 12°C, and summer temperatures can vary between 18° and 30°C. Annual precipitations usually range between 275 and 900 mm, the rain falls mainly during the three months of winter that is when some major storms occur (Gasith & Resh, 1999). This climate influences

greatly the hydrology of the fluvial ecosystems under study, causing typical episodes of floods and droughts.

1.2 Community ecology associated to the fluvial ecosystems

The organisms that form fluvial ecosystems are assembled in communities of various degrees of complexity. Communities are defined as groups of interacting populations that overlap in time and space (Clements & Newman, 2003).The **fluvial ecosystems integrate these biological interactions** with all of the environmental factors that collectively determine how systems function (Allan & Castillo, 2007).

Diversity in local communities can be regulated by local factors (competition, disturbance, abiotic conditions) as well as by regional ones (history of climate, evolution and migration) (Hillebrand & Blenckner, 2002). The assembly of a local community is the result of large sets of species after going through a series of filters, which represent historical as well as ecological constraints on the arrival and survival of organisms at a certain area. Environmental filtering is a process in which abiotic conditions select the organisms that are best adapted to survive to these prevailing conditions (Rath, Maheshwari & Rousk, 2019; Song et al., 2019). Alternatively, biotic filtering supports the hypothesis that competitive exclusion is the dominant force which structures community assembly; whereby, greater competition between similar species leads to the exclusion of species with similar niches (Rapport, Regier & Hutchinson, 1985) (Figure 2).

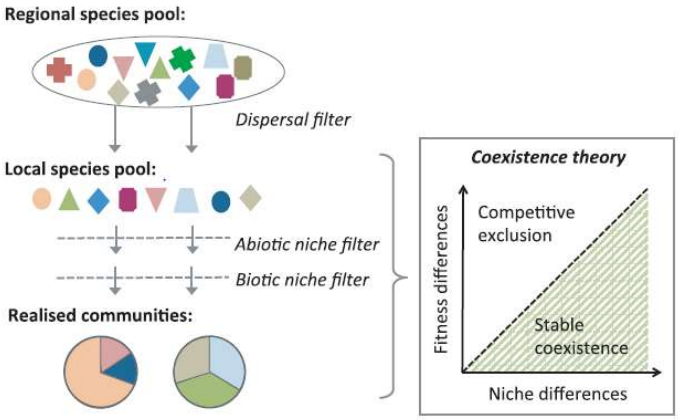


Figure 2. Diagram representation of ecology filtering cascade that shapes the local community. Niche and fitness differences will determine the presence and abundance of species in the local communities. Figure from Zurell (2017).

A variety of approaches have been developed by community ecologists to define and quantify species diversity (Clements & Newman, 2003). This thesis highlights two different measures of species diversity,

α-diversity, that refers to the species richness within a local area, and β-diversity, that is the change in species between adjacent habitats.

2. Stress ecology

2.1. Concepts and importance

Natural environments have always been hostile and unavoidable for organisms; in such a way that they can come to experience **stress**. The ecology of the stress defines stress as a condition suffered by an organism due to environmental factors that bring it near or over the edges of the reference range of its ecological function (ecological amplitude or ecological niche of the species) (Figure 3). These environmental factors are known as stressors. The stressors can be of a different nature, including chemical, physical or biological ones (Steinberg, 2012). The specific physiological response of an organism, induced by the stressor, is the stress response. Some organisms can survive temporarily outside its niche, although without growing nor reproducing. So, they need to be relieved from the stress by moving back to the niche (using behavioral mechanisms or suppressing the stressor), causing temporary physiological adaptations or changing the boundaries of the niche (by genetic adaptation) (Straalen, 2003).

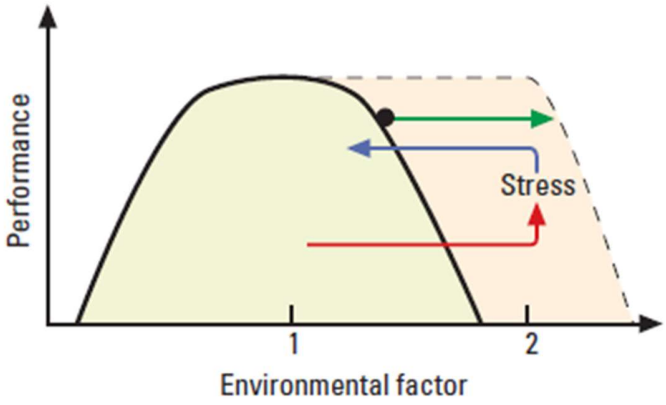


Figure 3. Scheme of niche-based definition of stress. Stress appears when an environmental factor passes from point 1 to 2, so the specie is forced out of ecological niche (red line). Stress responses provide temporary survival returning to its niche (blue line). The borders of the niche are extended by the specie's adaptation (green line).From Straalen (2003).

In addition, stress can be defined in a more realistic way, as a deviation from the normal operating range (NOR) in a multidimensional space (Figure 4). NOR is defined by Kersting (1984, 1988) as 95% of confidence space of undisturbed areas. This concept of multidimensional stress can be applied to the level of communities or even ecosystems.

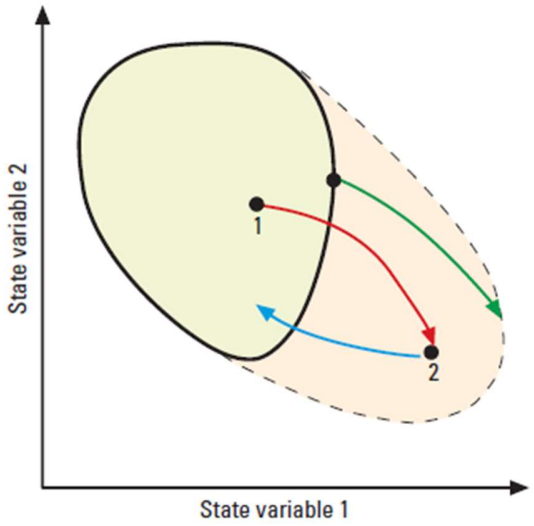


Figure 4. Multivariate stress in a community. The scheme follows the same pattern of colours and arrows as in Figure 3 but all the combinations of states in absence of the stress are defined as the NOR. In this figure the stress is defined by two states variables, but can generalizer to more variables. From Straalen (2003).

Nowadays with global change, the most common situation in fluvial ecosystems is the presence of multiple stressors, that include natural (biotic and abiotic factors) and human made disturbances that co-occur and interact. When this occurs, complex responses from additive to multiplicative can appear. Additive responses arise when the joint effect of two or more stressors equal the sum of individual effects. On the other hand, a multiplicative response takes place when the joint effect is greater (synergism) or lesser (antagonism) than the sum of individual effects (Jackson, Loewen, Vinebrooke, & Chimimba, 2016; Piggott, Townsend, & Matthaei, 2015).

Overall, several natural and anthropic stressors, acting at the same time, may become essential driving forces in the functioning of the ecosystems.

2.2. Metal pollution, a factor of stress

A stress factor of concern for the freshwater ecosystems is that caused by **metal pollution**. High concentrations of metals in freshwater can affect negatively to the environment. Natural or anthropogenic activities can be the sources of metals (Figure 5).

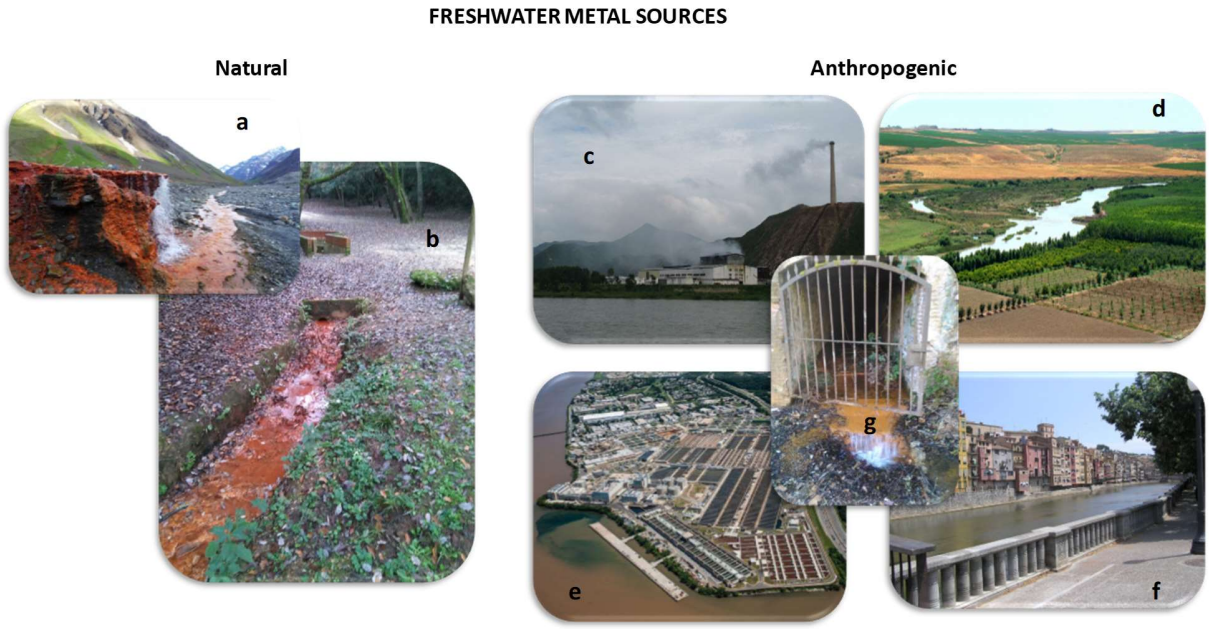


Figure 5. Metal sources to fluvial ecosystems. Natural sources: a) a natural weathering of sulfide rocks feeds acid and iron into the spring in Alaska from Andrew Mattox (http://www.groundtruthtrekking.org/) and b) Fe and CO_2 rich groundwater spring in the Llémena watershed (Girona, Spain) from María Argudo. Anthropogenic sources: c) a smelting plant across the Yalu River, Ji'an (China), d) Tigris River, an important river for agriculture just outside Diyarbakir (Turkey), e) Blue Plains Advances Wastewater Treatment Plant (Washington), f) Onyar River across Girona city from Wikimedia Commons and g) Osor Mine source at Osor River (Girona) from Carmen Espinosa.

Soils inherit trace metals from parent materials. Heavy metals can be released into natural waters due to mineral deposit-water interactions, such as weathering (Figure 5a). This increases the concentration of metals in the water causing the deterioration of the quality of the water in the surrounding areas (Kacmaz, 2020). For instance, Verplanck, Nordstrom, Bove, Plumlee & Runkel (2009) reported that metal-rich waters, produced by oxidative weathering and leaching of trace elements from pyritic rocks (with concentrations of 400 mg L^{-1} of Fe, 3.5 mg L^{-1} of Cu and 14.4 mg L^{-1} of Zn), affected adversely the quality of water in streams on the Southern Rocky Mountains. In other specific geological settings, such as those found in intraplate extensional regions, mantellic emanation of CO_2 in faults may lead to the release of metals, such as Fe and As from sediments and rocks. These metals, which are incorporated to the groundwater systems, have made important changes in the biota, limiting and/or inhibiting algal growth (Menció et al., 2016) (Figure 5b).

It is generally accepted, that although high concentrations of metals may occur in natural ecosystems, human activity is one of the major causes of high concentrations of metals in fluvial ecosystems, mainly due to metal manipulation and its wastes. The main sources of heavy metals are mines and industries (Figure 5c and g). For instance, a study of the environmental effects in the mining activity of Troya Mine on the fluvial ecosystems (Basque Country, Spain) (maximum concentrations of 16.09 mg L^{-1} of Zn and 0.34 mg L^{-1} of Fe in water and 6.84 mg g^{-1} and 13.97 mg g^{-1} in sediment, respectively) revealed a de-structuring of the macroinvertebrate community (Marqués, Martínez-Conde, & Rovira, 2003). A High concentration of Pb in water (3 mg L^{-1}), found near a Zn smelter plant in Brazil, reduced the prokaryotic biodiversity (Almeida et al., 2009). There are other sources for heavy metals, such as agriculture or urban pollution (Figure 5d, e and f). Mendiguchía, Moreno & García-Vargas (2007) associated the concentrations of dissolved Ni, found in the Guadalquivir River (average of 2.31 μg L^{-1}), with agricultural activity. However, data of metals from urban sources are not as readily available and very few studies have been carried out focusing on wastewater. For instance, Kahn et al. (2015) reported a concentration of Cr of 210 ± 30 μg L^{-1} which was above the limits deemed permitted by the Environmental Protection Agency (EPA, 2019).

Most western countries are carrying out water treatment and waste management programs to improve the quality of freshwater. In the United States, according to the EPA, the legislation establishes an aquatic life criterion for toxic chemicals, proposing limit values for the concentration of specific pollutants which include a great number of metals. These measures try to avoid deleterious effects on the majority of species in a given environment. In Europe, the Water Framework Directive (Directive 2008/105/EC) aims to achieve good surface water chemical status to avoid the loss of biodiversity, as well as to preserve human health. This directive highlights the importance of evaluating the effects of 33 pollutants, referred to as priority pollutants, composed mainly of metals, organic substances and emerging contaminants (European Comission, 2019). In Spain, the Royal Decree 817/2015 establishes the criteria for monitoring and evaluating the state of surface waters and environmental quality standards, by proposing priority substances (Ministry of Agriculture Food and Environment, 2015) in which metals such as Zn, Cu, and Cr are also included.

Although all of these measures generate an important improvement in the quality of water, metal pollution is still a problem in fluvial ecosystems due to its high biotoxicity, perdurability and bioaccumulation across the food chain (Zhang et al., 2014), which causes adverse effects on biota and contributes to the deterioration of fluvial ecosystems' integrity (Corcoll, Bonet, Leira & Guasch, 2011). It is important to highlight that nowadays the situation of chronic metal pollution, even at a low

concentration, is very common but causes serious environmental health effects on fluvial ecosystems in the long run. This type of pollution may lead to gradual effects, which are difficult to differentiate from those of natural environmental variations (Moore, 2002). This natural variation could depend on the source of pollution, on the hydrological regime or on the transfer processes from the water to other compartments (Guasch et al., 2010). For instance, in Mediterranean rivers, water scarcity can exacerbate the harmful effects of metal exposure due to low dilution (Guasch, Serra, Corcoll, Bonet & Leira, 2009). Therefore, it is very important to have the appropriate tools to detect and evaluate the effects of this chronic low metal pollution in fluvial ecosystems.

3. Specific tools to assess the impact of chronic metal pollution in fluvial ecosystems

3.1. Prokaryotic communities stand out as bioindicators inside epilithic biofilm

Fluvial biofilms are consortia of phototrophic (green algae, diatoms and bacteria) and heterotrophic (bacteria, fungi and protozoa) organisms which make up complex and well-structured assemblages embedded in a polysaccharide matrix (Sabater & Admiraal, 2005). Biofilm attached to rock surfaces is referred to as epilithic biofilm (Guasch & Sabater, 1994) which is studied in this thesis (Figure 6). **Epilithic biofilms** have a complex 3D structure and generally high algal biomass. However, in shaded environments, such as in forested rivers, heterotrophic biomass becomes more relevant (Romaní, 2010).

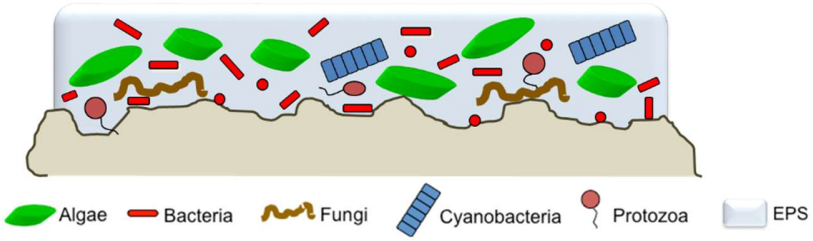


Figure 6. An idealized scheme of epilithic river biofilm components (mainly algae and prokaryotes) embedded in an exopolysaccharide matrix (EPS). Adapted from Mora-Gómez, Freixa, Perujo & Barral-Fraga (2016).

Biofilms are crucial in the ecosystem functionality in such a way that phototrophic organisms are able to carry out primary production (photosynthetic activity) (Underwood et al., 2005) that prokaryotes and fungi are also able to process organic matter (Bärlocher, 2005) and to contribute to biogeochemical cycles (Battin, Besemer, Bengtsson, Romani & Packmann, 2016). Moreover, biofilm communities are an essential source of matter and energy located in the base of the food-web (Lefrançois et al., 2011). Therefore, any perturbation in the community, for instance, could provoke a significant impact to higher trophic levels.

Biofilms are the first to interact with dissolved substances, such as metals, so they can easily be affected. Freshwater biofilms often accumulate metals at greater concentrations and even quicklier than in sediments. This metals can be transferred to higher trophic levels such as protozoa, invertebrates or fish, increasing their toxicity (Ancion, Lear, Dopheide & Lewis, 2013; Farag et al., 2007; Serra, Corcoll & Guasch, 2009). Moreover, fluvial biofilms are able to reflect historical and current effects of chemical stressors (Corcoll et al., 2015; Proia et al., 2016; Sabater et al., 2007). Thus, they can be used as biondicators of the effects that metals cause in the ecosystems.

The study of communities provides a much broader context for the assessment of environmental contamination than the study of individual species (Clements & Newman, 2003). In this thesis, **prokaryotic communities,** which form ephilitic biofilms, will be highlighted as a powerful biological model to assess the effects of metal pollution under different environmental scenarios. In order to emphasize the prokaryotic fraction within the microbial community of biofilm, the term “microbial community” will be used to refer to prokaryotic community hereinafter.

Microbial communities can be used as indicators of environmental pollution, since any environmental impact can be translated into structural and functional changes that can be clearly recognised (Shahsavari, Aburto-Medina, Khudur, Taha, & Ball, 2017). Moreover, the community response to environmental pollutants can be easily observed thanks to the fact that prokaryotes are very abundant, diverse (Figure 7a) and have a high growth rate (Figure 7b) (Lear & Lewis, 2009; Ma et al., 2015).

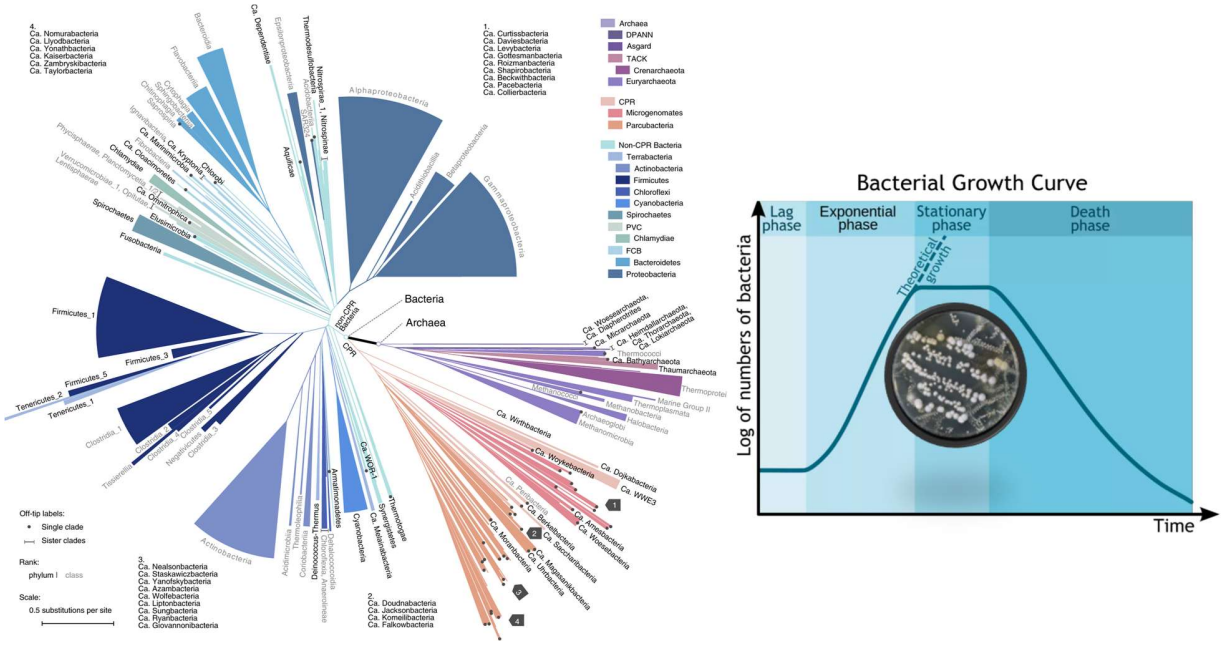


Figure 7. a) The prokaryotic phylogenetic tree based on individual trees of 381 globally sampled marker genes and branch lengths estimated based on 100 most conserved sites per gene (Zhu et al., 2019) b) The bacterial growth curve represents the number of living cells in population over time from Michal Komorniczak (Wikimedia Commons). The annexed image highlight bacteria growing exponentially in a Petri dish from Wladimir Bulgar/Science Photo Library/Getty Images.

A variety of molecular methods have been recently developed to investigate the microbial community structure and its potential function. Specifically, amplicon sequencing allows a high number of gene sequences to be recovered, since the sensitivity is increased by several orders of magnitude compared to previously used molecular techniques, such as cloning or fingerprinting methods (Lear et al., 2013; Qu et al., 2017; Wang, Sudduth, Wallenstein, Wright & Bernhardt, 2011). Amplicon sequencing is carried out following PCR amplification and further sequencing of any of the target genes of interest, such as the 16S rDNA gene, which codifies for the highly conserved 16S rRNA macromolecule, from which it is possible to infer phylogenetic and taxonomic information (Rodicio & Mendoza, 2004). Consequently, this powerful method can assess community responses at low taxonomic levels. This fact could highlight the important ecological attributes of these members that serve as potential biological indicators of ecosystems' health (Hermans, Buckley, Case, Curran-Cournane & Taylor, 2017; Siddig, Ellison, Ochs, Villar-Leeman & Lau, 2016).

3.2. Field studies

Nowadays, there is a lack of capacity to assess and predict the effects of toxicants on ecosystems at large spatial scales. Risk assessment is made under conditions of uncertainty (Beketov & Liess, 2012). In fact, the most common approaches are made in the labs at a small scale such as single species toxicity tests and microcosms experiments. These experiments provide high control on the variables of study and they are easily replicable even though they lose ecological realism (Figure 8). However, field studies are conducted at larger spatiotemporal scales and that allows ecologists to know about the complexity and dimension of the ecosystem; thus providing a better approach to reality. Despite the important advantages of field studies due to the understanding and the need to use this approach, they are less common since there is a lack of rigorous control and they are difficult to replicate; specially, field experiments that have many logistical difficulties to be carried out (Clements & Newman, 2003) (Figure 8). Moreover, the complexity of natural settings includes a myriad of confounding factors that may hinder our capacity to derive sound conclusions and link stressor effects to ecosystem responses (Romero, 2019).

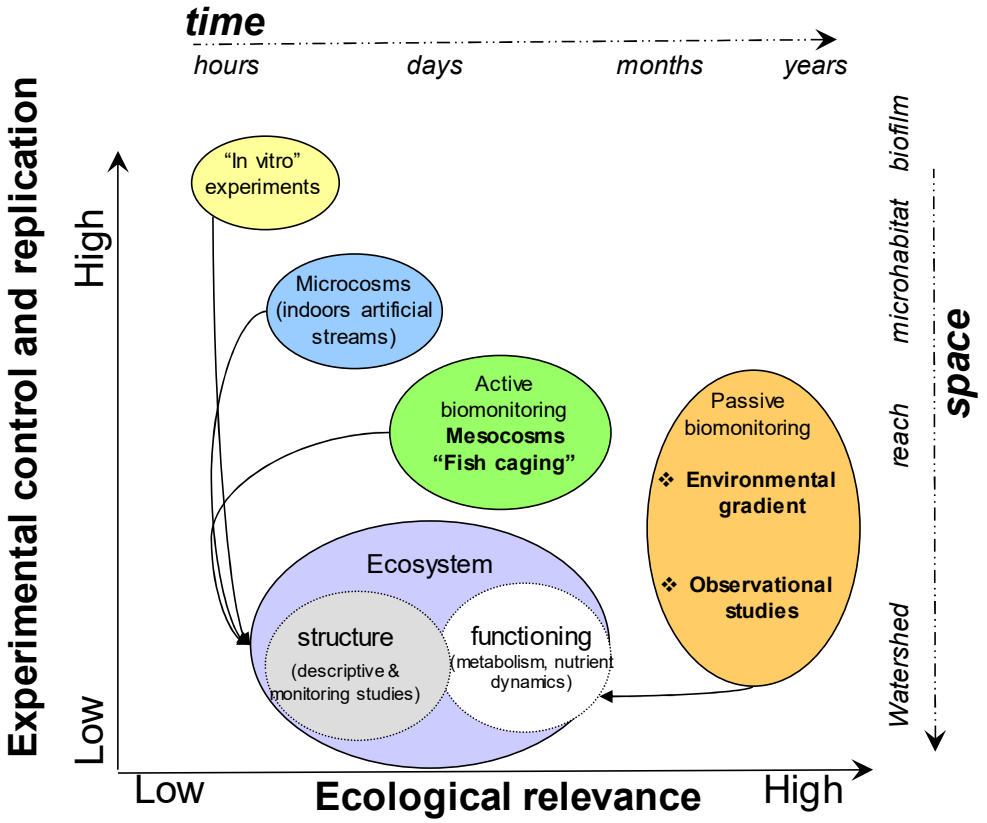


Figure 8. Relationship between ecological relevance and experimental control and replication in fluvial ecosystems studies. The field approaches in bold are carried out in this thesis. Modified from Clements & Newman (2003).

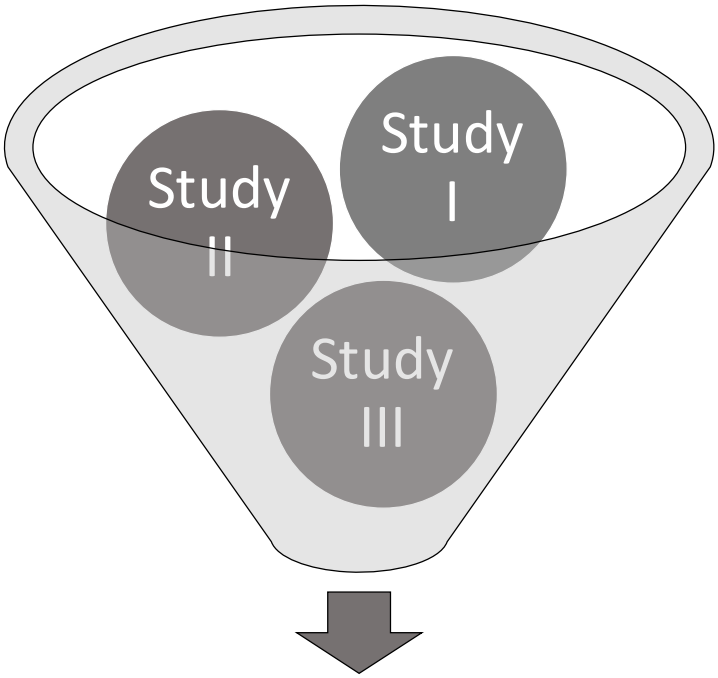
Focusing on the advantages of field studies, this thesis will study microbial communities following two field approaches: passive biomonitoring and active biomonitoring. The latest is going to include the use of mesocosms. Moreover, both approaches will be supported by multivariate analyses.

To assess pollution effects on aquatic ecosystems, biomonitoring (the study of biological responses of exposed organisms) seems to be an appropriate method (Lacroix et al., 2015). Two different strategies can be used in **biomonitoring: passive or active**. Passive biomonitoring consists of collecting organisms from the environment to analyse. The passive approach is very useful, it is simple and gives the possibility to set-up long-term measurements (Turley et al., 2016). On the other hand, active biomonitoring involves direct manipulation of organisms. The chemical and biological consequences of this manipulation can then be monitored in space and time to assess the effects of exposure on selected endpoints (Wang et al., 2011). This approach is specially valuable because it can show causation between stressors and biological responses and disentangle different drivers, allowing testing hypothesis (Clements & Newman, 2003).

Mesocosms are outdoor experimental systems that reduce the complexity and provide replicable and controlled test systems that allow ecologists to have the environment under more controlled conditions, providing a link between observational investigations and laboratory studies (Stewart et al., 2013). They have been widely used to study trophic interactions, such as top-down control exerted by fish (Flecker, 1996; Rodríguez-Lozano, Verkaik, Rieradevall & Prat, 2015; Rubio-Gracia et al., 2017; Winemiller et al., 2014). However, its application in fluvial ecotoxicology is less common (Gardham, Chariton & Hose, 2015; Roussel et al., 2007).

In order to interpret complex data obtained from biological communities, its direct and indirect relationship with the environment, as well as the effects of metal pollution, multivariate analyses are in many cases required. These analyses can perform joint ordination of several sets of physical, chemical and biological variables assembled from the field, and also define the distribution patterns of organisms according to the driving pressures in a given set of sites (Sabater, Barceló ,et al., 2016). This is a correlational approach with recognised weaknesses (Legendre and Legendre, 1998), but also powerful enough to define emerging patterns on the structure of ecological data (Legendre and Legendre, 1998). Moreover, the popularity of multivariate analyses is continuing to increase and their application to microbial ecological data has become technically simplified. However, the large amount of data needs more powerful statistic and bioinformatic tools that are being developed (Buttigieg & Ramette, 2014; Paliy & Shankar, 2016).

OBJECTIVES AND EXPECTED RESULTS



MAIN OBJECTIVE

The main aim of this thesis is to gain a better understanding of the effects of metals, from natural and anthropogenic sources, in the structure and function of the prokaryotic communities developed in epilithic biofilms of fluvial ecosystems. A holistic approach, based on the combination of concepts and molecular techniques from stress ecology to microbial communities' ecology, is used. This complex view requires the use of several endpoints related with diversity, taxonomy and activity of microbial communities.

To achieve this main goal, the following specific objectives and expectations are formulated through three field studies:

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

The specific objective of this study is to provide an interpretation of the molecular data, based on the RNA and DNA sequence biomarkers that contribute to the understanding of the effect of low metal chronic pollution and eutrophication on the prokaryotic communities.

It is expected that the RNA data will provide more precise information about the possible changes in the structure and potential function of fluvial biofilm's prokaryotes under different conditions of stress than the DNA data. Moreover, it is expected that mining metals would provoke clearer changes in the structure of prokaryotes (α and β-diversity) than other anthropogenic metal pollution sources or eutrophication.

Following these ideas, we want to focus on the effects of a higher concentration of mining metals, which occurs under low flow conditions, in the structure and function of microbial communities inside of the natural complexity of fluvial ecosystems when adding top-down control of macroconsumers, such as fish, by means of Study II.

Study II. Direct and indirect effects of multiple stressors on the microbial communities in a mining area

The specific aim of study II is to ascertain the impact of a multiple-stressed environment (metal pollution + hydrological alterations + nutrient enrichment) and its biological interactions in the structure and function of microbial communities that form fluvial biofilms.

It is expected that remarkable differences in the structure (β-diversity) and function (nutrient uptake) of biofilm microbial communities along a chemical gradient will be found. It is predicted that these differences would be the highest under multiple stresses situations, when the metal effluents and hydrological alterations coincide. In addition, it is hypothesized that the presence or absence of fish would affect the biofilm differently, i.e. the presence of fish could produce bioturbation to biofilm, and the absence of fish an accumulation of biomass. Furthermore, it is believed that fish effects would differ along the water scarcity and pollution gradient, reflecting the chronic effects of these multiple stressors in the different trophic levels and their interactions.

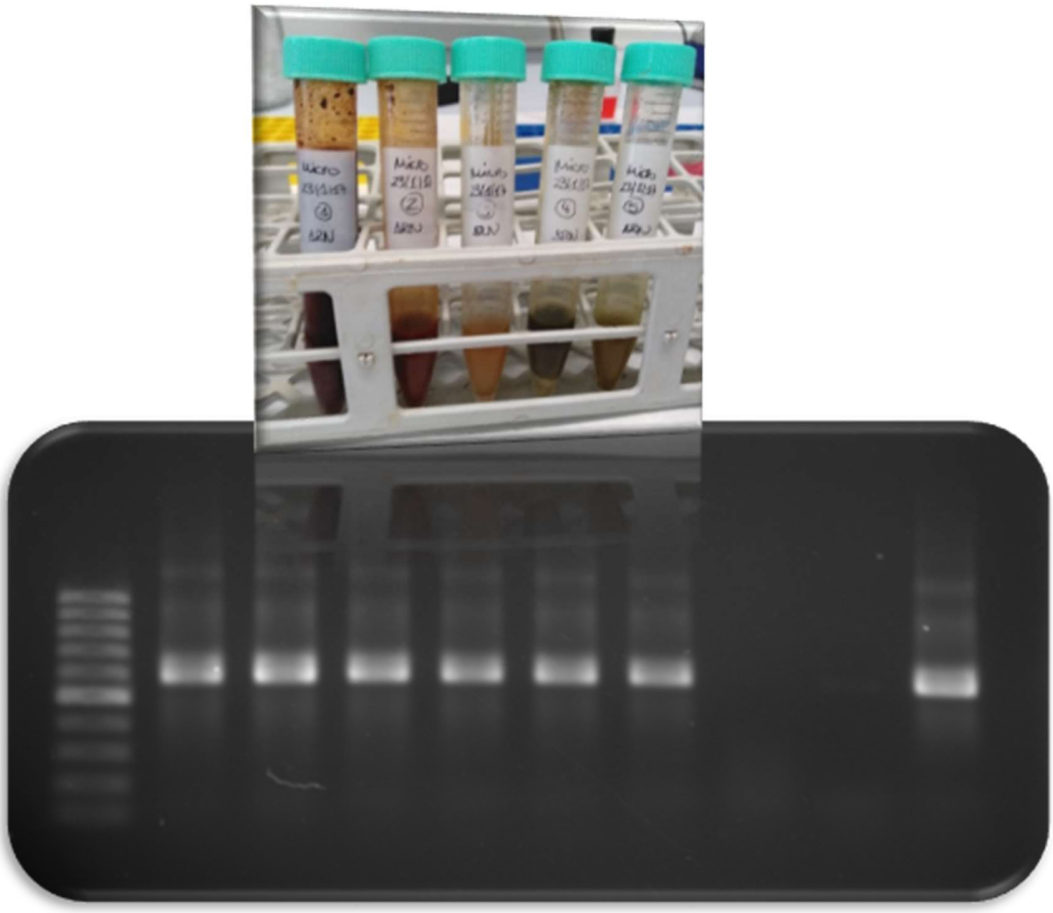
In this sense and with the prior knowledge acquired, our intention is to deepen the knowledge on the biotic and abiotic drivers that control the structure and functions of the microbial community of biofilm with a high metal concentration from a natural source. In this case, we want to apply a novel bioinformatic tool with a great taxonomic resolution (reaching genus or even species taxonomic levels) that could allow us to improve what we already know about the structure and potential functions of the prokaryotic fraction of biofilms along Study III.

Study III. Environmental drivers of microbial community structure in a high iron calcareous-spring

The goal of this study is to know the drivers of microbial community structure and function by the determination of α and β-diversities of prokaryotes, primary production of biofilm and leaf litter breakdown/decomposition from a natural iron (Fe) spring.

It is expected that a shift from environmental filtering to biotic filtering along a chemical gradient of decreasing Fe concentration will be found. It is assumed that the most extreme chemical conditions will reduce primary production and leaf litter decomposition rate and will exert a strong filter on microbial community composition selecting metal tolerant or adapted species. On the other hand, under low stress conditions, biotic factors will play a more important role. High primary production, leaf litter breakdown, oxygen concentration and nutrient availability will predominate. The main limits to growth will be the competition with other organisms, thus promoting the competitive exclusion.

GENERAL METHODOLOGY



1. Study sites

All the field studies within this doctoral thesis were carried out in the Ter River hydrographical basin. The Study I was carried out in the main course of the Ter River and in two of its tributaries the Llémena and Osor Rivers. The Study II was conducted in the Osor River and the Study III in a small Fe spring within the Llémena watershed (Figure 1).

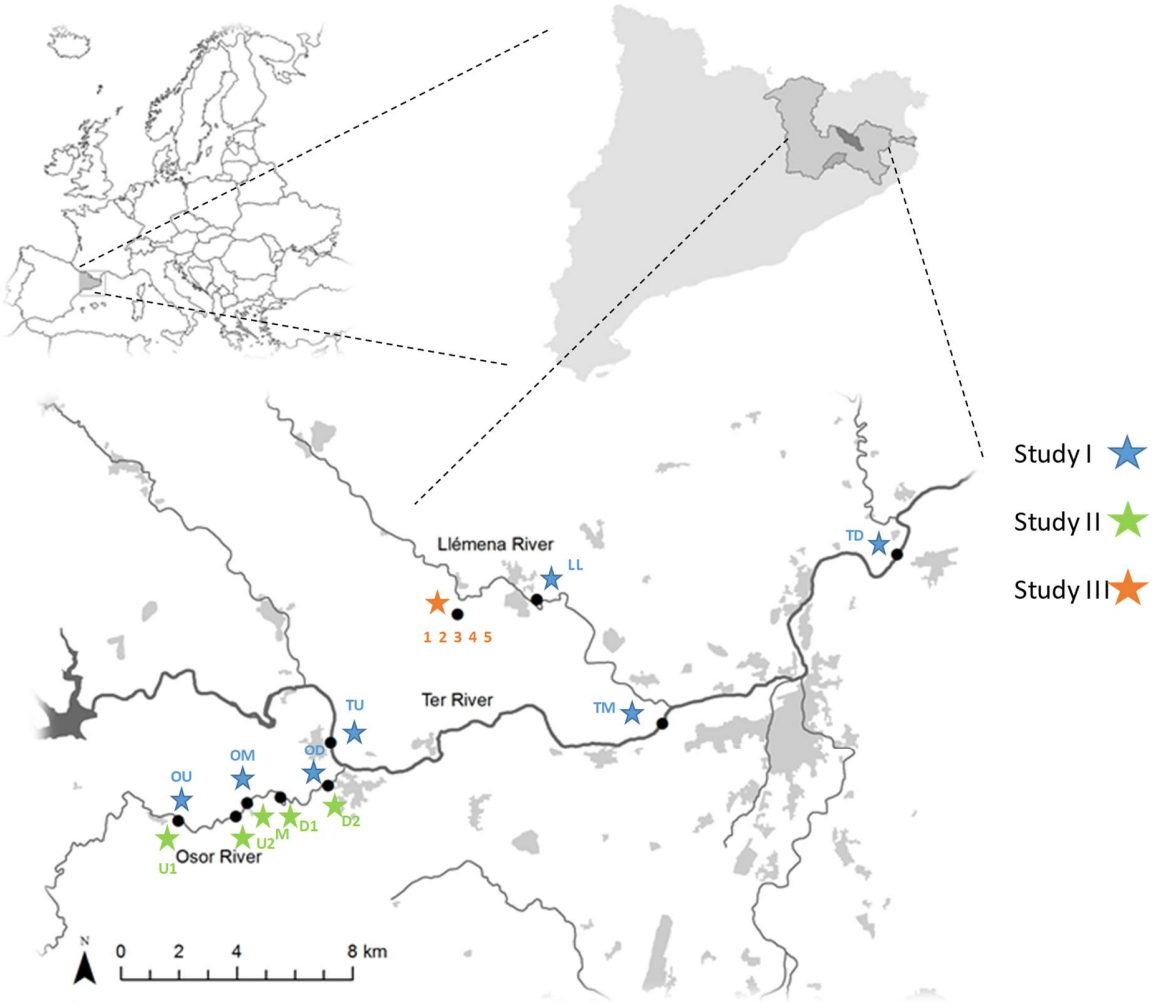


Figure 1. Study areas in the Ter River hydrographical basin (Catalonia, Spain), the stars of different colours represent the selected sampling points where physicochemical and biofilm samples were collected for the Studies I, II and III. From Lluis Zamora.

The source of the Ter River is in the middle of the Catalan Pyrenees (at 2,400 m) and the river flows into the Mediterranean Sea at the coastal town of L´Estartit. The Ter comprises 3,010 km^2 of basin surface and is 208 km long, being the longest and largest of the Catalan internal basins in the northeast of Spain. Its

drainage area is mainly calcareous except for the head, which is siliceous (Céspedes, Lacorte, Ginebreda & Barceló, 2008). It forms the Sau-Susqueda-Pasteral dam system which has a total capacity of 375 hm^3 of water and supplies the Barcelona city and its surroundings with raw water for drinking (Espadaler et al., 1997). Along its watercourse, it receives the direct impact of metallurgic, pulp mill, textile and tannery industries (Céspedes et al., 2008).

The Osor River is a second-order stream that flows into the Ter River. It is 23.5 km-long and drains a catchment area of 88.9 km^2 within the Guilleries Mountains (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), 2018). Urban pressures are relatively low, although it receives small amounts of residual sewage from Osor village (354 inhabitants). Moreover, a wastewater treatment plant is located upstream (St. Hilari Sacalm, 5,064 inhabitants) and the hydrology of the stream is altered due to a deviation of part of the flow towards a hydroelectric power station that is partially recovered in the lower part of the stream. The stream is also affected by effluents and runoff from a 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 mine effluent (Bonet, Corcoll, Tlili, Morin & Guasch, 2014). The concentration of Zn commonly exceeds, the toxicity threshold marked by the (EPA, 2019) (120 µg L^{-1} for acute and chronic exposure). Zn is not part of the priority substances in the European Directive 2008/105/EC and in Spain the threshold depends on water hardness (500 µg L^{-1} for $CaCO_3$ > 100 mg L^{-1}) (Ministry of Agriculture Food and Environment, 2015). Previous investigations demonstrated that this low but chronic Zn pollution causes many deleterious effects on fluvial biota. More precisely, it causes a clear decrease in diatom diversity in favour of cyanobacteria and green algae, an increase in malformed diatoms and also an increase in biofilm community tolerance (Corcoll et al., 2012; Tlili et al., 2011). Furthermore, Zn pollution reduce the seasonal patterns of antioxidant enzymes activities (AEA) and the AEA diversity (Bonet et al., 2014).

The Llémena River is also a tributary of the Ter River. It is a 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 the agriculture, livestock, and water diversion for irrigation, as well as, urban activity. In particular, in the Llémena basin, there is the Can Verdaguer spring, also called Fe spring. It is located above the Llorà fault. This fault puts in contact igneous and metamorphic rocks from the Ordovician period (zone mainly forested) and young materials from the Quaternary (zone mainly for agricultural and urban activities). Based on the chemical composition of water and the geology of the

zone, it can be deduced that the spring aquifer receives water from rain, which is enriched with Fe throughout water filtration in soil. Moreover, this water dissolves carbonates from carbonated deposits found in the lower part. Finally, the water rises to the surface from an artificial fountain. More precisely, the water flows through a 12.6 m long pipe and is discharged into a slightly modified water canal covered by natural sediments and vegetation debris mainly from the surrounding trees of *Quercus pubescens*. The canal is 62 m long, 0.93 m wide and 0.62 m deep (on average) (Guasch, Acosta, Urrea, & Bañeras, 2012; Menció et al., 2016). According to these authors the spring is permanent, with a stable flow of 0.4 L s^{-1} and the temperature of source water is 16ºC. The spring is characterized by high Fe concentration of 7,000 mg L^{-1} and has been classified as one of the CO_2 rich springs in this area.

2. Methods

This general section provides an overview of collection, storage and methods that this thesis employed to achieve the planned objectives through water and biofilm samples analyses. The protocols used for collecting and storing water and biofilm samples are common for the three studies. However, some techniques and analyses are different, as it is described below for each study (Figure 2, Table 2, Table 4). The design, sampling, some specific details of the laboratory analyses and statistical analyses were quite different, so they will be described within each study in order to facilitate the understanding of the results and discussion of each of them.

Figure 2. Images of some procedures followed to analyse water and biofilm samples. a) falcons for nutrient analysis, b) biofilm collection by scrapping, c) dissolved and bioaccumulated metal analysis, d) PCR products on agarose gel, e) Chl-a fluorescence measurements. From María Argudo and Helena Guasch.

2.1. Pre-treatment in the field

Water samples for inorganic nutrients were filtered through 0.7 µm pore-diameter glass fiber filters GFF (Whatman). Water samples for dissolved organic carbon (DOC) and dissolved organic matter (DOM) were filtered with pre-combusted filters (for 4 h at 450 ºC). Water samples for metal analysis were filtered through 0.2 µm pore-diameter nylon membrane filters (Whatman) and then the samples were acidified with 1% HNO_3 (65% Suprapur©, Merck). Finally, water samples for suspended solids (SS) and alkalinity (Alk) were not filtered. Afterwards, all the samples were frozen at -20 ºC until analysis.

Biofilm samples for ash free dry weight (AFDW), chlorophyll-a (Chl-a), C:N:P and metal bioaccumulation were scrapped with brushes, cell scrapers and collected by Pasteur pipettes. These samples were also stored at -20 ºC until analysis. On the other hand, biofilm samples for microbial community analysis were scrapped using sterilized sampling material (cell scrapers, Pasteur pipettes and gloves) (Figure 2b) and the samples were immediately preserved in liquid nitrogen and then stored at -80 ºC.

2.2. Physicochemical analysis of water samples

- Physicochemical parameters.
  - Temperature (Tª), pH, dissolved oxygen (O_2) and conductivity (Cond) were analysed "in situ" with a multi-parametric probe (WTW Meters, Weilheim, Germany).
  - Water velocity ($\vec{v}$) was measured with a flow probe (Schiltknecht 43221; MiniAir2).
  - Discharge was calculated with the measures of width, depth and water velocity of the stream channel.
- Inorganic ions (Figure 2a).
  - PO_4^{3-} was analysed following the method of Murphy & Riley (1962).
  - NH_4^+ was measured by Reardon, Foreman & Searcy (1966) method.
  - Other cations (K^+, Na^+, Ca^{2+} and Mg^{2+}) were analysed by ion chromatograph DIONEX ICS-5000.
  - Other anions (Cl^-, NO_3^{2-}, NO_2^- and SO_4^{2-}) were measured using ion-chromatography (761 Compact IC, Metrohm, Herisau, Switzarland) (Hach, 1992).
- SS were analysed according to standard methods of the American Public Health Association (APHA) (Elosegui & Butturini, 2009).
- DOC concentration was determined using a total organic carbon analyser Shimadzu TOC-V CSH (230V).

- DOM was determined indirectly as absorbance measures at 254 nm (UV-2401PC, Shimadzu) following the technique developed by Weishaar et al. (2003).
- Alk was measured following standard methods (Snoeyink, Jenkins & Jenkins, 1980).
- Dissolved metals were analysed by inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies, Inc., Wilmington, DE) (Figure 2c) and samples with high metal concentration were determined by inductively coupled plasma optical emission spectrometry (ICP-OES 5100 Agilent Technologies). The detection limits are showed in Table 1 for each study.

Table 1. Detection limits for dissolved metals in each study. In Study I the different values for each year of the study are shown. Half of these values' concentration were used to analyse these data. In Studies II and III, half of these values' concentration were used for data analyses only when the values are <15% of the total metal concentrations data (EPA Quality Staff, 2006). Values underlined show the detection limits of ICP-OES, the others are ICP-MS values.

	Study I		**Study II**	**Study III**
Year	**2016**	**2017**	**2017**	**2016-2017**
Zn (µg L^{-1})	0.90	0.28	0.40	3.36
Mn (µg L^{-1})	1.96	0.49	1.05	<u>0.47</u>
Fe (µg L^{-1})	2.14	0.41		<u>77.95</u>
Pb (µg L^{-1})	0.91	0.32		
Ni (µg L^{-1})	1.23	0.11	0.19	0.45
Cu (µg L^{-1})				0.34
B (µg L^{-1})				<u>7.02</u>
Sr (µg L^{-1})				<u>12.17</u>
Al (µg L^{-1})				12.91
Cr (µg L^{-1})				0.23
Co (µg L^{-1})				0.09
As (µg L^{-1})				0.16
Ba (µg L^{-1})				0.47

Table 2. Summary of different variables measured with the methods described before in water samples in each study of this thesis.

	Study I	**Study II**	**Study III**
Tª, pH, O_2, Cond			
$\vec{v}$			
Discharge			
PO_4^{3-}			
NH_4^+			
Other cations			
Other anions			
SS			
DOC			
DOM			
Alk			
Metal concentrations			

2.3. Biofilm analysis

- AFDW analysis was used as a measure of total biofilm biomass. The biofilm samples with water were filtered through 0.7 µm pore-diameter glass fiber filters GFF (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. Therefore, the organic fraction was obtained by the difference between mineral content and dry matter (Steinman, Leavitt & Uzarski, 2017).
- Chl-a fluorescence measurements were performed "in situ" by portable amplitude modulated fluorimeter (Mini-PAM fluorometer Walz, Effeltrich, Germany). The measurement was obtained by placing three to six small glass substrata (1.2 x1.2 cm) at the bottom of methacrylate boxes (9 x 15 cm), covered with a small quantity of water for 15-20 min (Figure 2e).
- Chl-a concentration was obtained by the method proposed by Jeffrey & Humphrey (1975). Chl-a was extracted from biofilm samples with 10 mL of 90% acetone at 4 °C for 24 h in dark conditions. Extracts were read at 430, 665 and 750 nm for the calculation of Chl-a content using a spectrophotometer (UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan)). This content was used as a measure of algal biomass.

- C:N:P was calculated in order to obtain C:N, C:P and N:P molar ratios. To measure C and N content, the biofilm samples were lyophilized, homogenized and analysed by elemental analyser (AE2400 Perkin Elmer). On the other hand, P content was determined after a basic digestion (NaOH + $K_2S_2O_8$ + H_3BO_3) in an autoclave (110 ºC for 90 min) (Grasshoff, 1983). Then the inorganic forms of P were analysed following the protocol described by Murphy & Riley (1962).
- Metal bioaccumulation concentration was obtained by lyophilizing, weighing and digesting biofilm samples with 4 mL of HNO_3 (65% Suprapur©, Merck) and 1 mL of H_2O_2 (30% Suprapur©, Merck) in a high performance microwave digestion unit (Milestone, Ethos Sel) using the following method: 85 ºC for 2 min, 145 ºC for 5 min, 210 ºC for 7 min and, finally, 210 ºC for 10 min. Thereafter, the samples were diluted to 15 mL with Milli-Q water. So, the liquid samples were analysed with the same criteria as dissolved metals (Figure 2c). The detection limits are showed in Table 3.

Table 3 Detection limits for bioaccumulated metals in each study. In Study I the different values for each year of the study are shown. Half of these values' concentration were used to analyse these data. In Studies II and III, half of these values' concentration were used for data analyses only when the values are <15% of the total metal concentrations data (EPA Quality Staff, 2006). Values underlined show the detection limits of ICP-OES, the others are ICP-MS values.

	Study I		**Study II**	**Study III**
Year	**2016**	**2017**	**2017**	**2016-2017**
Zn (µg L^{-1})	0.90	0.28	<u>0.66</u>	2.50
Mn (µg L^{-1})	1.96	0.49	<u>2.38</u>	<u>2.04</u>
Fe (µg L^{-1})	2.14	0.41	<u>2.27</u>	<u>3.36</u>
Cd (µg L^{-1})	2.02	1.09		
Pb (µg L^{-1})	0.91	0.32	0.31	
Cr (µg L^{-1})	1.47	1.36	1.03	0.48
Ni (µg L^{-1})	1.23	0.11	0.69	0.26
Cu (µg L^{-1})			0.75	
Co (µg L^{-1})				1.09
As (µg L^{-1})				1.12
Ba (µg L^{-1})				1.95
B (µg L^{-1})				<u>2.28</u>
Al (µg L^{-1})				<u>4.87</u>
Sr (µg L^{-1})				<u>0.69</u>

- The uptake of NO_3^-, $NH_4^+$and PO_4^{3-} was measured following an adaptation of Rubio-Gracia et al. (2017). This analysis will be described in more detail in the Study II.
- Biofilm samples used for molecular analysis of the microbial community (Figure 3) were thawed, centrifuged at 4,000 rpm for 10 minutes at 4 ºC and weighted in order to keep only the fresh pellet for nucleic acids extraction.

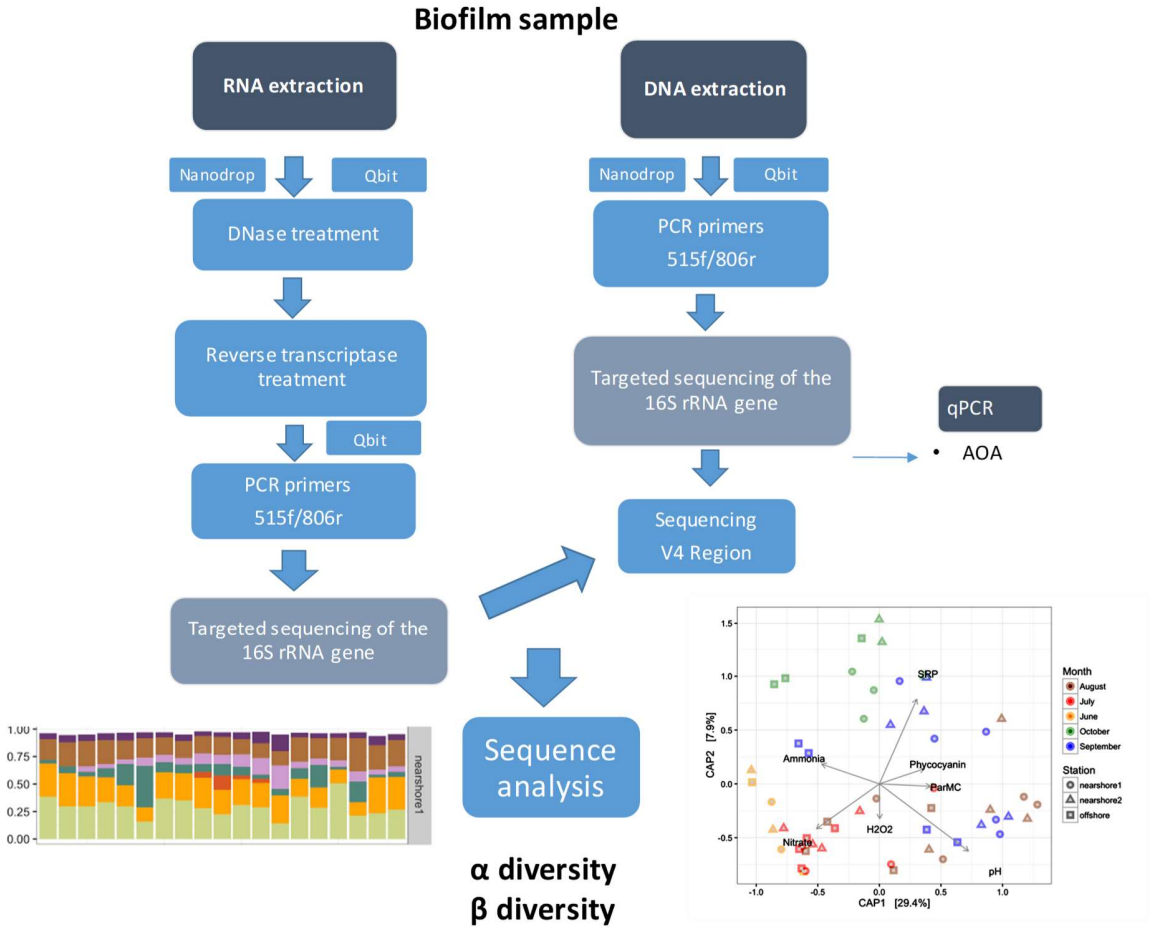


Figure 3. Scheme of the workflow of molecular analysis of the microbial community in the studies.

- DNA extraction was performed with different commercial kits such as Soil DNA isolation plus kit (Norgen Biotek, Ontario, Canada) for the Study I and DNAeasy® PowerBiofilm® Kit (Qiagen) for the Studies II and III. In both we included a step of mechanical cell disruptions (3 cycles at 5.5 power intensity for 30 sec) with FastPrep®-24 Instrument. The DNA extracts obtained were quantified with a Qubit® DNA Assay Kit (Thermo Fisher Scientific,

EEUU) and the quality of this nucleic acid was determined with a Nanodrop ND-1000 (NanoDrop Technologies Inc, New York, USA).

- RNA extraction was done using the AllPrep DNA/RNA Mini kit (Qiagen) in the Study I. The biofilm samples followed the same pre-treatment and mechanical cell disruptions as in DNA extraction. Each extract of RNA was treated with DNases using TURBO DNA-free Kit (Ambion, Inc) and quantified by Qubit® RNA Assay Kit (Thermo Fisher Scientific, EEUU). The quality was determined with a Nanodrop ND-1000 (NanoDrop Technologies Inc, New York, USA).
    - Retrotranscription of RNA extracts was carried out with SuperScript® III First-Strand Synthesis System for RT-PCR KIT (Invitrogen, EEUU), using random hexamers to synthesize first-strand cDNA. Then, this cDNA was quantified again by Qubit® cDNA Assay Kit (Thermo Fisher Scientific, EEUU).
- Control PCRs were made for DNA (positive) or RNA (negative) extracts to ensure that the samples contained DNA or did not, respectively. They were carried out with primers for the 16S rRNA gene, the 357F and 907R (Weisburg, Barns, Pelletier & Lane, 1991) using a PCR Core Kit (Qiagen). PCR amplifications were performed in a GeneAmp PCR System 9700 (Applied Biosystems) following these conditions: 94 ºC for 4 min, 10 cycles of 94 ºC for 30 s, 61 ºC for 45 s, 72 ºC for 1 min; 30 cycles of 94 ºC 30 s, 56 ºC for 45 s and 72 ºC for 1 min, finally 72 ºC for 10 min. PCR products were checked by an agarose gel (1.5%, w/v) electrophoresis with a loading buffer using the Marker GeneRuler 1,000 bp to check for the PCR product specificity (Figure 2d). The gel was stained in an ethidium bromide solution (0.2 µg mL^{-1}) for the DNA visualization on a transilluminator Herolab UVT-20M.
- Quantitative PCR (qPCR) was conducted in a Roche LightCycler® 96 System to determine copy numbers of amoA genes from ammonia-oxidizing archea (AOA), using the primers pair Crenamo-A23f/-Crenamo A616r (Tourna, Freitag, Nicol & Prosser, 2008). The reactions contained 10 µl LightCycler® 480 SYBR Green I Master (Roche Life Science, Basel, Switzerland), 1 µL of each primer (20 µM), 2 µL of DNA from samples at 5 ng μL^{-1} and 6 µL of Milli-Q water for a total volume of 20 µl. Calibration curves were prepared by serial dilutions (10^8-10^1 copies) of plasmid with AOA amoA gene fragments cloned in the 3,957 bp vector pCR®4-TOPO® (Invitrogen, Carlsbad, CA). Amplification was performed following conditions proposed by Tourna et al. (2008) with minor modifications. Controls without templates gave null or negligible values.

- Inhibition tests were performed for all samples supplementing the qPCR reaction mixture with plasmids at a known concentration (10^5 copies μL^{-1}). These plasmids harbour the M13 region, a target for M13F-20 and M13R primers, which is supplied by the pCR®4-TOPO® Cloning kit (Invitrogen, Carlsbad, CA). Plasmid quantifications in the presence of samples did not differ from plasmid controls devoid of samples.

➢ Analysis of 16S rRNA gene was carried out from the DNA and RNA extracts. Sequencing were performed at MSU Genomics Core (Michigan, USA) using a 2x250 bp paired-end Illumina MiSeq platform (Mardis, 2008). The V4 region of the 16S rRNA gene of the prokaryotes was amplified using the 515F/806R primer pair (Caporaso et al., 2011). Afterwards the quality of raw reads was initially checked using the FastQC application (Andrews, 2010). Raw sequences were treated following different protocols along the three studies to increase the resolution of taxonomy, so these will be explained carefully in each study. However, all of these treatments had the objective to calculate the α and β-diversity of prokaryotes.

Accordingly Gotelli & Chao (2013):

- α diversity was calculated with different indices as observed OTUs (Sobs) and Chao1.
  - Sobs indicates the total number of species, in this case OTUs or amplicon sequence variants (ASVs) presents in the sample.
  - Chao1 estimator uses only the numbers of singletons and doubletons (rare species), which complement Sobs to obtain a good estimation of richness.
- β-diversity was calculated with Shannon index (H') and Inverse Simpson index (1/D).
  - H' quantifies the uncertainty in the species identity of a randomly chosen individual in the assemblage.
  - 1/D measures the probability that two randomly chosen individuals (selected with replacement) belong to two different species.

Table 4. Summary of the different procedures used in biofilm samples in each study of this thesis.

	Study I	Study II	Study III
AFDW	■	■	
Chl-a fluorescence	■		■
Chl-a		■	■
C:N:P		■	
Metal bioaccumulation	■	■	■
Nutrient uptake		■	
DNA extraction and control PCR	■	■	■
RNA extraction and control PCR	■		
qPCR		■	
Analysis of 16S rRNA	■	■	■

RESULTS

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

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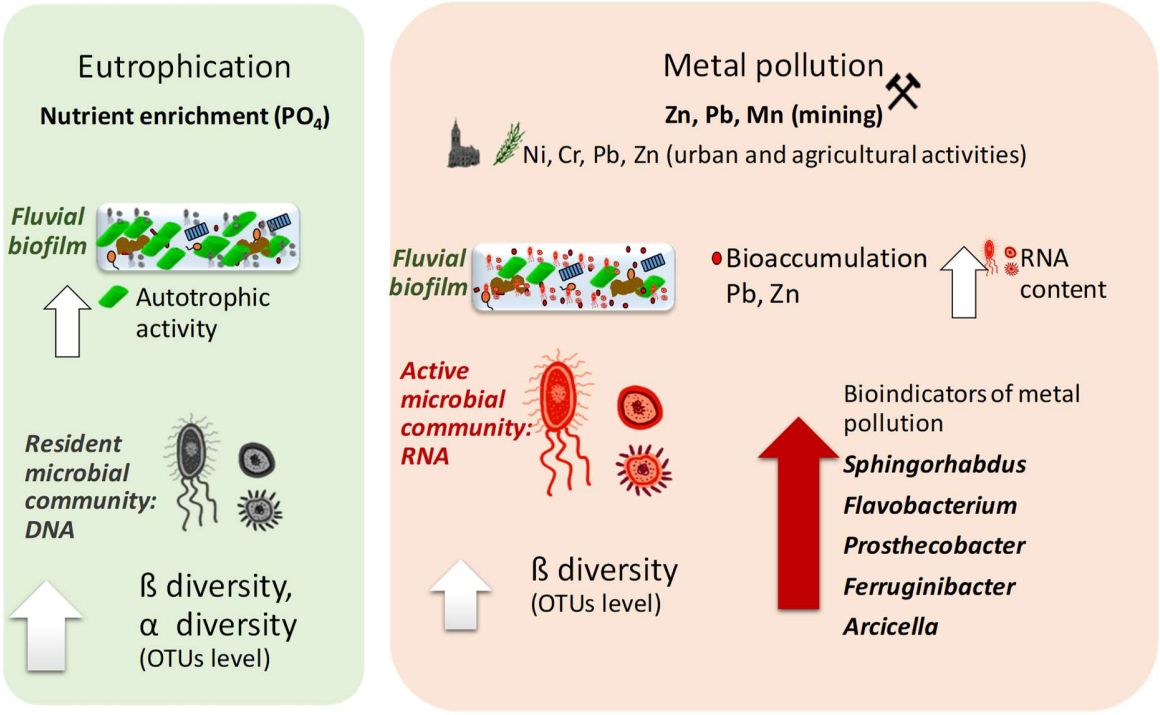


1. Contextualization

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, becoming a principal threat to the ecological integrity of river ecosystems (Allan, 2004). Overall, these activities generate multiple pollutants such as metals (Beasley & Kneale, 2004; Brunzel, Kellermann, Nachev, Sures, & Hering, 2018; Mance, 1987) and nutrients (Drury, Rosi-Marshall, & Kelly, 2013; Smith, Tilman, & Nekola, 1999; Withers & Lord, 2002) entering the river.

Nitrogen (N) and phosphorous (P) concentrations are of concern because they cause eutrophication that threatens the ecological status of the aquatic ecosystem (Lemm & 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, Finn, & Franz, 1979).

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

Metal pollution is a great concern due to its high biotoxicity, perdurability and bioaccumulation across 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 & 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 1,523.50, 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, Gunn, Wall, & Fanning, 2016). For instance, Mendiguchía et al. (2007) associated the concentrations of dissolved Ni found in the Guadalquivir River (average of 2.31 μg L^{-1}) with agricultural activity. However, data from urban sources of metals are not as readily available and there are very few studies. Such studies that show values of 210 ± 30 μg L^{-1} for Cr above the limits deemed permissible by the EPA (2019) 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 the understanding of the effects that these multiple-stress scenarios have (Sabater, Muñoz, García-Berthou, & Barceló, 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 ± 2,470 and 1,809 ± 200 μg 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, Martin-Laurent, & Pesce, 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. Amplicon sequencing has developed a high sensitivity to recover a high

number of gene sequences (Lear et al., 2013; Qu et al., 2017; Wang, Sudduth, et al., 2011). It 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 macromolecule 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 that is not present only in actively growing cells. More precisely, it includes 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, Algesten, Bergstrom, Jansson, & Tranvik, 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 existant microbial activity than DNA does, although some limitations have been recently considered (Blazewicz, Barnard, Daly, & Firestone, 2013).

Nowadays most of developed countries carry out freshwater water treatment and waste management programs, 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 from 2007 through the monitoring carried out by the ACA. Gaining a better understanding of the composition of fluvial biofilm prokaryotic communities, their major drivers and their response to anthropogenic pressures are of critical importance to obtain insights into ecosystem health and to preserve its biodiversity and function. Accordingly, 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-spring 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. Specific methods

2.1. Study sites

Seven sampling points along these rivers were selected (Figure 1 pag. 27). Three sampling points were located in the Osor River, the first (OU) upstream from the mine, the second (OM) downstream from the mine effluent and the third (OD) 12 km downstream from OM. Another sampling point was selected in the lower part of the Llémena River (Sant Gregori) (LL), which is expected to be moderately polluted as a result of urbanization, agricultural and farming activities. The Ter River was sampled first (TU) before the confluence of the Osor River (Cellera del Ter) and considered as a reference site, the (TM), after the confluence of the Osor River but before the confluence with the Llémena River, thus potentially impacted by the Osor River and finally (TD) 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) (95%), chemical oxygen demand (COD) (92%), N (75%) and P (97%) with a flow nearly 45,000 (m^3 day^{-1}) (ACA, 2017a).

2.2. Design and sampling

A passive biomonitoring with biofilm was conducted from late-February to mid-April for two consecutive years (2016 and 2017). Artificial substrata were used for biofilm growth (Figure 1a and b). 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 silicon sealant. Two cement cobbles were placed on the different streambeds at a depth of 20-30 cm to guarantee similar light and current conditions at each sampling site (Figure 1c).

Figure 1. Set up of biomonitoring study with biofilm. a) Artificial substrate before biofilm colonization, b) and after seven weeks of colonization, c) Artificial substrata on the streambead. From Carmen Espinosa and María Argudo.

Water samples were taken three times in 2016, whereas a more intense sampling was performed in 2017. In the two 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 (AFDW, metal bioaccumulation and DNA and RNA extraction for 16S rRNA analysis), explained in general methodology, biofilm was sampled once at the end of the study, 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.

Monthly rainfall data (February, March and April) were obtained from two observatories located near the study sites: Sant Gregori ("Estació meteorològica Sant Gregori (Gironés)", 2016, 2017) and Sant Hilari ("Meteoguilleries", 2016, 2017) in order to know if the two years were hydrologically comparable.

2.3. Data analysis

Raw microbial community data needed to be processed by bioinformatics prior to statistical analysis, so these data were treated separately. Raw sequence data from microbial community of this study were deposited in the short-read archive (SRA) via Biosample Submission Portal (National Center for Biotechnology Information (NCBI), 2019) under the accession number PRJNA523926. 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, Westcott, Baxter, Highlander

& Schloss, 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, α-diversity indices as (Sobs and Chao1) and (H' and 1/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) using MOTHUR. This matrix was used to perform a principal coordinate analysis (PCoA) by MOTHUR to ordinate sampling sites and the axes were thereafter related with environmental variables with Pearson correlation. A permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) 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 version 6 software (Anderson, Gorley & Clarke, 2008) and R software version 3.5.2 (R Core Team, 2018), respectively. In order to select potential biondicators of metal pollution; firstly, an nonmetric multidimensional scaling (NMDS) was generated with relative abundance at order taxonomic level by the PRIMER software; secondly, 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 with the phyloseq package (McMurdie & Holmes, 2013) of the R software. These OTUs were selected to identify potential biondicators of metal pollution.

A two-way ANOVA was used assuming independence between sampling sites to evaluate physicochemical parameters, inorganic nutrients, dissolved metals, monthly rainfalls, minimal fluorescence yield (F_0) and maximal or optimal quantum yield (Y_{max}) (biofilm parameters) differences among sites, between years and the interaction of annual temporality in each sampling site (site*year). Data was transformed when required to accomplish the assumptions of the model (normality and homocesdasticity) by neperian logarithm and square root. A post-hoc Bonferroni test was performed when significant differences ($p < 0.05$) were found between the sites in order to check exactly where significant differences were found. Moreover, a two-way ANOVA was performed with α-diversity indexes of microbial communities, among years and type of nucleic acid (factors). In addition, a one-way ANOVA was performed with the rest of biofilm characteristics (AFDW, metal bioacummulation, DNA and RNA concentration) to test differences among years of the study with a previous data transformation by neperian logarithm. Pearson correlations

were used to explore the relationship between environmental variables and α-diversity of prokaryotes. ANOVA and correlations analysis were done by SPSS version 25 and the R software.

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) ($p > 0.1$) showing that two years were hydrological comparable.

Physical and chemical water samples results are summarized in Table 1 and Supplementary material Figure 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 OU. Conductivity was higher in 2017, mainly at the Ter ($p < 0.001$). PO_4^{3-} and NH_4^+ concentrations were slightly higher in 2016 compared to 2017 ($p < 0.05$ and $p < 0.01$, 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 ($p < 0.05$). TD and OD had lower pH values in relation to LL, OU and OM ($p < 0.01$). O_2 concentration presented differences among sites ($p < 0.05$), which was statistically lower in TD compared to TU, TM and LL ($p < 0.05$). 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 (OU) being above the standard concentrations for a good ecological status (0.009-0.022 mg $P\text{-}PO_4^{3-}$ L^{-1}) (UK TAG, 2013), whereas LL had the lowest values of all the sampled sites ($p < 0.005$). 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 in average).

Metals dissolved in water showed temporal and site-specific variability (Table 1, Supplementary material Figure 1). Statistical differences in Zn concentrations were found among sites ($p < 0.05$) but not between years. The highest values were always found in OM, while LL had the lowest Zn concentration, which was statistically different from OM ($p < 0.05$). The Zn concentration found in OM exceeded the threshold set

up by EPA (2019) which is determined to be maximum concentration of 120 µg L^{-1} for acute and chronic exposure. Moreover, highest values for Ni, Pb and Mn were found at OM. Pb was only detected in 2016 with its highest concentration in OM. All sampling points in 2016 showed a Pb concentration above 2.5 µg L^{-1}, which is the limit for chronic exposure in freshwater systems (EPA, 2019). Ni was only present above detection limit in 2017 with three-times higher values in OM compared to OU. Concerning Mn, its concentration in OM was 3 and 10 times higher than in OU in 2016 and 2017, respectively. Fe content showed a significant reduction in 2017 ($p < 0.001$) for all sampling points with exception of LL. Cr and Cd were below the detection limit for all sampling sites in both years.

Table 1. Average and standard deviation of physicochemical parameters at each sampling site n = 49. A two-way ANOVA was performed to detect significant differences between year and sampling sites. Zn and Pb concentrations above the toxicity threshold according EPA (2019) (120 µg L^{-1}, 2.5 µg L^{-1}) and phosphate concentrations above good ecological status levels established (UK TAG, 2013) (0.022 mg P-PO_4^{3-} L^{-1}) are set in bold and underlined. bdl: below detection limit. Metals under detection limits are not displayed. Variables with blank holes in two way ANOVA did not accomplish assumptions.

Sample code	**OU**		**OM**		**OD**		**TU**		**TM**		**TD**		**LL**		**Two way ANOVA**		
Sample	**Osor upstream**		**Osor mine**		**Osor downstream**		**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[a] (ºC)	10.3 ± 3.2	9.7 ± 1.6	11.0 ± 2.6	11.7 ± 2.7	11.2 ± 2.7	11.1 ± 2.2	11.6 ± 1.7	11.3 ± 1.4	12.0 ± 1.5	13.4 ± 1.4	13.1 ± 1.6	14.7 ± 1.7	11.5 ± 2.7	13.6 ± 2.1	ns	ns	ns
pH	8.93 ± 0.38	8.60 ± 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 ± 1.10	10.08 ± 0.47	11.24 ± 0.91	9.88 ± 0.66	11.11 ± 0.93	10.35 ± 0.30	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
Cond (µS cm^{-1})	234 ± 17	227 ± 36	254 ± 23	240 ± 33	248 ± 21	240 ± 30	431 ± 37	518 ± 17	416 ± 4	485 ± 13	483 ± 13	554 ± 44	500 ± 25	500 ± 63	<0.001	<0.001	<0.001
SS (mg L^{-1})	82.3 ± 133.7	14.4 ± 22.0	75.2 ±127.4	5.0 ± 4.5	78.2 ± 117.1	1.4 ± 0.4	100.2 ± 170.3	149.3 ± 233.7	121.5 ± 206.3	19.4 ± 16.3	68.9 ± 110.4	13.2 ± 5.6	102.3 ± 110.7	27.1 ± 9.7			
NH_4^+ (mg N-NH_4^+ 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	0.02 ± 0.01	ns	0.006	ns
PO_4^{3-} (mg P- PO_4^{3-} 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**	**0.10 ± 0.01**	0.02 ± 0.00	0.01 ± 0.01	< 0.001	0.001	ns
NO_3^- (mg N-NO_3^- L^{-1})	1.51 ± 0.25	1.30 ± 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			
N/P ratio (molar)	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			
Zn (µg L^{-1})	69.06 ± 41.23	45.15 ± 27.45	**139.8 ± 45.8**	**292.1 ± 252.3**	96.46 ± 9.71	109.3 ± 109.1	26.25 ± 9.71	100.8 ± 49.1	8.00 ± 34.16	43.62 ± 17.15	35.91 ± 15.41	117.4 ± 128.2	22.17 ± 4.13	99.47 ± 65.98	0.016	ns	ns
Fe (µg L^{-1})	121.4 ± 91.0	49.86 ± 32.05	75.39 ± 24.55	24.36 ± 14.59	86.93 ± 37.72	37.13 ± 24.93	47.96 ± 34.28	31.10 ± 17.22	109.6 ± 77.9	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 (µg 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			
Mn (µg L^{-1})	15.00 ± 5.42	7.10 ± 2.26	32.57 ± 0.73	98.36 ± 7.34	24.17 ± 7.42	16.23 ± 8.20	9.21 ± 3.79	9.95 ± 2.70	8.56 ± 3.48	7.42 ± 0.99	15.01 ± 2.17	26.50 ± 3.37	5.07 ± 2.62	12.79 ± 8.41			
Ni (µg 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 ± 0.4	bdl	1.39 ± 0.26	bdl	1.85 ± 1.36			

3.2. Biofilm characterization

Biofilm measurements are shown in Table 2 and Supplementary material Figure 2. The biofilms did not present significant differences between their principal characteristics in relation to the year of evaluation ($p > 0.05$). F_0 did not show significantly differences between sampling sites ($p > 0.1$) but Y_{max} was significantly higher in OU, TU and OD sampling points ($p < 0.01$). Although differences among sites could not be demonstrated statistically for the rest of characteristics due to the lack of replication, it was possible to point out some patterns. Regarding RNA, the results obtained in OM stand out remarkably because of high values of almost ten times more than in the other sampling sites. Moreover, the RNA values in the Llémena and the Ter rivers showed a notably temporal difference with higher values in 2016 mainly in TU and TD (up and downstream).

Neither bioaccumulated metals showed significant differences between years ($p > 0.05$) but some differences between sampling sites were detected. Cd bioaccumulation was mostly below the detection limit, except in OM, where it was detected in both years. In fact, sampling site OM differs from the rest of the sites mainly by its bioaccumulated metals (Zn, Mn, Pb and Cd) provided by the mine (Table 2). In addition, Zn exceeded the threshold (150 μg g^{-1}) proposed by Corcoll (2012).

Table 2. Average and standard deviation of biofilm characteristics for two years in the different sampling sites (n = 7 in 2016 and n = 12 in 2017). Values above the toxicity threshold (150 µg Zn g^{-1}) according to structural and functional changes in biofilms (Corcoll, 2012) are shown in bold and underlined. mv: missing value, bdl: below detection limit.

Sample code	**OU**		**OM**		**OD**		**TU**		**TM**		**TD**		**LL**	
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	115.2	**<u>2438</u>**	**<u>2678 ± 840</u>**	**<u>1139</u>**	**<u>538.3 ± 167.9</u>**	30.77	50.27 ± 0.76	97.47	**<u>178.0 ± 68.5</u>**	98.11	**<u>283.1</u>**	38.12	29.76 ± 0.23
Fe (µg g^{-1})	15234	18345	17215	14984 ± 9679	15586	17354 ± 10330	3784	9193 ± 2293	69.14	10088 ± 3941	6935	20800	13452	10395 ± 1201
Pb (µg g^{-1})	19.72	23.72	225.0	214.8 ± 100.0	145.2	110.0 ± 51.0	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})	835.7	539.9	2773	1040 ± 706	1619	657.2 ± 298.5	387.3	699.1 ± 212.4	856.4	632.8 ± 77.1	577.9	1144	361.6	461.97 ± 65.18
Cr (µg g^{-1})	7.50	7.86	10.97	7.62 ± 4.18	9.30	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	4.50	9.94	12.11 ± 7.70	6.53	5.78 ± 2.35	5.00	10.37 ± 1.44	6.26	6.64 ± 1.20	9.02	21.60	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
F_0 final	223	386	15	476	452	139	644	22	28	339	83	121	10	349
Ymax	0.533	0.541	mv	0.430	0.573	0.559	0.609	0.470	mv	0.471	mv	0.449	mv	0.459
AFDW (mg cm^{-2})	2.61	1.18	0.67	0.81	2.44	1.59	0.60	1.62	0.70	0.62	1.66	0.52	mv	0.53
DNA (ng mg^{-1})	4.7	5.1	6.4	9.3	6.7	4.9	5.6	8.9	1.3	4.7	9.3	6.6	3.5	2.0
RNA (ng mg^{-1})	13.0	14.2	177.4	188.0	17.2	65.5	117.6	22.4	86.1	61.0	89.5	16.3	59.7	18.9

3.2.1. Microbial community response

Microbial community was studied based on the 16S rRNA gene sequences. A total of 4,488,081 sequences passed quality trimming and 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 OTUs at a 97% taxonomic cut-off ranged from 484 to 6,134 OTUs per sample. The prokaryotic richness (Sobs and Chao1) and diversity (H' and 1/D) were significantly higher in the RNA fraction (active community) than in the DNA fraction (resident community) with $p < 0.005$ (means 3,729-2,147), $p < 0.0001$ (means 6,291-3,075) for richness and $p < 0.05$ (means 5.84-5.25), $p < 0.1$ (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, OU and OD had a high richness and diversity mainly in the active fraction (Table 3). In contrast, richness and diversity were lower in OM, principally in the RNA fraction but also in the DNA fraction. The Ter upstream was very similar to OU and OD sampling points in terms of the richness and diversity values. On the other hand, the TM and the LL had the lowest values of richness in DNA and RNA fractions, respectively. TD was very different between years with the lowest values for diversity found in 2016 in both resident and active communities.

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 material Table 1).

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

DNA parameters														
Sample code	**OU**		**OM**		**OD**		**TU**		**TM**		**TD**		**LL**	
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**
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	3323	5061	1894	2291 ± 863	4154	8650 ± 201	4464	2791 ± 528	722	826 ± 18	1102	3026	4400	792 ± 283
1/D	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
H'	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
RNA parameters														
Sample code	**OU**		**OM**		**OD**		**TU**		**TM**		**TD**		**LL**	
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**
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	9277	4993	4592 ± 118	5561.	8415 ± 2119	6023	7362 ± 2128	2981	6761 ± 1245	2999	10745	2207	6732 ± 1035
1/D	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
H'	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

Most of the sequences identified in the samples corresponded to the Bacterial domain (4,703,891 sequences) and only 1,377 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 material Figure 3).

The differences in the structure of both resident and active microbial communities were analysed using a PCoA (Figure 2, Figure 3). The PCoA based on the total OTUs abundance of the microbial community corresponding to the resident community explained 48.8% of the observed variance. The first axis explained 30.87% of the variance and was positively correlated with water Tª ($r = 0.614$, $p < 0.05$), Ni (dissolved) and Ni (bioaccumulated) ($r = 0.482$, $p < 0.05$ and $r = 0.516$, $p < 0.05$, respectively) and Cond ($r = 0.528$, $p < 0.05$) 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 (dissolved) ($r = 0.458$, $p < 0.05$) 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 to resident community (Figure 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 ($r = 0.511$, $p < 0.05$), SS and negatively with Mn (bioacummulated) ($r = -0.542$, $p < 0.05$), Pb (bioaccumulated) ($r = -0.589$, $p < 0.005$), Zn (bioaccumulated) ($r = -0.527$, $p < 0.05$) and PO_4^{3-} ($r = -0.603$, $p < 0.01$), 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 shown in Table 4 and in Supplementary material Figure 4 at order level. The most abundant genera found only in metal polluted sites were identified *Sphingorhabdus, Flavobacterium, Prosthecobacter, Ferruginibacter* and *Arcicella*.

β-diversity differences between resident and active communities were checked out by PERMANOVA and Mantel ´s test analysis. The resident community was different for site ($p < 0.05$), year ($p < 0.0516$) and site * year ($p < 0.05$), while it was only different for site ($p < 0.05$) in the active community (Supplementary material Table 2). Moreover, β-diversity of DNA and RNA fractions were not significantly correlated ($r = 0.06$, p-value > 0.1, permutations = 999).

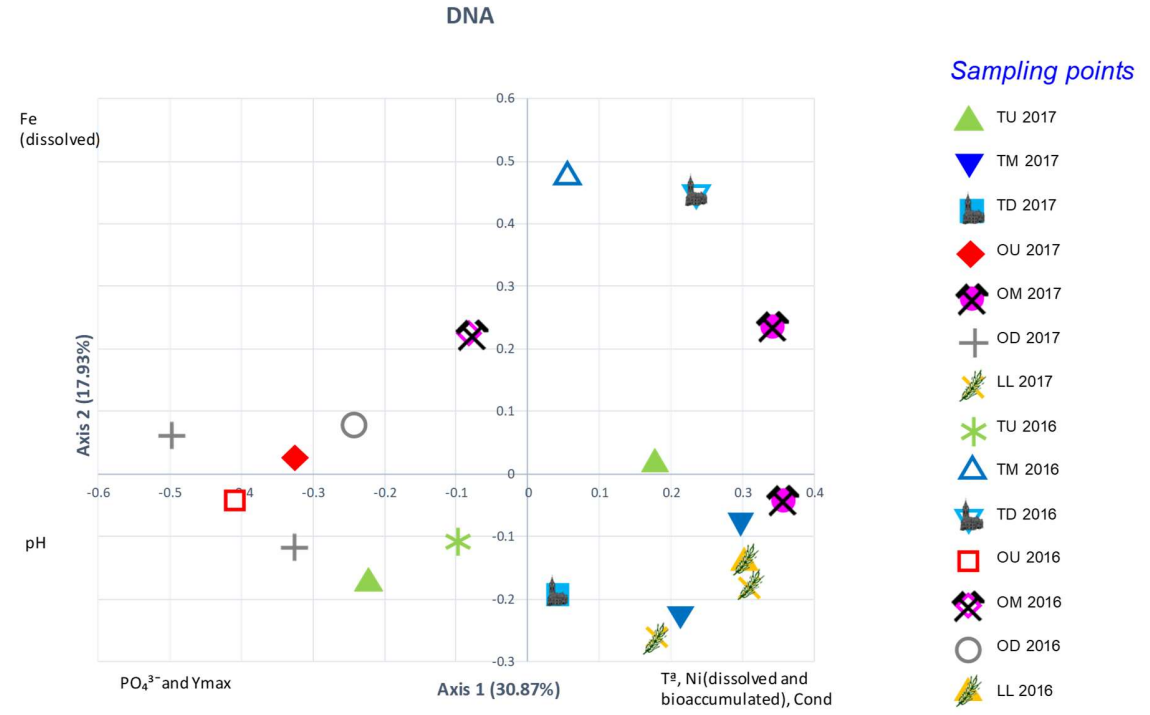


Figure 2. PCoA of the dissimilarities between samples positions respect to the total OTUs of resident community using thetaYC distances and correlations with the environmental and biofilm parameters.

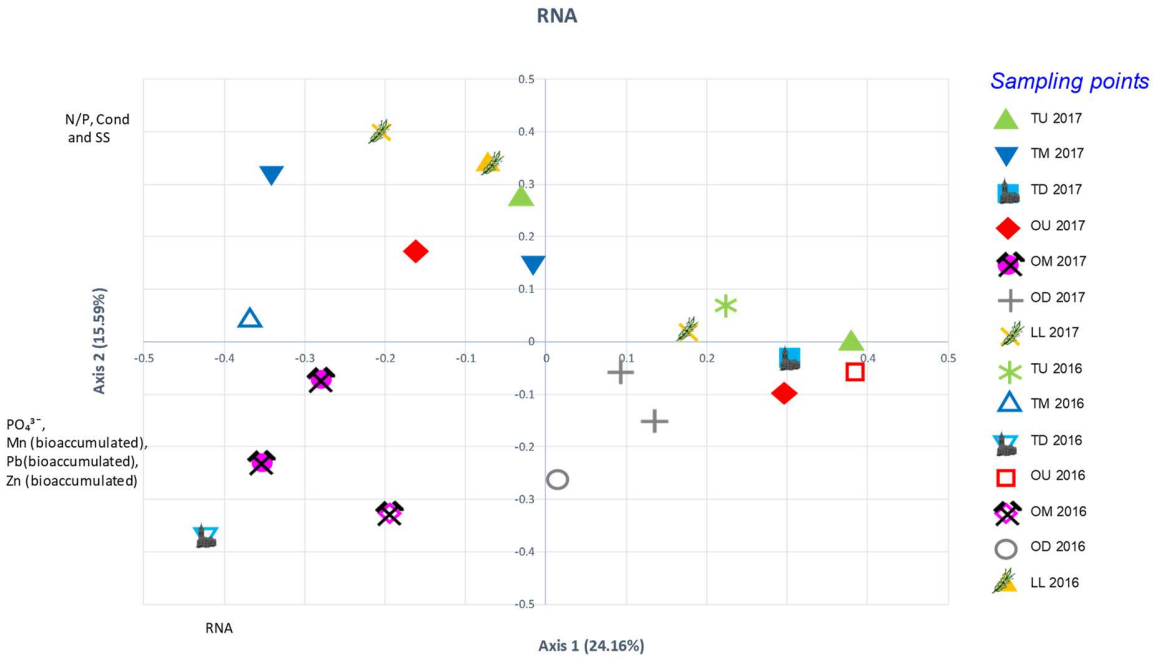


Figure 3. PCoA of the dissimilarities between samples positions respect to the total OTUs of active community using thetaYC distances and correlations with the environmental and biofilm parameters.

Table 4. Specific metal polluted and nonmetal polluted OTUs of active microbial community (RNA fraction) from a taxonomic selection of 20 OTUs with higher abundance. Metal polluted sites correspond to OM 2016, OM 2017 and TD 2016 and nonmetal polluted sites to OU 2016, OU 2017, TU 2016 and TU 2017.

Phylum	Metal polluted sites	Non metal polluted sites
Acidobacteria		• **Acidobacteria GP3**
Bacteroidetes	• ***Ferruginibacter*** *(Fam. Chitinophagaceae, Ord. Sphingobacteriales).* • ***Flavobacterium*** *(*Fam.*Flavobacteriaceae,* Ord. *Flavobacteriales).* • ***Arcicella*** *(*Fam. *Cytophagaceae,* Ord. *Cytophagales).*	• **Fam*. Flavobacteriaceae*** *(*Ord. *Flavobacteriales)*
Gemmatimonadetes		• ***Gemmatimonas*** *(*Fam. *Gemmatimonadaceae,* Ord. *Gemmatinomonadales).*
Alphaproteobacteria	• ***Sphingorhabdus*** *(*Fam.*Sphingomonadaceae,* Ord. *Sphingomonadales)* • **Fam*. Sphingomonadaceae*** *(*Ord. *Sphingomonadales)*	• **Fam*. Acetobacteraceae*** *(*Ord.*Rhodospirillaes)*
Betaproteobacteria		• **Fam*. Comamonadaceae*** *(*Ord. *Burkholderiales)*
Deltaproteobacteria		• **Fam. *Polyangiaceae*** *(*Ord. *Myxococcales)*
Gammaproteobacteria		• **Fam. *Pseudomonadaceae*** *(Ord. Pseudomonadales)* • ***Haliea*** *(Fam. Alteromonadaceae, 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 β-diversity of prokaryotic communities provided different and complementary information about the ecological integrity of the ecosystem. More precisely, Mantel´s test and PERMANOVA showed it since both data sets were not statistically correlated.

The information provided by the DNA fraction, that represents the taxa that is present in the biofilm, including spores, dormant or non-growing active cells and dead cells (Blagodatskaya & Kuzyakov, 2013) was not very conclusive in terms of bioindication of metal pollution, since it was mainly attributed to ecological differences among river sites (i.e. upstream-downstream gradients of mineralization and nutrient contents). On the contrary, the RNA fraction which reflects the active community members was related to chronic but low metal pollution (Figure 3). However, there are some constraints in the interpretation of the RNA values as indicators 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 & Binder, 2003). In addition, dormant cells can contain higher number of ribosomes than in the vegetative state (Sukenik, Kaplan-Levy, Welch & Post, 2012). Overall, these considerations should be contemplated when RNA is used as a proxy of cell activity.

Based on the assumption of the information associated with RNA represents only a fraction of that from the resident community i.e. that only active cells (growing or non-growing) contain significant amounts of RNA while all cells being active or not harbour genomic DNA, one would expect higher diversity and richness in the DNA fraction (Lennon, Muscarella, Placella & Lehmkuhl, 2018). Contrarily in this study, it is noteworthy to highlight that the richness and diversity were significantly higher in the active community than in the resident community supporting other studies such as Baubin et al. (2019); Gill, Lee & McGuire (2017). This could be explained because rare bacterial taxa could be disproportionately more active than common taxa, accordingly to what Jones & Lennon (2010) reported. However, as stated by Gill et al. (2017) we can not rule out the effect of alternative splicing in post-transcriptional processes which may be the cause that expressed transcripts seem more diverse than their corresponding DNA templates.

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

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 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 the 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 parameters 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 concentration 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 WWTP in Sant Hilari Sacalm and Osor, leading to concentrations 10-20 times higher than background phosphate concentration in undisturbed streams which are around 0.003 mg $P\text{-}PO_4^{3-}$ L^{-1} (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). The PO_4^3 concentration was the lowest in the calcareous stream (Llémena) where chemical removal of phosphate was expected due to co-precipitation of with carbonate (Otsuki & Wetzel, 1972). It is interesting to highlight that phosphate and conductivity were correlated with the first axis of the PCoA of the resident microbial community which would explain the 30.87% of variance in community composition (Figure 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 (Figure 3). These 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 & Dahm, 2011; Wakelin, Colloff & Kookana, 2008), with a low 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, Gołębiewski, Morawska-Płoskonka, Kuduk, & Niklińska, 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, Brauns & Pusch, 2006). This effect is shown mainly in the Osor, except in OM 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 to determining the composition of the active microbial community as discussed below.

α-diversity of prokaryotes was correlated negatively with conductivity and water temperature and positively with Y_{max} (Supplementary material Table 1). 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 & 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 among study streams have a great influence on the resident community as reported in Findlay & 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) were measured in the Osor downstream from the mine effluent (mainly in OM but also in OD). Ni, Cr, Pb and Zn were measured in biofilms in TD, mainly in 2017 and Ni and Cr in the biofilms of the LL, 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 & Chen, 2013; Victoria & Gómez, 2010). The lowest concentrations of metals measured in the OU and in the Ter upstream from the reservoirs (TU), are within the range of background metal contents reported elsewhere (Bonet et al., 2014; Vishnivetskaya et al., 2011; J. 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 cause-effect relationship between Ni and the community composition since the values reported (maximum 29.31 μg Ni g^{-1}) were moderate.

Metals from the mine effluent (Pb and Zn accumulated in biofilm) are correlated with the PCoA of the active microbial community (Figure 3), supporting our expectation of the effects of metal pollution on the composition of the community. 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 and TD 2016 were separated from the rest. The sites affected by mining metals had values of Zn concentration above the 150 μg g^{-1} toxicity threshold in accordance with Corcoll (2012). These values, detected in previous studies, are shown to inhibit antioxidant enzyme mechanisms such as glutathione-S-transferase (Bonet et al., 2014), decrease photosynthetic efficiency, enhance protection mechanisms through the xanthophyll cycle, modify the diatom community (Corcoll et al., 2012) and exert structural pressure by selecting the most metal-tolerant species (Tlili et al., 2011). Focusing on DNA values (Table 2), which is a proxy of the biomass of the whole microbial community (in this case, including autotrophic and heterotrophic organisms) the values measured in metal-polluted sites were relatively high, indicating that metal toxicity was mainly affecting the accrual of the biomass of the autotrophic component of the biofilm. Moreover, RNA content was even higher (in relative numbers) than DNA values, indicating the presence of a very active microbial community (Besaury, Ghiglione & Quillet, 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 increase of metal pollution unlike β-diversity as in Yang, Huang, Wu, Zhang & Liu (2013). In fact, Ter downstream of the Girona city was very different between years with respect to this parameter, the diversity was the lowest in 2016 appearing with sampling sites affected by mining metals, although this could be attributed to different urban waste not analysed in this study like antibiotics as Kümmerer (2009) who suggested the possibility of these substances reduce the number of bacteria in hospitals´sewage systems. Although some differences were observed between sites

located in the same stream. Microbial richness decreased downstream from OM with respect to the sampling sites on the Osor River. This result is in agreement with results from previous studies that 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, Lear, & Lewis, 2010; Kavamura & Esposito, 2010; Singh et al., 2014).

In addition, RNA content was correlated negatively with the richness and diversity of active microbial community. 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 & Madamwar, 2007).

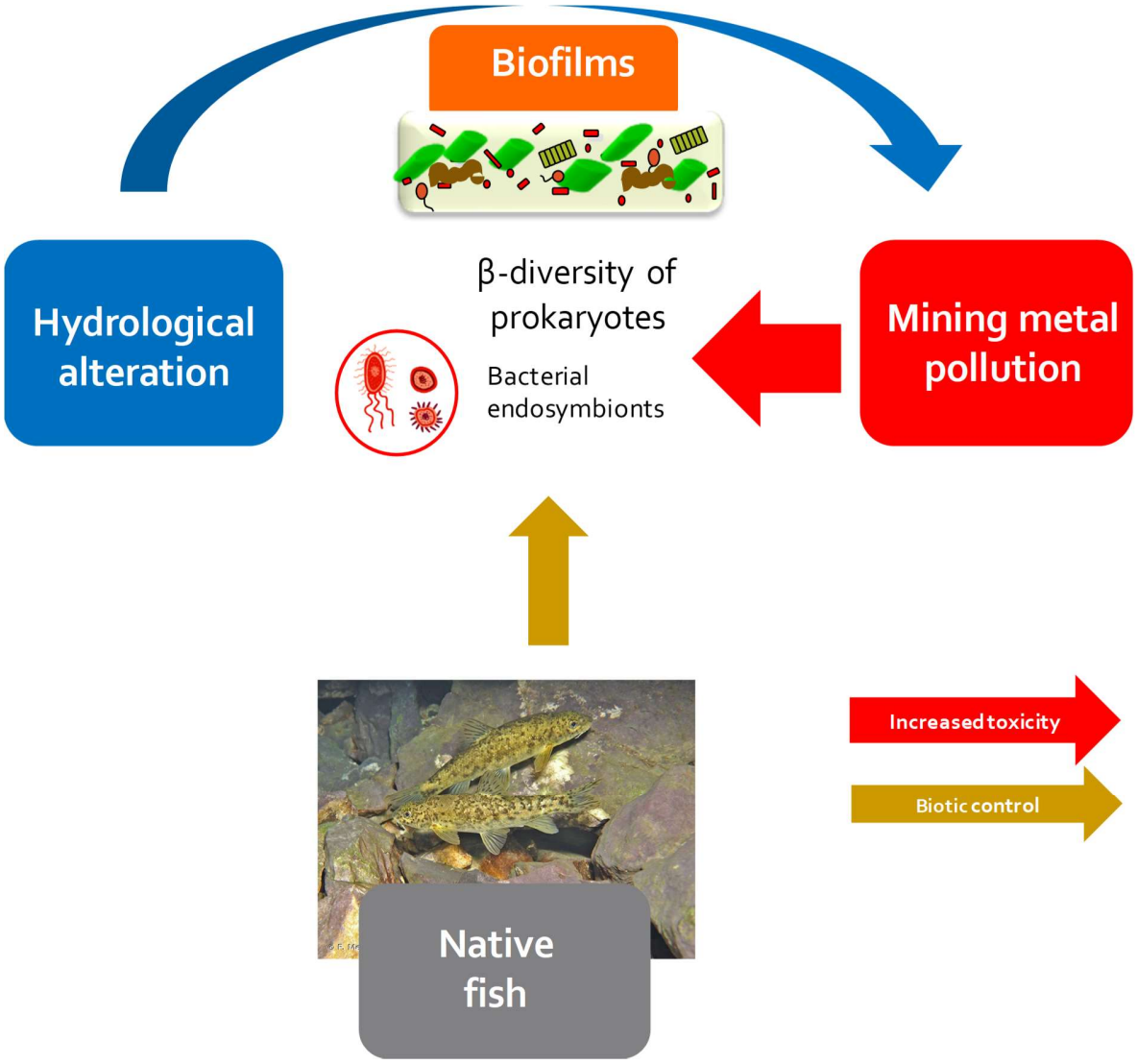
Once the effects of heavy metals on the microbial communities were detected, it was possible to identify 5 bacterial genera proposed as bioindicators of the heavy metal contamination. More precisely, *Sphingorhabdus* which has been described by Jogler, Chen, Simon, Rohde & Busse (2013) is known for their ability to cope with various metals because contain multiple genes associated with resistance to Cu, Co, Zn, Cd and Hg (Silva, Lago-Lestón, Costa & Keller-Costa, 2018). In fact, the family *Sphingomonadaceae* harbours members with known ability to biodegrade pollutants and generate exopolysaccharides (Mahmoud, Goulder & Carvalho, 2005). Concerning *Flavobacterium*, Maja, Menke, Höckner & 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 with metals (Najiah et al., 2009; Zhao et al., 2019). The genus *Prosthecobacter* is very common in freshwaters (Bao et al., 2017), but also is known as indicator of metal pollution in soils (Maja et al., 2019). *Ferruginibacter* can reduce Fe (III) and was found as an abundant genus in riparian soils adjacent to mine drainage settling pond of Pb-Zn smelter (Fan et al., 2016) and although *Arcicella* is a non-dominant bacteria in freshwaters, Londono, Donovan, Shi, Geisler & Liang (2019) noticed that increased with metals as Ti and Zn.

5. Concluding remarks

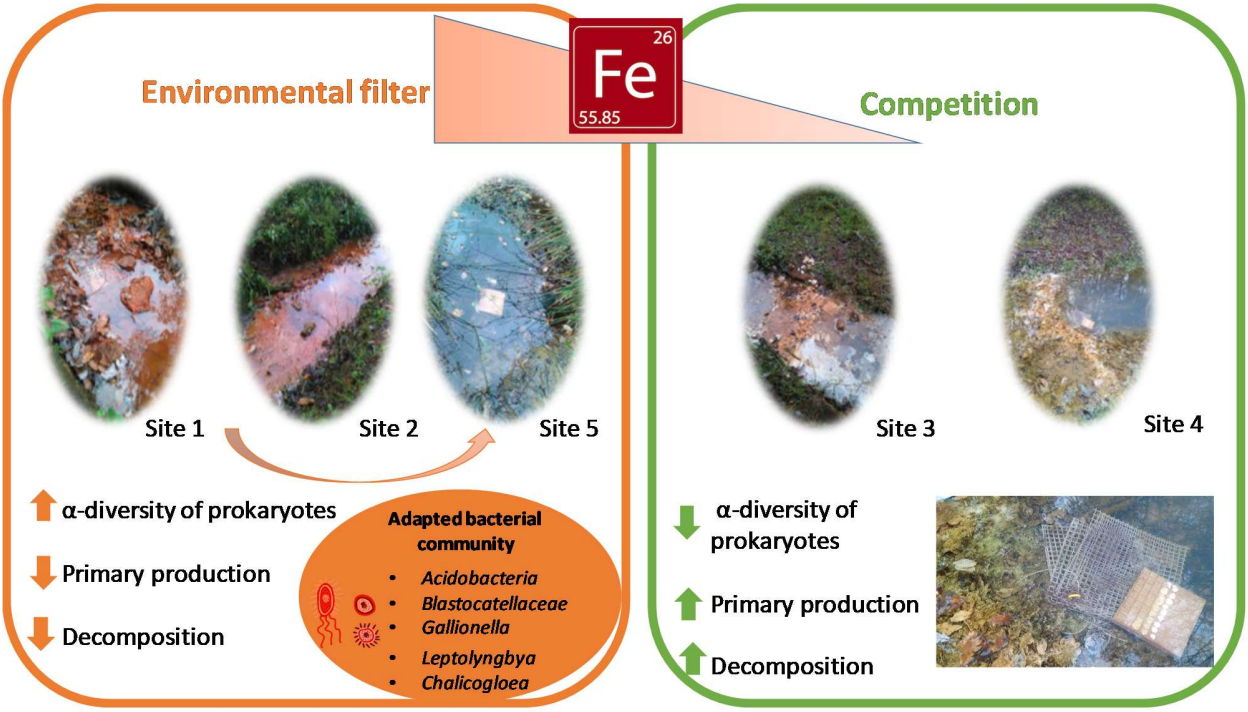
We conclude that in Mediterranean rivers such as the Ter, Osor and Llémena subjected eutrophication and chronic but low metal pollution, nutrients and conductivity were the main driving factors behind the diversity and composition of the microbial communities. These driving factors were very clear in terms of resident community, but also affected the active one. Moreover, 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. However, the effects of mining were mainly seen on the structure of active microbial community (β-diversity). 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.

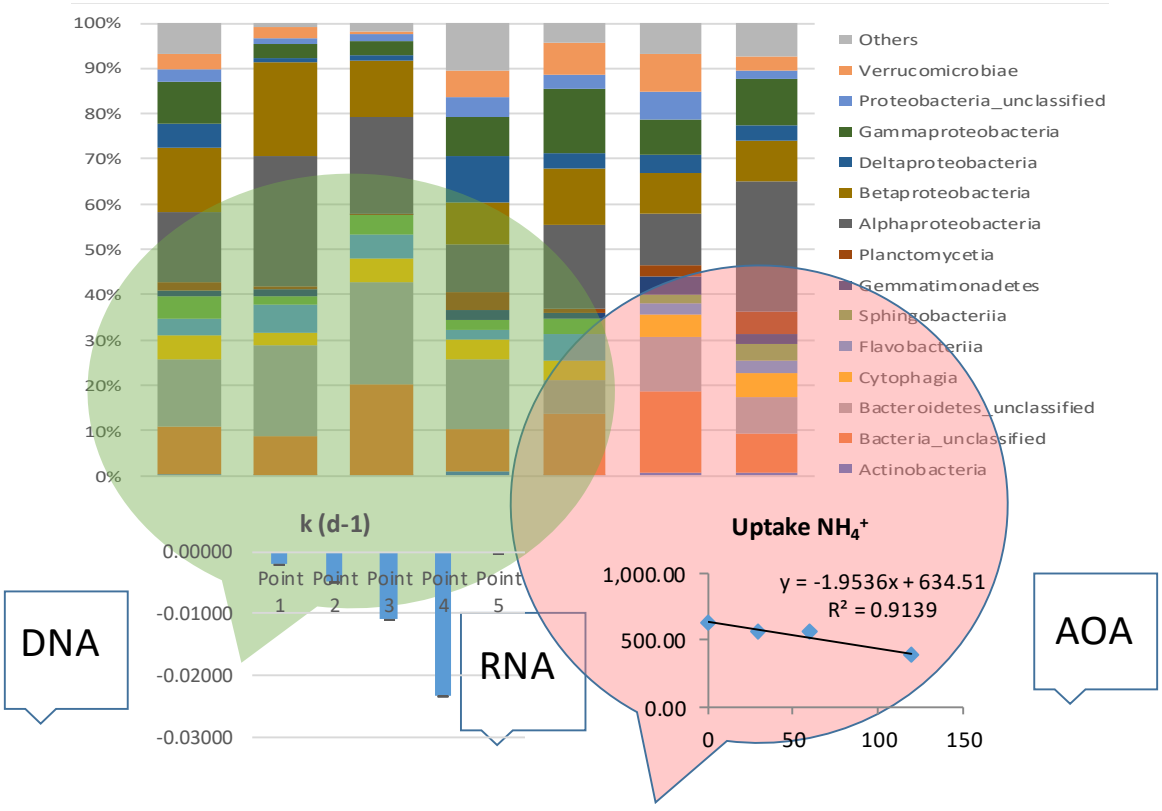
★ Study II: Direct and indirect effects of multiple stressors on the microbial communities in a mining area



★Study III: Environmental drivers of microbial community structure in a high iron calcareous-spring



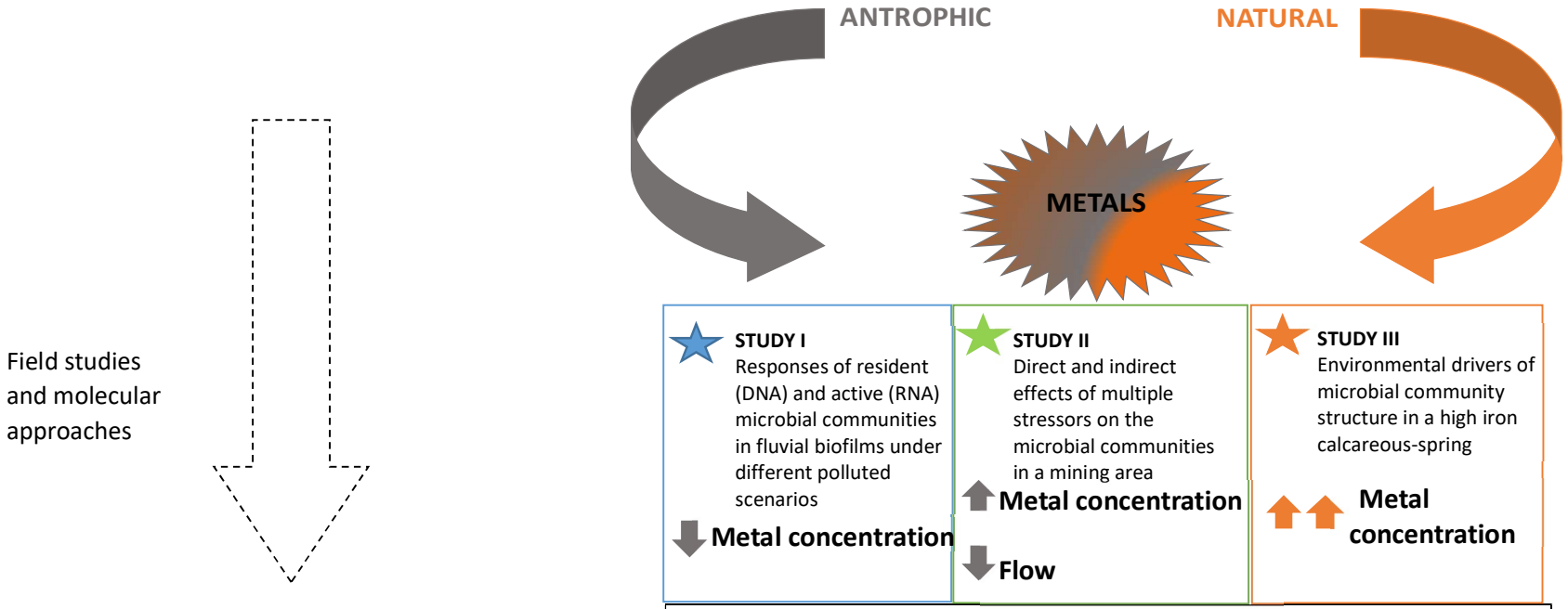
GENERAL DISCUSSION



Microorganisms are essential actors in the functioning of the ecosystem. The evaluation of the pollution effects on microorganisms is of paramount importance since their response may serve as a proxy to report the negative effects on the ecosystem, as well as its recovery capacity (Cravo-Laureau, Lauga, Cagnon, & Duran, 2017). This fact motivated the development of microbial ecotoxicology, an emergent multidisciplinary field that integrates microbial ecology, microbial toxicology, chemistry and physics (Shahsavari et al., 2017). Microbial ecotoxicology offers great potential in the assessment of the impact of pollution on the structure and function of microbial communities. However, there are still conceptual and methodological challenges to design studies that link the structure and function of microbial communities (Bier et al., 2015). Molecular technologies allow us to identify microbes and their activity throughout their genes but does this information enable us to understand, predict and assess the functioning of the ecosystem under metal stress? The technical and methodological advances help us define the role of microorganisms, which is a complex task because metabolic flexibility and diversity of microbes are greater than we can imagine (Blagodatskaya & Kuzyakov, 2013; Prosser, 2012).

In view of these challenges, the main objective of this thesis was to determine the responses/changes to metal stress from different sources in the structure and function of the prokaryotic communities in epilithic biofilms. We wanted to study these responses at an ecosystem-scale, combining traditional ecological methodologies and recent molecular microbial ecology methods.

Results from this thesis have revealed that microbial communities are able to respond to changes in metal concentrations. When the concentration of metals was low, changes in active bacteria (RNA fraction, which indicate **potential function**) were detected mainly in β-diversity, at OTU level and in an increment of RNA content. These changes allowed us to classify some genera as metal pollution indicators, but the resident community (DNA fraction) was unaffected (Study I) (Figure 1). However, a further increase in the concentration of metals, due to low flow conditions, affected mainly the community **structure.** Remarkable changes in the composition of bacterial communities over a metal pollution gradient (β-diversity at OTU level) were detected and bacteria indicators were selected in each site. In this case, there were hardly any changes in functions related to the nutrient cycling (Study II) (Figure 1).



				MICROBIAL COMMUNITIES RESPONSES					
STRUCTURE		**FUNCTION**		STRUCTURE	FUNCTION	STRUCTURE	FUNCTION	STRUCTURE	FUNCTION
DNA content		RNA content		**X**	✓	**X**		**X**	
DNA α-diversity		RNA α-diversity		**X**	~	**X**		**X**	
DNA β-diversity	Phylum	RNA β-diversity	Phylum	**X**	**X**	**X**		✓	
	Order		Order	**X**	~	**X**		✓	
	OTUS		OTUS	**X**	✓	✓			
	ASVs		ASVs					✓	
DNA Indicators	Family	RNA Indicators	Family			✓			
	Genera		Genera	**X**	✓			✓	
		AOA					**X**		
		Nutrient uptake					~		
		Leaf litter decomposition							✓

Figure 1. Synthetic figure of principal results obtained along this thesis highlighting the metal stress condition and the responses of microbial communities. Gray colour symbolize anthropic source and orange natural source of metals. The tick mark describes the metal affected properties (structure or function) of microbial communities, the cross mark indicates those do not affect and the diacritical mark, an unclear response. The empty spaces show that these variables have not been analysed in the study.

On the other hand, a higher concentration of metals, generated by extreme natural gradient of metals, affected the **structure and function** of bacteria. The bacteria showed a high variation of taxa over the metal gradient at phylum level (β-diversity). These dramatic changes in the composition let us identify a large number of resistant genera. Respect to the function, the leaf litter decomposition showed a clear inhibition in sites with higher concentration of metals (Study III) (Figure 1).

In this general discussion, the possible links between the structure and the function of microbial communities and the pros and cons of different molecular approaches, followed to accomplish the principal goal of this thesis, will be presented and discussed combining and commenting the different results obtained. Future perspectives will be also integrated.

1. Linking structure and function

The strong relationship between structure and function into complex microbial community of fluvial biofilm required an approach to complement the use of structural and functional descriptors to assess potential effects of stressors on the fluvial ecosystem. Therefore, the use of a multi-marker approach in ecotoxicology studies is very useful (Bonnineau et al., 2010; Sabater et al., 2007). Following the same argument, in this thesis a multi-descriptor approach has been used to cover both functional and structural aspects of biofilm communities mainly adapted to prokaryotic communities. However, even today, one of the main goals of microbial ecology is to identify possible links between microbial community structure and microbial processes (Bier et al., 2015).

Several researches have shown that some alteration of environmental variables can cause shifts in both, the structure and function of microbial communities (Galand, Pereira, Hochart, Auguet, & Debroas, 2018; Reed & Martiny, 2013; Vishnivetskaya et al., 2011). The identification of both responses could depend on the conditions and techniques used in each study (Shade et al., 2012) or on the time-scale over which measurements occur. It is easier to link structure and function in organisms with narrow phylogenetic distributions, like nitrifying microbial communities (Suarez et al., 2019). In the Study III of this thesis, the structure and function of microbial communities were affected, and we associated it to the extremely high concentration of metals.

On the contrary, in literature, there are lots of studies in which the structure and function appear uncoupled. Several authors observed that microbial communities functional responses to stressors were detected earlier than responses in the structure or in both, the structure and functioning of microbial

communities (Bier et al., 2015; Comte, Fauteux, & Giorgio, 2013; Ruiz-González, Lefort, Massana, Simó, & Gasol, 2012). In this thesis, the structure and function also appear unlinked (Study I and Study II), but we related these situations mainly to the concentrations of metals. While potential functional β-diversity of the active community changed in low metal concentrations sites, the resident community (DNA fraction) characteristics were not affected by these concentrations (Study I). However, Jacquiod et al. (2018) showed that the DNA fraction in river sediment microbiome had the discriminate power to tell the difference between metal concentration sites and non-metal concentration sites (with concentrations of Zn 10-fold higher compare to the highest concentrations of Zn in biofilm at Study I). Accordingly, in this thesis, the β-diversity of the resident fraction of communities suffered a change when the concentration of metals increased (Study II) without noticing important changes in nutrient cycling. Therefore, the changes in the microbial resident fraction are mainly affected to a high concentration of metals.

Bier et al. (2015) gave several reasons for the lack of linking between structure and function. He mentions microbial dormancy, horizontal gene transfer, functional redundancy, priority effects and neutral assembly processes. In the Study II of this thesis, we suggested that the fact that nutrient uptake was not affected by metals could be due to the functional redundancy or to the compensatory effects of microbial communities, which is more probable if diversity is high in the ecosystem (Yachi & Loreau, 1999).

Overall, according to our interpretations of the results of the three studies about the link between the structure and the function of microbial communities, it was observed that the link was associated with different concentration of metals. In fact, Eng & Borenstein (2018) stated that it could vary depending on the environment.

2. Pros and cons of the molecular and traditional approaches carried out in this thesis

In this section, we are going to discuss the methods carried out in this thesis and the advantages and disadvantages in microbial ecotoxicology. The following arguments will be debated based on the study of the structure and function of microbial communities.

2.1 Structure

The prokaryotic identification based on sequencing of gene encoding **16S rRNA** polyribonucleotide has lots of advantages and it has allowed us to make good progress on microbial ecology and, consequently, on microbial ecotoxicology (Table 1). Current editions of the two fundamental treatises, Bergey's Manual of Systematic Bacteriology (Whitman, 2015) and The Prokaryotes (Dworkin & Falkow, 2006) base the structure of the prokaryotic world on the phylogenetic relationships established with the 16S rRNA

macromolecule. Rodicio & Mendoza (2004) explained that it is present in all the current bacteria and archaea, so it constitutes a universal target for identification. Moreover, its structure and function have remained constant for a long time; changes occur slowly enough to provide information about all prokaryotes over their evolutionary scale. Furthermore, it has been demonstrated that a precise identification of prokaryotes does not always require amplification and subsequent sequencing of the full 16S rDNA gene. Generally, some partial amplicons are used in most studies, such as the v4 region, also used in this thesis.

Cultivation-independent genome approaches of 16S rDNA amplicon have revealed an unexpected huge diversity of microorganisms (Hug et al., 2016; Jay T Lennon & Locey, 2016). The study of this diversity is crucial in microbial community ecotoxicology. In this thesis, changes in the α and β-diversity have allowed us to know about the important influence of metals in fluvial ecosystems. In fact, several studies have looked into the impact of pollutants, such as metals on the diversity of microbial communities, and shifts have been reported in the community structure (richness and evenness) when there are similar environments (Ancion et al., 2010; Fan et al., 2016; Zhang et al., 2018; Zhang et al., 2019; Zhu et al., 2013).

Furthermore, Gibson et al. (2015), among other authors, revealed that amplicon sequencing can provide more detailed taxonomic information than the conventional morphological analysis. High-throughput sequencing offers a greater sequencing depth with rapid and relatively easy taxonomic characterization of microbial communities at a high level of resolution (Liu et al., 2012). High taxonomic resolution can be used to select bioindicators, such as metal-resistant bacteria (Leon et al., 2018), which are associated to some metals or sites with high metal concentrations (Bao et al., 2017; Guo, Nasir, Lv, Dai, & Gao, 2017; Londono et al., 2019). This characteristic is very useful in microbial ecotoxicology and has been considered in this thesis.

The fast development of microbial eco-genomics provides well established lab protocols, commercial nucleic extraction kits and bioinformatic pipelines, such as MiSEQ SOP (Schloss et al., 2009), Phyloseq (manual) (Callahan, Sankaran, et al., 2016) or Usearch manual (Edgar, 2016), which were used in this thesis to process raw 16S rRNA gene sequences in order to obtain data on diversity and on the composition of prokaryotic communities.

Bacterial identification, based on 16S rDNA gene sequence analysis, is supplied by many companies. For example, Illumina Company (www.illumina.com) provides a line of products and services that serve the

sequencing, genotyping and gene expression markets. This technology has reduced the cost of sequencing.

However, this approach also has some disadvantages or limitations. These limitations must be taken into account when making a correct interpretation of the results (Table 1).

This approach requires making decisions between different options, which can change the results, such as the choice of the region targeted. Whiteley et al. (2012) clarify the importance of using longer fragments of 16S rDNA gene, such as V3 fragments (200 bp) where the 80% of sequences were accurately classified to known taxa. However, when using shorter fragments as V6 (100 bp), 95% of the total reads were classified to the bacterial root.

Moreover, the bioinformatic process of amplicon sequencing is subject to various levels of sequencing error. Different pipelines may produce significant different results. The choice of algorithms for quality filtering, OTU clustering and taxonomic assignment with reference database, may affect the downstream analysis of the taxonomic composition of microbial communities (Kopylova et al., 2016; Whelan & Surette, 2017). Somboonna et al. (2014) advised to use more than one database to determine whether they lead to the same result.

Another key method to study the structure of microbial communities followed in this thesis was looking into the communities at **different bacterial taxonomic levels** (Table 1). There are many tools to quantify and compare the composition of communities adapted to different conditions. Common approaches use the notion of clustering all 16S rRNA gene sequences with a similarity of 97% and then assigning these to "OTUs" from reference trees (Caporaso et al., 2010; Schloss et al., 2009). Recently, new atomic units that infer the sequences before the introduction of amplification and sequencing errors and distinguish sequence variants with a single different nucleotide, such as ASVs, have been develop. ASV methods show a higher resolution than the OTUs methods, which improve the capacity to discriminate ecological patterns (Callahan, McMurdie, & Holmes, 2017). These approaches were followed in this thesis and have let us compare microbial communities at different taxonomic levels to find one, which could detect responses of bacteria to metal stress (Table 1). When the environmental differences are very large, changes in the community can be detected at phylum level, which occurs in the Study III of this thesis. Following the same line, important differences in bacterial composition at phylum level are found in some samples located at different sites under extreme environment, Río Tinto (Sánchez-Andrea et al., 2011) or when comparing microbial communities of river sediments with a great different concentration of loads

of metals (Zhu et al., 2013). However, when the changes in the environmental variables are less sharp, it is necessary to increase the taxonomic resolution at OTU level. The study II of this thesis evidence that metals affected the community composition as it was also shown in the results obtained by Gołebiewski, Deja-Sikora, Cichosz, Tretyn, & Wróbel (2014), in which OTU level was the best option since it allowed the demonstration of Zn influence on soil bacterial communities. Moreover, assessing community responses at low taxonomic levels, such as the genus level, could highlight important trends that might not always be observed in the higher taxonomic ranks (Dohrmann et al., 2013).

Unfortunately, using different approaches and statistical analyses, such as in this thesis, does not allow us to compare the results of the different studies, nor to study statistically the possible link between structure and function as Bier et al. (2015) pointed out (Table 1).

In the Study III of this thesis, the ASV approach was used and rare species were eliminated because there are some errors in the amplicon data processing, such as chimeras/artefacts/contaminants that they can be understood as real variants like singleston-doubleston types (Callahan, Sankaran, et al., 2016). Including rare species in diversity analyses can almost entirely drive richness estimates to nonsensical values. However, this receives a lot of criticism since rare members are assumed to play an important role harbouring ecologically critical functions in the ecosystem (Pester, Bittner, Deevong, Wagner, & Loy, 2010; Tsementzi, Poretsky, Rodriguez-R, Luo, & Konstantinidis, 2014). So far, there is not a universal scientific consensus to calculate the richness and it is an issue widely discussed in popular forums about metagenomics.

Table 1. Summary of the main potentials and drawbacks of the molecular methods used in this thesis for the study of the composition and diversity (structure) of prokaryotes.

	APPROACHES	PROS		CONS	
STRUCTURE	16S rRNA (DNA fraction)	• Target for identification • Huge diversity • High taxonomic resolution • Laboratory protocols and procedures available • Standard bioinformatic pipelines • Availability of sequencing commercial systems	• Rodicio & Mendoza, 2004 • Hug et al., 2016; Jay T Lennon & Locey, 2016 • Gibson et al., 2015 • Callahan, Sankaran, et al., 2016; Edgar, 2016; Schloss et al., 2009	• Chosen target region • Bioinformatic choices and errors	• Whiteley et al., 2012 • Kopylova et al., 2016; Whelan & Surette, 2017.
	Different taxonomic levels for β-diversity	• Phylum • Genus • OTU/ASV	• Sánchez-Andrea et al., 2011; Zhu et al., 2013 • Dohrmann et al., 2013 • Caporaso et al., 2010; Gołębiewski et al., 2014/Callahan et al., 2017; Dohrmann et al., 2013	• No comparison analysis among Study I, Study II and Study III • Rare members (singletons and doubletons)	• Bier et al., 2015 • Pester et al., 2010; Tsementzi et al., 2014
	Indicator species	• High sensitivity • Low site disturbance • Environmental risk assessment	• Hermans et al., 2017; Lear & Lewis, 2009; Shahsavari et al., 2017	• Environment complexity • Subjective election criteria • Methodology difficulties	• Lindenmayer & Likens, 2011 • Siddig et al., 2016 • Urban, Swihart, Malloy, & Dunning, 2012

The search for **indicator species** (Table 1) was used in this thesis as the first step to develop microbial metal-pollution indices targeted to assess environmental risk. Microorganisms, such as bacteria, are highly sensitive, play crucial roles in biogeochemical cycles, have fast growth rates and respond quickly to changes in the environment. Hermans et al. (2017), among others, confirmed that microbial community indicators and specific taxa can reflect changes in the environment due to anthropogenic activities and therefore, sites can be classify into contaminated and noncontaminated sites. Bacteria have significant advantages as indicators in terms of relative speed, ease of data analysis and minimization of site disturbance during sampling collection but the use of these indicators is not as common as the use of macroorganisms such as plants, waterbirds, fish and invertebrates (Yang, Li, Gao, Chen, & Zhan, 2019).

Despite the increasing popularity and the advantages of using indicator species, several limitations have been described (Table 1). A single population rarely reflects the complexity of the environment; needless to say single species (Lindenmayer & Likens, 2011). Siddig et al. (2016) argued that election criteria for indicators are subjective. Many times they are just selected because they are locally abundant or ecologically significant. The term "indicator" is ambiguous. There are lots of terms use to refer to this word: ecological indicator, indicator species, bioindicator... Additionally, methodological difficulties like experimental protocols and statistical methods may bias results (Urban et al., 2012).

2.2 Function

In this thesis, **16S rRNA gene amplicon ARN** sequencing was also performed. Apart from having all the advantages commented before for DNA sequencing (Table 1), it is also an approximation to the function of the communities in the sense that it reflects the active fraction of community members (Jacquiod et al., 2018) (Table 2). This approach is a better strategy than the DNA sequencing for predicting the actual performance of an ecosystem under metal stress.

However, this approach has some limitations (Table 2). The RNA extraction is very difficult to obtain from natural environments because this molecule is very unstable and degrades easily. In addition, there are some constraints in the interpretation of the RNA values as indicators of microbial activity that should be considered. For instance, the growth rate of many prokaryotes is not always simply correlated with the RNA content (Worden & Binder, 2003). In fact, the relationship between the RNA concentration and the growth rate can be significantly different among taxa (Blazewicz et al., 2013). Dormant cells can contain higher number of ribosomes than in the cells in vegetative state (Sukenik et al., 2012).

A second approach used to determine the function of microbial communities in this thesis was the quantification of **functional target genes**. This allows to study in depth the functions of interest.

As it has been exposed in this thesis, microbial communities play an important role in nutrient cycling. Denitrification, nitrification, anammox and dissimilatory nitrate reduction to ammonia metabolic pathways are well known. It is also known that some can be affected by metals such as nitrification. Nitrification is a key process in the cycling of nitrogen in terrestrial and aquatic ecosystems. The first rate-limiting step of nitrification is the oxidation of ammonia to nitrite, which is carried out by AOB and AOA and it is catalyzed by the enzyme ammonia monooxigenase. The subunit A of the *amoA* gene is the most commonly used marked for tracing ammonia oxidizers in environmental samples by means of gene sequencing and qPCR (Fernàndez-Guerra & Casamayor, 2012; Merbt et al., 2012). These prokaryotes can be very interesting for ecotoxicology field because they can be indicators of metal pollution. In the Study II of this thesis AOA were quantified and they did not seem to be affected by metal concentrations. In fact, Wang et al. (2014) indicated that these effects on ammonia oxidizers are complex and they depend not only on metal concentrations but also on the physiological role of each metal element in the microorganisms and on the environmental conditions.

In this thesis, the study of these genes was carried out by qPCR following the standardized protocols previously published. This technique is preferentially used due to its high specificity, sensitivity, wide dynamic range for huge variety of functional genes and its relatively low cost (Ginzinger, 2002) (Table 2).

However, qPCR requires a previous and thorough study to understand the complex, very diverse and in some cases even unknown metabolic pathways of prokaryotes. This includes primer design for these genes, which is sometimes a complex task due to primer optimization (Cantos-Parra, Ramió-Pujol, Colprim, Puig, & Bañeras, 2018). Moreover, the usefulness and interpretation of qPCR results depend heavily on a number of factors, including the quality and quantity of extracted samples, random errors in experiments and the reference gene selected for normalization and comparison (Klein, 2002) (Table 2).

Furthermore, in this thesis we also used some traditional ecological methodologies to study the effect of metal concentration on microbial functions such as nutrient uptake and decomposition. The use of these endpoints also has benefits and drawbacks (Table 2).

Nutrient cycling is a central aspect of stream ecosystem functioning in which prokaryotes play an important role as we have commented before. For this reason, several researchers have used a variety of methods to quantify related parameters to describe nutrient retention (isotopic methods, short-term nutrient addition experiments, chambers experiments) (Dodds et al., 2002; Mulholland et al., 2000; Niyogi, Simon, & Townsend, 2004).

Nutrient cycling is mostly affected by metal pollution as a functional descriptor for microbial ecotoxicology. This concept has been largely studied in soils (Kandeler, Kampichler, & Horak, 1996; Liu, Xue, Yu, & Li, 2019; Plante, 2007). In this thesis, experiments with crystallizers and stream cobbles were used in order to study **nutrient uptake** of microbial communities affected by metal stress in streams. In the Study II, we mainly looked for the effect of heavy metal on nitrification. This approach is largely known and the main advantage is that these methods are easy to conduct, replicable and inexpensive, so they are available to a wider research community (O'Brien & Dodds, 2008).

However, in many streams, nutrient concentration is relatively stable over a time frame of hours during baseflow conditions and there is little net uptake. That is to say that, the nutrient uptake method is slow. It takes at least 2 hours to carry out. Moreover, it is difficult to scale measurements up to the whole system. On the contrary, O'Brien & Dodds (2008) did not find significant differences between chamber experiments and short-term nutrient addition experiments but a selection of representative components of the stream was necessary to obtain that result.

Leaf litter decomposition was addressed in this thesis as a model of key microbial activity under metal stress in the same way as Ferreira et al. (2016) and Sridhar & Bärlocher (2011) did. They found negative effects of heavy metals on this decomposition. Leaf litter is a dominant component of coarse particulate organic matter in streams and its decomposition has received considerable attention. Gessner & Chauvet (1994) and Mora-Gómez et al. (2015) determined the drivers of this decomposition. Duarte, Pascoal, Alves, Correia, & Cássio, 201) studied the succession of organisms that take part in this degradation. Furthermore, Artigas, Romaní, Gaudes, Muñoz, & Sabater (2009) and Romaní et al. (2013) looked into how climatic and hydrological dynamics affect it. As this concept has been largely studied, there is a standard procedure with litter bags and exponential decay model to obtain a decomposition rate (Bärlocher, 2005).

The critical role of faunal community composition in leaf decomposition has been demonstrated using different mesh size litter bags to control exposure of litter to different faunal size classes (Alp, Cucherousset, Buoro, & Lecerf, 2016; Handa et al., 2014). In this thesis, the breakdown was studied by using of a mesh with the of size 1 cm^2. The decomposition was also explored as the extreme environmental conditions did not allow macro and meiofauna to survive (Study III). However, to center the study on prokaryotes and fungi is usually necessary the use of litter bags with a finer mesh size (<1mm^2). This decomposition could occur at a slower rate than when using litter bags with a larger mesh size. Moreover, it is dominated by dissolution processes (Lecerf, 2017).

Table 2. Summary of the main potentials/benefits and drawbacks of using molecular methods and other endpoints in this thesis to study the function of prokaryotes.

	APPROACHES	PROS		CONS	
FUNCTION	16S rRNA (RNA fraction)	• Active fraction	• Jacquiod et al., 2018	• Costly extraction • No correlation to growth rate • Dormant cells	• Blazewicz et al., 2013; Worden & Binder, 2003 • Sukenik et al., 2012
	Functional genes	• Ecophysiology and habitat distributions of ammonia oxidizing microorganisms • Available molecular standardized protocols for genes of interest qPCR	• Fernàndez-Guerra & Casamayor, 2012; Merbt et al., 2012; Wang et al., 2014 • Ginzinger, 2002	• Complex metabolic pathways. Primers design • qPCR method errors	• Cantos-Parra et al., 2018 • Klein, 2002
	Nutrient uptake	• Widely applied method • Easy to conduct and reproducible • Inexpensive	• Kandeler et al., 1996; O'Brien & Dodds, 2008; Plante, 2007	• Stable nutrient concentration • Difficult scaling up measurements	• O'Brien & Dodds, 2008
	Decomposition/ breakdown	• Standard procedure (litter bags)	• Bärlocher, 2005	• Mesh size <1mm^2 to center the study on prokaryotes and fungi • The velocity of rate vary among streams	• Lecerf, 2017 • Menéndez et al., 2001

In addition, the decomposition rate of a given leaf species can vary greatly depending on the streams, so the classification concerning velocity of rate has limitations subject to the environmental characteristics of the stream (Bärlocher, 2005; Menéndez, Martinez, Hernández, & Comín, 2001).

3. Future perspectives

This thesis, through its three studies, mainly observational, has generated a deeper insight into the effects of different metals on epilithic microbial communities. These observations give us the basis for the following questions that might require a more controlled approach.

- Is there a link between the resident community structure and its functioning?
  - Future experiments with manipulation of the structure of microbial communities could potentially help assess directly if the composition alter the function under controlled conditions.
- Which genes within active microbial communities are expressed? And more specifically, how do the genes involved in metal resistance or metal detoxification respond under these different metal concentrations?
  - Laboratory experiments with biofilm microcosms simulating these different environmental conditions analyzed with the new molecular methods, such as shotgun metagenomics and metatranscriptomics, would allow us to check the identification and expression patterns of these resistant genes and quantify their gene expression in order to compare them.
- In this context, how do bacterial metal indicators at genus or family taxonomic levels selected in this thesis respond to multiple stress? Could they serve as Mediterranean indicators?
  - It would be interesting to define certain experimental conditions simulating different Mediterranean regions in channels that permit the growth of some indicator species, such as genera Sphingorhabdus, Flavobacterium, Prosthecobacter, Ferruginibacter, Arcicella (Study I) or family Family XII, TRA3-20, Caedibacteraceae, Paracaedibacteraceae, Rickettsiaceae or Diplorictttsiaceae (Study II). Then, it could add an inoculum of experimental microbial community with only one of the indicator species commented before and verify through monitoring if this specie can be always detected in a significant number.

GENERAL CONCLUSIONS

- The nutrients and the conductivity conditions of the three streams in the Ter River basin had a great influence on the α and β-diversity of the resident prokaryotic community of epilithic biofilms under chronic and low metal stress.

- Chronic exposure to low levels of metal pollution (mainly Zn) changed the composition of the active prokaryotic communities (β-diversity) without reducing the number of species (α-diversity) favouring metal adapted communites in epilithic fluvial biofilms. Metal-impacted communities were very active, indicating a close link with the stress deal with, probably related to the stimulation of detoxification processes.

- Low flow conditions associated with water scarcity increased the dissolved and bioaccumulated metal concentration in the Osor River (mainly 400 μg Zn L^{-1} and 17,000 μg Zn g^{-1}) causing great effects on the β-diversity of the resident microbial community of epilithic biofilms.

- High toxicity of chronic metals caused by interactions between hydrological and chemical alterations led surprisingly to the presence of endosymbiotic bacteria, probably related to the adaptation of the community to metal pollution.

- Abrupt changes in metal concentrations at short space scale (Fe spring) affected both, the resident structure and the function of microbial community at a higher taxonomic level (phylum) than those previously reported in epilithic biofilms sampled at a larger scale in the Osor, Llémena and Ter Rivers.

- Natural high metal concentrations increased the richness and diversity of prokaryotes generating a singular environment dominated by chemolithotrophic bacteria. However, low metal concentrations decreased the richness and diversity of prokaryotes, changing their composition and suggesting that eukaryotic communities (algae and fungi) compete with prokaryotic ones.

- The methodologic approaches followed in this thesis, with field studies and the use of molecular tools, has shed light on the metal chronic stress which fluvial ecosystems suffer in the Mediterranean regions.

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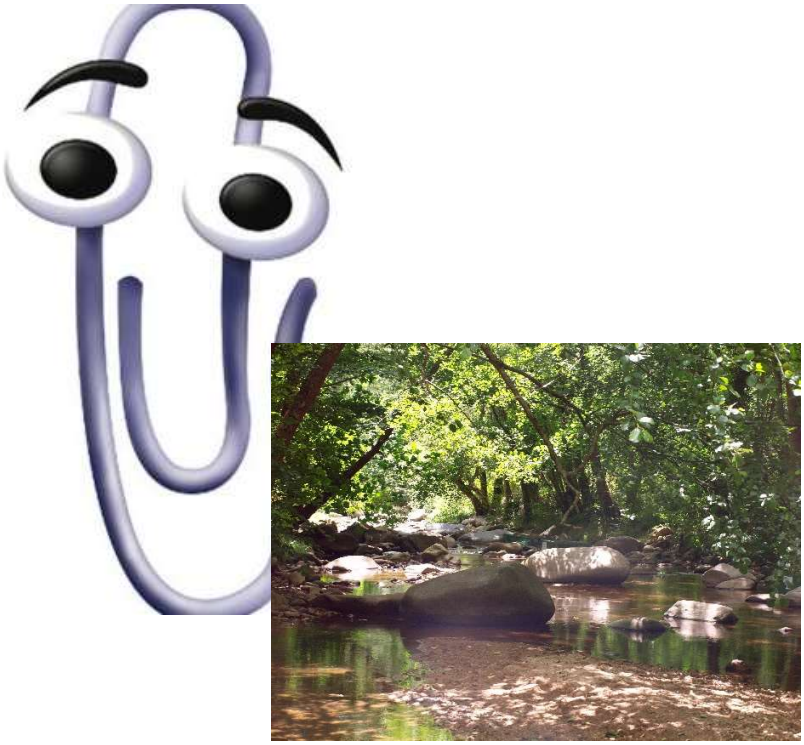
SUPPLEMENTARY MATERIAL



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

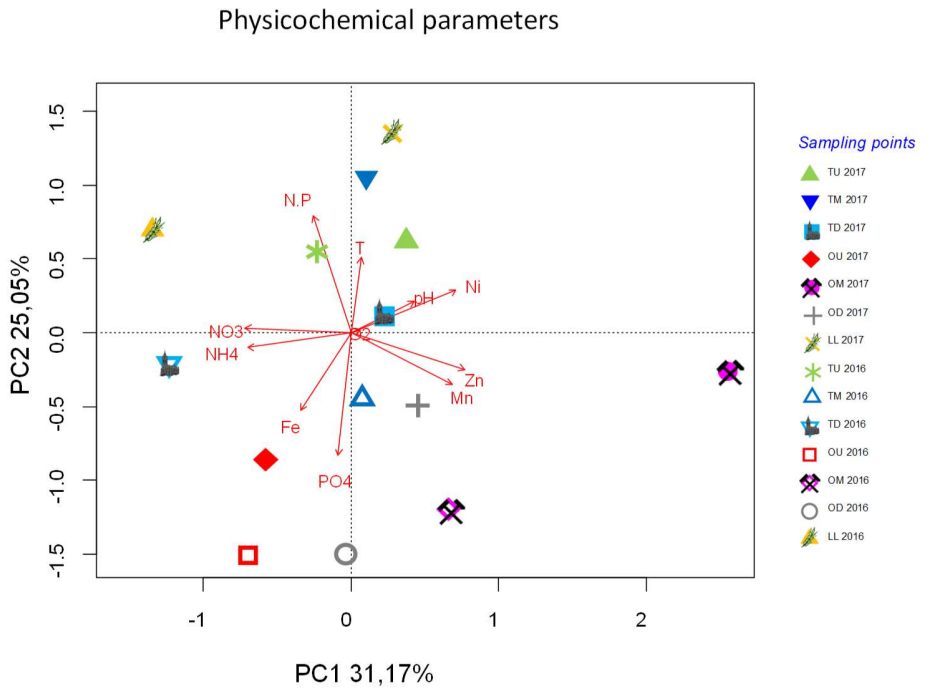


Figure 1. Principal component analysis (PCA) of the different physicochemical parameters in each sampling site and year (shown in legend).

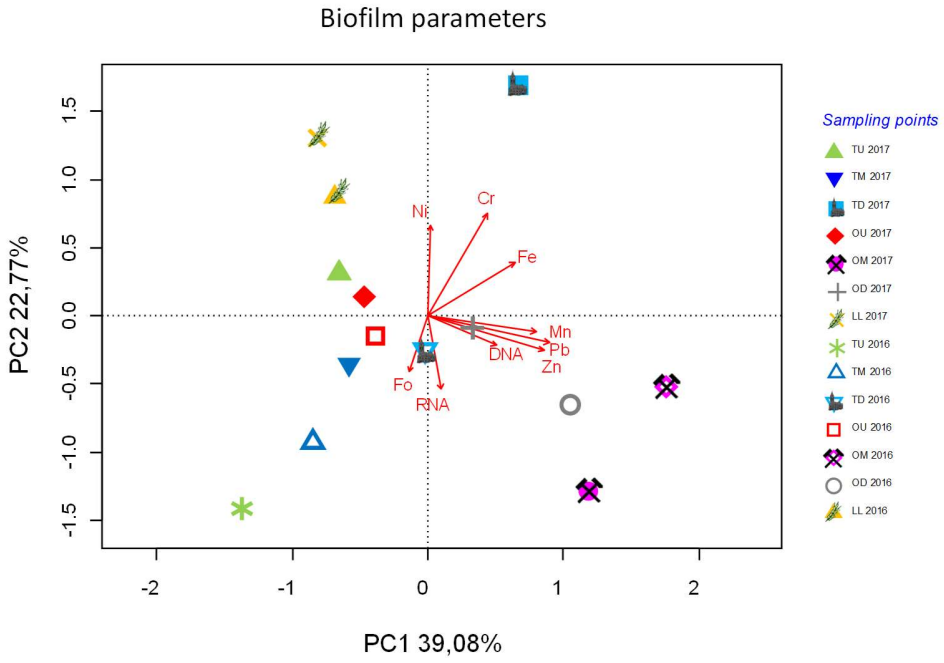


Figure 2. PCA of the different biofilm parameters in each sampling site and year (shown in legend).

Table 1. Pearson correlations between some environmental variables and α-diversity of microbial communities (those that are considered more important). Coefficients (r) and significance levels (p) (top and bottom values, respectively) are given.

	DNA				RNA			
Environmental variables	**Sobs**	**Chao1**	**1/D**	**H'**	**Sobs**	**Chao1**	**1/D**	**H'**
T (ºC)	-0.56 (0.01)	-0.54 (0.02)	-0.48 (0.04)	-0.48 (0.04)				
Cond (µS cm^{-1})	-0.49 (0.03)	-0.49 (0.03)						
Ni (µg L^{-1})				-0.47 (0.04)				
Cr (µg g^{-1})					0.48 (0.04)	0.52 (0.02)		
Fe (µg g^{-1})	0.47 (0.05)				0.52 (0.02)	0.52 (0.02)		
Ymax					0.68 (0.00)	0.68 (0.00)	0.54 (0.02)	0.57 (0.01)
RNA (ng mg^{-1})					-0.47 (0.04)	-0.47 (0.04)	-0.46 (0.05)	-0.55 (0.02)

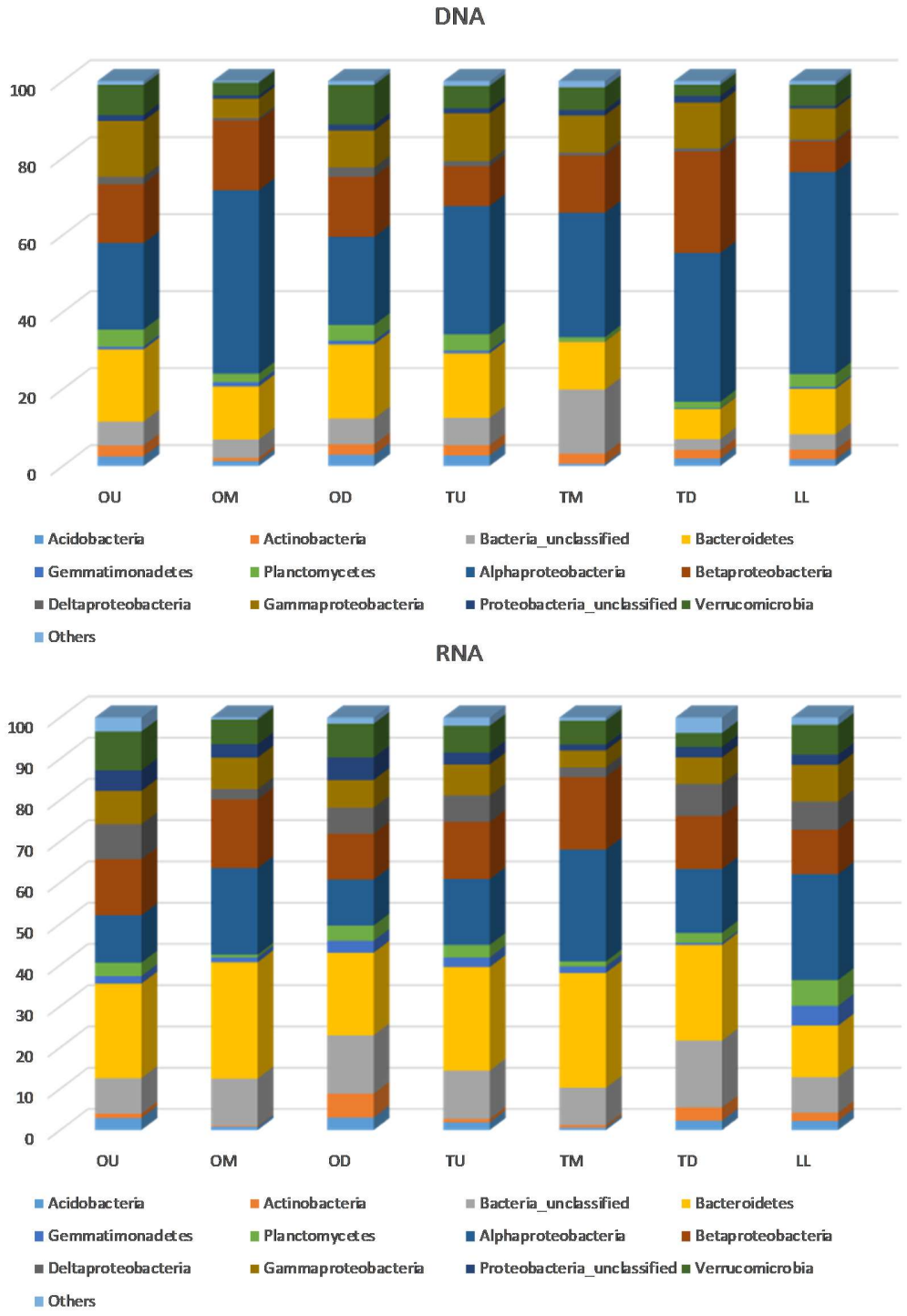


Figure 3. Relative abundance at phylum (or class) level of the DNA fraction (above) and RNA fraction (below) of microbial communities in the studied sites, average of 2016 and 2017 sampling sites n=21. The group "Others" represents the 1.7% of the total sequences.

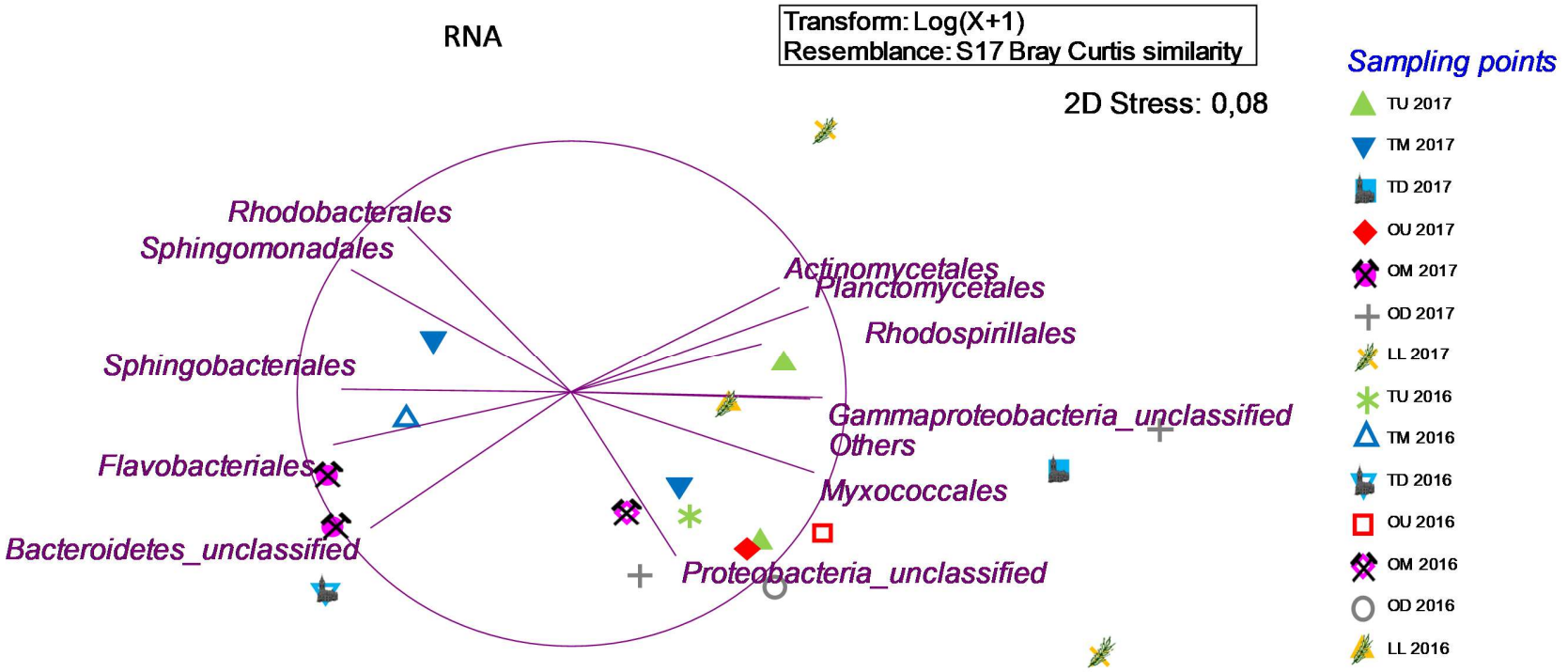


Figure 4.The NMDS represents the RNA fraction respect to the bacteria relative abundance at order level with a 0.7 Pearson correlation based on Bray-Curtis distance measures.

Table 2. PERMANOVA results show significant differences between years and sampling sites. Significant p-values (at a level of α = 0.05 with 999 permutations) are highlighted in bold.

	PERMANOVA	Site	Year	Site*Year	PERMDISP	Site	Year
DNA	p-value	**0.005**	**0.006**	**0.034**		0.554	0.511
	Signification	*	*	*			
RNA	p-value	**0.026**	0.186	0.57		0.614	0.892
	Signification	*					

Study II: Direct and indirect effects of multiple stressors on the microbial communities in a mining area

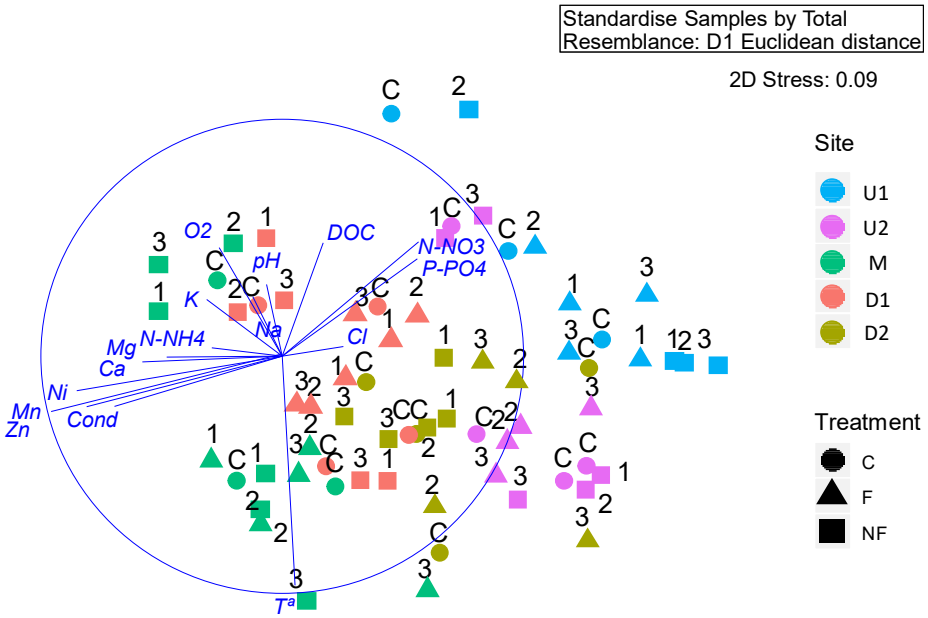


Figure 1. An NMDS analysis of physicochemical parameters (Pearson correlations > 0.2) shows differences between sites and no differences between treatments. Numbers represents replicates of each site (different enclosures). C: river samples (control), NF: treatment without fish, F: treatment with fish.

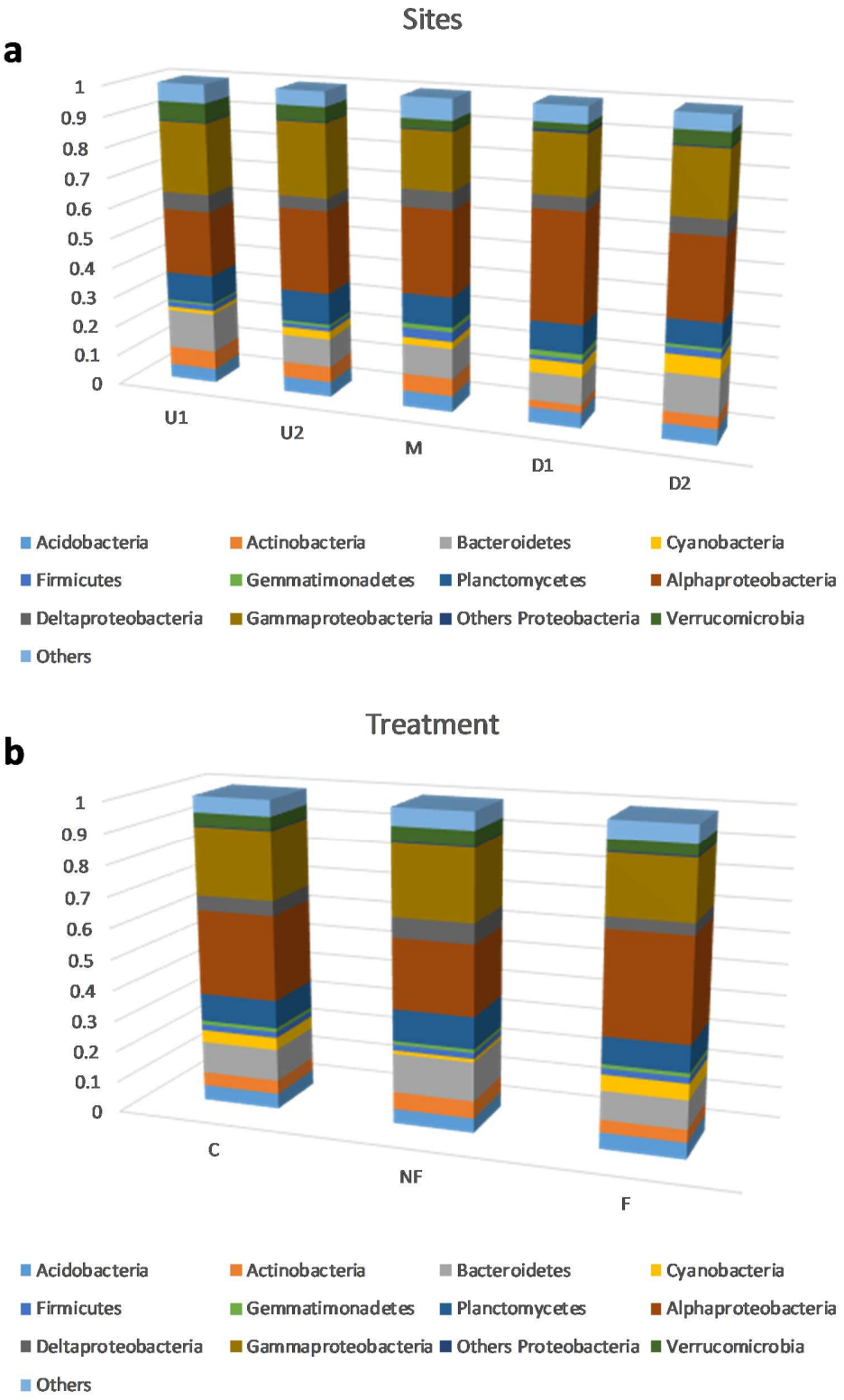


Figure 2. Relative abundance of bacterial community at phylum level (or class) by sites (a) and treatment (b). C: river samples (control), NF: treatment without fish, F: treatment with fish.

Table 1 Average and standard deviation of biofilm parameters at each sampling site, n= 40.

	U1	U2	M	D1	D2
Uptake PO_4^{3-} ($\mu g\ P\text{-}PO_4^{3-}\ cm^{-2}$)	0.001 ± 0.016	-0.010 ± 0.044	-0.021 ± 0.023	0.004 ± 0.022	0.008 ± 0.017
Uptake NO_3^- ($\mu g\ N\text{-}NO_3^-\ cm^{-2}$)	0.020 ± 0.132	-0.005 ± 0.110	-0.010 ± 0.158	-0.106 ± 0.145	0.037 ± 0.062
Uptake NH_4^+ ($\mu g\ N\text{-}NH_4^+\ cm^{-2}$)	0.010 ± 0.10	0.002 ± 0.008	-0.008 ± 0.016	-0.001 ± 0.011	-0.006 ± 0.008
C/N	9.90 ± 1.71	9.09 ± 1.09	9.86 ± 0.68	8.89 ± 0.77	8.75 ± 1.09
C/P	1155 ± 513	1746 ± 1163	1244 ± 458	1570 ± 533	1465 ± 904
N/P	128.6 ± 81.6	195.5 ± 142.6	126.2 ± 46.0	179.7 ± 67.0	171.8 ± 118.3
AOA (gene copies mg^{-1} biofilm)	1981.3 ± 1508.8	1552.6 ± 1084.5	856.5 ± 326.5	1481.7 ± 1016.7	3320 ± 859.2
DNA yield (ng DNA mg^{-1} biofilm)	20.8± 1.8	23.6 ± 5.6	14.0. ± 6.3	23.4 ± 7.9	33.1 ± 13.4
AFDW ($mg\ cm^{-2}$)	0.74 ± 0.58	0.66 ± 0.36	0.87 ± 0.73	0.76 ± 0.36	1.40 ± 1.12
Chl-a ($\mu g\ cm^{-2}$)	1.21 ± 0.96	2.09 ± 2.35	2.74 ± 2.62	4.07 ± 3.38	6.92 ± 6.49
Autotrophic index	0.73 ± 0.34	0.52 ± 0.36	0.42 ± 0.20	0.25 ± 0.12	0.25 ± 0.08
H’	6.73 ± 0.14	6.54 ± 0.24	6.66 ± 0.28	6.29 ± 0.23	6.66 ± 0.31
1/D	247.37 ± 81.44	164.17 ± 58.63	223.87 ± 97.23	142.64 ± 29.71	194.81 ± 101.30
Sobs	4184 ± 687	4171 ± 387	4181 ± 500	3499 ± 412	4601 ± 371
Chao1	5745 ± 1332	5869 ± 577	5636 ± 875	4885 ± 681	6562 ± 503

Table 2. Average and standard deviation of biofilm parameters for the three different treatments. C: river samples (control), NF: treatment without fish, F: treatment with fish, n =40.

	C	NF	F
Uptake PO_4^{3-} (µg P-PO_4^{3-} cm^{-2})	-0.008 ± 0.021	0.001 ± 0.036	-0.006 ± 0.021
Uptake NO_3^- (µg N-NO_3^- cm^{-2})	0.033 ± 0.159	-0.062 ± 0.066	0.005± 0.147
Uptake NH_4^+ (µg N-NH_4^+ cm^{-2})	0.002 ± 0.011	0.000 ± 0.016	-0.003 ± 0.009
C/N	9.03 ± 1.33	10.17 ± 0.62	8.61 ± 0.98
C/P	1593 ± 1079	1317 ± 501	1451 ± 749
N/P	186.1 ± 136.5	129.6 ± 48.3	173.9 ± 100.2
AOA (gene copies mg^{-1} biofilm)	1995.6 ± 1585.1	2457.6 ± 1103.3	1114.5 ± 882.9
DNA yield. (ng DNA mg^{-1} biofilm)	23.1± 5.6	22.4 ± 10.2	23.5 ± 11.9
AFDW (mg cm^{-2})	0.87± 0.41	1.38 ± 0.87	0.40 ± 0.10
Chl-a (µg cm^{-2})	4.96± 5.24	4.57 ± 4.27	1.21 ± 0.70
Autotrophic index	0.41 ± 0.36	0.42 ± 0.26	0.46 ± 0.30
H’	6.57 ± 0.36	6.71 ± 0.21	6.45 ± 0.24
1/D	194.15 ± 96.46	240.05 ± 79.48	149.37 ± 52.76
Sobs	4194 ± 696	4106 ± 639	4105 ± 406
Chao1	5987± 985	5550 ± 1221	5824 ± 661

Study III: Environmental drivers of microbial community structure in a high iron calcareous-spring

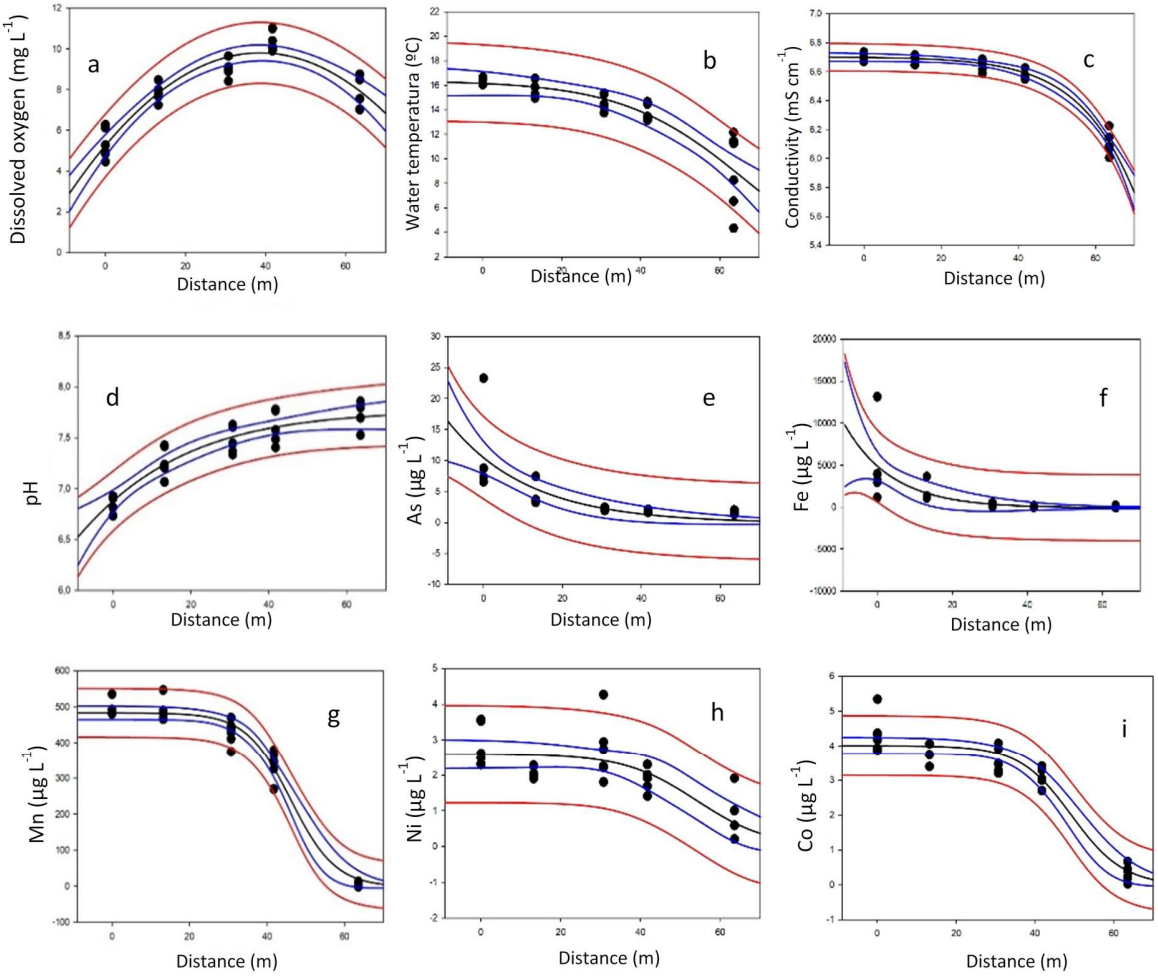


Figure 1. Changes along the canal in a) dissolved oxygen (cuadratic curve); b-d) water temperature, conductivity and pH (sigmoidal curve); e-f) As and Fe (exp decay); g-i) Mn, Ni and Co (sigmoidal curve). See tables 1 and 2 for fitting results.

Table 1. Biofilm metal contents in sampling sites.

Site	1	2	3	4	5
Al (µg/g)	2827	1059	424.6	506.3	15631
Cr (µg/g)	5.48	2.28	2.74	2.51	310.5
Fe (µg/g)	167288	182250	23338	5740	11845
Mn (µg/g)	222.5	615.5	1355.7	820.5	355.5
Sr (ug/g)	591.7	837.7	888.8	694.6	497.3
As (µg/g)	279.7	287.4	51.22	9.20	8.78
B (µg/g)	172.5	189.4	21.99	4.54	129.4
Ba (µg/g)	227.7	255.4	69.0	33.2	154.3
Co (µg/g)	3.77	5.47	7.64	4.11	7.10
Ni (µg/g)	7.48	7.00	4.86	2.44	17.15
Zn (µg/g)	16.39	16.68	14.94	7.90	36.29

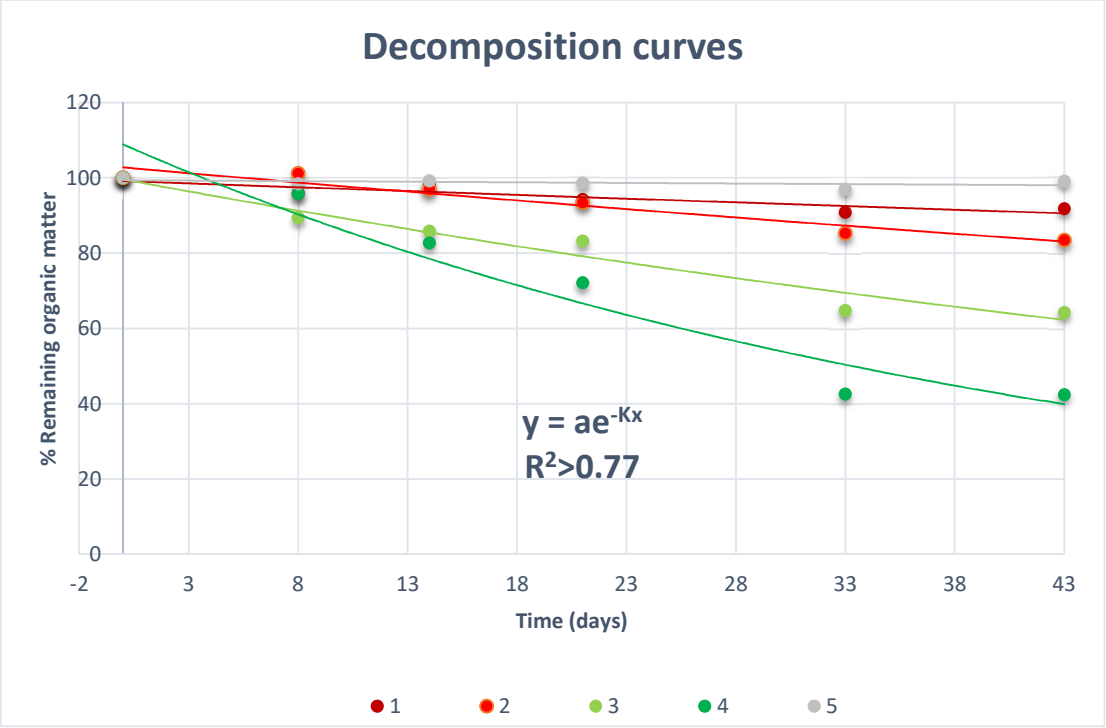


Figure 2. Decomposition curves of percentage of Remaining organic matter vs Time let calculate the decomposition or breakdown rates in each site.

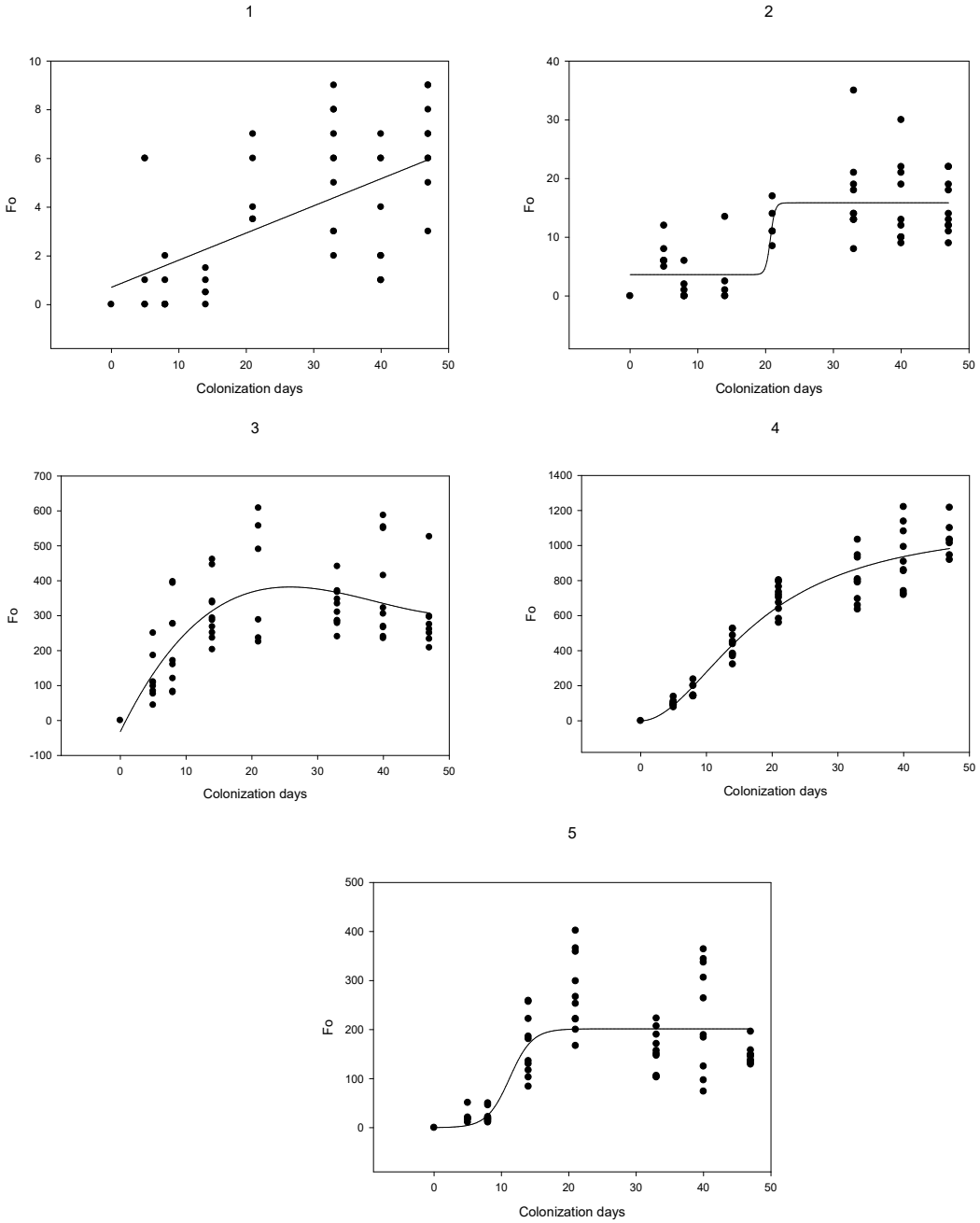


Figure 3. Different fittings of F_0 vs Colonization days in each site. The area below the curves allow us to calculate primary production.

Table 2. Report of fitting of Figure 3.

SITE 1				
R	**Rsqr**	**Adj Rsqr**	**Standard Error of Estimate**	
0.9788	0.958	0.9454	9.8296	
	Coefficient	**Std. Error**	**t**	**P**
a	732.181	52.5845	13.9239	<0.0001
b	0.1915	0.0173	11.0463	<0.0001
x0	5.9551	0.0934	63.7357	<0.0001
y0	4.1764	3.5212	1.1861	0.263
SITE 3				
R	**Rsqr**	**Adj Rsqr**	**Standard Error of Estimate**	
0.9704	0.9417	0.9242	7.7805	
	Coefficient	**Std. Error**	**t**	**P**
a	456.2929	38.779	11.7665	<0.0001
b	0.1999	0.0206	9.7096	<0.0001
x0	5.514	0.1087	50.7491	<0.0001
y0	6.6644	2.7322	2.4392	0.0349
SITE 4				
R	**Rsqr**	**Adj Rsqr**	**Standard Error of Estimate**	
0.9948	0.9896	0.9864	2.7678	
	Coefficient	**Std. Error**	**t**	**P**
a	365.9259	12.3974	29.5164	<0.0001
b	0.2985	0.0133	22.5225	<0.0001
x0	5.7479	0.0682	84.309	<0.0001
y0	1.769	1.2574	1.4068	0.1898
SITE 5				
R	**Rsqr**	**Adj Rsqr**	**Standard Error of Estimate**	
0.9927	0.9854	0.981	4	
	Coefficient	**Std. Error**	**t**	**P**
a	483.0472	19.4701	24.8097	<0.0001
b	0.2501	0.013	19.2855	<0.0001
x0	6.0542	0.0705	85.8605	<0.0001
y0	3.3823	1.6537	2.0453	0.068

Table 3. Fe-tolerant ASVs with a presence > 90% in site 1.

Acidobacteria

Blastocatella
Aridibacter famidurans
Stenotrophobacter
RB41
Geothrix

Actinobacteria

Actinoplanes
Microbacteriaceae
Cellulomonas
Nocardioides glacieisoli
Crossiella
Lapillicoccus
Gaiella
Paenarthrobacter
Rubrobacter

Bacteroidetes

Dysgonomonadaceae
Macellibacteroides
Flavisolibacter
IheB3-7
Paludibacteraceae
WCHB1-32
Niastella
ST-12K33
Prolixibacteraceae

Cyanobacteria

Leptolyngbya_ANT.L52.2
EcFYyy-200
Scytonema_UTEX_2349
Chalicogloea_CCALA_975
Mastigocladopsis_PCC-10914
Aliterella_CENA595
Pleurocapsa_PCC-7319

Spirochaetes

RBG-16-49-21
Spirochaetaceae
Salinispira

Alphaproteobacteria

Ellin6055
Qipengyuania
Sphingomonas lutea
Sphingomonas parvus
Rubellimicrobium
Devosia geojensis
Phenylobacterium mobile
Xanthobacteraceae
Rhodoplanes
Mesorhizobium
Hirschia
Beijerinckiaceae

Deltaproteobacteria

Oligoflexus
Myxococcaceae
Desulfovibrio mexicanus

Gammaproteobacteria

Gallionella
Candidatus_Nitrotoga
Massilia
Piscinibacter

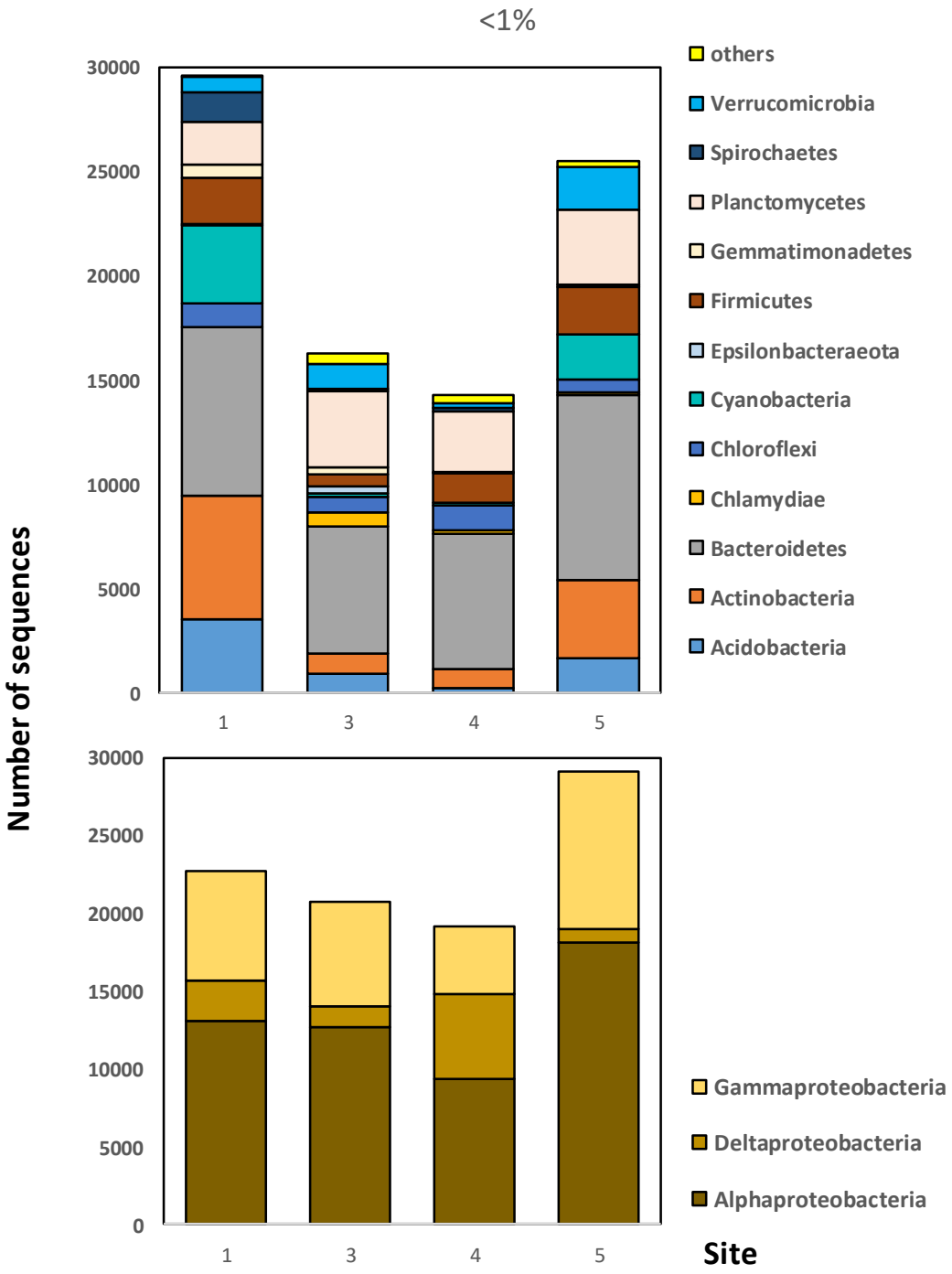


Figure 4. ASVs representing < 1% of total number of sequences in different sites.

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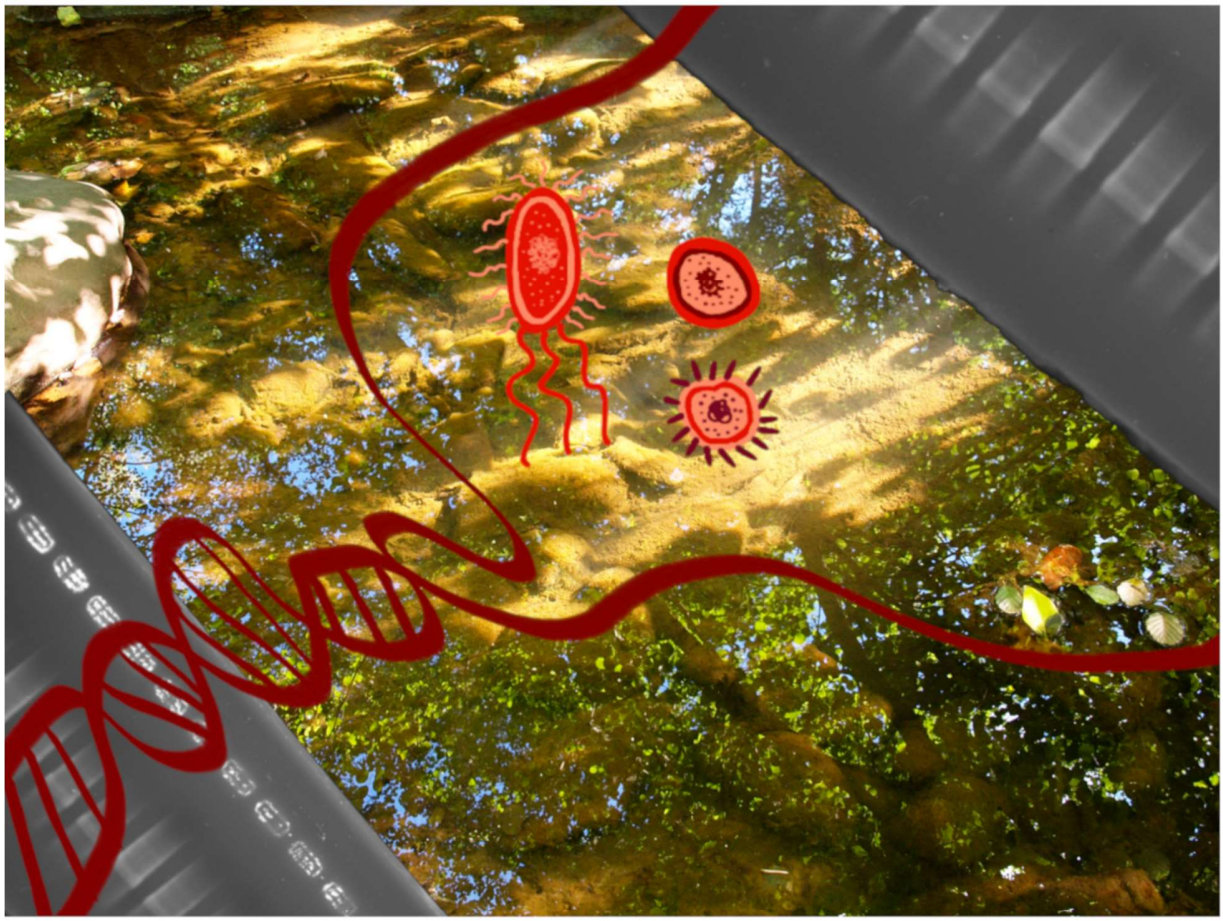
Molecular



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