

Exploring the Diversity and Bioremediation Potential of Epilithic Biofilms Living in an Acid Mine Drainage-affected Mountain Stream

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Sarah Zecchin*, Evelien Jongepier[°], Gigliola Borgonovo*, Leonardo Scaglioni*, Nicoletta Guerrieri', Gerard Muyzer[°], Lucia Cavalca*

*Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente (DeFENS), Università degli Studi di Milano (Italy); [°]Microbial Systems Ecology, Department of Freshwater and Marine Ecology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam (The Netherlands); 'Water Research Institute, National Research Council of Italy (CNR-IRSA), Verbania Pallanza (Italy)

INTRODUCTION & AIMS

Due to the increasing of Epilithic biofilms and to lower nitrate/nitrite, in Italian Italy streams in natural water sites several low levels of 20 mg kg⁻¹ and 10 mg L⁻¹ respectively (Fig. 1) (Zecchin et al., 2020). In the Amosio Valley (Fig. 1), an acid mine drainage is present in the stream bed and some harmful acid mine leachate affect the Rio Rosso stream (Fig. 2).

THE STRUCTURE OF THE RIO ROSSO BIOPILMS MICROBIOTA

The microbial community living in the Rio Rosso location was characterized by 16S rDNA amplicon sequencing, targeting the V3-V4 region of bacterial and archaeal 16S rDNA genes.

The rare work was processed with QIIME 2 (Caporaso et al., 2010) and the SILVA 132 reference database (Silva 2013) (Silva et al., 2012) to identify the obtained Amplicon Sequence Variants (ASVs).

In both Mio and Rosso streams, Bacteria species richness was higher than Archaea (Figures 3 and 4). In the Rosso both bacterial and archaeal communities were richer with respect to the Mio biofilms. Bacterial and archaeal evenness showed the opposite trend in the same sampling sites.

THE RIO ROSSO BIOPILMS MICROBIOME

Mio and Rosso biofilms samples were characterized by Illumina shotgun sequencing, processed on a MiSeq platform. Rarefaction plots showed that the 250,000 reads pipeline (Kover et al., 2020).

De novo assembly was performed with SPAdes (Banik et al., 2017) and the sequences were mapped on the SRA-KAT server (Eaton-Bornstein et al., 2019) to get an overview of the microbiome.

Reconstruction of the microbial communities living in the Rio Rosso epilithic biofilms, in both samples, Prokaryotes to use the recent database pangenome, while in Mio and Rosso biofilms were, in both samples and Prokaryotes were the recent meta-database, respectively (Figure 11).

CULTIVABLE MICROORGANISMS

Low and sulfur-oxidizing bacteria living in the biofilms were enriched with low nitrate/nitrite values, as reported in Amosio and Mio (Fig. 1) (Figure 10). The microorganisms cultured in the gradient tubes were mostly affiliated to aerobic sulfur-oxidizing genera (Figure 17) and contributed to the organization of streams with high nitrate.

WATER & BIOPILM CHEMISTRY

The Rio Rosso epilithic biofilms were characterized by the water (20 mg sample) and approximately 1.7 km² distance from the mine (Rosso sample) (Figure 15).

CONCLUSIONS

The Rio Rosso epilithic biofilms represent greater complexity when microorganisms are well adapted to high concentrations of arsenic and heavy metals. Such metabolic properties and the presence of EPS ensure that contaminated water resulting out of the mine is filtered by the microbial canopy and reduce biofilms that represent a natural sink for arsenic and heavy metals.

The tandem studies with arsenic and heavy metal resistances might be exploited in bioremediation strategies applied to arsenic-contaminated environments.

CHAT | ABSTRACT | REFERENCES | CONTACT AUTHOR

Sarah Zecchin*, Evelien Jongepier[°], Gigliola Borgonovo*, Leonardo Scaglioni*,
Nicoletta Guerrieri', Gerard Muyzer[°], Lucia Cavalca*

*Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente (DeFENS), Università degli Studi di Milano (Italy); [°]Microbial Systems Ecology, Department of Freshwater and Marine Ecology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam (The Netherlands); 'Water Research Institute, National Research Council of Italy (CNR-IRSA), Verbania Pallanza (Italy)



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INTRODUCTION & AIMS

Due to the mineralogy of Alpine bedrock and to former mining activities, in Northern Italy arsenic in soil and water often exceeds law limits of 20 mg kg^{-1} , and 10 mg L^{-1} , respectively (D.Lgs 152/2006). In the Anzasca Valley (Piedmont, Figure 1), an acid mine drainage originated from a former gold mine heavily affects the Rio Rosso stream (Figure 2).

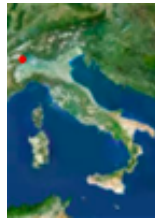


Figure 1. Location of the Anzasca Valley



Figure 2. Rio Rosso stream at approximately 1 km from the mine

The stream is characterized by the presence of extensive epilithic biofilms at the opening of the mine and throughout the contaminated waterbed (Figure 3).



Figure 3. Epilithic biofilm growing on the rocks in the Rio Rosso stream waterbed

The aim of this study is to characterize the microbial communities inhabiting in the Rio Rosso epilithic biofilm and to elucidate the microbial metabolic properties allowing these microorganisms to survive extreme metal concentrations

WATER & BIOFILM CHEMISTRY

The Rio Rosso epilithic biofilm was characterized at the mine (**Mine sample**) and approximately 1.7 km downstream the mine (**Stream sample**) (Figure 4).

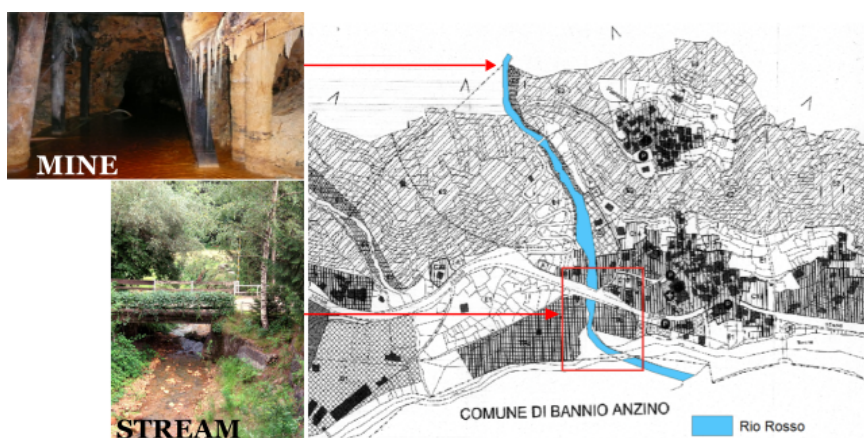


Figure 4. Location of the sampling points

The water sampled at the **Mine** point was **acidic, sulfur-rich** and with **negligible organic carbon**, while in the **Stream** sampling point the water was **neutral** and with **higher organic carbon, nitrate and ammonia**.

Table 1. Physico-chemical parameters measured in the Rio Rosso water at the Mine and Stream sampling points

	T (° C)	pH	TOC (mg L ⁻¹)	SO ₄ ²⁻ (mg L ⁻¹)	NO ₃ ⁻ (µg L ⁻¹)	NH ₄ ⁺ (µg L ⁻¹)
Water at gold mine	8	2.63	n.d.	1580.00	0	n.d.
Downstream water	16.7	7.62	0.28	74.60	207	3

In the water at both sampling points all metals and metalloids exceeded the law limits.

The epilithic biofilms accumulated high amounts of all metals and metalloids.

Table 2. Metals and metalloids measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) in water and biofilm samples

	Al	As	Cu	Fe	Mn	Ni	Zn
Water at gold mine (mg L ⁻¹)	35.82	10.6	2.70	149.79	4.25	3.53	9.63
Downstream water (mg L ⁻¹)	0.75	0.10	0.03	1.31	0.15	0.01	0.22
Mine epilithic biofilm (mg kg ⁻¹)	59740	93430	30	184810	70	0	170
Downstream epilithic biofilm (mg kg ⁻¹)	n.d.	7170	2540	129690	1380	70	10710

Microorganisms living in the biofilms produced extracellular polymeric substances (EPS) including different monosaccharides (Figure 5 and Table 3), that putatively contributed to accumulate metals and metalloids.

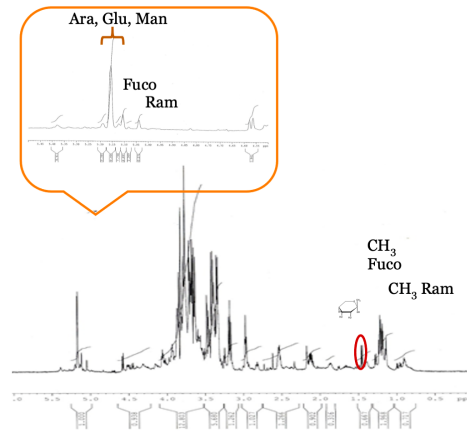


Figure 5. Monosaccharide composition of the EPS characterized by Nuclear Magnetic Resonance (NMR) after hydrolysis with trifluoroacetic acid (TFA) and 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization

Table 3. Percentage of monosaccharides present in the EPS extracted from the Rio Rosso biofilm, determined by High-Performance Liquid Chromatography (HPLC)

Monosaccharides	%
Mannose	8.15
Rhamnose	3.37
Glucose	67.14
Unknown	8.15
Arabinose	9.57
Fucose	3.62

THE STRUCTURE OF THE RIO ROSSO BIOFILMS MICROBIOTA

The microbial communities living in the Rio Rosso biofilms were characterized by MiSeq Illumina amplicon sequencing, targeting the V3-V4 region of bacterial and archaeal 16S rRNA genes.

The raw reads were processed with QIIME2 (<https://qiime2.org/>); Bolyen et al., 2019) and the SILVA SSU reference database version 138 (<https://www.arb-silva.de/>); Pedregosa et al., 2011) was used to classify the obtained Amplicon Sequence Variants (ASVs).

In both Mine and Stream biofilms, Bacteria species richness was higher than Archaea (Figures 6 and 7). In the Stream both bacterial and archaeal communities were richer with respect to the Mine biofilm. Bacterial and archaeal evenness showed an opposite trend in the two sampling sites.

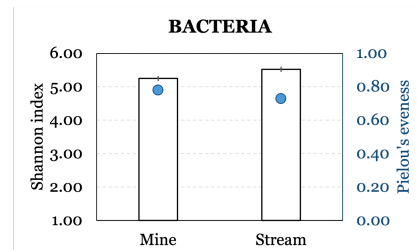


Figure 6. Alpha diversity of the bacterial communities

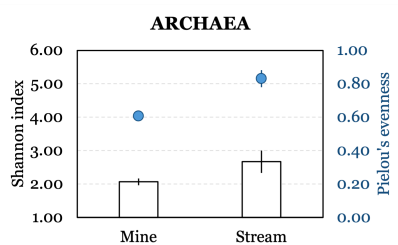


Figure 7. Alpha diversity of the archaeal communities

Principal Component Analysis (PCoA) revealed that the microbial communities in the two sampling sites were completely different and that Stream replicates were more variable with respect to the Mine replicates (Figures 8 and 9).

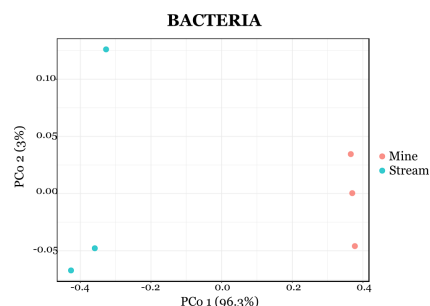
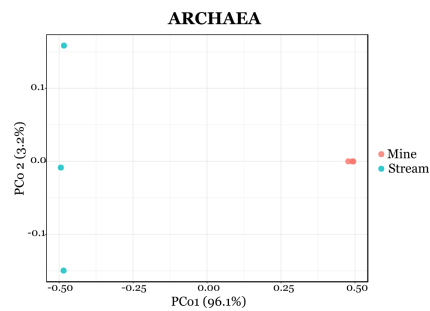
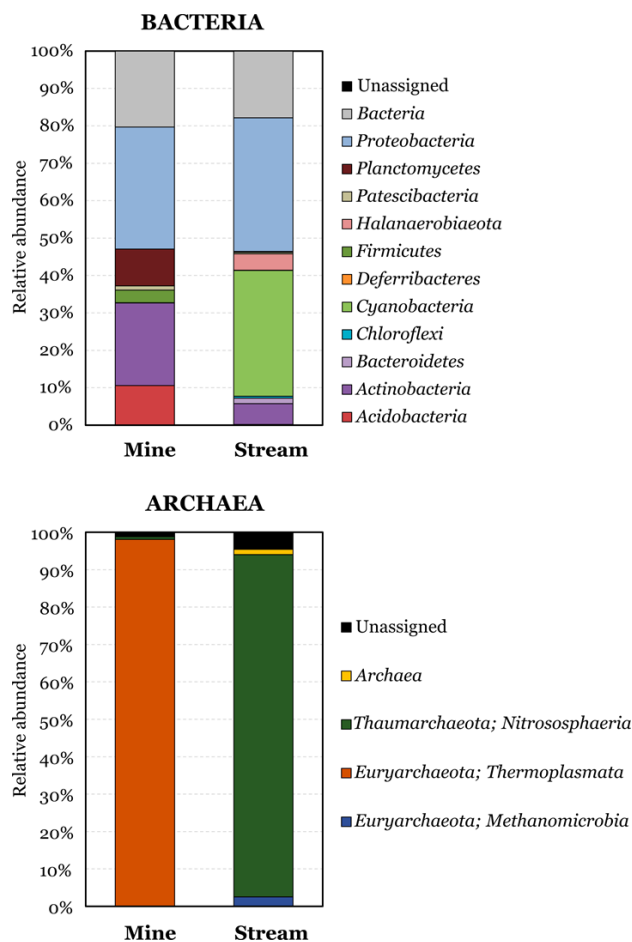


Figure 8. PCoA performed on the Bray Curtis dissimilarities in the bacterial communities**Figure 9.** PCoA performed on the Bray Curtis dissimilarities in the archaeal communities

Half of both Mine and Stream biofilms was characterized by unclassified Bacteria and by *Proteobacteria* (Figure 10). The other half of the community was different in the two biofilms: the Mine biofilm was dominated by *Planctomycetes*, *Actinobacteria* and *Acidobacteria*, while the Stream biofilm was dominated by *Cyanobacteria*.

Concerning Archaea, the Mine biofilm was dominated by *Thermoplasmata*, while the Stream biofilm was dominated by *Nitrososphaeria*.

**Figure 10.** Relative abundance at phylum and class level of bacterial and archaeal communities living in the Rio Rosso biofilms

THE RIO ROSSO BIOFILMS MICROBIOME

Mine and Stream biofilm samples were characterized by Illumina shotgun sequencing, performed on a MiSeq platform. Raw reads were processed following the ATLAS pipeline (Kieser et al., 2020).

De novo assembly was performed with metaSPAdes (Nurk et al., 2017) and the sequences were uploaded on the MG-RAST server (<https://www.mg-rast.org/> (<https://www.mg-rast.org/>), Meyer et al., 2008) to get an overview of the metagenomes.

Bacteria dominated the microbial communities living in the Rio Rosso epilithic biofilms. In both samples, *Proteobacteria* was the most abundant phylum, while in Mine and Stream biofilms where *Acidobacteria* and *Cyanobacteria* were the second most abundant phyla, respectively (Figure 11).

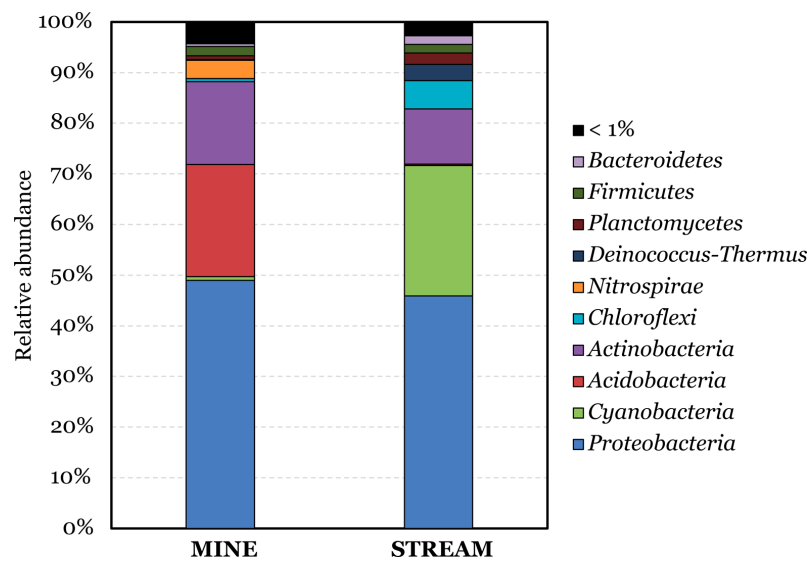


Figure 11. Taxonomic composition of the Mine and Stream metagenomes

The abundance of genes involved in arsenic transformation was similar in the two samples, where arsenic resistance was mostly mediated by arsenate reduction to arsenite (Figure 12). Only a few sequences aligned with genes encoding the arsenite oxidase enzyme, suggesting that arsenite oxidation was not a dominant strategy in the biofilm.

Genes involved in heavy metal resistance were significantly more abundant in the Stream biofilm (Figure 12).

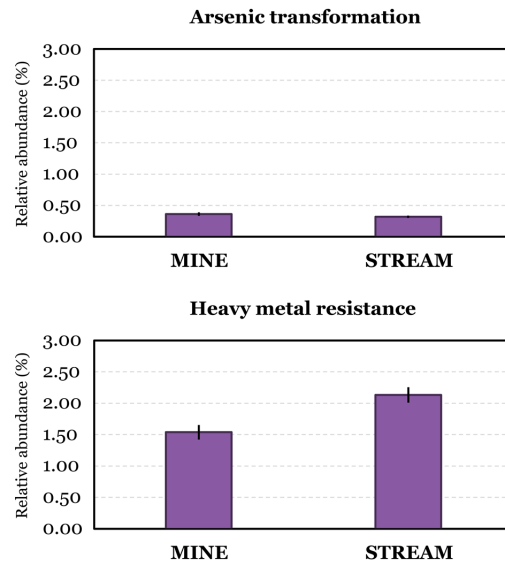


Figure 12. Relative abundance of genes involved in arsenic and heavy metal resistance in the Rio Rosso biofilms

Genome binning was performed using metabat2 and maxbin2 (Kang et al., 2019; Wu et al., 2016) to extract the most representative Metagenome-Assembled Genomes (MAGs) from Mine and Stream metagenomes.

Respectively 15 and 8 MAGs were retrieved from Mine and Stream metagenomes, mostly belonging to previously uncharacterized bacterial taxa (Figures 13 and 14).

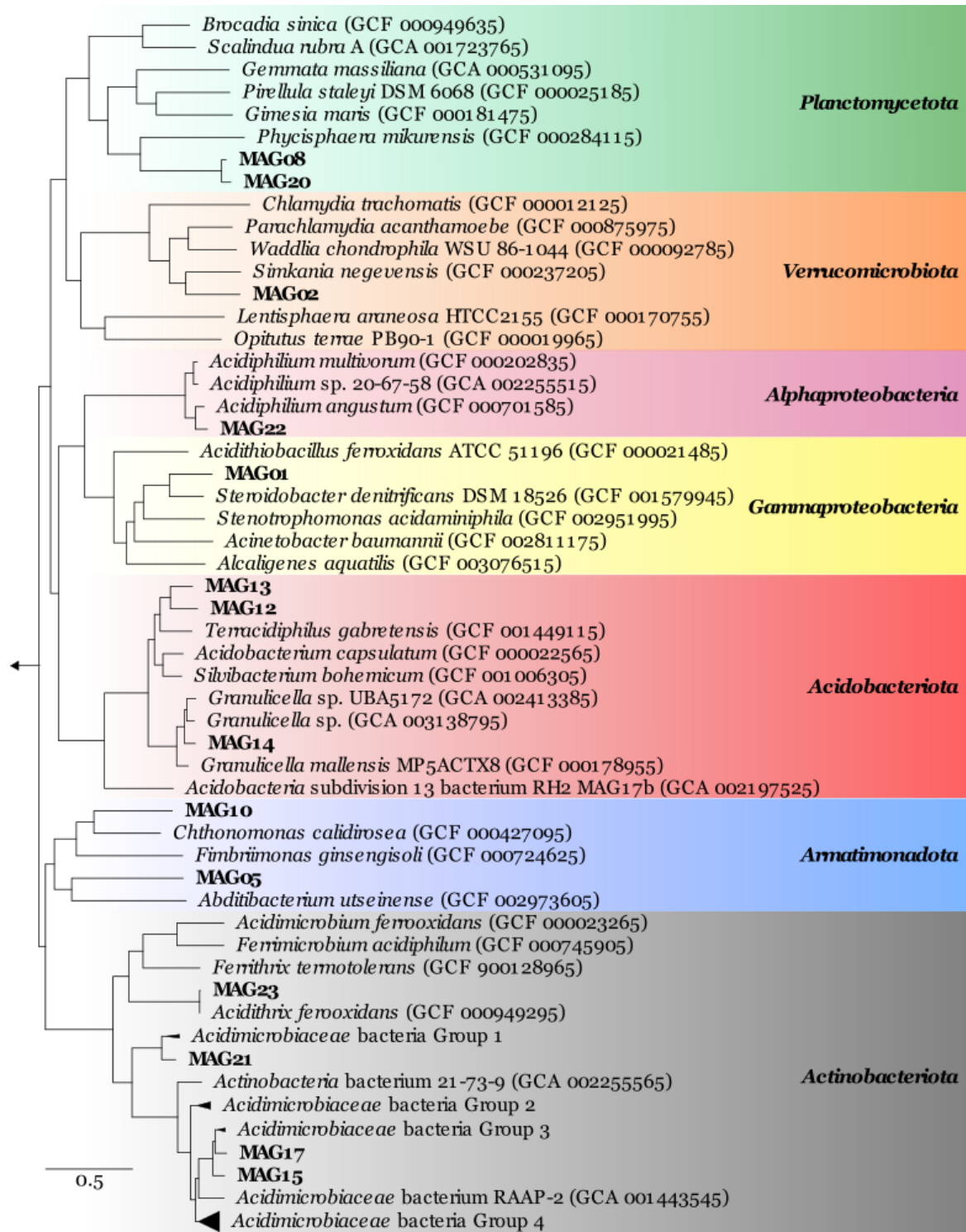


Figure 13. Phylogenetic analysis of Mine MAGs

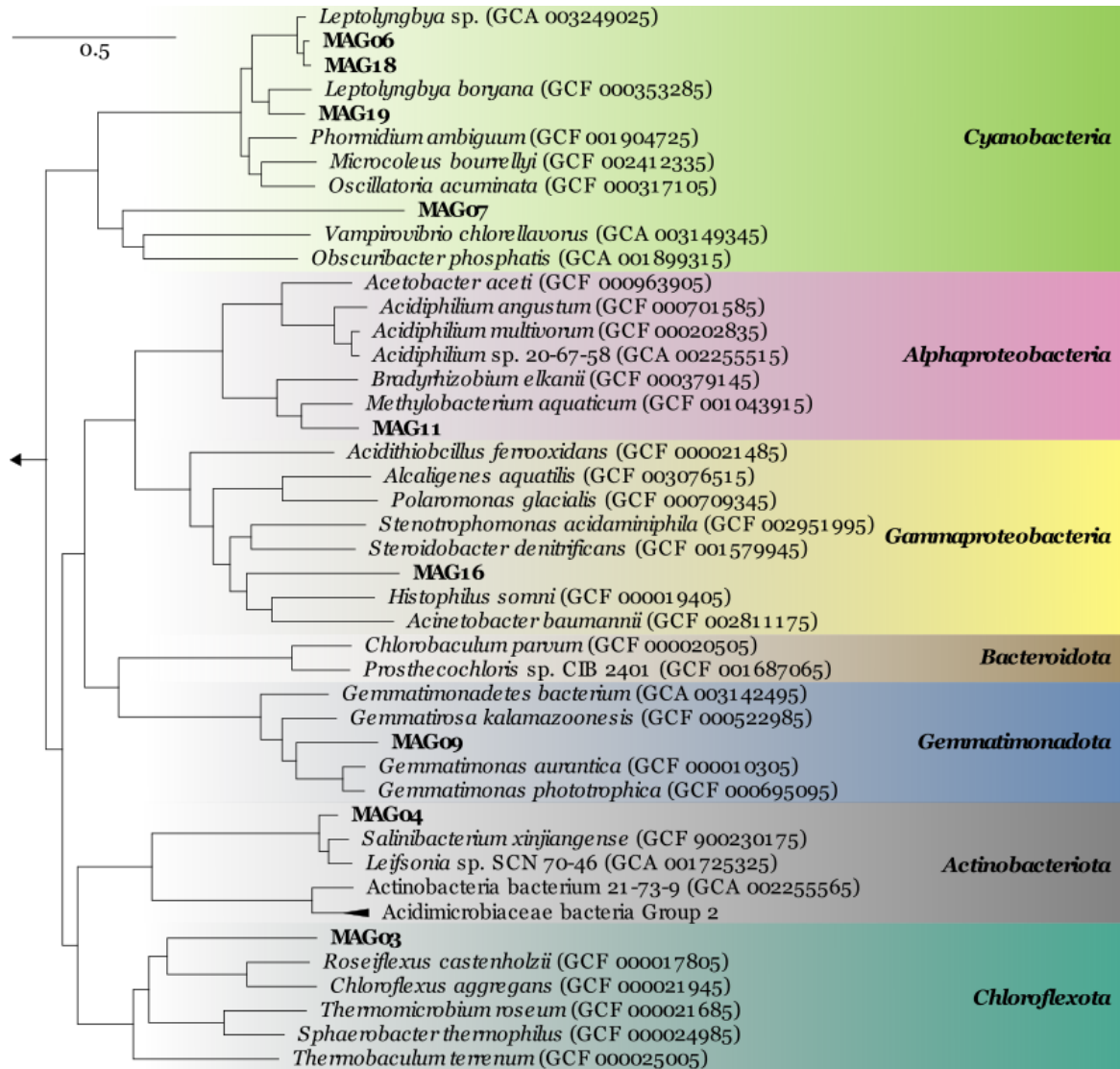


Figure 14. Phylogenetic analysis of Stream MAGs

According to gene prediction performed with Prodigal (Hyatt et al., 2010) and mapping to the eggNOG catalogue v5 (Huerta-Cepas et al., 2019), all MAGs included genes involved in metal resistance (Figure 15). Arsenic resistance was mediated by arsenate reduction followed by arsenite extrusion, while only MAG11 and MAG22 included genes putatively encoding the arsenite oxidase enzyme. This outcome confirms that arsenate reduction is the dominant resistance strategy in the Rio Rosso biofilms.

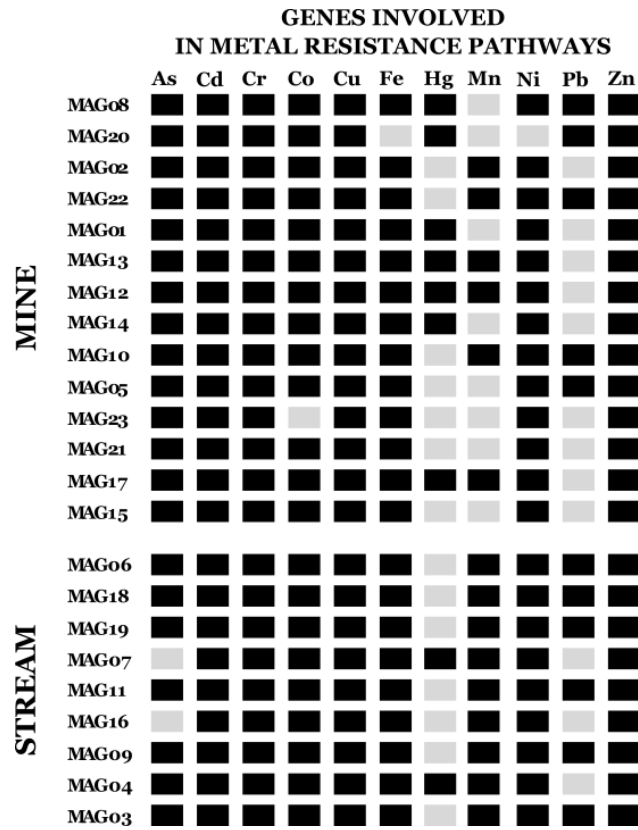


Figure 15. Presence of genes involved in metal resistance pathways in the MAGs

CULTIVABLE MICROORGANISMS

Iron- and sulfur-oxidizing bacteria living in the biofilm were enriched with iron sulfide gradient tubes set up according to Emerson and Moyer (1997, Figure 16). The microorganisms retrieved in the gradient tubes were mostly affiliated to versatile sulfur-oxidizing genera (Figure 17) and contributed to the coprecipitation of arsenic with Fe-S minerals.

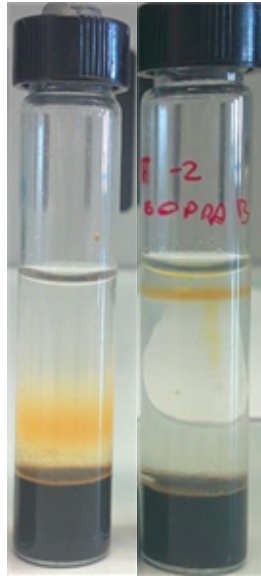


Figure 16. Non-inoculated (left) and inoculated (right) iron sulfide gradient tubes

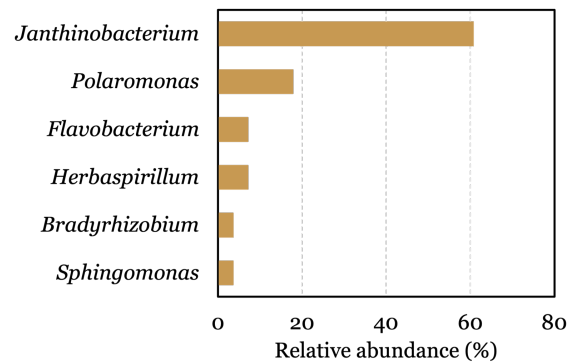


Figure 17. Relative abundance of bacterial genera retrieved in FeS gradient tubes

A total number of 67 heterotrophic bacteria, 10 autotrophic bacteria and 15 algae strains were isolated from the Stream biofilm. Heterotrophic and autotrophic bacterial strains were able to reduce arsenate and to oxidize arsenite, respectively (Figure 18). Algal strains were not able to transform arsenic, but they were able to resist to the metalloid, likely by the production of EPS.

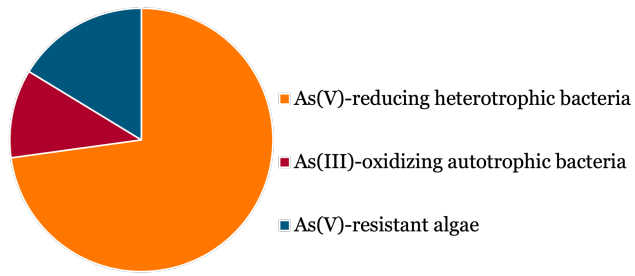


Figure 18. Arsenic transformation capacities in the microbial strains isolated from the Stream biofilm

CONCLUSIONS

The Rio Rosso epilithic biofilms represents peculiar ecosystems where microorganisms are well adapted to high concentrations of arsenic and heavy metals.

Such metabolic properties and the presence of EPS ensure that contaminated water running out of the mine is filtered by the microbial stream bed rocks biofilm that represents a natural sink for arsenic and heavy metals.

The isolated strains with arsenic and heavy metal resistances might be exploited in bioremediation strategies applied to arsenic-contaminated environments.

ABSTRACT

Given the mineralogy of the Alpine bedrock and former mining activities, several Northern Italy areas show arsenic concentrations above the Italian law limit of 20 mg kg⁻¹ of soil. In the Anzasca Valley (Piedmont), an acid mine drainage originated from an abandoned gold mine heavily affects the Rio Rosso stream. Due to the oxidative dissolution of pyrite and arsenopyrite, arsenic and other heavy metals are continuously released into the stream, contaminating the surrounding area. On the whole riverbed, the stream is characterized by the presence of an extensive reddish epilithic biofilm.

This study aimed at analyzing the composition and metabolic properties of the Rio Rosso epilithic biofilms to unveil their strategies to cope with extreme metal contamination and evaluate possible metabolic features exploitable for bioremediation purposes.

The epilithic biofilms in the vicinity of the mine and at 1.7 km downstream the mine were characterized by 16S amplicon and metagenomic sequencing in relation to the main physicochemical parameters of the biofilm and the surrounding water. In parallel, autotrophic and heterotrophic microorganisms were characterized by enrichment cultivation, isolation and testing for their ability to perform arsenic transformation.

Both mine and downstream biofilms were affected by metal contamination, with arsenic accounting for 93.43 and 8.66 g kg⁻¹, respectively. Other heavy metals were accumulated in the downstream biofilm, ranging from 3.59 mg kg⁻¹ to 10 g kg⁻¹. The mine biofilm was further affected by a pH of 2 and dominated by acidophilic iron- and sulfur-oxidizing microorganisms belonging to *Acidithrix*, *Acidiphilium* and uncharacterized Planctomycetota. Downstream the mine, a sub-neutral pH (pH 6.7) promoted the establishment of a significantly more diverse community driven by Cyanobacteria.

Both metagenomics and cultivation experiments revealed that arsenic resistance was mainly mediated by arsenate reduction to arsenite and consequent extrusion from the cell. Extruded arsenic was likely embedded in an extracellular polysaccharide matrix. To a lesser extent, arsenite oxidation was revealed by the presence of arsenite oxidase genes and the isolation of autotrophic arsenite-oxidizing bacterial strains.

These outcomes revealed that the acid mine drainage-affected Rio Rosso epilithic biofilm is a natural sink for arsenic and heavy metals, characterized by the assemblage of bacterial species with a great potential to be exploited for arsenic and heavy metal bioremediation in freshwater environments.

REFERENCES

- Bolyen et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37: 852–857. <https://doi.org/10.1038/s41587-019-0209-9>
- Emerson and Moyer (1997). Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. *Appl. Environ. Microbiol.* 63(12):4784–4792
- Huerta-Cepas et al. (2019). eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Res.* 47(D1): D309-D314. <https://doi.org/10.1093/nar/gky1085>
- Hyatt et al. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC bioinformatics* 11(1): 1-11. <https://doi.org/10.1186/1471-2105-11-119>
- Kang et al. (2019). MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *Peer J.* 7: e7359. <https://doi.org/10.7717/peerj.7359>
- Kieser et al. (2020). ATLAS: a Snakemake workflow for assembly, annotation, and genomic binning of metagenome sequence data. *BMC Bioinformatics* 21(1): 1-8. <https://doi.org/10.1186/s12859-020-03585-4>
- Meyer et al. (2008). The metagenomics RAST server—a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC bioinformatics*, 9(1), 1-8. <https://doi.org/10.1186/1471-2105-9-386>
- Nurk et al. (2017). metaSPAdes: a new versatile metagenomic assembler. *Genome Res.* 27(5): 824-834. <https://doi.org/10.1101/gr.213959.116>
- Pedregosa et al. (2011). Scikit-learn: Machine learning in Python. *J. Mach. Learn. Res.* 12: 2825-2830.
- Wu et al. (2016). MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets. *Bioinformatics* 32(4): 605-7. <https://doi.org/10.1093/bioinformatics/btv638>