Bacterial ecology of biofilms sustaining pollution by acid mine drainage near mining areas in Mpumalanga Province – South Africa

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Abstract

The ecology of biofilms collected from sediments and efflorescent crusts along the AMD near coal mine in the Mpumalanga Province was determined using the 16S bacterial metagenomy.

The results revealed a dominating presence in the biofilms of selected acidophilic chemoautotrophs that are known to contribute to AMD formation. The water samples had low pH (2.4 to 3.5), total acidity as $CaCO_3$ (2440 to 7040 mg/L), high level of metals (Al, Ca, Fe, Mg and Ni) and sulphate (3899 to 8874 mg/L). PHREEQC model allowed to predict the dominance of free hydrated speciated forms of Fe and Ni in the AMD.

Keywords: AMD, biofilm, efflorescent crusts, metagenomy, acidophiles, PHREEQC

Introduction

Acid and metal tolerant microorganisms are likely to proliferate in the AMD through formation of chemoautotrophically-based biofilms which are sustained by electron donors from sulphide minerals as well as CO_2, O_2 and N_2 derived from air and phosphate liberated by water-rock interaction (Baker and Banfield, 2003). These microorganisms which are present in biofilms attached to coals at the bank of AMD have the potential to increase or sustain AMD formation. The most common acidophilic bacteria have been identified in the AMD worldwide and these include the first isolated microbe from AMD called *Acidithiobacillus ferrooxidans* (Colmer et al., 1950) and *Leptosprillum ferrooxidans*. Although the iron and sulfur oxidizing chemoautotrophic prokaryotes dominate primary production in acidophilic environment, other species of microorganisms such as iron-oxidizing heterotrophs, obligate-heterotrophs and moderate acidophiles have also been identified in AMD (Johnson, 2007; Hallberg, 2010). Acidophilic archaea have also been detected in AMD with the most common being the mesophilic *Ferroplasma acidiphilum*, while the other are mostly thermophilic (Colmer et al., 1950). A number of microorganisms found in AMD have been reported to grow with ferrous as electron donor and therefore consider as primary producers of AMD water while fixing CO_2 for the synthesis of organic matters. The acidophilic heterotrophs on the other hand benefit from these organic matters and in return produce CO_2 use by the autotrophs and also degrade the organic matters in the soil that can be toxic to the primary producers (Johnson and Hallberg, 2008). Acting in co-operation, the primary producers and heterotrophs continuously influence the geochemistry of the AMD through the production of acidity and mobilization of metals which increase the pollution of the environment. The geochemistry of acid mine drainage varies from site to site, implying that a diverse communities of acidophilic microorganisms with varying physiological properties can occur in AMD across geological areas. Spatial distribution at the local level may also consider the differentiation of the influence of microorganisms in planctonic state and in biofilms on metal sulphides. Biofilms are communities of microorganisms embedded in a matrix of extracellular polymeric substances (EPS) (Fosso-Kankeu et al., 2010). The attachment of chemoautotrophic leaching microorganisms at the surfaces of minerals plays a significant role in their dissolution and therefore on the generation of AMD (Bellenberg et al., 2012; Vera et al., 2013; Zhang et al., 2014). Study by Sanhueza et al. (1999) revealed that attachment of cells of *A. ferrooxidans* to pyrite correlated with the degree of pyrite crystallization.

The determination of the microbial ecology of biofilms found in AMD is therefore important to predict the persistence of acidification and release of toxic metals in the aqueous system.

Study area

Emalahleni is situated in Mpumalanga province in South Africa with geological coordinates [25.8728° S, 29.2553° E]. This area is 2 678 km^2 in size with a reported population of approximately 395,466 people in 2011 (Statistics South Africa, 2011), and is approximately 140 km from Johannesburg. Emalahleni is characterised as a semi-arid area with a grassland biome. The coal mining activities situated in this region feeds into the Olifants catchment area. Samples were collected near the mining areas and from a stream tributary of the Brugspruit River at 15 km to the east of the perennial Olifants River into which the Brugspruit River feeds (Figure 1). Upstream activities include an abandoned and operating underground coal mines as well as a ferrochrome processing plant, resulting in severe water pollution as reported by Netshitungulwana et al. (2013).

Methodology

Sample collection

Water samples along the AMD effluent from the mine canal and dam through the extended paths discharged in the nearby residential area, were collected, stored, transported and analyzed as discussed in our previous paper (Fosso-Kankeu et al., 2017).

Biofilms were collected at the surface of the efflorescent crusts and at the bank of AMD (attached to the rocks) using spatula. The dislodged biofilms were collected into sterile glass bottles and transported in a cooler box containing ice packs and stored in the fridge (4°C) in the water laboratory at the School of Chemical and Minerals Engineering at the North-West University. After 16 h the biofilms were used for molecular analysis.

Water analysis

At the sampling site, physico-chemical parameters such as pH, conductivity, redOx potential (ORP) and temperature were determined at each sampling point using appropriate probes and meters (Hanna Instrument Inc, USA): temperature (°C), pH, electrical conductivity (mS/cm) and redOx potential (mV). The pH meter was calibrated before analysis in the field, using reference buffer solutions. The analysis of cations and anions was performed by external accredited laboratory.

Molecular identification of microorganisms

The Genomic DNA was extracted from the biofilms using the ZR Soil Microbe DNA Mini Prep kit™ according to the manufacturer's instruction. DNA concentrations were determined spectrophotometrically with a Nano-Drop spectrophotometer (Thermo Scientific). Then extracted DNA samples were sent to Inqaba Biotechnical Industries (Pty) Ltd for sequencing. Briefly, genomic DNA samples were PCR amplified using a universal primer pair (341F and 785R - targeting V3 and V4 of the 16S rRNA gene). Resulting amplicons were gel purified, end repaired and illumina specific adapter sequence were ligated to each amplicon. Following quantification, the samples were individually indexed, and another purification step was performed. Amplicons were then sequenced on illumina's MiSeq platform, using a MiSeq v3 (600 cycle) kit. 20Mb of data (2×300bp long paired end reads) were produced for each sample. The BLAST-based data analysis was performed using an Inqaba in-house developed data analysis pipeline; 1) Data was trimmed and only >q20 (i.e high quality) reads were used; 2) Every read was BLASTED and the result file saved; 3) The top hit for every BLAST result (i.e genus and species name) was counted and a record was kept of how many times each species appeared as a hit.

Results and discussion

Microbial communities in the biofilms

Visible pellicles of biofilms of brownish to greenish colors were collected at four locations along the AMD. After DNA sequenc-

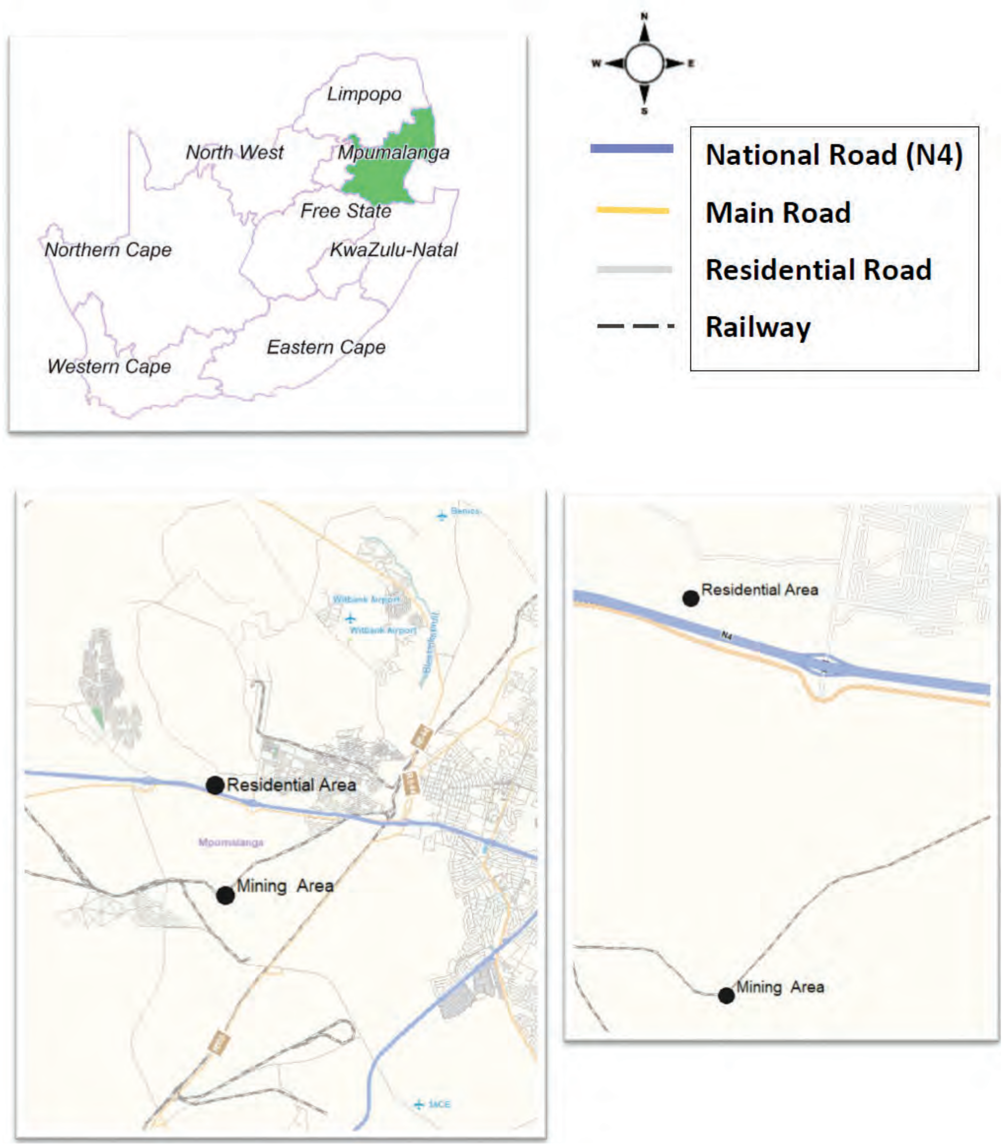

Figure 1 *Map of the sampling area in Emalahleni, Mpumalanga Province of South Africa*

ing, the results showed that the biofilms were harboring complex assemblages of microorganisms significantly dominated by bacterial community over the range of samples; previous reports have also shown that bacteria tend to dominate over archaea and eukarya in the mining wastes (Kock and Schippers, 2008). The abundant species were found to be acidophilic chemoautrophic bacteria such as *Acidithrix*, *L. ferrooxidans*, *A. ferrooxidans*, *Leptospirillum*, *Acidimicrobiaceae*, *Sulfobacillus*, *Acidiphilium* and *Acidithiobacillus* sp (Figure 2). These bacteria have been previously identified in environments of lower pH and higher ionic strength such as AMD (Bond et al., 2000). Acidophilic chemoautotroph bacteria group has been reported to dominate the community of bacteria in AMD, responsible of the acceleration of metal sulphide dissolution and formation or sustaining of AMD (Baker and Banfield, 2003). Sampling point Dt2 was found to contain most of the bacteria except *Sulfobacillus*, with *L. ferrooxidans* and *A. ferrooxidans* almost exclusively found in this sample. On the other hand, *Sulfobacillus* and *Acidithiobacillus* sp were dominants in sample D where they occur at 98.5 and 93% respectively. The last two species have been often detected in AMD and are known to enhance acid production in metal-leaching environments by oxidizing Fe^{2+} and replenishing the oxidant Fe^{3+} (Sand et al., 1995; Bond et al., 2000). *Acidimicrobiaceae* was found to be well represented in all the four samples. This is expected as it constitutes the family of *Acidimicrobiaceae* which harbors five monospecific genera, including *Acidimicrobium*, *Ferrimicrobium* and *Ferrithrix*, *Aciditerrimonas* and *Ilumatobacter* (Clark and Norris, 1996; Johnson et al., 2009; Matsumoto et al., 2009; Itoh et al., 2011; Stackebrandt, 2014). Although the profile of bacteria in the biofilms from all the different sites varied, all the sites had *Acidiphillium* and *Acidimicrobiacreae* in common, and most of them had at least one acidophilic chemoautotroph capable to oxidize sulphide minerals and generate acid. This could probably be the reason why water samples collected from sampling points Dt1, Dt2 and Dt3, located at few kilometers from the mine still had a very low pH. The involvement of iron-oxidizing chemoautotrophs in sulphide dissolution and acid generation has already been reported with implication on the environment. However, it was quite interesting to notice that the biofilms collected at the bank of the Dam's entrance (sample D) were mostly attached to the efflorescent crusts which is dominated by secondary minerals easily oxidizable. In our recent investigation (not published) it was shown that acidophilic chemoautotrophs from these biofilms accelerate the rate of oxidation of efflorescent crusts by several order of magnitude compared to the oxidation of coal tailings. This clearly shows that the presence of biofilms on efflorescent crusts at the bank of AMD can accelerate the process of acid generation therefore sustaining AMD pollution.

Water quality and metals speciation

After collection of water samples, some variables such as pH, electrical conductivity (EC) and redOx potential (Eh) were measured in-situ. The results (Table 1) show that all the samples were acidic with pH lower than 3.5, while high EC was also recorded indicating higher ionic strength which is characteristic of AMD. Although the sulphate content was high in all samples, higher concentrations were recorded in samples collected at the vicinity of the mine (PM and BB); this was also related to the large amount of minerals available at those points.

A multitude of metals were detected in the water samples at various concentrations. The major elements mainly included Al, Ca, Fe and Mg while those occurring in lower concentrations included Co, Cr, Ni and Zn among which Ni was the most abundant (Table 2). The concentration of Fe was ten times lowered

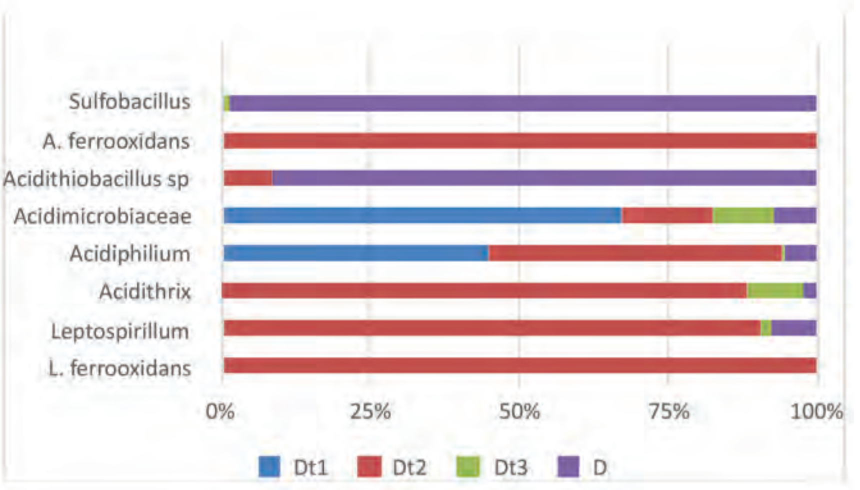

Figure 2 *Microbial profile in the biofilms collected from river bank and efflorescent crusts*

Table 1 Physico-chemical characteristics of water

Sampling points	In-situ variables			Major anions (mg/L)	
	pH	EC (mS/cm)	Eh (mV)	SO_4^{2-}	Cl^-
PM	2,7	750	220	8680	8
BB	2,7	718	220	8874	8
D	2,6	697	225	6776	11
AD	3,5	560	194	4911	29
UB	2,5	627	229	5624	14
Dt1	2,4	503	247	4069	14
Dt2	2,5	487	246	3899	13
Dt3	2,4	502	249	4008	14

when exiting the dam which implies that part of the Fe precipitated in the dam probably as a result of some form of treatment, but the concentration was increased further downstream, confirming that mobilization of metals is occurring along the AMD stream. It must also be noted that compared to Dt1, Dt2 and Dt3, higher concentration of Fe in the dam may have contributed to the growth of bacteria such as Sulphobacillus, Leptospirillum and Acidithiobacillus which required ferrous as electron donor. The continuous oxidation of minerals along the AMD stream can be partly attributed to the acidophilic chemoautotrophic bacteria present in the biofilms at the bank or attached to the efflorescent crusts.

To predict the potential of dispersion of mobilized metals from the coal tailings, the PHREEQC geochemical speciation model was used. The results of the prediction of metal species was presented in the form of free hydrated species or inorganic complexes (Table 3). It was observed that for both Fe and Ni, free hydrated species dominated over the inorganic complexes in all the water samples. The percentage range of the free hydrated speciated forms of Fe and Ni was between 68 to 74.8% and 52 to 61.8% respectively, while the percentage range of the inorganic complexes speciated forms of Fe and Ni was between 25.2 to 31.7% and 38.1 to 47.7% respectively. The free hydrated are likely to be bioavailable, while the inorganic complexes restrict the availability of free aqua metal ionic species to low concentration therefore reducing their bioavailability to the aquatic organisms which otherwise will be highly toxic (Korfali and Davies, 2004; Fosso-Kankeu et al., 2017). The dominance of free hydrated metal ionic species supported by lower pH contributed by acidophilic chemoautotrophic bacteria in the biofilms in this study therefore enhances the bioavailability and toxicity of metals to the aquatic life.

Table 2 Major and trace elements in water

Sampling points	Concentration of ions in water mg/L							
	Major elements				Trace elements			
	Al	Ca	Fe	Mg	Co	Cr	Ni	Zn
PM	261	461,8	2909	357,1	0,59	0,02	19,29	2,10
BB	261,7	454,6	2796	365,2	0,53	0,02	19,95	1,76
D	233,5	453,3	2514	387,7	0,52	0,02	17,63	1,52
AD	265,6	491,5	226,4	490	0,91	0,00	1,13	0,39
UB	208,7	453,6	1678	303,2	0,50	0,02	13,85	1,54
Dt1	110,1	402,8	598	227,8	0,27	0,01	5,70	1,01
Dt2	111,2	397,9	627,2	235,8	0,26	0,01	5,86	0,97
Dt3	114,1	401,3	582,9	238,7	0,27	0,02	5,76	1,00

Table 3 *Percentages of dominant speciated forms of metals in water*

Sampling point	% of species			
	Fe		Ni	
	Fe^{2+}	FeSO4	Ni^{2+}	NiSO4
AD	69	31	60.4	39.5
BB	71	28.9	52	47.7
D	74.8	25.2	59.4	40.5
Dt1	68	32	61	39
Dt2	68.5	31.5	61.8	38.1
Dt3	68.3	31.7	61.5	38.4
PM	72	28	53	46.8
UB	72.7	27.3	61	39

Conclusion

This study has demonstrated that the biofilms at the bank of AMD streams and those attached to the efflorescent crusts harbor complex assemblages of acidophilic chemoautotrophic bacteria with the potential to dissolve sulphide mineral and generate acid. The investigation of the quality of the AMD water along the stream further demonstrated that Fe was further mobilized downstream, clearly showing that the pollution is regenerated or sustain along the stream. The prediction of metals speciation revealed a dominance of free hydrated elements which are more likely to disperse across the stream and very importantly are bioavailable and more toxics.

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