Chemolithotrophic sulfide oxidizers in mine environment

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Abstract Microbial communities in waste rock waters from Mine A were compared to process water sample microbial communities from Mines B and C, with special attention to microbes active in sulfur and iron cycling. It turned out that in Mine A waste rock waters, neutrophilic, psychrotolerant proteobacteria represented the majority of sulfur oxidizers while in Mine B and C process samples, acidophilic sulfur oxidizers dominated. Data presented in this paper emphasize the interaction between environmental conditions and microbial clusters.

Key words chemolithotrophic microbes, molecular techniques, waste rock and process waters, sulfur and iron oxidation and reduction, bioleaching, NRD and ARD

Introduction

Sulfide minerals are exposed to both physicochemical and microbiological oxidation, releasing sulfate and metals from the ore (Nordstrom & Southam 1997, Lindsay et al. 2009). This process is desirable in bioleaching processes and neutral in traditional mining, but represents a challenge when occurring in waste rock piles. Depending on the neutralization capacity of the waste rock, microbial bioleaching results in Neutral Rock Drainage (NRD) or Acid Rock Drainage (ARD) (Singer & Stumm 1970, Blowes et al. 2003).

Molecular microbiological techniques enable the characterization of microbial communities, especially sulfur oxidizing microbes, and analysis of the effect of environmental conditions on microbial community structure and *vice versa*.

Methods

Mines A, B and C are Finnish operational mines, Mine A representing a copper and nickel mine in Lapland province, Mine B a zinc and copper mine in Oulu province and Mine C a zinc mine in Oulu province.

Altogether 57 individual waste rock water samples were collected at two locations (WR120 and WR118) from Mine A in December 2015 (winter) and July-September 2016 (summer). Average values for the two locations are shown in Tables 1a and 2a while Fig. 1a-1b represent averages of the two locations. In addition, one process water sample (PL7) from Mine A was collected in December 2015. Waste rock water microbial communities are compared to process samples taken from Mines B and C. From Mine B, one process water sample (PL1), one filament sample (PF1) and one solid rock (PS1) sample were collected in December 2014 (winter). From Mine C, process water samples were collected from two locations (P318 and PE1) in February 2013 (winter) and two locations (PKP1 and PKP2) in July 2016 (summer).

Microbial levels in mine waters were measured by quantitative polymerase chain reaction (qPCR) using broad-range primers represented in (Nadkarni et al. 2002) while microbial communities were characterized with sequencing covering the V3-V4 variable region of the 16S rRNA gene. For Mine C winter samples, a shot-gun Sanger sequencing based on cloning of partial 16 sequences covering the V3-V5 variable region to *E.coli* vector was applied. For all other samples, Next Generation Sequencing with Illumina MiSeq 2x300bp sequencing lane was carried out.

At sequence quality check, poor-quality sequences were discarded. High-quality sequences were clustered to Operational Taxonomic Units (OTUs) at similarity level 97% and resulting OTUs were identified using RDP database (Wang et al. 2007).

Results

Tables 1a-c and Figs. 1a, 1a, 1c, 1e, 1g and 1i show composition of microbial community in Mines A, B and C in relation to taxonomic clustering. Proteobacteria representes a major cluster in all samples in all three mines (Tables 1a-c and Figs. 1a, 1c, 1e, 1g and 1i), followed by bacteroidetes in Mine A. In contrast, in Mines B and C, the proportion of bacteroidetes remained below 0.1% in all samples and in hence, not shown in Tables 1b-c nor Figs. 1a, 1c, 1e, 1g and 1i for simplicity. Bacteroidetes are included in 'Others' in Figs. 1a and 1c. Actinobacteria and firmicutes represented a minor cluster in all three mines. Mine C showed higher proportions of firmicutes than Mines A and B. Nitrospirae were detected in significant proportions from Mines B and C. Even though taxonomic phylum level clustering depicts certain differences between mines and samples, it does not shed light on the functional activity of microbes in the samples.

Tables 2a-c and Figs. 1b, 1d, 1f, 1h and 1j show clustering of microbes into functional groups relevant to sulfur and iron oxidation in microbial leaching of ores and waste rocks. Mine A waste rock water samples show high proportion of microbes representing typical environmental microbial clusters within α- and β-proteobacteria and bacteroidetes (included in 'Other' in Table 2a and Figs. 1b and 1d) and also a high proportion of methane oxidizers. These microbes do not oxidize or reduce sulfur and iron compounds and are hence totally passive in microbial bioleaching. In the process water sample P7 from Mine A, these microbes show a much lower proportion, indicating that they most likely originate from microbial community in the peat surrounding waste rock area as waste rock water passed though peat before reaching the collection point. Process samples from Mines B and C practically lack these microbes, emphasizing their irrelevance in sulfur and iron cycling.

In Mine A waste rock water samples, neutrophilic and psychrotolerant sulfur oxidizing β-proteobacteria represented the majority of bacteria active in sulfur and iron cycling with a proportion from a few percentages to 12% of total bacteria. Major genera were *Gallionella*, *Thiobacillus*, *Sulfuritalea* and *Sulfuricella*. Process water samples from Mines A (P7 in Table 2a) and B (PL1 in Table 2b) as well as filament and solid samples PF1 and PS1 from Mine B showed even higher proportions (from 21 % up to 71 %) of

this cluster. In contrast, in Mine C, neutrophilic, psychrotolerant sulfur oxidizers were not detected. Neutrophilic, thermophilic sulfur oxidizers are shown for symmetry even though they remained negligible in all samples from all three mines and are hence not discussed further.

In Mine A, acidophilic sulfur oxidizers (both psychrotolerant and thermophilic) showed a low proportion in all samples (Table 2a) while in Mines B and C acidophilic sulfur oxidizers represented major clusters (Tables 2b-c). Process water samples PL1 from Mine B and P318 from Mine C collected in winter time showed a high proportion of acidophilic psychrotolerant sulfur oxidizers combined with a low proportion low proportion of acidophilic thermophilic sulfur oxidizers (Table 2b) while process water sample PKP2 from Mine C collected in summer time showed a clear dominance of acidophilic thermophilic sulfur oxidizers (Table 2c), potentially reflecting differences in temperature between winter and summer. Nevertheless, it should be acknowledged that no samples were collected from Mine B during summer time and winter samples from Mine C were collected from different locations than summer samples from Mine C. Therefore, these data cannot be reliably connected to annual temperature fluctuations in Mines B and C. But comparison of samples collected at the same time indicates that temperature was higher in solid sample PS1 than in process water sample PL1 from Mine B (higher proportion of thermophilic sulfur oxidizers were detected from PS1 than PL1 in Table 2b), possibly indicating that within biofilms attached to rock surfaces, temperatures increase due to intense thermophilic reactions. Also comparison of thermophilic vs. psychrotolerant sulfur oxidizers in Mine C process waters suggests that a more effective community prevailed at PE1 than P318 and especially at PKP2 than PKP1 (Table 2c), an observation in line with process measurements in Mine C.

Table 1a *Taxonomic clustering of microbial community in Mine A process (P7) and waste rock (WR120 and WR118) water. '+' indicates a value greater than 0% but smaller than 1%.*

Taxonomic phylum level cluster	Mine A WR120 in winter	Mine A WR118 in winter	Mine A P7 in winter	Mine A WR120 in summer	Mine A WR118 in summer
Actinobacteria	5%	10%	3%	4%	3%
Bacteroidetes	3%	12%	1%	20%	19%
Firmicutes	3%	3%	+	6%	5%
Nitrospirae	0%	1%	+	+	+
Proteobacteria	82%	64%	95%	56%	59%
Other	6%	11%	+	14%	15%

Table 1b *Taxonomic clustering of microbial community in Mine B process samples PL1 = liquid, PF1 = filament and PS1 = solid. '+' indicates a value greater than 0% but smaller than 1%.*

Taxonomic phylum level cluster	Mine B PL1 in winter	Mine B PF1 in winter	Mine B PS1 in winter
Actinobacteria	+	17%	13%
Firmicutes	+	1%	0%
Nitrospirae	6%	3%	50%
Proteobacteria	92%	79%	35%
Other	1%	+	1%

Table 1c *Taxonomic clustering of microbial community in Mine C process water (P318, PE1, PKP1 and PKP2). '+' indicates a value greater than 0% but smaller than 1%.*

Taxonomic phylum level cluster	Mine C P318 in winter	Mine C PE1 in winter	Mine C PKP1 in summer	Mine CPKP2 in summer
Actinobacteria	0%	4%	4%	3%
Firmicutes	9%	26%	10%	12%
Nitrospirae	0%	6%	17%	28%
Proteobacteria	91%	64%	69%	56%
Other	0%	0%	+	+

Table 2a *Functional clustering of microbial community in Mine A process (P7) and waste rock (WR120 and WR118) water. '+' indicates a value greater than 0% but smaller than 1%.*

Functional cluster	Mine A WR120 in winter	Mine A WR118 in winter	Mine A P7 in winter	Mine A WR120 in summer	Mine A WR118 in summer
Neutrophilic,psychro-tolerant S oxidizers	12%	4%	61%	4%	4%
Neutrophilic,thermo-philic S oxidizers	0%	+	+	0%	0%
Acidophilic,psychro-tolerant S oxidizers	0%	+	1%	+	+
Acidophilic,thermo-philic S oxidizers	0%	2%	+	+	+
Iron reducers	0%	+	+	+	+
SO_4 reducers	1%	2%	+	1%	1%
CH_4 oxidizers	28%	16%	1%	29%	32%
Other	59%	76%	37%	66%	63%

Table 2b *Functional clustering of microbial community in Mine B process samples PL1 = liquid, PF1 = filament and PS1 = solid. '+' indicates a value greater than 0% but smaller than 1%.*

Functional cluster	Mine B PL1 in winter	Mine B PF1 in winter	Mine B PS1 in winter
Neutrophilic,psychro-tolerant S oxidizers	29%	71%	21%
Neutrophilic,thermo-philic S oxidizers	0%	0%	0%
Acidophilic,psychro-tolerant S oxidizers	37%	+	1%
Acidophilic,thermo-philic S oxidizers	6%	19%	60%
Iron reducers	+	+	+
SO_4 reducers	24%	+	+
CH_4 oxidizers	+	0%	0%
Other	3%	10%	18%

Table 2c *Functional clustering of microbial community in Mine C process water (P318, PE1, PKP1 and PKP2). '+' indicates a value greater than 0% but smaller than 1%.*

Functional cluster	Mine C P318 in winter	Mine C PE1 in winter	Mine C PKP1 in summer	Mine CPKP2 in summer
Neutrophilic,psychro-tolerant S oxidizers	0%	0%	0%	0%
Neutrophilic,thermo-philic S oxidizers	0%	0%	0%	0%
Acidophilic,psychro-tolerant S oxidizers	88%	36%	44%	2%
Acidophilic,thermo-philic S oxidizers	6%	45%	36%	89%
Iron reducers	3%	12%	1%	4%
SO_4 reducers	3%	7%	16%	1%
CH_4 oxidizers	0%	0%	+	0%
Other	0%	0%	4%	4%

Mine A is the only mine in these data with samples from the same locations in winter and summer time. Both in winter and in summer, neutrophilic, psychrotolerant sulfur oxidizers dominated the sulfur oxidizing community and no logical difference in neutrophilic vs. acidophilic nor psychrotolerant vs. thermophilic sulfur oxidizers could be seen in Mine A waste rock waters (Table 2a and Figs. 1b and 1d). Nevertheless, interestingly, the total proportion of sulfur oxidizers was higher in winter than in summer. Most likely, this is due to the fact that during winter time, water volumes in waste rock pile remain low as rain falls in the form of snow which does not enter the waste rock pile as water. Microbial data indicate that

during summer time, there were no local acidic spots in waste rock pile but during winter time, a proportion of the pile turns acidic due to microbiological sulfur oxidation within the pile, allowing the growth of acidophilic sulfur oxidizers.

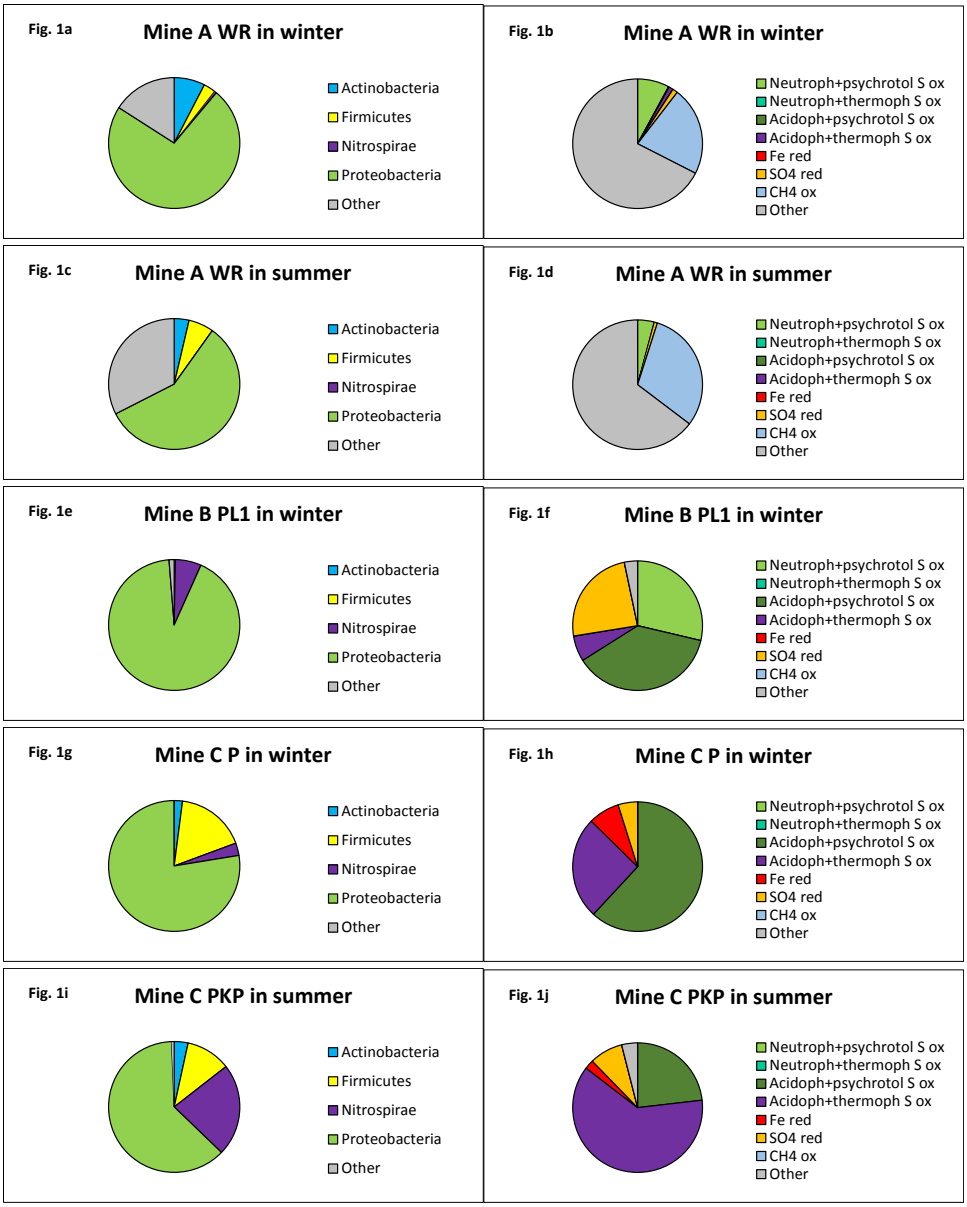

Figure 1 *Microbial community in mine waters. .Fig. 1a,1c,1e,1g and 1i show taxonomic and Figs. 1b,1d,1f,1h and 1j functional classification. Neutroph = neutrophilic, acidoph = acidophilic, psychrotol = psychrotolerant, thermoph = thermophilic, S = sulfur, Fe = iron, SO4 = sulfate, CH4 = methane, ox = oxidizers and red = reducers.*

The opposite reactions to oxidation of sulfur/sulfide and iron are reduction of sulfate and ferric ion. The proportion of iron and sulfate reducers hence is an indirect indication of the intensity of sulfur and iron oxidation as the latter provides the former with substrates, namely ferric iron and sulfate. In Mine A, sulfur oxidizers represented a smaller proportion of the microbial community (Table 2a) than in Mines B and C (Tables 2b and 2c) and consistently, also iron and sulfate reducers showed a lower proportion in Mine A waste rock water than in Mine B and C process water samples. In Mine B, process water sample PL1 showed a significant proportion of sulfate reducers while in filament and solid samples PF1 and PS1, only hints of sulfate and iron reducers could be detected (Table 2b). In Mine C, all process water samples showed both iron and sulfate reducers. Direct comparison of sulfur and iron oxidation vs. sulfate and iron reduction suggests a higher overall efficiency of sulfur oxidation for P318 (94% vs. 6%) than PE1 (81% vs. 19%) and for PKP2 (91% vs. 4%) than PKP1 (80% vs. 17%) for Mine C. It should, however, be noticed that such comparison is oversimplified as different sulfur oxidizers show different reaction kinetics and differ in relation to RedOx requirements (strict aerobes vs. facultative anaerobes) and carbon metabolism (obligate autotrophs vs. autotrophs/heterotrophs vs. obligate heterotrophs). Classification of microbial community taking into account also these factors results in more a complicated, but also a complex picture. It appears that strict aerobes, such as *Alicyclobacillus* spp. and *Leptospirillum* spp., occur mainly in conditions where external, mechanical aeration is provided, *i.e.* in mining processes based on bioleaching. Another observation is that obligate heterotrophs (*e.g. Ferrimicrobium*/*Ferrithrix* spp.) are detected when a mature biofilm providing organic carbon is present. A more detailed analysis of these factors, however, is not possible within the scope of this paper.

Conclusions

Sulfur and iron oxidizing microbes prevail whenever suitable substrates, such as sulfides in ores, are available. Nevertheless, in waste rock piles, the intensity of sulfur oxidation remains low resulting in only a moderate decrease in pH. The circumneutral pH does not allow growth of acidophilic microbes with potentially more efficient sulfur oxidizing capacity. Mine A has a lower ore sulfur content than Mine B and C ores, which further explains the observed differences in microbial communities between waste rock waters from Mine A and process waters from Mines B and C.

One motivation for this work was to evaluate the risk that Mine A waste rock area turns into ARD. Microbial data combined with chemical measurements, estimated dissolution rates, ore composition and acid-base balance calculations indeed indicated that ARD is not likely in Mine A conditions, but the main challenge probably is to manage elevated sulfate and metal concentrations in drainage water.

Acknowledgements

The authors thank Alan Delaney, Ville Heikkinen, Mikael Korte and Ulla Syrjälä for reviewing the text and providing constructive critique and suggestions to improve.

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