Bacterial Community of Neutral Mine Drainage of Elizabeth's Shaft (Slovinky, Slovakia)

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Abstract

Neutral mine drainage is the less frequent subject of the interest than acid mine drainage but it can have adverse environmental effects caused mainly by precipitation of dissolved Fe. The aim of the study was to characterize the composition of bacterial population in environment with high concentration of iron and sulfur compounds represented by neutral mine drainage water of Elizabeth's shaft, Slovinky (Slovakia). The pH value of drainage water decreased from 7.1 to 6.5 during the years 2008–2014. Direct microscopic observations, cultivation methods, and 454 pyrosequencing of the 16S rRNA gene amplicons were used to examine the bacterial population. Microscopic observations identified iron–oxidizing Proteobacteria of the genera Gallionella and Leptothrix which occurrence was not changed during the years 2008–2014. Using 454 pyrosequencing, there were identified members of 204 bacterial genera that belonged to 25 phyla. Proteobacteria (69.55%), followed by Chloroflexi (10.31%) and Actinobacteria (4.24%) dominated the bacterial community. Genera Azotobacter (24.52%) and Pseudomonas (14.15%), followed by iron–oxidizing Proteobacteria Dechloromonas (11%) and Methyloversatilis (8.53%) were most abundant within bacterial community. Typical sulfur bacteria were detected with lower frequency, e.g., Desulfobacteraceae (0.25%), Desulfovibrionaceae (0.16%), or Desulfobulbaceae (0.11%). Our data indicate that the composition of bacterial community of the Elizabeth's shaft drainage water reflects observed neutral pH, high level of iron and sulfur ions in this aquatic habitat.

Keywords: bacterial community, neutral mine drainage, high-throughput sequencing

Introduction

Mining activities and ore processing result in irreversible changes in landscape in the form of heaps and sewage sludge beds of waste material. These deposits are permanent source of toxic substances, especially heavy metals that contaminate all environmental compounds. Changes of water and soil quality affect also biodiversity of mining area (Angelovicova et al., 2012; Xu et al., 2016). The properties of drainage water depend on many factors, including mineralogical, geochemical properties, hydrogeological conditions, and the activity of lithoautotrophic microorganisms. Oxidative dissolution of sulfide minerals generates acidity and releases sulfate, iron, and associated metals to pore waters.

This phenomenon is known as acid mine drainage (AMD). However, neutral mine drainage (NMD) conditions may persist in an abundance of carbonate minerals (Lindsay et al., 2009; Lindsay et al. 2015).

Slovinky mining area (north part of Slovak Ore Mountains territory, Spišská Nová Ves district, Slovakia) is considered the largest source of cooper ores in Slovak region but it is also rich in iron ores (e.g. chalcopyrite, cuprite, siderite, pyrite). The most frequent secondary and tertiary minerals are iron oxides such as goethite or crystalline hydrous ferric oxide (Kucerova et al., 2014). Mining in Slovinky was stopped in 1999 but due to sludge bed vulnerability represent high-risk area threatening all environmental components. The mine dumps are near-neutral or slightly alkaline (pH 7.2–8.8) because the acidity generated by the decomposition of the sulfide ores is efficiently neutralized by the abundant carbonate minerals (Angelovicova et al., 2012; Kucerova et al., 2014). Elizabeth's shaft was built in 1900 and it works as mining drainage water system. Sulfide minerals exposed by mining and erosion are unstable in the presence of atmospheric oxygen and water; the resulting oxidation of sulfides can release sulfate and iron ions into the drainage water (Lindsay et al., 2015). Regular monitoring of physico-chemicals parameters of mine drainage water demonstrated that an average iron concentration did not exceed 0.5 mg/l for many years. In addition, a high concentration of SO_4, Mn, As, and Sb have been long-term monitored (Bajtoš et al., 2015). Based on these characteristics, we assumed a high incidence of iron and sulfur bacteria within bacterial community in drainage water.

The original aim of this study was to examine the occurrence of iron-sulfur bacteria in a neutral mine drainage of Elizabeth's shaft using direct microscopy and cultivation methods and to investigate their seasonal dynamics over several years. Since some changes in pH of drainage water have been recorded during the period of investigation, we decided to analyze the structure of bacterial community in NMD using a high-throughput sequencing technique in order to detect bacteria that could contribute to (or responsible for) these

Tab. 1. Physico-chemicals parameters and microscopic observation of abioseston and bioseston of mine drainage water from Elizabeth's shaft measured in the years 2008–2014 (Slovinky, Slovakia)

Tab. 1. Parametry fizyko-chemiczne i obserwacja mikroskopowa abiosestonu i biosestonu wody kopalnianej z szybu Elżbieta mierzona w latach 2008–2014 (Slovinky, Słowacja)

Years	2008 (n = 2)	2009 (n = 2)	2010 (n = 2)	2011 (n = 2)	2012 (n = 4)	2013 (n = 4)	2014 (n = 4)	P value
pH[a]	7.1 ± 0.3	7 ± 0.1	6.9 ± 0.1	6.9 ± 0.3	6.8 ± 0.3	6.7 ± 0.2	6.5 ± 0.2	> 0.05
EC [mS/m][a]	42 ± 1.4	50 ± 1.4	49 ± 4.2	54 ± 2.8	57.3 ± 2.3	61.5 ± 1.3	63.5 ± 1.3	< 0.001
TDS [mg/l][a]	172 ± 2.8	181 ± 1.4	185 ± 2.8	198 ± 4.2	201.5 ± 3.1	200.3 ± 1.7	220.3 ± 2.6	< 0.001
Gallionella spp.[b]	1 ± 0.0	2 ± 0.0	1 ± 0.0	1 ± 0.0	1.5 ± 0.7	1 ± 0.0	1 ± 0.0	> 0.05
Leptothrix spp.[b]	1 ± 0.0	2 ± 0.0	2 ± 0.0	2 ± 0.0	1 ± 0.0	1.5 ± 0.7	1 ± 0.0	> 0.05

[a]Values are expressed as arithmetic mean value ± standard deviation of measurements in a given year (n)
[b]Values are expressed as arithmetic mean value ± standard deviation of the percentage of coverage of 10 different microscopic fields measurements in a given year (n)
EC - electric conductivity, TDS – total dissolved solids, P value - result of Kruskal-Wallis test (one-way ANOVA) electric conductivity, TDS – total dissolved solids, P value - result of Kruskal-Wallis test (one-way ANOVA)

changes. The overall results of this study are published by Kisková et al. (2018) (in press).

Materials and methods

Mine drainage water samples were collected directly from the water flowing out from Elizabeth's shaft in Slovinky village, Slovakia (48°52′43″N 20°50′38″E). Fivehundred microliters of water was taken two times a year in the period from 2008 to 2011 (first week in March and October), and four times a year in the years 2012–2014 (first week in March, June, August, and October) into sterile bottles. The water was transported under cold, dark conditions to the laboratory. Physico-chemical parameters (pH and electric conductivity) were measured directly in the field with WTW Multi 340i instrument (WTW Gmbh, Weilheim, Germany) equipped with a pH electrode WTW Sen Tix 31-3 and standard WTW TetraCon 325 electrode for an electric conductivity (total dissolved solids, TDS) measurement.

Direct microscopic observations, cultivation, bacteria determination and high-throughput sequencing analysis of 16S rRNA gene were performed as described Kisková et al. (2018) (in press). The next generation 454 pyrosequencing was performed using Roche 454 GS-FLX Titanium instrument (LaRoche, USA) and reagents according to the manufacturer's guidelines. DNA sequencing data were processed using the Mothur bioinformatics software package (version 1.36.1) (Schloss et al., 2009).

Non-parametrical Kruskal–Wallis test (one-way ANOVA) was used to analyze the changes in physico-chemical parameters and occurrence of iron bacteria and/or other microorganisms detected by microscopic observation during the period of investigation.

Results and discussion

Drainage water flowing from Elizabeth's shaft is characterized by high concentration of iron, manganese, arsenic, and sulfate ions (Bajtoš et al., 2015). Concentration of copper and zinc did not overexpress limit values according of Regulation of the Government of the Slovak Republic. Continual increase of TDS concentration is probably due to the gradual release of metal(loid)s during sulfide mineral oxidation and bacterial metabolic activity. The Elizabeth's shaft drainage water could be defined as circumneutral, since acid generated via sulfide mineral oxidation (e.g. pyrite or chalcopyrite) is neutralized by the dissolution of carbonate minerals as siderite or malachite. On the other side, our results indicate a slight shift to the acidic pH during the examined period (Table 1). Slow reduction of pH of drainage water is supported by earlier studies demonstrating pH value of 8.24 in 1999 and 7.56 in 2000 (Bajtoš and Záhorová, 2007). Therefore, we performed an analysis of the bacterial community using a high-throughput sequencing technique to find out bacteria that could be involved in reducing pH of drainage water.

Despite expectations, direct microscopic observations and the high-throughput analysis have shown a low abundance of *Gallionella* spp. (percentage of coverage ranged from 1.0 to 2%) (Table 1). Even *Gallionella* spp. was not detected by 454 pyrosequencing. The low occurrence of these bacteria may be due to preference for more acidic conditions as well as other environmental factors (e.g. heavy metals). *Leptothrix* spp. are heterotrophic Fe/Mn-oxidizing inhabitant of aqueous environments (Kunoh et al., 2015).

Acidithiobacillus species was detected neither by cultivation methods nor by 454 pyrosequencing. These findings confirmed the preference of the genus *Acidithiobacillus* of acidic environment, despite the high sulfur and iron content in neutral mine drainage water.

Cultivation analysis on the selective medium confirmed the presence of *Ferribacterium* species in each sampling during the period of investigation. *Ferribacterium* is a genus of the family Rhodocyclaceae and up to now, only one species is known (*F. limneticum*). This bacterium belongs to Fe(III)-reducing bacteria and was first isolated from mining-impacted fresh lake sediments containing heavy metals (Cumming and McCann, 1978). Its presence in drainage water of Elizabeth's shaft was confirmed by cultivation as well as 454 pyrosequencing.

During the years 2008–2014, the salinity (TDS) of Elizabeth's shaft drainage water significantly increased from 172 to 220.25 mg/l ($pH < 0.001$) and pH value slightly degreased from 7.1 to 6.5 ($P > 0.05$) but these changes did not affect the incidence of observed bacteria (Table 1).

After trimming, denoising, quality, and length filtering and removing chimeras, a total of 7095 high-quality sequenc-

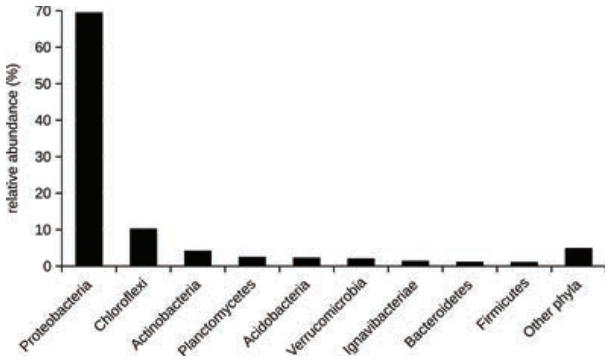

Fig. 1. Bacterial composition at the phylogenetic phylum level of mine drainage water of Elizabeth's shaft (Slovinky, Slovakia). The category "Other phyla" groups bacterial phyla whose relative abundance was below 1%

Rys. 1. Skład bakteryjny na poziomie budowy filogenetycznej wody drenażowej szybu Elizabeth (Slovinky, Słowacja). Kategoria „Inne rodzaje" obejmuje gromadę bakteryjną, której względna z zawartość była poniżej 1%

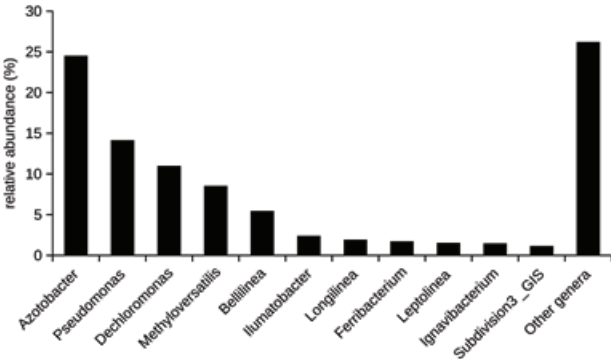

Fig. 2. Bacterial composition at the phylogenetic genus level of mine drainage water of Elizabeth's shaft (Slovinky, Slovakia). The category "Other genera" groups bacterial genera whose relative abundance was below 1%

Ryc. 2. Skład bakteryjny na poziomie rodzaju filogenetycznego wody drenażowej szybu Elizabeth (Slovinky, Słowacja). Kategoria „Inne rodzaje" grupuje rodzaje bakterii, których względna zawartość była poniżej 1%

es were obtained. Sequences were clustered into 813 OTUs, while 414 were non-singletons. One OTU was classified as Archaea, phylum Aigarchaeota (represented by two sequences). Other sequences were classified into 22 known bacterial phyla and three candidate phyla. The majority of sequences belonged to Proteobacteria (69.55%) followed by Chloroflexi (10.31%), Actinobacteria (4.24%), Planctomycetes (2.57%), Acidobacteria (2.35%), and Verrucomicrobia (2.14%). Other phyla were represented by less than 2% sequences (Figure 1).

Genus *Azotobacter* was most abundant genus (24.52%) followed by *Pseudomonas* (14.15%), *Dechloromonas* (11%), *Methyloversatilis* (8.53%), and *Bellilinea* (5.46%). Other genera were represented by less than 5% of sequences (Figure 2).

Proteobacteria such as *Dechloromonas* spp. (11%) and *Ferribacterium* spp. (1.73%) dominated within iron bacteria. Other most abundant iron bacteria were Chloroflexi such as *Bellilinea* spp. (5.46%), *Longilinea* spp. (1.93%), and *Leptolinea* spp. (1.54%).

Generally, Proteobacteria, Nitrospirae, Actinobacteria, and Firmicutes are most frequently detected phyla in AMD (Kadnikov et al., 2016; Travisany et al., 2012). Proteobacteria, Deinococcus/ Thermus, Gemmatimonadetes, Acidobacteria, and Actinobacteria were found with high frequency also in neutral mine drainage (Pereira et al., 2015)]. While lithotrophic genera such as *Acidithiobacillus*, *Acidiphilum*, *Ferrovum*, *Leptospirillum*, *Gallionella*, and *Sulfobacillus* dominate AMD environments (Kadnikov et al., 2016; Travisany et al., 2012), heterotrophic Proteobacteria such as *Pseudomonas* spp., *Bacillus* spp., and *Stenotrophomonas* spp. were found with high abundance in neutral copper mine drainage (da Costa et al., 2016) and other mining samples (Choudhary and Sar, 2011).

Pereira et al. (2014) assumed that the abundance of tolerant bacteria in areas of extreme environmental conditions increases, while that of more sensitive microorganism's decreases. Analysis of microbial composition of wastewater of Elizabeth's shaft showed a trend to increase the abundance of tolerant bacteria leading to a reduction of the total bacterial diversity. Proteobacteria represent almost 70% of the total diversity of bacterial community. These bacteria have been found to be predominant phylum in many mine environments indicating the high adaptability of members to extreme mining environments (Halter et al., 2001; Pereira et al., 2014).

Genus *Pseudomonas* are characterized by great deal of metabolic diversity and they are able to colonize a wide range of environments. A number of studies have been demonstrated its resistance to heavy metals and its capability to degrade a wide range of pollutants (Kumar et al., 2013; Naz et al., 2016).

Azotobacter spp. were found mainly in neutral soil and aquatic environments and they are capable of atmospheric nitrogen fixation due to iron requiring enzymatic system and can survive in contaminated environments by heavy metals.

Thus, isolates resistant to heavy metals could be employed in bioremediation processes (Abo-Amer et al., 2014).

Rhodocyclales, Rhizobiales, Rhodobacterales, and Rhodospirillales formed relatively large group within Proteobacteria. Many members of these taxa exhibit very versatile metabolic capabilities allowing them survive under various extreme environmental conditions (Frigaard and Dahl, 2009). They were found with high frequency in neutral mine drainage (Pereira et al., 2015), in slightly alkaline mine sediments (Halter et al., 2001) as well as in AMD (Kadnikov et al., 2016). In this study, the highest number of sequences was affiliated to the genus *Dechloromonas*. These bacteria are known as nitrate-dependent neutrophilic iron-oxidizers and perchlorate reducer (Chakraborty and Picardal, 2013). Bacteria belonging to this genus were found in soil high concentration of iron also in circumneutral or slightly acidic mine waters contaminated by many different heavy metals (Drewniak et al., 2016; Watanabe et al., 2013).

The phylum Chloroflexi with the predominant family Anaerolineaceae represents about 10% diversity of bacterial community in this study. Similarly, phylum showed a high abundance in alkaline river sediments contaminated with heavy metals (Reis et al., 2013) and in neutral copper mine drainage (Pereira et al., 2014), but was not detected in AMD (Kadnikov et al., 2016).

While sulfate-reducing bacteria have been commonly identified in tailings deposits and sulfide mine wastes (Lindsay et al., 2009; Schippers et al., 2010), representatives of this bacterial group (Desulfobacterales, Desulfovibrionales, Desulfuromonadales, and Syntrophobacterales) were detected with relative low frequency in this study. In addition, typical sulfur-oxidizing bacteria (e.g. *Acidiferrobacter* spp. *Thiofaba* spp., *Thiococcus* spp.) were found with low abundance.

Conclusion

In conclusion, relatively low abundance of typical iron and sulfur-bacteria in microbial community indicates that in addition to high concentration of iron and sulfur, other environmental factors significantly affect the composition of bacterial community and bacterial species with a great metabolic diversity dominate among bacteria. The pH of the drainage water is still nearly neutral, however we detected slightly shift to acidic. Sulfide mineral oxidation and possibly metabolic activity of iron/sulfur oxidizers could lead to the continual decrease of pH and to the deterioration of environmental impact of mine drainage of Elizabeth's shaft. Monitoring of the pH value continues and any changes in the bacterial community will be verified by further metagenomic analysis.

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Bakteryjna wspólnota neutralnej kopalni Drenaż szybu Elizabeth's (Slovinky, Słowacja)

Odciek kopalniany o odczynie obojętnym jest rzadszym przedmiotem zainteresowania niż odciek kopalniany kwaśny, ale może mieć niekorzystne skutki środowiskowe spowodowane głównie przez wytrącanie rozpuszczonego Fe. Celem artykułu jest scharakteryzowanie składu bakterii w środowisku o wysokim stężeniu związków żelaza i siarki reprezentowanych przez obojętne wody drenażowe kopalni szybu Elizabeth, Slovinky (Słowacja). Wartość pH wody drenażowej spadła z 7,1 do 6,5 w latach 2008–2014. Bezpośrednie obserwacje mikroskopowe, metody hodowli i pirosekwencjonowanie amplikonów genu 16S rRNA wykorzystano do zbadania populacji bakterii. Obserwacje mikroskopowe zidentyfikowały proteobakterie utleniające żelazo z rodzajów Gallionella i Leptothrix, których występowanie nie uległo zmianie w latach 2008–2014. Przy użyciu pirosekwencjonowania 454 zidentyfikowano 204 rodzajów bakterii należących do 25 typów. Proteobakterie (69,55%), a następnie Chloroflexi (10,31%) i aktynobakterie (4,24%) zdominowały społeczność bakteryjną. Rodzaje Azotobacter (24,52%) i Pseudomonas (14,15%), a następnie proetobakterie żelazo utleniające Dechloromonas (11%) i Methyloversatilis (8,53%) były najbardziej rozpowszechnione w społeczności bakteryjnej. Typowe bakterie siarkowe wykryto z mniejszą częstotliwością, np. Desulfobacteraceae (0,25%), Desulfovibrionaceae (0,16%) lub Desulfobulbaceae (0,11%). Uzyskane dane wskazują, że skład flory bakteryjnej wody drenażowej szybu Elżbieta odzwierciedla obserwowane neutralne pH, wysoki poziom zawartości jonów żelaza i siarki w środowisku wodnym.

Słowa kluczowe: flora bakteryjna, neutralny drenaż kopalniany, sekwencjonowanie