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Article

Characterization of the Bacterial and Sulphate Reducing Community in the Alkaline and Constantly Cold Water of the Closed Kotalahti Mine

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Abstract: Drainage from metal-sulphide rich rocks may cause considerable environmental stress in the form of elevated sulphate and heavy metal contamination of the environment. Mine draining effects from closed mines may be abated using indigenous and introduced microbial communities for sulphate reduction and metal precipitation at the mining site. Here we characterized the general and sulphate reducing bacterial (SRB) community of Kotalahti Mine (Finland). The mine was flooded after closure and sulphate reduction and metal precipitation was induced by addition of pig manure sludge into the Vehkankuilu shaft. Water was sampled from Vehkankuilu and Ollinkuilu shafts from depths −10, −30, −70 and −100 m 15 years after the treatment. The water in the shafts differed from each other biologically and geochemically. The shafts are not directly connected except by some fracture zones, and the Ollinkuilu shaft is used as a reference for environmental monitoring. The detected bacterial communities from both shafts contained methylotrophic γ-*Proteobacteria*, hydrogenotrophic and methylotrophic β-*Proteobacteria* and fermenting bacterial clades. The concentration of SRB was low, at most 4.0×10^3 *dsr*B genes·mL^{-1}, and the SRB affiliated with *Desulfobulbus* and *Thermoanaerobacteriales* clades. Despite the obvious success of the mine as an *in situ* bioreactor for increasing water pH and removing sulphate and heavy metals by induced sulphate reduction under suboptimal temperature, only a small portion, less than 0.5%, of the bacterial population in the mine water was SRB.

Keywords: mine drainage; abandoned mine; SRB; heavy metals; methylotroph; manure sludge; *in situ* bioreactor

1. Introduction

Drainage from mines may have great effects on the environment and cause problems in the vicinity of closed mines. It has been shown that heavy metal and sulphate rich discharge may impact receiving water bodies (rivers, lakes, drinking water wells) at several kilometres from the mining site [1]. However, the impact of mine drainage can be decreased by treating the mine water to induce, for example, sulphate reduction, which decreases the sulphate and metal concentration of the water e.g., [2].

Mining allows the introduction of oxygen and water to the otherwise reduced geological environment. The oxidation of certain sulfidic minerals leads to the solubilisation of metals and (possibly) often to the production of net acidity. The oxidation of pyrite can occur through several pathways, and reactions including interactions with dissolved O_2, Fe^{3+}, other mineral catalysts (Mn^{4+}) and nitrate have been reported [3–5]. Some of the most common reactions are [3]):

$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2Fe^{2+} + 4SO_4^{2-} + 4H^+ \quad (1)$$

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \quad (2)$$

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (3)$$

Many rock drainage reactions require the presence of oxygen (Reactions 1 and 2). Nitrate reduction can be coupled to pyrite oxidation in anoxic environment. The role of ferric iron under anoxic conditions is less clear. Typically the pH value should be below 3.0 in order for ferric iron to remain in significant quantities in solution [5]. The production of mine drainage may continue for a considerable time after mine closure. In addition to the generally known acid mine drainage (AMD) where the discharge waters are highly acidic, mine water discharges may also be pH neutral to alkaline. Alkalinity may be available as bicarbonate in the groundwater or in mineral phases, such as carbonate minerals [6]. Furthermore, denitrification, methanogenesis, sulphate reduction, iron reduction, manganese reduction, and ammonification are microbiological processes, which can subsequently increase the pH of the mine waters [7]. Underground mines are often flooded in order to prevent sulphide mineral oxidation and AMD formation through isolating the sulphides from atmospheric oxygen. When dewatering pumps are turned off after mine closure, the mined voids are flooded with ground- or surface water [8,9]. Mineral-oxidation and microbial activity consume dissolved oxygen present in the flooding waters [7]. In addition, flooded open pits and mine shafts can be utilized as large sulphate reducing bioreactors to treat AMD *in situ*.

Even though sulphate-reducing bacteria (SRB) play a crucial role in the *in situ* mine water treatment, only little is known about the abundance and biodiversity of microorganisms in flooded mine shafts, especially in cold and alkaline conditions. In this study, the microbial communities of two flooded mine shafts were characterized at different depths between −10 and −100 m below ground level. Vehkankuilu shaft has been operated as *in situ* biological reactor for mine water treatment at 5–8 °C for approximately 15 years, and Ollinkuilu shaft outside the actual ore body is monitored as an

environmental reference for possible water seepage between the shafts. Of special interest was the abundance and diversity of the SRB in the shafts, as these microorganisms treat the mine water containing elevated concentrations of metals and sulphate. Microbial community analysis enhances the understanding of the *in situ* flooded reactor in Kotalahti, Finland.

2. Experimental Section

2.1. Sampling Site

The Kotalahti Mine is situated in the municipality of Leppävirta, Finland (N 62.579944, E 27.608083). The volume of the mine is 3.5 million m^3 and its deepest parts reach a depth of 800 m. The mine was active during 1957–1987, after which it was gradually filled with water until 1994. The most commonly mined ores were sphalerite, chalcopyrite and pyrite.

The rock of the area is 2800 Ma old Archean nickel-bearing mafic and ultramafic plutonic rocks of banded granitodic gneiss with inliers of Archean metasedimentary and metavolcanic rocks [10]. Kotalahti is part of the Main Sulphide Ore Belt of Finland reaching from Lake Ladoga to the Bothnian Bay (Northern Baltic Sea) [11]. This study comprises two mine shafts, the Vehkankuilu (VK) and the Ollinkuilu (OK) shafts, situated approximately 1 km apart. The Vehkankuilu shaft spans the Kotalahti ore body and has an amphibole-rich pyroxenite zone in the upper part of the shaft. The rock of Ollinkuilu is mainly Archaean tonalite gneiss [12]. The pH of the mine waters in Vehkankuilu mine shaft after closure was only slightly acidic with the lowest pH of 6.3 measured at 100 m below ground level in 1997 (Figure 1). The concentrations of sulphate and iron at −100 m depth of the Vehkankuilu mine shaft were high, 1100 $mg \cdot L^{-1}$ and 23 $mg \cdot L^{-1}$, respectively. The Ni concentration at −100 m was 3.2 $mg \cdot L^{-1}$ in 1996 and 1.8 $mg \cdot L^{-1}$ in 1997. Between 1996 and 1997, 635 m^3 pig manure sludge and silage was added to the Vehkankuilu shaft as a source of bacteria, nutrients and organic carbon to enhance the sulphate reduction process [9]. In the Ollinkuilu mine shaft, the pH is high, between pH 9.5 and 10, probably due to concrete reinforcements and construction in the mine shaft. The sulphate concentration has remained below 4 $mg \cdot L^{-1}$ since 2009, the concentration of nickel has stayed below 0.5 $mg \cdot L^{-1}$, but the concentration of iron has been higher than in Vehkankuilu mine shaft, varying from 19 $mg \cdot L^{-1}$ at −70 m to 61 $mg \cdot L^{-1}$ at −30 m in 2012 (Figure 2, Supplementary Table S1). The environmental permit limits for discharging water from the mine site have been 2.0 $mg \cdot L^{-1}$ Fe; 1.0 $mg \cdot L^{-1}$ Ni (from 2014 onwards 0.7 $mg \cdot L^{-1}$ Ni); 10.0 $mg \cdot L^{-1}$ suspended solids and pH 6–9. The target value for Ni set by the regional environmental administration is below 0.4 $mg \cdot L^{-1}$ [13].

2.2. Sampling

Groundwater samples were obtained from two water-filled shafts from the Kotalahti Mine, the Ollinkuilu and the Vehkankuilu shafts in November 2012. Ollinkuilu (OK) samples were collected using a stainless steel sampler (1 L) and Vehkankuilu (VK) samples were obtained with a Limnos sampler (2 L). Samples were collected from four depths, −10, −30, −70 and −100 m, by sampling the −10 m depth first and the −100 m depth last. It has been estimated that the −30 and −70 m samples represent best the runoff water, which is released through fractures to Mertakoski pond and further to the environment [13]. Vehkankuilu water was clear and Ollinkuilu water was dark brown. From each

sampling depth, biomass from 100 and 50 mL water sample were collected by filtering the water through Sterivex™-GP 0.22-µm pore-size filter units equipped with Luer-lock fittings (Merck Millipore, Billerica, MA, USA), using a sterile 60 mL syringe. The 100 mL samples were filtered directly after sample retrieval on site, and stored on ice for 5 h during transportation to the laboratory. The 50 mL samples were filtered the next day from 60–100 mL samples that had been collected using a sterile syringe equipped with sterile hypodermic needle. The sample was inserted into anaerobic acid-washed sterile 120 mL infusion bottles equipped with butyl rubber stoppers and transported chilled to the laboratory protected from light.

2.3. Physicochemical Measurements

Physicochemical measurements in Kotalahti mine have been done throughout the years according to the environmental permit, which requires measurements of pH, oxygen, sulphate, iron, nickel, copper, nitrogen compounds, phosphorus and coliform bacteria, but not carbon content [14]. The temperature of the sample water was measured directly from the sample water on site. In addition, oxygen concentration and pH were measured on site applying the Finnish Standards Association's (SFF) SFS-EN 25813 [15] and SFS 3012 [16] standard protocols, respectively. Total concentration of N and P was measured using a modified SFS-EN ISO 11905-1 [17] and SFS-EN 1189 [18] method, respectively. The sulphate concentration was determined by ion chromatography according to the SFS-EN ISO 10304-1 [19] standard. Iron, copper and nickel concentrations were determined using ICP-MS according to the SFS-EN ISO 17294-1 and -2 [20,21] and SFS-EN ISO 15587-2 [22] standards.

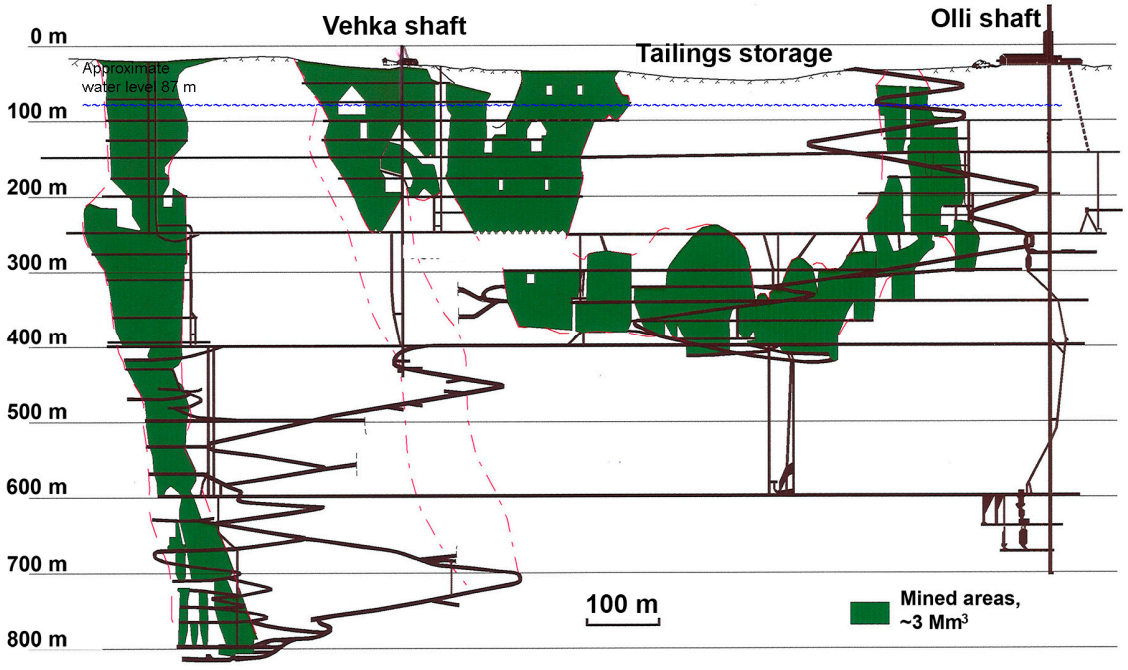

Figure 1. Schematic cross section of Kotalahti Mine showing Vehkankuilu and Ollinkuilu shafts. The green areas show the excavated parts of the mine, the red lines outline the ore body in the mafic and ultramafic complex. The two shafts, Vehkankuilu and Ollinkuilu, are situated approximately 1 km apart. Modified from [9,10]. Depths are given as below ground level. The water level is indicated with a blue line.

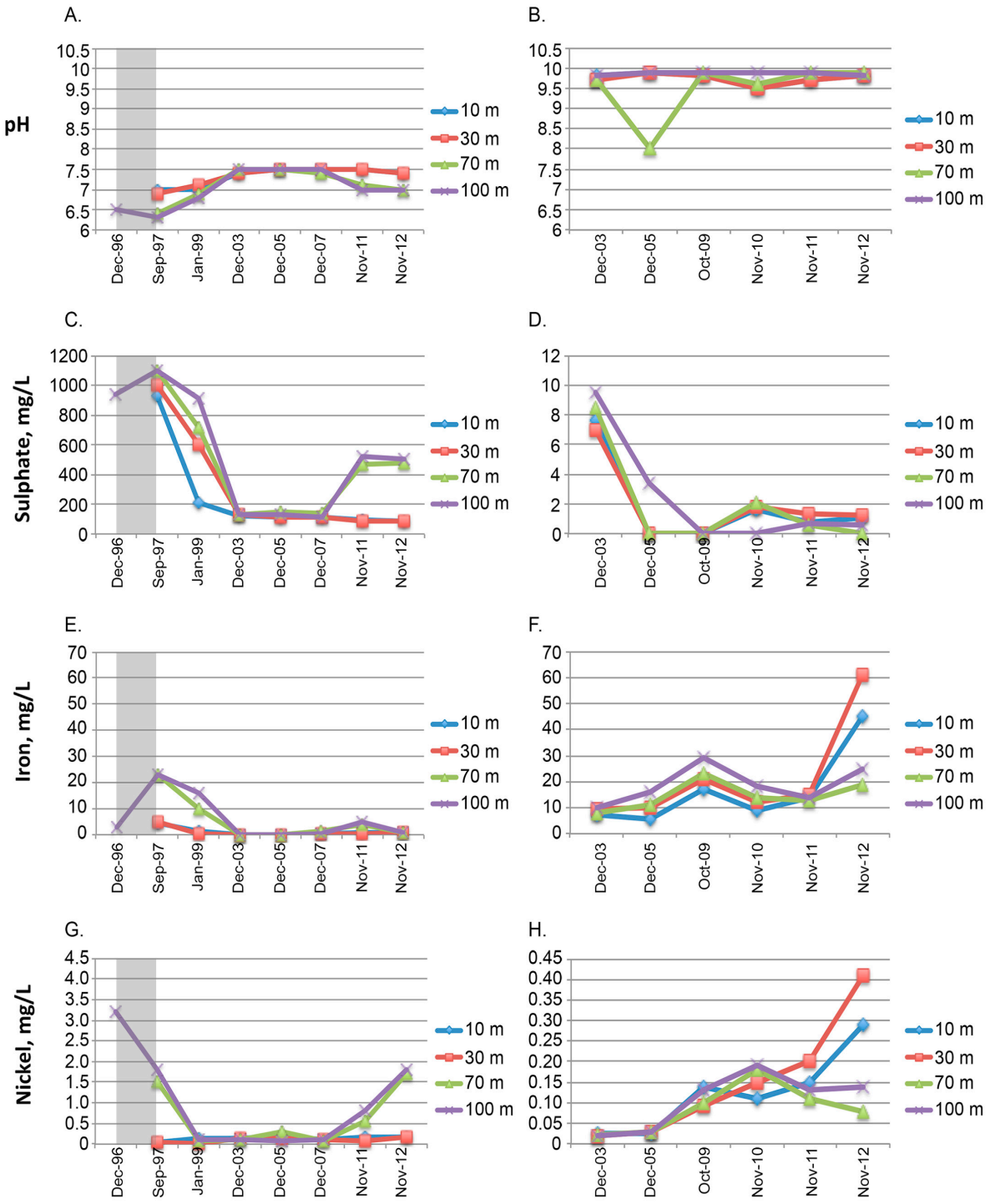

Figure 2. Evolution of the (**A**, **B**) Ph; and the concentration of (**C**, **D**) sulphate; (**E**, **F**) iron and (**G**, **H**) nickel in the water of Vehkankuilu shaft (**A**, **C**, **E**, **G**) from 1996 to 2012 and Ollinkuilu shaft (**B**, **D**, **F**, **H**) from 2003–2012. Pig manure sludge and silage was added to Vehkankuilu shaft as a source of bacteria, nutrients and organic carbon in order to induce sulphate reduction and metal precipitation in 1996 and 1997 (shading in **A**, **C**, **E**, **H**). The data from 1996 to 2007 for Vehkankuilu are from [9], and the data from 2011 and 2012 as well as from Ollinkuilu shaft is from this study. Depths are measured as below ground level.

2.4. Total Number of Microbial Cells

The number of microbial cells in the samples was determined by fluorescence-activated cell sorting (FACS). A 1 mL subsample of each sample was dyed with Syto16 dye (Life Technologies, Paisley, UK) according to the manufacturer's instructions. The cells were stained over night at 4 °C before analysis by FACS. Non-stained background samples of each of the samples were also prepared. The samples were analysed with a flow rate of 10 µL/min over a collection time of 15 s (OK 10–100 m), 30 s (VK 10–30 m) or 60 s (VK 70–100 m).

2.5. DNA Extraction

DNA was extracted from the Sterivex™-GP filter units using the MoBio PowerWater Sterivex kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Negative extraction controls, *i.e.*, a clean Sterivex™-GP filter, was treated as the samples and included in all downstream Polymerase Chain Reaction (PCR) and quantitative Polymerase Chain Reaction (qPCR) applications as background control. The DNA content of the extracts was examined with the Nanodrop-1000 spectrophotometer.

2.6. dsrB qPCR

The abundance of bacterial *dsr*B genes was determined by qPCR with KAPA™ SYBR® Fast 2× Master mix for Roche LightCycler 480 (Kapa Biosystems, Inc., Boston, MA, USA). Reactions were performed in triplicate for each sample. Each reaction contained 1 µL of extracted DNA as template and 5 pmol of primers *dsr*2060F and *dsr*4R [23,24]. The qPCR was performed on a Roche LightCycler 480 (Roche Applied Science, Penzberg, Germany) on white 96-well plates (Roche Applied Science) sealed with transparent adhesive seals (4titude, Surrey, UK). The qPCR conditions consisted of an initial denaturation at 95 °C for 10 min followed by 45 amplification cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C with a quantification measurement at the end of each elongation. A final extension step of three minutes at 72 °C was performed prior to a melting curve analysis. The melting curve analysis consisted of a denaturation step for 10 s at 95 °C followed by an annealing step at 65 °C for one minute prior to a gradual temperature rise to 95 °C at a rate of 0.11 $°C \cdot s^{-1}$ during which the fluorescence was continuously measured. The amplification level of *dsr*B genes in the samples was compared to a standard curve. The standard curve consisted of a dilution series of *dsr*B gene fragments of *Desulfobulbus propionicus* in a plasmid. The dilution series reached 1.5×10^6 to 1.5×10^0 copies of *dsr*B genes per reaction and each dilution was run in triplicate reactions. The standard curve was calculated from the mean value of the three replicate reactions. The mean amplification level of each sample was compared to the standard curve and converted to concentration of *dsr*B gene copies mL^{-1} water. No amplification was detected in the PCR or qPCR assays from the background control DNA extracts used in this study.

2.7. DGGE Analyses

The bacterial communities of the samples were determined by denaturing gradient gel electrophoresis (DGGE). A 473 bp fragment covering the V6–V8 variable sites of the bacterial 16S

rRNA gene was amplified with primers U968f-GC-f and U1401r [25]. PCR amplification was carried out in 50-μL mixtures containing 1× reaction buffer 0.2 mM of each deoxynucleoside triphosphate, 50 pmol of each primer and 1U Maxima DNA polymerase (Thermo Scientific, Espoo, Finland) and 1 μL 1:10 diluted template DNA. The PCR program consisted of 5 min initial denaturation at 94 °C followed by 30 cycles of 30 s at 94 °C, 20 s at 55 °C and 40 s at 72 °C and final elongation at 72 °C for 7 min. The amplified gene fragments were resolved on DGGE as described in [26]. DGGE gels were stained with Sybr Green II (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's recommendations and imaged with a Bio-Rad GelDoc imager (Bio-Rad, Hercules, CA, USA) using UV light.

A 470 bp fragment of the *dsr*B gene was amplified with primers *dsr*2060F + GC and *dsr*4R [23,24]. The PCR reaction mixture was as described above, and the PCR profile consisted of a 5 min initial denaturation at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C and final elongation at 72 °C for 20 min. PCR products were separated on a DGGE gel containing 8% acrylamide and 40%–70% denaturing gradient at 85 V and 60 °C for 20 h. DGGE gels were imaged as described above.

2.8. Sequencing

The most prominent bands were excised from the Sybr Green II stained DGGE gel under UV light using a sterile scalpel. Each band was incubated overnight in 20 μL of ultra clean water at 4 °C. The DGGE bands were reamplified using 2 μL o/n extracted DNA as template using the same PCR conditions and primers as for the original PCR, except without the GC-clamp. The reamplified DGGE bands were sent for purification and sequencing to Macrogen Inc., Seoul, Korea. The 16S rRNA fragments were sequenced with the U1401r primer and the *dsr*B gene fragments with primer *dsr*4R.

2.9. Phylogenetic Analyses

The phylogenetic analyses were performed with GENEIOUS PRO (Biomatters Ltd., Auckland, New Zealand). All sequences were imported into GENEIOUS PRO, and aligned to reference sequences and most closely matching sequences determined with the blastn tool in GENEIOUS PRO. The alignments were performed with the MAFFT tool in GENEIOUS PRO with default settings and the alignments were edited by hand. Phylogenetic analyses were performed on the alignments using PhyML [27] with the Jukes-Cantor69 substitution model [28]. Bootstrap support for nodes were calculated based on 1000 random repeats.

Accession numbers. One representative sequence of each Operational Taxonomic Unit (OTU) was deposited in Genebank under accession numbers KP192130–KP192166 (bacterial 16S) and KP192241–KP192246 (*dsr*B, only sequences >200 bp).

2.10. Statistical Analyses

The non-metric multi-dimensional scaling (NMDS) analysis was done using PAST v. 3.0 [29].

3. Results and Discussion

Drainage and water discharge from mining areas may have severe environmental effects in form of acidification and increase in sulphate and heavy metal concentration in the environment in the vicinity of mining sites [1]. However, these effects have effectively been decreased by induced biological sulphate reduction in anaerobic pit lakes and flooded underground mines [9]. Sulphate reducing bacteria (SRB) play key roles in these processes. Most known SRB are mesophilic [30,31], but nevertheless, psychrophilic, thermophilic and hyperthermophilic species have been described [30,31]. Psychrophilic SRB are able to grow even below 0 °C [32,33]. However, sulphate reducing rates and activities tend to be low in cold environments. For example, Isaksen and Jorgensen [34] showed that the sulphate reduction activities of permanently cold sediment were 4%–10% at 0 °C and 10%–29% at 5 °C of maximal sulphate reduction activity, respectively.

In Finland, mine waters have successfully been treated by inducing biological sulphate reduction. In the closed Kotalahti Mine sulphate reduction was induced by adding pig manure sludge to the flooded mine [9]. Despite the permanently cold water of the mine, sulphate and heavy metal concentration decreased and pH increased within a few years after substrate addition (Figure 2) [9]. In addition, acceptable water quality was maintained for 15 years after the original heavy metal and sulphate concentrations decreased and the pH increased, showing that the mine worked well as an *in situ* bioreactor. The physicochemical conditions in Vehkankuilu and Ollinkuilu shafts 15 years after initiation of the induced sulphate reduction are presented in Table 1. The temperature of the water was low and suboptimal for sulphate reduction. The pH in Vehkankuilu was neutral to slightly alkaline, while the Ollinkuilu water was clearly alkaline varying between pH 9 and 9.8. The Vehkankuilu water showed high concentrations of sulphate especially in the two anaerobic deeper samples where the sulphate concentrations reached 500 $mg \cdot L^{-1}$. In Ollinkuilu water, the sulphate concentrations varied between <0.5 to 1.2 $mg \cdot L^{-1}$. Compared to the Vehkankuilu mine shaft, Ollinkuilu water had elevated concentrations of total nitrogen, phosphorus, iron and copper. Vehkankuilu, on the other hand, showed elevated concentrations of nickel, especially in anaerobic deeper water.

Table 1. Groundwater measurements from Vehkankuilu (VK) and Ollinkuilu (OK) samples. Data provided by Savo-Karjaan Ympäristötutkimus Oy (Kuopio, Finland). Time of sampling: 5 November 2012.

Parameter	VK 10 m	VK 30 m	VK 70 m	VK 100 m	OK 10 m	OK 30 m	OK 70 m	OK 100 m
T °C	5.70	5.70	5.60	5.6	7.7	6.90	6.5	6.9
O_2 mg/L	0	0.34	0.84	0.48	0	0.19	0.13	0
O_2%	0	2.70	6.70	3.8	0	1.6	1.1	0
pH	7.4	7.4	7	7	9.80	9.80	9.0	9.80
Tot N mg/L	0.16	0.16	0.42	0.39	4.7	4.7	4.7	4.7
Tot P µg/L	31	32	9	9	76	86	46	58
SO_4^{2-} mg/L	86	85	480	500	1.1	1.2	<0.5	0.54
Fe mg/L	1	1	1	1	45	61	19	25
Cu µg/L	1.50	1.80	1.10	1.10	17	29	6.60	3.4
Ni µg/L	180	180	1700	1800	290	410	79	140

The number of microbial cells in the different samples varied from 6×10^4 to 4.8×10^5 mL^{-1} in the Vehkankuilu water, while the number in the Ollinkuilu water was between 9.8×10^5 to 1.3×10^6 mL^{-1} (Figure 3A,B, Table 2). Despite the indication of active sulphate reduction in the flooded Kotalahti Mine in the form of reduction in sulphate and metal concentration [9], the SRB constituted only a small part of the microbial communities in the uppermost 100 m of the water column in the flooded shafts. In Vehkankuilu water, the number of SRB, as determined by the number of *dsr*B genes mL^{-1}, varied between 4.8×10^5 mL^{-1} in the upper samples to approximately 6×10^4 mL^{-1} in the deeper samples (Figure 3C). In Ollinkuilu the concentration of *dsr*B genes mL^{-1} was 6–16 $\times 10^2$ mL^{-1} in the two upper samples and 2.8–3.8 $\times 10^3$ mL^{-1} in the two deeper samples (Figure 3D). The proportion of SRB in relation to the total microbial community as determined by the ratio of *dsr*B genes to the total number of microbial cells was generally low (Figure 3E,F). If the number of *dsr*B gene copies is used as a proxy for the number of SRB present, in Vehkankuilu that was inoculated with swine manure 15 years ago at −70 and −100 m SRB contributed with 0.1% to 0.5% of the total microbial community. In Ollinkuilu, the SRB contributed with 0.2% of the total microbial community at −70 and −100 m depth. These values are significantly lower than in recent studies, which have typically shown the clear dominance of sulphate reducers in the reactors treating sulphate and metal rich waters [35], also in *in situ* reactors [36] and at low temperatures (9 °C) [37]. However, it is plausible that the most effective sulphate reduction is taking place at the bottom of the pit at 800 m depth to where the organic substrates and sulphate reducers have sedimentated. No samples were analysed from this depth in the present study.

In the upper samples (−10 and −30 m) of both shafts, the relative abundance of SRB was high (0.3%) in Vehkankuilu at −10 m, but, in all other shallow samples, the abundance of SRB was 10 folds lower than in the deeper samples. The SRB population in the *in situ* mine reactor has most likely been formed on the bottom and walls of the pit and may thus in reality represent a much higher proportion of the microbial communities attached in biofilms and sediment, where the sulphate reduction might be more active. The inoculation of the flooded mine reactor with swine manure sludge and silage occurred approximately 15 years ago, but, unfortunately at the time, no microbiological analyses were done. However, the abundance of SRB in the inoculant was examined and Cook *et al.* [38] found that SRB contribute typically with at most 1% of the bacterial population in the swine manure slurry.

The bacterial communities in the Vehkankuilu mine shaft differed markedly from that of the Ollinkuilu shaft. The amplified DGGE bands belonged to 27 different operational taxonomic units (OTUs) (Table 3, Figure 4). Vehkankuilu samples from −10 and −30 m depth showed similar DGGE banding profiles and had all together nine different DGGE bands (Figure 4). The DGGE profiles of the bacterial communities from −70 and −100 m were similar to each other but differed from the upper samples. The bacterial communities detected from Ollinkuilu samples were clearly different from those in Vehkankuilu and the DGGE profiles also showed more DGGE bands and more similar banding profiles throughout the depth profile than those of the Vehkankuilu samples. The −10 and −30 m samples from Ollinkuilu had identical banding profiles (total 12 OTUs) slightly differing from those of the −70 and −100 m samples (total 12 OTUs). The community profiles corresponded well to the major groups of bacteria detected from the Vehkankuilu and Ollinkuilu shafts by high throughput sequencing [39].

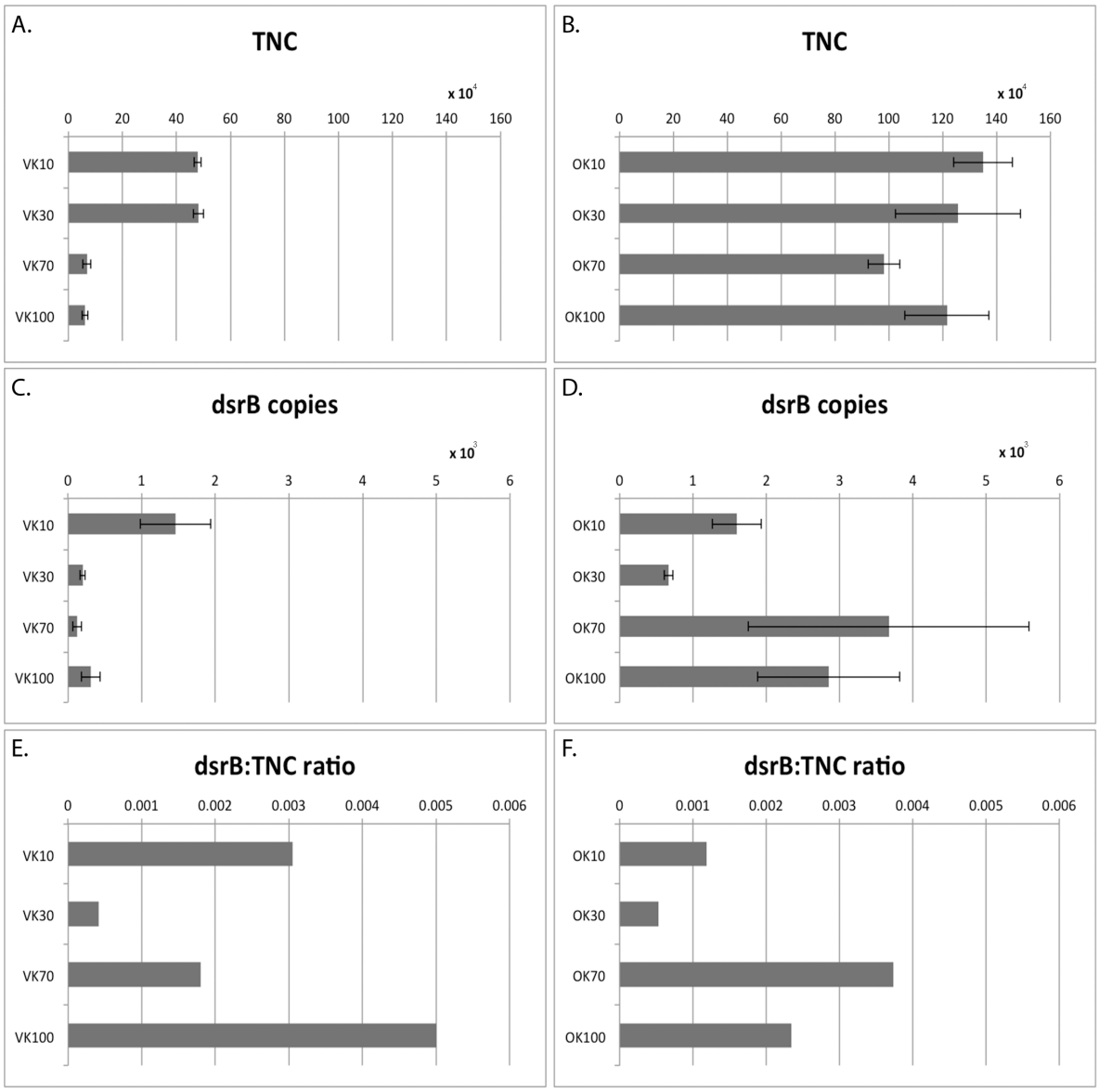

Figure 3. The number of bacterial cells mL^{-1} in (**A**) Vehkankuilu and (**B**) Ollinkuilu water according to sampling depth (10, 30, 70 and 100 m); and the number of *dsr*B gene copies of the SRB mL^{-1} in (**C**) Vehkankuilu and (**D**) Ollinkuilu samples; (**E**,**F**) displays the ratio on *dsr*B genes to the total number of microbial cells detected in the samples from Vehkankuilu and Ollinkuilu, respectively.

Table 2. The number of microbial cells, *dsr*B gene copies of the SRB and the ratio of *dsr*B genes: total number of microbial cells (TNC) in Vehkankuilu (VK) and Ollinkuilu (OK) samples. SEM describes standard error of mean.

Parameter	VK 10 m	VK 30 m	VK 70 m	VK 100 m	OK 10 m	OK 30 m	OK 70 m	OK 100 m
TNC mL^{-1}	4.8×10^5	4.8×10^5	6.8×10^4	6.2×10^4	1.4×10^6	1.3×10^6	9.8×10^5	1.2×10^6
SEM	1.2×10^4	1.9×10^4	1.5×10^4	1.1×10^4	1.1×10^5	2.3×10^5	5.9×10^4	1.6×10^5
*dsr*B mL^{-1}	1.5×10^3	2.0×10^2	1.2×10^2	3.1×10^2	1.6×10^3	6.7×10^2	3.7×10^3	2.9×10^3
SEM	4.78×10^2	3.38×10^1	6.03×10^1	1.26×10^2	3.36×10^2	6.01×10^1	1.91×10^3	9.7×10^2
*dsr*B/TNC ratio	0.0030	0.0004	0.0018	0.0050	0.0012	0.0005	0.0027	0.0023

Table 3. The taxonomic groupings of each sequenced DGGE band. DGGE bands with the same mobility in the DGGE gel were determined to belong to the same operational taxonomic unit (OTU). The dots in the table indicate presence of DGGE bands in this position that were not sequenced.

Phylogenetic Group	OTU	VK 10	VK 30	VK 70	VK 100	OK 10	OK 30	OK 70	OK 100
ε-*Proteobacteria/Sulfurimonas*	1	●	a1						
ε-*Proteobacteria/Sulfurimonas*	2	●	b1						
β-*Proteobacteria/Comamonadaceae*	3					●	g2	●	c4
β-*Proteobacteria*/unclassified	4	●	c1						
β-*Proteobacteria*/unclassified	5			●	b2				
β-*Proteobacteria*/unclassified	6	●	d1						
γ-*Proteobacteria*/*Methylobacter*	7	●	e1						
β-*Proteobacteria*/unclassified	8					●	a3		
β-*Proteobacteria*/unclassified	9							●	e4
Ignavibacter	10					●	h2	●	d4
β-*Proteobacteria/Curvibacter*	11					●	b3		
γ-*Proteobacteria/Methylobacter*	12	●	f1	●	c2				
β-*Proteobacteria/unclassified*	13					●	c3	●	f4
γ-*Proteobacteria/Methylobacter*	14				d2				
β-*Proteobacteria/Methylotenera*	15			●	e2				
β-*Proteobacteria/Methylotenera*	16	●	g1	●		●	d3		
β-*Proteobacteria/Methylobacter*	17							●	g4
β-*Proteobacteria/Methylotenera*	18	●	h1	●	f2				
β-*Proteobacteria/Methylobacter*	19					●	e3		
β-*Proteobacteria/Hydrogenophaga*	20							●	h4
β-*Proteobacteria/Hydrogenophaga*	21					●	f3	●	a5
β-*Proteobacteria/Rhodocyclaceae*	22					●	g3	●	b5
Thermodesulfovibrio	23					●	h3	●	c5
Bacillus	24								d5
Bacillus	25					●	a4	●	e5
α-*Proteobacteria/Rubritepida*	26					●	b4	●	f5
β-*Proteobacteria*	27		a2	●	●				

According to the bacterial community profiles, methane or methylated compounds may play a great role as carbon source in Vehkankuilu shaft water since the majority of the bacteria detected in Vehkankuilu were similar to one-carbon compound-oxidizing taxa. Putatively methanotrophic γ-*Proteobacteria* belonging to the *Methylobacter* were represented by three OTUs (7, 12 and 14), and were found at all depths in Vehkankuilu (Table 3, Figure 5). β-*Proteobacteria* were represented by OTUs 15, 16 and 18 (*Methylotenera*) and an uncultured additional β-proteobacterial clade (OTU 27). Members of the *Methylobacter* oxidize methane as sole carbon and energy source and nitrate and ammonium as nitrogen source [40,41]. Some methylobacter species have also been shown to reduce nitrate [41]. Methanol may be oxidized by the facultative methylotrophic *Methylotenera*, which have the ability to use methylamine and methanol as sole carbon sources [42,43]. An abundance of inorganic sulphur oxidizing bacteria were detected in Vehkankuilu at −10 and −30 m depth. These bacteria belonged to the genus *Sulfurimonas* (OTUs 1 and 2), which have been shown to oxidize

sulphur, sulphide and thiosulphate [44]. The abundance of these bacteria indicates that the sulphur cycle in the −10 and −30 m deep water of the Vehkankuilu shaft may lean more towards sulphate production than sulphate reduction. Specific *Sulfurimonas* species are also known to oxidize hydrogen and use CO_2 as sole carbon source and nitrate or ammonium as nitrogen source [45].

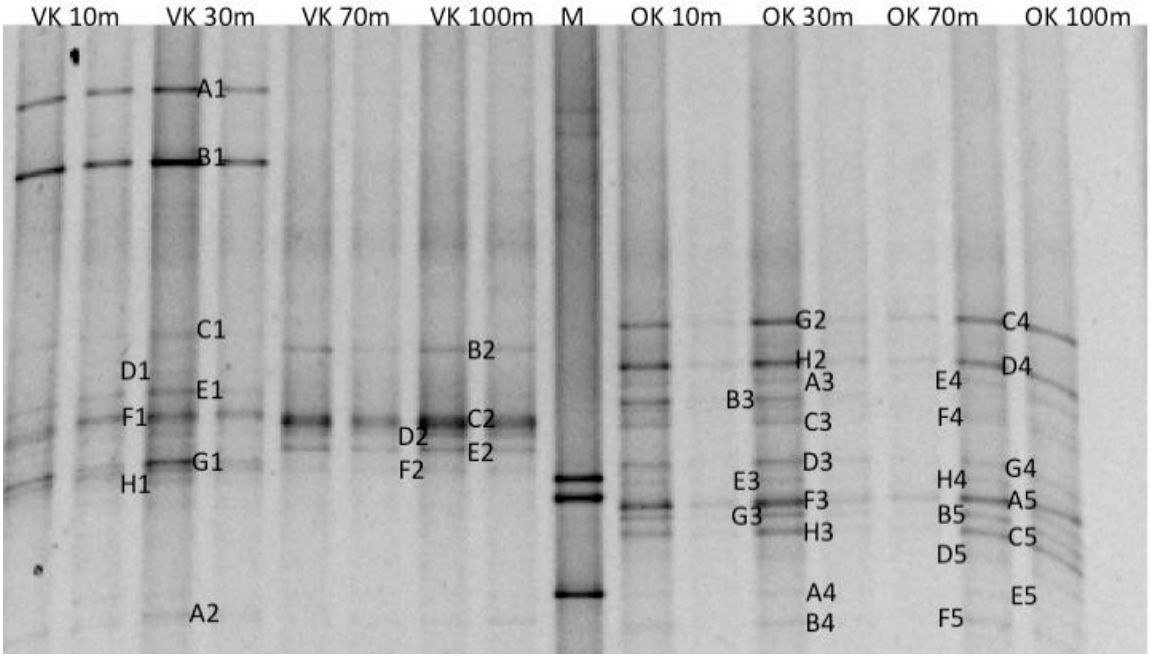

Figure 4. The DGGE banding profiles of the bacterial communities of Vehkankuilu (VK) to the left and Ollinkuilu (OK) samples to the right, at 10, 30, 70 and 100 m depth. Each depth is represented by two samples. The bands marked with a LetterNumber combination have successfully been identified by sequencing. M stands for molecular marker, and has been used as a standard for the DGGE.

The bacterial communities in Ollinkuilu were dominated by β-*Proteobacteria* belonging to the *Comamonas*, including the genera *Hydrogenophaga* (OTUs 20 and 21), *Curvibacter* (OTU 11) and unclassified *Comamonadaceae* β-*Proteobacteria* (OTU 3). Putatively methylotrophic β-*Proteobacteria* affiliating to *Methylotenera* was found at −10 to −30 m depth (OTU 16). β-*Proteobacteria* belonging to the *Rhodocyclaceae* were present in all samples (OTU 22). *Rhodocyclaceae* β-*Proteobacteria* are mostly denitrifying, *i.e.*, remove nitrogen by production of N_2. They play a significant role in remediation of contaminated waste- and groundwater, as they are able to degrade aromatic hydrocarbons [46,47]. γ-*Proteobacteria* most resembling the *Methylobacter* were present at all depths, represented by OTU 19 at −10 to −30 m and OTU 17 at −70 to −100 m. α-*Proteobacteria* affiliated with the *Rubritepida* were represented by OTU 26, which was present throughout the studied depth profile. The *Rubritepida* bacteria found at all four studied depths in Ollinkuilu shaft were most similar to the cultured species *Rubritepida flocculans*. *R. flocculans* has the ability to oxidize thiosulphate to sulphate [48]. In addition, *Bacillus* represented by OTUs 24 and 25 were found at all depths. *Bacillus* species have also been shown to degrade hydrocarbons, such as *n*-hexadecane and naphthalene [49]. Heterotrophic fermenting *Ignavibacter* [50] (OTU 10) was detected from Ollinkuilu from all depths. The only sulphate reducing taxa found based on the 16S

rRNA gene analysis belonged to the *Thermodesulfovibrio* and was present in Ollinkuilu at all depths (OTU 26). This bacterium has the ability to reduce at least sulphate, sulphite, thiosulphate and Fe(III) nitrilotriacetate and generally grows at high temperatures, such as 55 °C and above [51]. Nevertheless, our results indicate that these thermophiles can inhabit constantly cold environments. This is in accordance with Isaksen *et al.* [52], who detected a thermophilic sulphate reducer, *Desulfotomaculum*, in constantly cold marine sediment.

The two mine shafts are situated approximately 1 km from each other (Figure 1). Vehkankuilu spans the nickel-bearing ore body of amphibole-rich pyroxenite, while Ollinkuilu runs through tonalite gneiss. The differences in geology can be seen already in the difference in pH and colour of the water in the two shafts (Figure 3). After addition of carbon substrate, the pH of Vehkankuilu increased from acidic to neutral. In Ollinkuilu, the pH has always been highly alkaline. This is also reflected in the bacterial communities detected in the two shafts. The seven times higher concentration of microbial cells, the abundance of fermenting bacteria as well as the high concentration of nitrogen in the water may indicate higher organic carbon content in Ollinkuilu water than that in Vehkankuilu. Vehkankuilu bacterial profiles also consisted of bacteria often found in nutrient-poor conditions. The sulphate concentrations at −70 m and below in Vehkankuilu were high, which was also the case with the metal ions. Carbon substrates for the SRB may be limited, which could reduce the activity of SRB and subsequently the amount of biologically produced hydrogen sulfide for metal precipitation. The upper water was also inhabited by autotrophic, sulphur oxidizing and nitrate reducing bacteria, which indicates that the conditions for sulphate reduction in Vehkankuilu are not optimal. The estimated number of sulphate reducers mL^{-1} was higher in Ollinkuilu water than in Vehkankuilu, although, Ollinkuilu had only low concentrations of sulphate (up to 1.2 $mg \cdot L^{-1}$) while in Vehkankuilu water the sulphate concentration increased to 500 $mg \cdot L^{-1}$ at 100 m. Different factors appear to limit the microbial communities in the two shafts. In Ollinkuilu, sulphate may be a limiting factor, since nitrogen and phosphorus levels are high. In Vehkankuilu, nitrogen appears to be the limiting factor. The concentration of available carbon substrates is also likely to affect the efficiency of sulphate reduction, and measurement of total organic carbon in the mine water has been added to the monitoring of the mine water from 2015. In addition, it would be beneficial to follow the dissolved sulfide and intermediate sulfur species in the water to confirm the activity of SRB.

In the NMDS plot (Figure 6) the elevated Ni and SO_4^{2-} concentrations had the highest influence on the bacterial communities at −70 and −100 m depth in Vehkankuilu. In contrast, pH, temperature, elevated concentrations of N, PO_4^{3-}, Fe and Cu determined the bacterial community of the Ollinkuilu −10 and −30 m samples. The bacterial communities in Vehkankuilu samples from −10 and −30 m and Ollinkuilu samples from −70 and −100 m were not as clearly influenced by the measured chemical and physical characteristics.

SRB were detected by *dsr*B gene targeted PCR-DGGE from Vehkankuilu −30 and −70 m depth and from Ollinkuilu −10 and −30 m depth (Figure 7). In Vehkankuilu, five different *dsr*B-OTUs (OTUs 2, 3, 4, 5, 6) were successfully identified and they all belonged to *Desulfobulbus* sp. (Figures 6 and 7). In Ollinkuilu, three different *dsr*B-OTUs were identified of which OTU 1 belonged to an uncultured group of sulphate reducers, OTU 3 to *Desulfobulbus* sp. and OTU 7 to *Desulfotomaculum* sp. (Figures 7 and 8). In addition, the majority of the *dsr*B clone sequences from the swine manure storage systems were associated with *Desulfobulbus* species, which were one of the main SRB groups detected

in our study. *Desulfobulbus* sp. has been shown to tolerate oxygen and utilize NO_3^- and O_2 instead of SO_4^{2-} as a terminal electron acceptor [30]. Some oxygen was detected in the water of the shafts, which may have given the *Desulfobulbus* sp. a competitive edge compared to other SRB (Table 1).

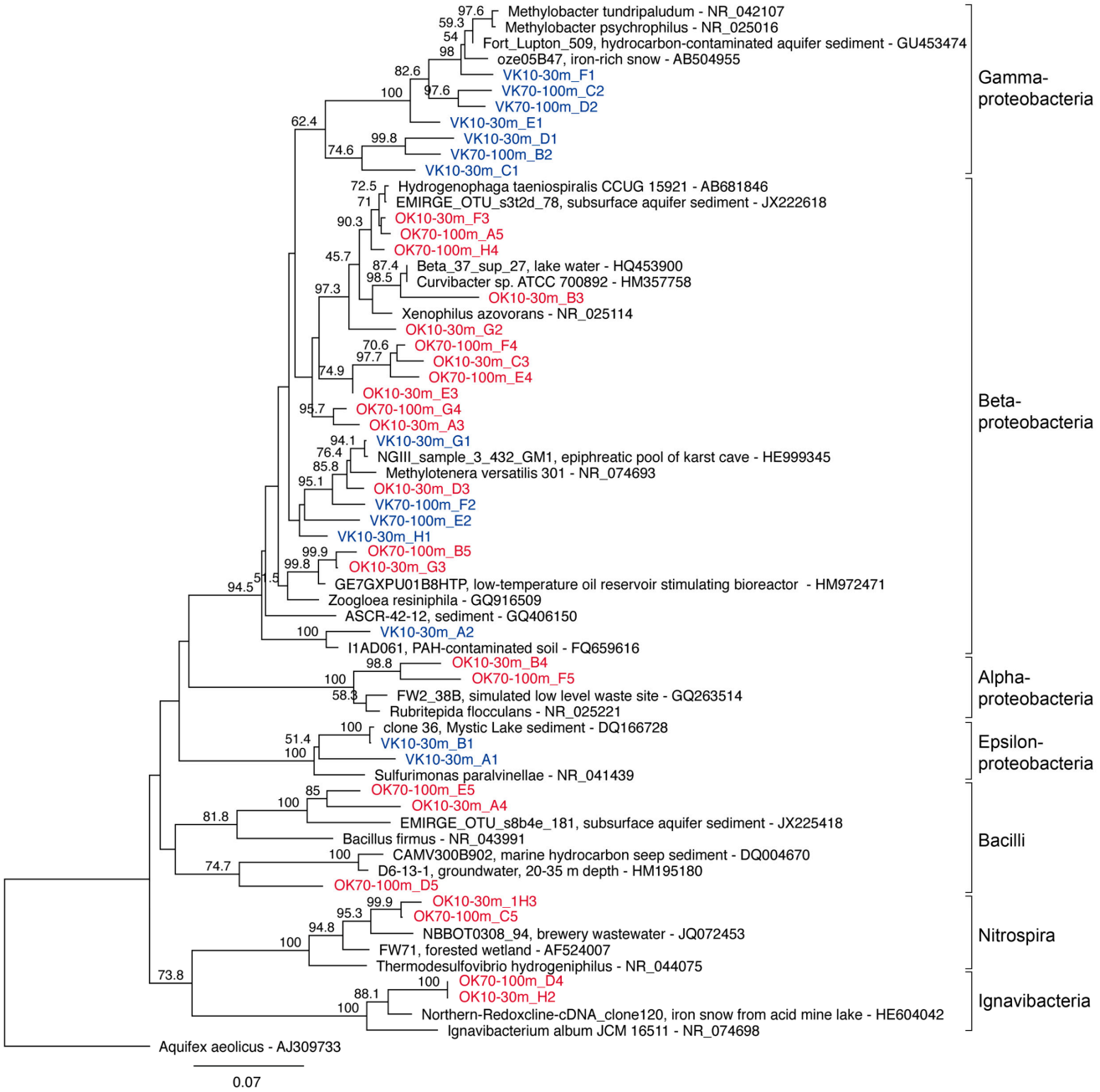

Figure 5. The bacteria detected from Vehkankuilu and Ollinkuilu samples determined by their 16S rRNA genes. Vehkankuilu sequences are shown in blue and Ollinkuilu in red. The LetterNumber combination at the end of the sequence names correspond to those in Figure 4 and Table 3. Bootstrap values from 1000 random repeats are shown for nodes with at least 50% support. The scale bare indicates number of nucleotide substitutions.

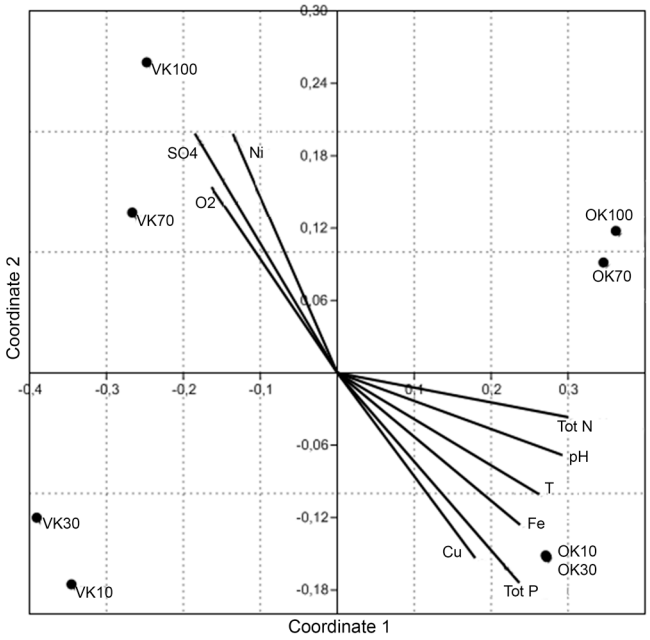

Figure 6. NMDS plot displaying the relationship between the physicochemical parameters and the bacterial community profiles of the samples.

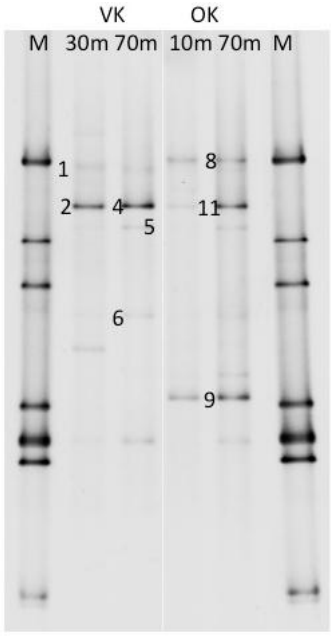

Figure 7. DGGE banding profiles of the *dsr*B gene fragments obtained from Vehkankuilu (VK) and Ollinkuilu (OK) samples. DGGE band indicated with a number was successfully sequenced. M stands for molecular marker.

No typically psychrophilic SRB were detected in this study (Figure 8). The temperatures at the sampling sites in this study were 5.6–7.7 °C, thus being close or even below the lower temperature limits of the detected mesophilic SRB. For example, the growth of *Desulfobulbus propionicus* has been reported to occur in the temperature range of 10–43 °C [23]. However, Rabus *et al.* [53] showed that mesophilic SRB could be acclimated to tolerate lower temperatures, although according to Nevatalo *et al.* [54], the sulphate reduction and electron donor oxidation rate of mesophilic SRB

decrease at suboptimal temperatures. Furthermore, the competitiveness of bacteria under *in situ* conditions is more important in demonstrating the successful adaptation to low temperatures than operation at optimal temperature. The temperature for highest sulphate reduction rates may significantly differ from the measured optimal temperature of the sulphate-reducers [24].

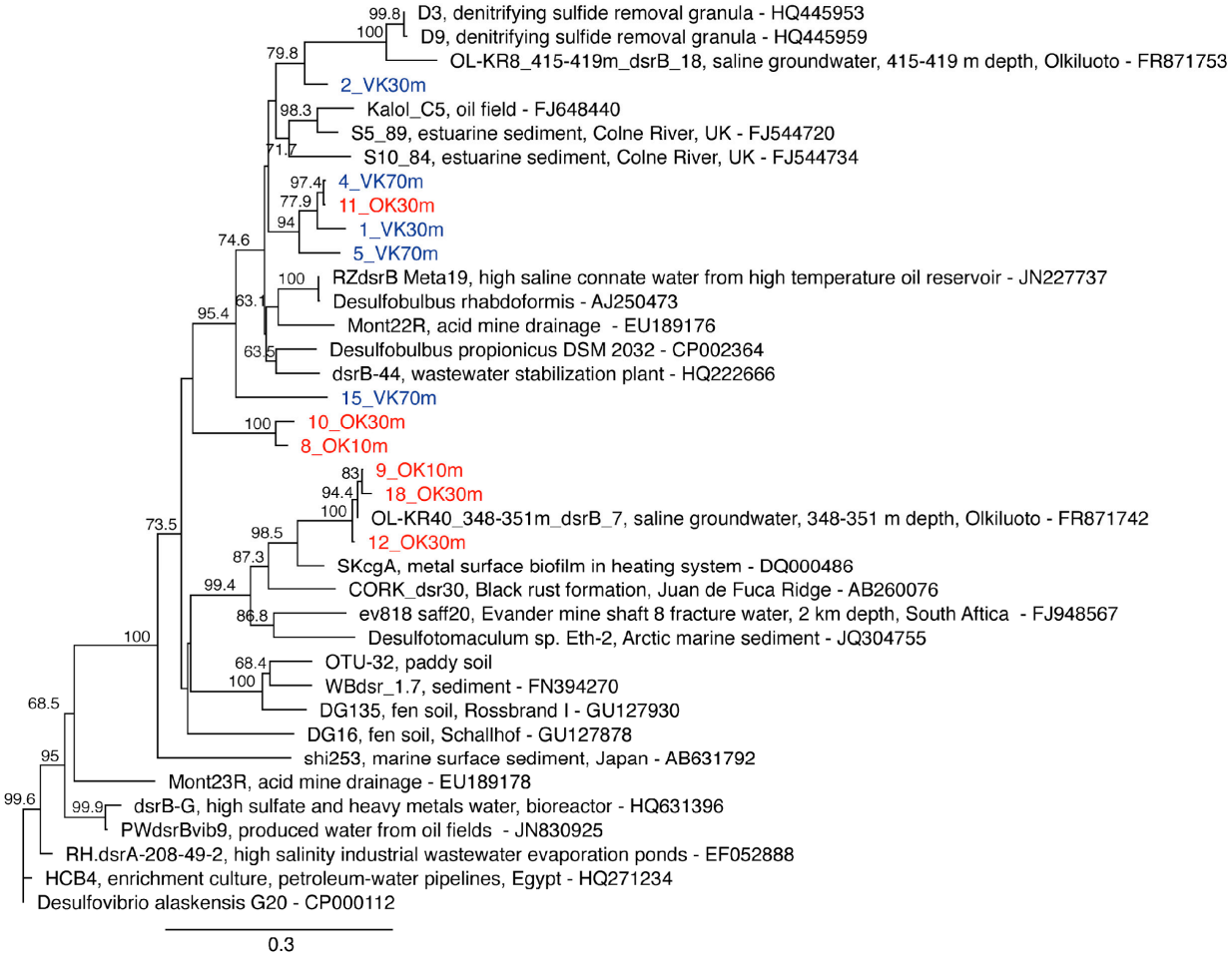

Figure 8. The sulphate reducing bacteria detected from Vehkankuilu and Ollinkuilu samples as determined by their *dsr*B genes. Vehkankuilu *dsr*B are shown in blue and Ollinkuilu in red. The number in front of each *dsr*B sequence corresponds to those in Figure 7. Bootstrap values from 1000 random repeats are shown for nodes with at least 50% support. The scale bar indicates number of nucleotide substitutions.

4. Conclusions

In general, the removal of heavy metals and sulphate as a result of the activity of sulphate reducing bacteria in the water of the closed Kotalahti Mine has been shown to be successful. However, 15 years after the introduction of carbon substrates and SRB inoculum (e.g., manure sludge) the activity was seen to decrease followed by a decrease in pH in the deeper water layers in Vehkankuilu as well as increase in the concentration of sulphate and heavy metals in the water. The mine has been able to sustain the sulphate reduction activity for a relatively long time after inoculation, which is in accordance with the results obtained also e.g., in the Lilly/Orphan Boy Mine *in situ* bioreactor [55]. The majority of the bacterial communities in the flooded shafts of Kotalahti mine 15 years post

inoculation belonged to non-SRB taxa. The bacterial communities in the Vehkankuilu shaft were clearly different from that of the reference shaft Ollinkuilu. The microbial communities of Vehkankuilu consisted mostly of methanotrophic and methylotrophic bacteria (*Methylobacter*, *Methylotenera*) and sulphur-, sulphide- and thiosulphate-oxidizing bacteria (*Sulfurimonas*). In Ollinkuilu, the bacterial communities belonged to H_2-oxidizing (*Hydrogenophaga*), denitrifying (*Rhodocyclaceae*), and thiosulphate-oxidizing (*Rubritepida*) bacteria as well as many organic matter-degrading bacteria (*Bacillus*, *Ignavibacter*) and a sulphate reducing clade (*Thermodesulfovibrio*). The reason for this striking difference in bacterial communities between the two mine shafts may be explained by the significant differences in the physicochemical properties of their water and due to the fact that Vehkankuilu received manure sludge in 1996–1997.

Various abiotic phenomena occur in the shaft in addition to biological processes. However, the water in mine shafts reached environmental limits only after the addition of organic matter and inoculum of SRB. The role of biological phenomena will most likely be confirmed in the coming years, when there will be renewed additions of swine manure at the site.

Based on the number of *dsr*B gene copies, SRB contributed only with at most 0.1% to 0.5% of the total microbial community in Vehkankuilu, and at most with 0.2% in the water of Ollinkuilu reference shaft. However, the *in situ* reactor formed in Vehkankuilu has for the last 15 years kept heavy metal concentrations below the targeted environmental limits despite the constantly sub-optimal temperature and the above optimum pH. At the moment the sulphate reduction capacity of especially Vehkankuilu shaft may be hindered by the lack of organic carbon, which has now been added to the environmental monitoring program [13]. This is indicated by the increasing concentrations of sulphate and metals as well as slightly decreasing pH. One alternative for the future operation of the flooded *in situ* bioreactor would be a new addition of carbon substrates and inoculation of psychrophilic and psychrotolerant SRB. These SRB may have the potential of more effective sulphate reduction at constantly low temperatures. In addition, genes for copper and nickel resistance belonging to non-SRB microorganisms have been detected in the mine water [31], which may play a role in the efficient removal of heavy metals from the mine water. To our knowledge, this study and [31] are the first to analyse the microbial populations in the Kotalahti Mine shafts and can as such be used as references, when the effect of new inoculations will be monitored.

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Author Contributions

All authors conceived the idea for the paper and wrote the paper. Mona Arnold provided funding for the initial work. Malin Bomberg performed the analyses and interpreted the results. Päivi Kinnunen contributed with experience and knowledge of acid mine drainage.

Conflicts of Interest

The authors declare no conflict of interest.

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