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Microbial community diversity and changes associated with a mine drainage gradient at the Dexing copper mine, China

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ABSTRACT: Water samples were collected from 6 sites in the Dexing copper mine, one of the largest open-cast copper mines in China. Each corresponding habitat exhibited distinct geochemical characteristics allowing us to correlate microbial community structure to environmental conditions. We examined the molecular diversity of 16S rRNA genes in water samples from these sites using a PCR-based cloning approach. A total of 68 operational taxonomic units of 16S rRNA genes from 814 screened clones were obtained. The sequenced clones fell into 5 main phylogenetic divisions. The majority (79.1%) of the clones were affiliated with the *Gammaproteobacteria* (60%), *Acidobacteria* (8.8%) and *Nitrospira* (10.3%). However, these sites showed great differences in composition of microbial communities. Moreover, the environmental variables differed greatly across sites; iron, sulfur and calcium concentrations were the variables significantly correlated to the microbial community composition, indicating that Fe^{2+}, S and Ca are possible drivers in shaping microbial community structure. pH was also significantly correlated to microbial community composition at 2 sites.

KEY WORDS: 16S rRNA genes · Aquatic microbial communities · Environmental variables

INTRODUCTION

The oxidative dissolution of exposed minerals (principally sulfides) gives rise to acidic, metal-enriched waters generally referred to as 'acid mine drainage' (AMD). AMD-impacted sites derive from a heterogeneous mix of ecosystems and result in different environmental characteristics which potentially lead to unique microbial community assemblages (Baker & Banfield 2003). Studies of environmental (chemical and physical) gradients and their associated microbial communities have proved to be a promising approach to reveal how environmental variables determine the spatial structure and composition of microbial communities. For example, Bond et al. (2000a) compared the microbial communities in physically and geochemically distinct ecosystems in Iron Mountain, CA, USA; the results suggested that the highly acidic, metal-rich environments are the primary factors in determining the microbial communities.

The Dexing copper mine (Jiangxi Province, China) is the largest open-cast copper mine in China and the world's third largest. It has been mined for copper since the Tang Dynasty, approximately 800 years ago. Its recent ore production exceeds 100 000 tons d^{-1} (Wen & Allen 1999). A large quantity of AMD is formed through the combination of metal sulfide waste discharging and microbial activity. Approximately 10 years ago, an irrigation-type bioleaching operation was established to recover copper in Dexing mine. A treatment station was also built to neutralize acid and reduce the concentration of metal ions. The Dawu River, which is located downstream of this mining area, also carries various pollutants, including metal

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ions. These sites represent highly heterogeneous geochemical environments (especially with respect to pH and metals) and have provided an opportunity to investigate the relationships between environmental variables and microbial communities. Physiological responses of a single organism to heavy metals are useful for the study of metal resistance mechanism, but the long-term effects of heavy metal exposure within ecosystems may lead to a better understanding of its contribution to community composition. Our objectives were to determine (1) the microbial community structure along a pH and metals gradient, and (2) if environmental variables such as pH and metals affect microbial community alterations in the Dexing copper mine along this gradient.

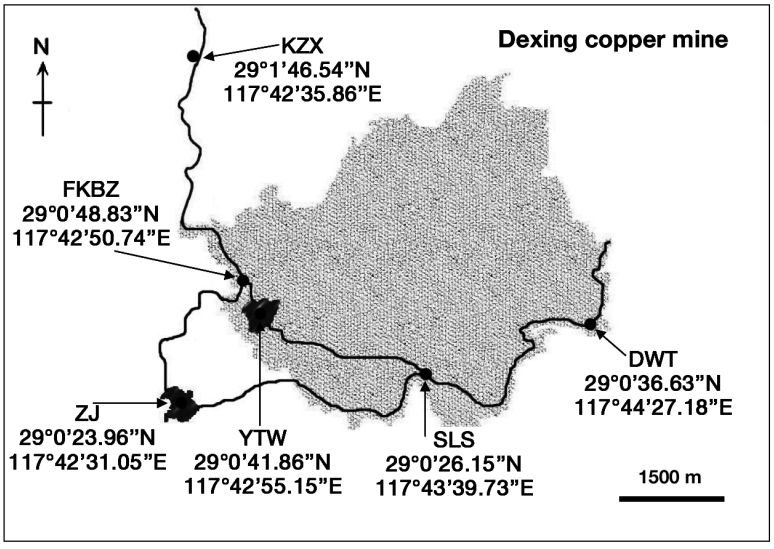

Fig. 1. Sampling sites in the Dexing copper mine (main mining area shaded grey). DWT, SLS, YTW, ZJ, FKBZ and KZX represent the Dawutou, Shuilongshan, Yangtaowu, Zujia, Fangkengbongzhan and Dawu River sites, respectively

MATERIALS AND METHODS

Site description and sample collection. Water samples for both microbial community and geochemical property analysis were collected from 6 different sites in the Dexing copper mine in July 2005 (Fig. 1). The Dawutou and the Shuilongshan sites (DWT and SLS) are located in the upper part of the copper district. The 2 sites were formed through oxidative dissolution of sulfide minerals. The Zujia (ZJ) and Yangtaowu (YTW) sites are reservoirs filled with mixture of AMD, infiltration water and rain. Our Fangkengbongzhan (FKBZ) samples were taken from a large wastewater treatment station. The FKBZ samples represent a mixture of waters from the prior 4 sites, but we do not have information showing the relative amounts from each source. The remaining water sample was taken from the treatment plant downstream of the Dawu River (KZX).

From each site, we collected about 150 l of water from just below (0 to 10 cm) the water surface in July 2005. Six water samples (ca. 900 l in total) were filtered through a 0.2 µm nylon filter and the biomass on the filter was immediately transferred to a bottle and kept in a cooler at approximately 0 to 4°C. The coolers were then transferred to the laboratory and stored in a –20°C walk-in freezer prior to molecular analysis. Moreover, 100 ml of the original water sample at each site was used for geochemical analysis.

Geochemical analysis. For water samples, we measured 25 elements, including total iron, using inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Baird Plasma Spectrovac PS-6 (N+1)) (Table 1). The ferrous iron concentration, pH and reduction-oxidation (redox) potential were determined during sampling. The ferrous iron concentration was measured using a potassium bichromate titration method (Vogel 1962). The redox potential was measured using a standard redox potential meter (Cany Precision Instruments).

Community DNA extraction, purification, amplification, and cloning of 16S rRNA genes. The crude community DNA for all 6 sites was extracted from the filtered biomass recovered from the water samples using Zhou's method (Zhou et al. 1996). The crude DNA was then purified by E ZNA^{TM} Gel Extraction Kit and quantified by ethidium bromide-UV detection on an agarose gel.

The extracted DNA was then used as template for PCR amplification of the 16S rRNA genes. The reaction mixtures contained about 50 to 100 ng DNA µl^{-1}, 1 × PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2 mM MgCl, and 0.001% [wt/vol] gelatin), 2 mM dNTPs (deoxyribonucleotide triphosphate), 2.5 mM $MgCl_2$, 5 pM each of the forward and reverse primers, and 0.025 U AmpliTaq Gold µl^{-1} (Perkin Elmer). Bovine serum albumin (400 ng µl^{-1}) was added to the reaction to promote the amplification of templates with high G+C content. The reverse primer was the universal 1387R (5′-GGCGGWGTGTACAAGGC-3′) and the forward primer was the Bacteria universal 63F (5′-CAGGCCTAACACATGCAAGTC-3′) (Marchesi et al. 1998). The initial denaturing step of 5 min at 94°C was followed by 30 cycles of 45 s at 94°C, 45 s at 55°C and 90 s at 72°C with a final extension step of 7 min at 72°C. The resulting PCR products were run on a 1.0% low-

Table. 1. Biogeochemical variables for the 6 sites (DWT, SLS, YTW, ZJ, FKBZ and KZX; see Fig. 1) and diversity indices based on *Hinp*I-*Msp*I RFLP phylotypes in 16S rDNA clone libraries. All biogeochemical variables are in mg l^{-1}. Data are means ± SD

	DWT	SLS	YTW	ZJ	FKBZ	KZX
Mg	61 ± 6.5	203.93 ± 32	1102 ± 102.3	3085 ± 310.5	4141 ± 370	166 ± 20.5
Cu	3.26 ± 0.6	12.3 ± 1.2	100.19 ± 14.0	213.2 ± 26	51.16 ± 10.3	2.34 ± 1
Mn	17.48 ± 2.2	13.83 ± 2.2	64.38 ± 8.8	156.66 ± 20.2	212.53 ± 30.4	6.79 ± 1.2
S	818 ± 40.6	2449.19 ± 103.6	4401 ± 300	7147 ± 500	8709 ± 620	481 ± 24
Mo	0.1	0.32	0.59 ± 0.2	0.85	0.95 ± 0.4	0.04
Fe	514.37 ± 20.3	1500.73 ± 88.9	981.83 ± 60.3	198.61 ± 17.9	563.93 ± 60.3	0.21
Fe^{2+}	182.7 ± 15.6	262.7 ± 16.8	125 ± 15.3	65.7 ± 5.8	116.65 ± 12.3	0.11
Al	120.33 ± 22.5	450.88 ± 30.7	944 ± 69.5	1588 ± 206	1906 ± 140	29.67 ± 3.2
Ca	122.08 ± 10.9	166.65 ± 20.3	336.35 ± 48	397 ± 45	446 ± 36	246.31 ± 14.6
pH	2 ± 0.1	2 ± 0.1	3 ± 0.2	3 ± 0.1	3 ± 0.2	5 ± 0.2
Redox potential (mV)	732 ± 30	727 ± 28	699 ± 26	627 ± 37	648 ± 35	560 ± 20
H[a]	4.28	4.28	4.16	2.23	4.20	4.22

[a]H was calculated as follows: $H = -\sum (pi)(\log_2 pi)$, where *pi* is the proportion of the phylotype

melting-point agarose gel. Amplicons of the expected size (approximately 1.3 kb) were then excised and purified with the Promega purification columns in accordance with the manufacturer's instructions.

The purified PCR products were cloned into vector PCR 2.1 TOPO and *Escherichia coli* TOP10F′competent cells according to the manufacturer's instructions (Invitrogen). We randomly selected about 120 clones from most samples and the small subunit (SSU) DNA from each insert-positive clone was then screened by digestion with 1 U each of the 4-base-specific restriction endonucleases *Hin*PI and *Msp*I in 1 × buffer (New England Biolabs) overnight at 37°C (John et al. 2000). The resulting restriction fragment length polymorphism (RFLP) products were separated on a 3% agarose gel. Bands were visualized by staining with ethidium bromide and UV illumination.

To determine similarity among the banding patterns and select clones for sequencing, Jaccard coefficients were used for all pairwise comparisons of the RFLP banding patterns and dendrograms were constructed with the unweighted pair group mean average method in Molecular Analyst (version 1.1, Bio-Rad). Cohesive groupings of highly similar, although not necessarily identical, RFLP banding patterns were identified, and a representative clone was selected for nucleotide sequence determination.

Sequencing and phylogenetic analysis. A total of 50RFLP patterns (the 16S rRNA gene) were selected for partial sequencing. The sequencing was performed with an ABI PRISM BigDye terminator cycle sequencing Ready Reaction kit (Applied Biosystems) and an ABI PRISM 3700 DNA analyzer (Applied Biosystems). Sequence identification was estimated initially by the BLASTN algorithm from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/). Then, 16S rDNA sequences were selected for alignments using ClustalW (with the following parameters: gap opening penalty = 5.00; gap extension penalty = 0.05) (EBI Tools). The final phylogenetic tree was constructed with the software Mega III (Kumar et al. 2004). Pairwise distances were calculated with the distance only option and the phylogeny tree was constructed using the Neighbor-Joining method (bootstrap test of the phylogeny: 500 replicates, seed = 70 189).

Statistical analysis. In order to analyze differences in diversity among the sites, the operational taxonomic units (OTUs) were defined through grouping of highly similar patterns. The Shannon-Weaver diversity index (*H*) for each site was computed. At the same time, we also performed a rarefaction analysis to check if the clone number was sufficient to detect community diversity. The relationship of the abundances of OTUs to the ranks of the corresponding OTUs was fitted with nonlinear statistical models (SigmaPlot version 8.0):

$$y = a \times (1 - e^{(-b \times x)})$$

where *y* is the abundance of an OTU, *x* is the rank of the corresponding OTU, and *a* and *b* are regression parameters (Zhou et al. 2004).

To determine the relationships between communities and the environmental variables, canonical correspondence analysis (CCA) was performed using PC-ORD (version 5; MJM Software Design) and Canoco for Windows 4.5. In this study, we used OTUs to represent clades. The percentage of each OTU in the libraries was used as input for the CCA analysis. The environmental variables presented in Table 1 were also used in the CCA analysis. CCA is a multivariate ordination method that combines correspondence analysis (CA) and multiple regression using environmental variables to 'constrain' the ordination leading

to a more realistic direct gradient analysis associated with biological variables (Gauch 1982, Legendre & Gallagher 2001). Our first step was to determine the environmental variables most important in shaping microbial community structure, using a stepwise CCA that helped to eliminate weakly correlated variables. While it is not possible to guarantee that other variables or overlapping variables are not responsible for the patterns seen, special attention was paid to the total 'inertia' in the CCA output. This number represents an estimation of the amount of variance in the community diversity data which can be explained by the environmental variables measured. If this number is low (<0.4), then much of the inherent variance in the community diversity data is unaffected by the variables chosen. To test the significance of the ordination output, we used a Monte Carlo Permutation test also using PC-ORD (version 5; MJM Software Design) in order to reveal the unique explanatory power of the environmental variables and the covariation. Canoco for Windows 4.5 was used for partial CCA analysis. In stepwise CCA, pH and Fe^{2+} were used for the first set of explanatory environmental variables, and Ca and S were used for the second set of explanatory environmental variables in the partial CCA analysis. The variation partitioning on 2 sets of explanatory variables was performed by the approach of Borcard (1992).

Nucleotide sequence accession numbers. All of the 16S rDNAs described in this study have been submitted to GenBank with accession numbers DQ457999–DQ458048, DQ646482–DQ646485, DQ646517 and DQ646518.

RESULTS

Biogeochemical characterization of sites

The pH for the water samples ranged from 2.0 to 5.0 (Table 1). Sites SLS and DWT had the lowest pH values (2.0). The pH values at YTW and ZJ were about 3.0, and the highest pH was observed in the discharge site, KZX (5.0). The sites also showed great differences in calcium concentration. For most of the geochemical parameters, the KZX site had the lowest levels and FKBZ the highest. In most cases, the differences among these 2 sites were very large as the discharge site (KZX) typically exhibited levels several-fold less than the site with the highest amounts. The differences were most pronounced for sulfur (481.00 to 8709.00 mg l^{-1}), aluminum (29.67 to 1906.0 mg l^{-1}) and magnesium (61.00 to 4141.0 mg l^{-1}) (Table 1). However, there was one major exception, Fe^{2+}. The KZX site had the lowest concentration (0.11 mg l^{-1}), but instead of FKBZ, SLS had the highest amount (KZX < ZJ < FKBZ < DWT < SLS) (Table 1). Redox potential was high at DWT (732 mV) and SLS (727 mV), but low at FKBZ (648 mV) and ZJ (627 mV).

RFLP analysis

Clones (n = 154 to 200) were screened from each site and 10 to 37 unique 16S rDNA fragments were detected from each sample using RFLP analysis. Five RFLP patterns (DX1, DX 3, DX 4, DX 11 and DX 29) (Table 2) were common to all 6 sites, and 3 to 4 domi-

Table 2. Distribution (%) and affiliation of some dominant clones in the 16s rDNA libraries. DWT, SLS, YTW, ZJ, FKBZ and KZX are site abbreviations (see Fig. 1)

	DWT	SLS	YTW	ZJ	FKBZ	KZX	Affiliation
DX1	15.1	16.9	22.1	27.47	13.1	0.5	*Acidithiobacillus ferrooxidans*
DX3	5.56	5.15	4.58	1.099	0.77	2.5	*Acidobacterium capsulatum*
DX4	4.76	5.88	3.82	3.297	0.77	0.5	*Leptospirillum ferriphium*
DX5	5.56	5.88	0	4.396	4.62	7	*Gammaproteobacteria* WJ-2
DX8	3.97	0	0	0	1.54	5	Uncultured bacterium clone RCP-2-96
DX11	1.59	1.47	1.53	43.86	19.2	16	*Gammaproteobacteria* WJ-2
DX12	0	0	0.76	0	4.62	5.5	*Acidithiobacillus ferrooxidans*
DX21	11.9	11.8	2.29	0	0.77	0.5	*Leptospirillum ferrooxidans*
DX29	1.59	2.94	7.63	15.38	16.2	4	*Gammaproteobacteria* WJ-2
DX36	0	0.74	0	0	0.77	6	Uncultured bacterium clone RCP-2-96
DX41	9.52	7.35	0	0	0	0	Uncultured bacterium clone RCP-2-96
DX49	0	3.68	0	0	0	7.5	*Pseudomonas pseudoalcaligenes*
DX51	0	0	4.58	0	0	5	*Pantoea agglomerans*
DX52	3.97	0	9.92	0	0	0	*Stenotrophomonas maltophilia*
DX53	0	1.47	6.11	0	0	0	*Acidobacterium capsulatum*
DX57	0	0.74	5.34	0	0	8	*Pantoea agglomerans*
DX63	0	1.47	0	0	0	6	*Pseudomonas pseudoalcaligenes*
DX64	0	9.56	0	0	0	3.5	*Pseudomonas pseudoalcaligenes*
Others	36.48	24.97	31.34	0.5	37.64	22.5	
Total OTUs	29	32	29	10	37	26	

nant patterns were detected for each site. The lowest number of unique 16S rDNA patterns was found in the samples taken from site ZJ (10 OTUs) where DX11 accounted for 43.86% of the clone library (Table 2). The greatest number of unique clones was obtained from FKBZ (37 OTUs). DX1 was dominant in DWT (15.1%), SLS (16.9%), YTW (22.1%) and FKBZ (13.1%). It also showed significance in ZJ (27.48%; Table 2).

The diversity index (*H*) was calculated for each site from the clone data. The result showed that the ZJ site had the lowest diversity, while the diversity in site DWT had the highest, more than twice that of the ZJ site (Table 1). The rarefaction curves suggested that the number of OTUs at all sites was close to the asymptotic level (Fig. 2).

Phylogenetic analysis

Representative 16S rDNA clones that occurred more than once in a given library, as well as representatives of some of the unique OTUs determined by cluster analysis on RFLP patterns, were partially sequenced. The phylogenetic distribution of the 6 sites fell into 5 putative phylogenetic divisions (Fig. 3). The majority (79.1%) of the clones were affiliated with 3 groups: *Gammaproteobacteria* (60%), *Nitrospira* (10.3%) and *Acidobacteria* (8.8%). A small percentage of the clones were affiliated with the *Actinobacteria* (1.8%) and *Alphaproteobacteria* (3.6%) (Table 3).

All of the clones affiliated with *Nitrospira* fell into 2 major groups. The more abundant group (85.4%) was affiliated with *Leptospirillum* (97 to 99% similarity). Within this group, 2 species (*L. ferrooxidans* and *L. ferriphilum*) occurred in 5 sites but none were found at the ZJ site. Only one OTU (DX27) belongs to the other group.

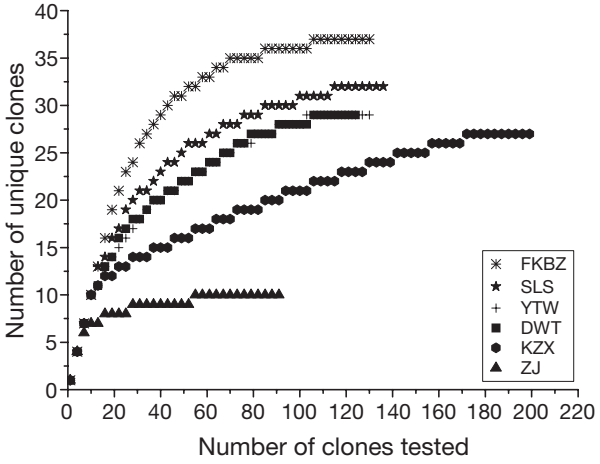

Fig. 2. Evaluation of the representation of the clones obtained from the 6 samples by rarefaction analysis. See Fig. 1 for site abbreviations

Clones associated with *Proteobacteria* were the most dominant and fell into *Gamma-* and *Alphaproteobacteria* (Fig. 3). All the clones related to *Gammaproteobacteria* fell into 4 major groups (I, II, III, IV); DX54, DX14, DX12, DX62, DX1, DX19 and DX18 were clustered well within group IV of *Gammaproteobacteria* and separated well from the 2 subgroups (one subgroup was related to *Acidithiobacillus ferrooxidans*, strain ATCC23270; the other was related to *Acidithiobacillus ferrooxidans* strain TFD) (Fig. 3). The clones exhibited 92 to 99% similarity to *A. ferrooxidans.*

Clones affiliated with *Actinobacteria* were divided into 2 groups. DX58, found only at YTW and SLS, was a group with only 83% similarity with the *Ferrimicrobium acidiphilum*. The second group (DX38, DX67 and DX65; Fig. 3) was affiliated with iron-oxidizing bacterium CS11 with 93 to 99% similarity. Phylogenetic analysis indicates that members of *Acidobacteria* were found at all 6 sites (Fig. 3).

The distribution of clones showed differences among all 6 sites. A high percentage of clones similar to *Acidithiobacillus ferrooxidans* were found at DWT, SLS, YTW, ZJ and FKBZ (17.5, 16.9, 28.2, 27.5 and 20.8%, respectively) (Table 3). Clones similar to *Leptospirillum ferrooxidans* were dominant at DWT and SLS (23 and 19.9%, respectively) but represented a much smaller fraction at other sites (<8%). Clones similar to iron-oxidizing bacteria were found in the slime matrix of acid-streamers (WJ-2) (a possible new genus or species) (Hallberg & Johnson 2003). These clones comprise a significant fraction of clones at FKBZ and ZJ and were observed at the other 4 sites. Clones similar to *Pseudomonas* spp. (13.5% of the total of clones) were primarily found at KZX.

Microbial communities and environmental variables

Stepwise CCA revealed that pH, Fe^{2+}, Ca and S were highly correlated to the microbial community structure at all 6 sites. Result of the CCA showed that the first 2 axes were significant ($p < 0.02$) and captured over 50% of the variation explained by the environmental variables. Axis 1 was defined as the gradient of pH and Fe^{2+} where pH decreases as Fe^{2+} increases (Fig. 4). Axis 2 was the gradient of Ca and S, both of which increase in the same direction (Fig. 4). These 5 (including Fe) environmental variables were almost exclusively correlated to either Axis 1 or 2, which means that Fe and pH were, at most, only weakly correlated to Ca and S.

Not surprisingly, the environmental variables most strongly associated with the microbial community structure varied among the sites (Fig. 4). The microbial

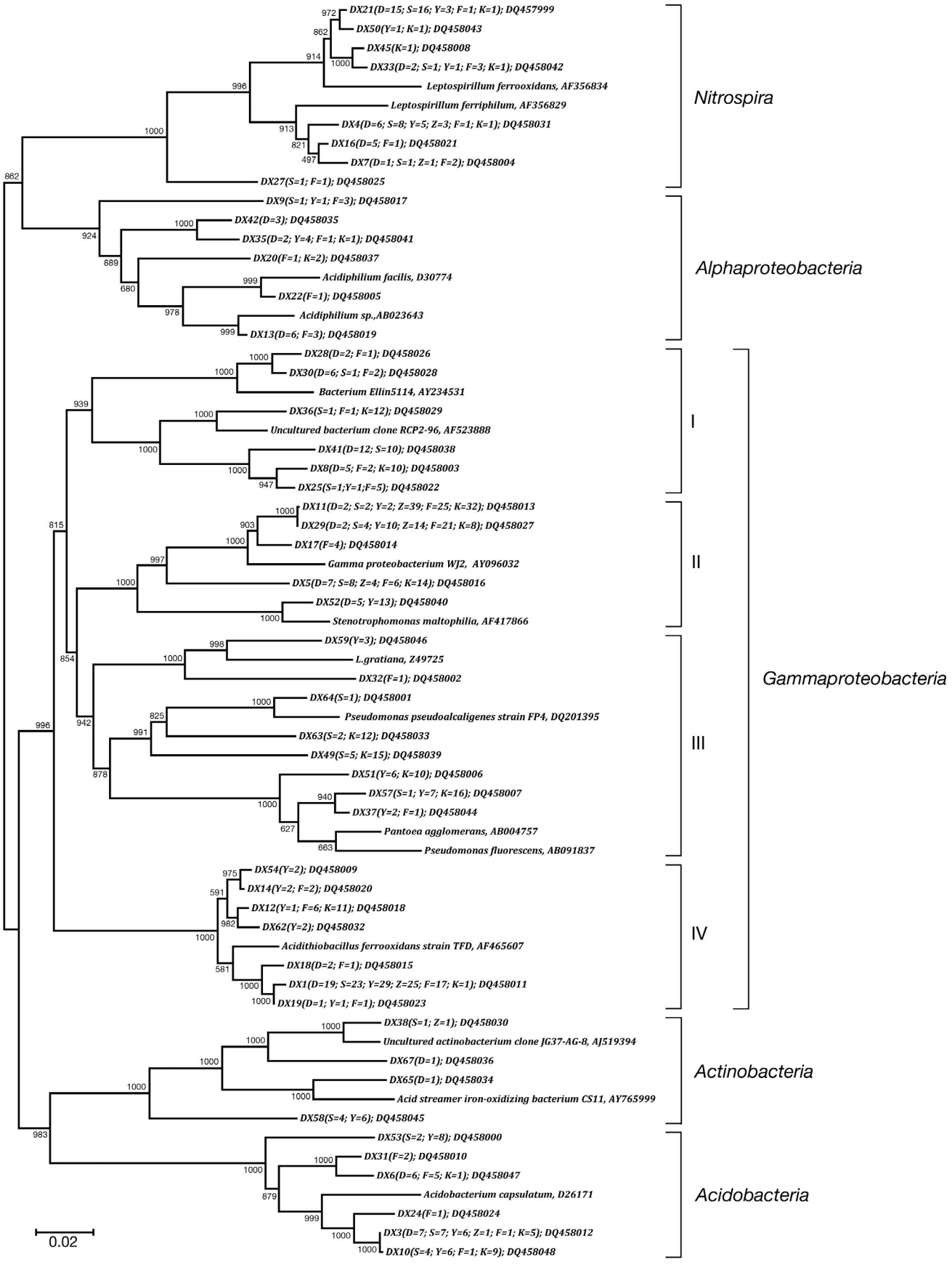
DX21(D=15; S=16; Y=3; F=1; K=1); DQ457999
DX50(Y=1; K=1); DQ458043
DX45(K=1); DQ458008
DX33(D=2; S=1; Y=1; F=3; K=1); DQ458042
Leptospirillum ferrooxidans, AF356834
Leptospirillum ferriphilum, AF356829
DX4(D=6; S=8; Y=5; Z=3; F=1; K=1); DQ458031
DX16(D=5; F=1); DQ458021
DX7(D=1; S=1; Z=1; F=2); DQ458004
DX27(S=1; F=1); DQ458025
Nitrospira
DX9(S=1; Y=1; F=3); DQ458017
DX42(D=3); DQ458035
DX35(D=2; Y=4; F=1; K=1); DQ458041
DX20(F=1; K=2); DQ458037
Acidiphilium facilis, D30774
DX22(F=1); DQ458005
Acidiphilium sp.,AB023643
DX13(D=6; F=3); DQ458019
Alphaproteobacteria
DX28(D=2; F=1); DQ458026
DX30(D=6; S=1; F=2); DQ458028
Bacterium Ellin5114, AY234531
DX36(S=1; F=1; K=12); DQ458029
Uncultured bacterium clone RCP2-96, AF523888
DX41(D=12; S=10); DQ458038
DX8(D=5; F=2; K=10); DQ458003
DX25(S=1;Y=1;F=5); DQ458022
I
DX11(D=2; S=2; Y=2; Z=39; F=25; K=32); DQ458013
DX29(D=2; S=4; Y=10; Z=14; F=21; K=8); DQ458027
DX17(F=4); DQ458014
Gamma proteobacterium WJ2, AY096032
DX5(D=7; S=8; Z=4; F=6; K=14); DQ458016
DX52(D=5; Y=13); DQ458040
Stenotrophomonas maltophilia, AF417866
II
DX59(Y=3); DQ458046
L.gratiana, Z49725
DX32(F=1); DQ458002
DX64(S=1); DQ458001
Pseudomonas pseudoalcaligenes strain FP4, DQ201395
DX63(S=2; K=12); DQ458033
DX49(S=5; K=15); DQ458039
DX51(Y=6; K=10); DQ458006
DX57(S=1; Y=7; K=16); DQ458007
DX37(Y=2; F=1); DQ458044
Pantoea agglomerans, AB004757
Pseudomonas fluorescens, AB091837
III
DX54(Y=2); DQ458009
DX14(Y=2; F=2); DQ458020
DX12(Y=1; F=6; K=11); DQ458018
DX62(Y=2); DQ458032
Acidithiobacillus ferrooxidans strain TFD, AF465607
DX18(D=2; F=1); DQ458015
DX1(D=19; S=23; Y=29; Z=25; F=17; K=1); DQ458011
DX19(D=1; Y=1; F=1); DQ458023
IV
Gammaproteobacteria
DX38(S=1; Z=1); DQ458030
Uncultured actinobacterium clone JG37-AG-8, AJ519394
DX67(D=1); DQ458036
DX65(D=1); DQ458034
Acid streamer iron-oxidizing bacterium CS11, AY765999
DX58(S=4; Y=6); DQ458045
Actinobacteria
DX53(S=2; Y=8); DQ458000
DX31(F=2); DQ458010
DX6(D=6; F=5; K=1); DQ458047
Acidobacterium capsulatum, D26171
DX24(F=1); DQ458024
DX3(D=7; S=7; Y=6; Z=1; F=1; K=5); DQ458012
DX10(S=4; Y=6; F=1; K=9); DQ458048
Acidobacteria
0.02

Fig. 3. Phylogenetic analysis of recovered 16S rDNA sequences from 6 sites at Dexing copper mine and selected sequences recovered from different environmental sources and formally described species. The clone composition of each site is provided. I, II, III and IV represent 4 groups of *Gammaproteobacteria*. Cloned sequences from 6 sites are given in parentheses (Site DWT = D; SLS = S; YTW = Y; ZJ = Z; FKBZ = F; KZX = K; see Fig. 1 for site details)

Table 3. Affiliation of the sequenced clones and percentage found at each site. See Fig. 1 for details of sites

	% (total # clones)	DWT (%)	SLS (%)	YTW (%)	ZJ (%)	FKBZ (%)	KZX (%)
Nitrospira	10.30 (126)	23	19.9	7.6	1	6.2	2.5
Alphaproteobacteria	3.60 (136)	8.7	0.7	3.8	0	6.9	1.5
Actinobacteria	1.80 (131)	1.6	3.7	4.6	1	0	0
Acidobacteria	8.80 (90)	10.3	9.6	15	1	7.7	7.5
Gammaproteobacteria	60 (130)	50	42.6	61	90.1	73	64.5
Acidithiobacillus ferrooxidans	17.9 (200)	17.5	16.9	28.2	27.5	20.8	6

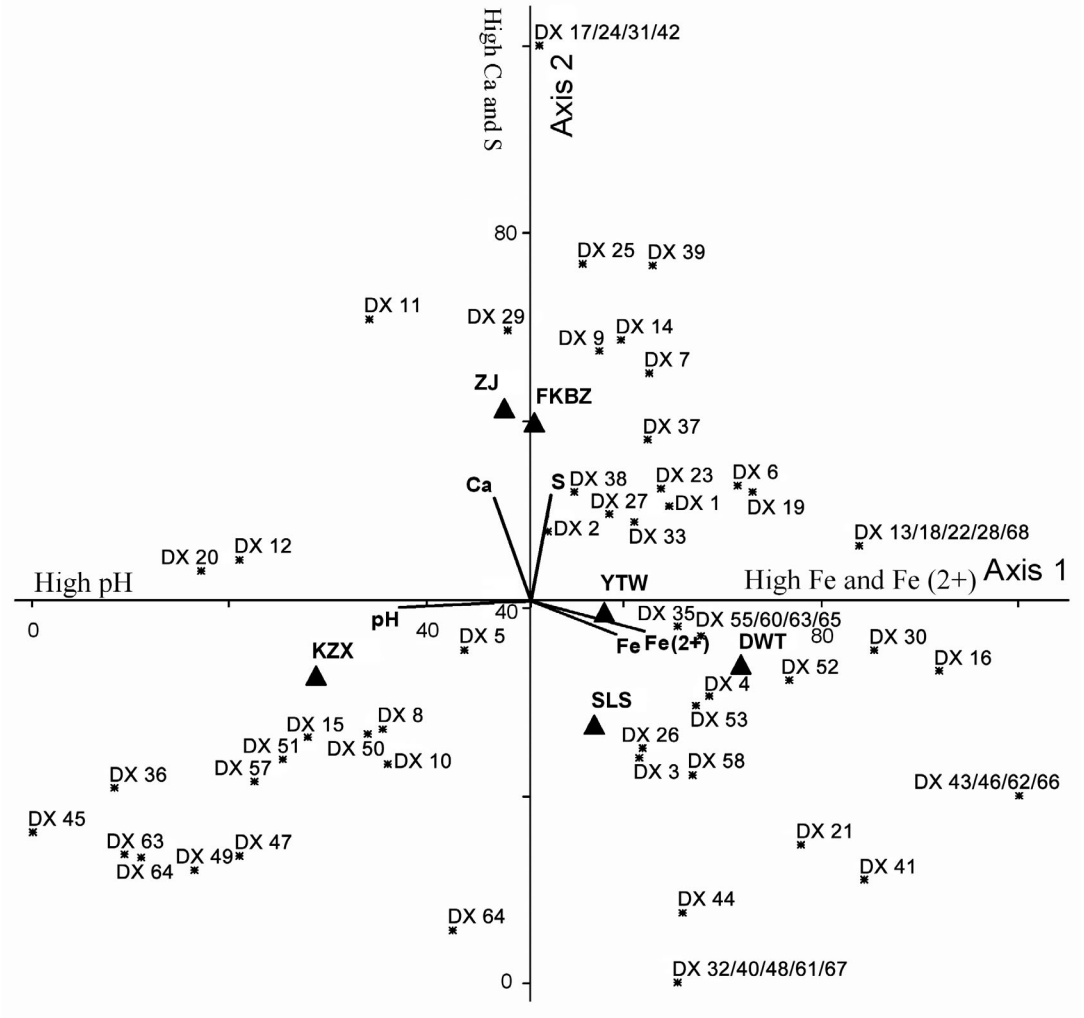

Fig. 4. Canonical correspondence analysis (CCA) split plot graph. ▲: sites; ✱: different OTUs. The percentage of each OTU in the libraries was used as input for the CCA analysis. Axis 1 was related to a gradient of pH and Fe2+. Axis 2 was related to a gradient of Ca and S. Axis 1 and 2 respectively explained 35.1 and 26.9% of variance of the species–environment relationship. Total variance ('inertia') in the species data was 1.3767 and the sum of all canonical eigenvalues was 1.195

community of KZX was most highly correlated to higher pH and low Fe^{2+} while that of DWT was associated with high Fe and low pH. The microbial community of SLS was weakly associated with increasing amounts of Fe and low pH. Both ZJ and FKBZ were highly correlated to Axis 2 and associated with increasing amounts of Ca and S. Both DWT and SLS were negatively associated with Axis 2. This may be caused by the lower amounts of Ca and S found at these sites. Another interpretation is that an environmental variable or a set of variables were exerting influence on the microbial communities of these sites. As all of the metals except Fe were autocorrelated, it would be difficult to determine if one or many of these were the variables influencing DWT and SLS in this direction.

In order to reveal the exact explanatory power of the environmental variables concerned, variation partitioning analysis was performed. The results showed that the environmental variables pH and Fe^{2+} together explained 37.9% of the total inertia, and Ca and S together explained 39.4%; 9.4% was the amount of covariation of these variables. Moreover, 13% of the total inertia was not explained by these variables. In all, these results suggested that pH, Fe^{2+}, Ca and S were significantly correlated to the microbial community.

DISCUSSION

AMD sites representing heterogeneous, highly different geochemical environments have provided an opportunity to examine the relationships between environmental variables and the microbial community structure (Bond et al. 2000a, Baker & Banfield 2003). As these sites are comprised of more simplistic microbial community assemblages, some have suggested that they represent a model system to investigate linkages between the microbial community and geochemistry (Bond et al. 2000b, Baker & Banfield 2003). From previous studies, microorganisms associated with these sites fell mainly into *Actinobacteria*, *Nitrospira*, *Firmicutes*, and *Acidobacteria* with a few from the *Proteobacteria* families. However, the heterogeneity of AMD sites may demonstrate vast dissimilarity in microbial community assemblages. Determining microbial community assemblage and relating this to geochemistry gradients may lead to a clearer understanding of the important variables that structure microbial communities in these sites.

Using water from multiple sites geographically linked and differentially impacted by AMD, we were able to compare the composition and structure of the microbial communities as well as correlate these communities to both the environmental variables of the site and potential linkages among geographical location. In general, the bacteria found belong to *Acidobacteria*, *Actinobacteria*, *Nitrospira*, *Alphaproteobacteria* and *Gammaproteobacteria*; no clones affiliated with *Firmicutes* were observed. The lack of *Firmicutes* may result from insufficient cracking of the thick Gram-positive cell walls during DNA extractions. In the AMD environments, archaea (such as *Acidianus*) are a minor component of the microbial community, especially at the lower temperature of 40°C (Baker & Banfield 2003). In the present study, strong correlations between microbial community structure and environmental variables were revealed, suggesting that the environment strongly affects the communities found in these sites. Bond et al. (2000a) also suggested that the environmental variables in AMD are the primary factors affecting the microbial communities inhabiting Iron Mountain.

Our results, mirroring assumptions in previous studies (e.g. Nordstrom & Southam 1997), found that the environmental variables pH and Fe concentration strongly correlate with the microbial community assemblages at KZX (pH), DWT, SLS, and YTW (primarily Fe). Lopez-Archilla et al. (2001) also found a close relationship between pH, metal concentrations and the abundance of chemolithotrophic bacteria in the Tinto River, Spain. Therefore, acidic environments and the presence of high iron boost growth of acidophilic bacteria able to use Fe^{2+} as an electron donor. Furthermore, S and Ca were also strongly correlated to the communities found at FKBZ and ZJ. Although sulfur reduction was not determined (this was likely an effect of the PCR bias), this correlation suggests that it may be an important process within these sites. In other AMD sites, anaerobic sulphate-reducing bacteria have been found, also suggesting this possibility (Redburn & Patel 1993).

Alternatively, Schrenk et al. (1998) suggested that the distribution of *Leptospirillum* may be more closely associated with lower pH. Edwards et al. (1999b) used fluorescent *in situ* hybridization (FISH) probes to quantify the relative abundances of *Leptospirillum* within the Richmond Mine at Iron Mountain. They found that *Leptospirillum* is more abundant in environments with higher temperatures and lower pH compared with environments where *Acidithiobacillus ferrooxidans* is the dominant group. Though the pH is not as low as the sites at Iron Mountain, the DWT and SLS sites have the lowest pH value among the sites investigated and the dominant clones were from *Leptospirillum*. As the pH increased, fewer clones of *Leptospirillum* were found. Interestingly, the DWT and SLS sites, both with a pH of 2.0, exhibited a similar number of clones affiliated with the *Leptospirillum*, but the level of Fe^{2+} in the SLS site was much higher than that at DWT, even

though *Leptospirillum* are restricted to using Fe^{2+} as an electron donor (Johnson 2001). This partially supports the notion that the distribution of *Leptospirillum* versus *A. ferrooxidans* could be caused by the effects of pH.

Many studies suggest that the physiological process of high heavy metals would lead to low microbial diversity (Sandaa et al. 2001, Feris et al. 2003). If this is true, then the assumption would be that as heavy metal concentration increases, diversity across this environmental gradient decreases. We found that the sites with the lowest pH and highest iron content (DWT and SLS) possess the greatest diversity. Not unexpectedly, at ZJ, where metal loads were high, diversity was the lowest. But surprisingly, FKBZ, with similar metal loads and pH to ZJ, had a much higher diversity index. In fact, the diversity indices along the gradient, with the exception of ZJ, did not change significantly. Similar results were found by Feris et al. (2003), suggesting that diversity may not be as dependent on stress as once thought. Microbial community structure and diversity is likely affected by a complex interaction between and among environmental variables and organisms. However, further interpretation would require analysis across temporal scales to confirm that these relationships are robust and not an accident of timing in sampling.

Karavaiko et al. (2003) divided *Acidithiobacillus ferrooxidans* strains into 3 groups, and suggested that these groups were correlated with their original geographical sites. This led to the assumption that these organisms are restricted geographically through microevolutionary trends, limitations in colonization ability, or a combination of both (Peng et al. 2006). Our study found that *A. ferrooxidans* fell into 2 groups (similar to ATCC23270, Group I and TFD, Group III). While each of these 2 groups showed different proportions among sites, this only provides part of the evidence suggesting a geographic correlation of *A. ferrooxidans* strains for these sites. A large-scale investigation covering the sites mentioned above of *A. ferrooxidans* strains in relation to their habitat might provide insight into their evolution, colonization and competitive abilities.

While the heterotrophic *Acidiphilium*, a genus of the *Alphaproteobacteria,* has been shown to be the third dominant genus in a study of the Tinto River (Erlich 1996), it was detected in low numbers in our study, perhaps due to differences in environmental variables. Of course, physiological and ecological analysis would need to be carried out to confirm the role that *Acidiphilium* plays in AMD-impacted sites. Other acidophilic microorganisms were detected in this study, such as *Ferrimicrobium acidiphilum*, which can oxidize iron under aerobic conditions or reduce it under anaerobic conditions. We also found a clone (<1% in DWT) affiliated with the uncultured *Acidimicrobium sp.* clone SK314 found at Rainbow and Joseph's Coat Hot Springs in Yellowstone National Park. Interestingly, we detected 2 sequences (clones DX11, DX29) with 98% similarity to the WJ-2 strain which is capable of autotrophic and heterotrophic growth. This organism may represent an unknown species and possibly a novel genus (Hallberg & Johnson 2003). At the highest representation, 59% of clones at ZJ were associated with the WJ-2 strain. The clones affiliated with this strain represented 20.3% of the total within the 6 clone libraries. Edwards et al. (1999b) also directly obtained the bacterial clone (98% similarity with DX11 and DX29) from highly acidic water in an abandoned pyrite mine at Iron Mountain, with pH < 1.0, temperature > 35°C. Edwards et al. (1999b) suggested that the newly described acidophilic microorganism was correlated with pyrite (FeS_2) dissolution.

The present and previous studies have shown that the microbial communities among the Dexing copper mine and other sites are influenced by a number of variables, including geochemistry and competitive interactions (Nordstrom & Southam 1997, Marchesi et al. 1998, Edwards et al. 1999a, Bond et al. 2000a, Gonzalez-Toril et al. 2003) Because AMD-impacted sites vary greatly in origination and geochemistry of the underlying strata, these results provide important insights into a major and previously unexamined AMD region. Our results suggest that, using CCA analysis, we will be able to examine the changes in microbial communities along an environmental gradient, as well as postulate (using correlations) potential structuring mechanisms of these communities. Though models such as CCA provide us with little information of the metabolic ability of these organisms, we are able to elucidate the gradients potentially affecting the distribution of microbial communities, which leads to greater precision in hypothesis testing within these sites. However, the information provided by CCA analysis is also limited by the environmental variables collected. More improvements should be made in environmental variable selection and analysis methodology in future studies. In the present paper, the proportion of each OTU in 6 libraries was examined through PCR-RFLP analysis, which is also the basis for all the successive CCA and partial CCA analysis. However, bias may exist because of the intrinsic differences in the amplification efficiency of templates (Polz & Cavanaugh 1998) and the amplification inhibition caused by the self-annealing of the most abundant templates in the late stages of amplification (Suzuki & Giovannoni 1996). To reduce this bias to the minimum level, methods with less PCR-related bias are recommended for

future study, such as FISH and real-time quantitative PCR. However, this study does present data from a novel site, which expands the global view of AMD environments.

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