The Journal of Microbiology (2012) Vol. 50, No. 2, pp. 207–217
DOI 10.1007/s12275-012-1342-z

Molecular Analysis of Spatial Variation of Iron-Reducing Bacteria in Riverine Alluvial Aquifers of the Mankyeong River§

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(Received July 18, 2011 / Accepted December 14, 2011)

Alluvial aquifers are one of the mainwater resources in many countries. Iron reduction in alluvial aquifers is often a major anaerobic process involved in bioremediation or causing problems, including the release of As trapped in Fe(III) oxide. We investigated the distribution of potential iron-reducing bacteria (IRB) in riverine alluvial aquifers (B1, B3, and B6 sites) at the Mankyeong River, Republic of Korea. Inactive iron reduction zones, the diversity and abundance of IRB can be examined using a clone library and quantitative PCR analysis of 16S rRNA genes. *Geobacter* spp. are potential IRB in the iron-reducing zone at the B6 (9 m) site, where high Fe(II) and arsenic (As) concentrations were observed. At the B3 (16 m) site, where low iron reduction activity was predicted, a dominant clone (10.6%) was 99% identical in 16S rRNA gene sequence with *Rhodoferax ferrireducens*. Although a major clone belonging to *Clostridium* spp. was found, possible IRB candidates could not be unambiguously determined at the B1 (18 m) site. Acanonical correspondence analysis demonstrated that, among potential IRB, only the *Geobacteraceae* were well correlated with Fe(II) and As concentrations. Our results indicate high environmental heterogeneity, and thus high spatial variability, in thedistribution of potential IRB in the riverine alluvial aquifersnear the Mankyeong River.

Keywords: iron reduction, riverineaquifer, arsenic, *Geobacter* spp., *Rhodoferax ferrireducens*

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§Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Introduction

Alluvial aquifers are being developed extensively to supply a significant amount of water in many countries (Bourg and Bertin, 1993; Hiscock and Grischek, 2002; Tufenkji *et al.*, 2002; Min *et al.*, 2003). Alluvial aquifers are vulnerable to river flow change and surface water pollution, because they are hydrologically connected to surface and river water. Thus, anthropogenic pollutants such as fertilizers, agricultural chemicals, and lime often impede development of alluvial aquifers (Kelly, 1997).

Dissimilatory Fe(III) reduction is an important respiratory process in subsurface environments, because this process is coupled to the oxidation of organic matter (Lovley *et al.*, 2004). When organic pollutants permeate into the groundwater, they can be oxidized by the Fe(III)-reduction process, which can also be employed for bioremediation (Lovley, 1995). On the other hand, the Fe(III) reduction process itself often causes problems by generating a substantial amount of Fe(II). As a consequence of Fe(III) reduction, high concentrations (10 to 100 mg/L) of dissolved Fe(II) are frequently observed in anaerobic aquifer environments (Norris and Matthews, 1994; Choi *et al.*, 2007). When the water is pumped to the surface and contacts oxygen, Fe(III) oxides are formed and these result in bad taste, odor, and color of the water, and in addition stain fabric (Bourg and Bertin, 1993; Lovley *et al.*, 2004). Fe(II) oxidation makes surface soil acidic and hampers crop cultivation (Brown *et al.*, 1999). The accumulation of Fe(III) oxides may clog pipelines and filters, thereby reducing the system's flow rate and hampering development of the water resources (Lovley *et al.*, 2004).

High concentration of Fe(II), which is frequently connected with arsenic (As) contamination, is a common, worldwide problem in alluvial aquifer development (Kim *et al.*, 2008). Arsenic immobilized by Fe(III) oxide can be released when the iron is reduced by dissimilatory iron-reducing bacteria (IRB) (Islam *et al.*, 2004). This might be a common process leading to As contamination of groundwater in many countries such as Bangladesh and the West Bengal area of India (Nickson *et al.*, 2000; Kim *et al.*, 2009). Arsenic concentrations in alluvial aquifers are characterized by high spatial variability. However, the iron-reducing processes causing the spatial variability in As concentration are still poorly understood.

Iron-reducing capability of prokaryotes is widespread throughout phylogenetic groups. Each IRB group has specificity regarding the substrate iron types [soluble (chelated) iron, ferrihydrite, goethite, etc.] (Lovley *et al.*, 2004; Weber *et al.*, 2006) and some IRB, such as *Geobacter* spp., *Shewanella* spp., and *Geothrix* spp., are able to use solid phase iron,

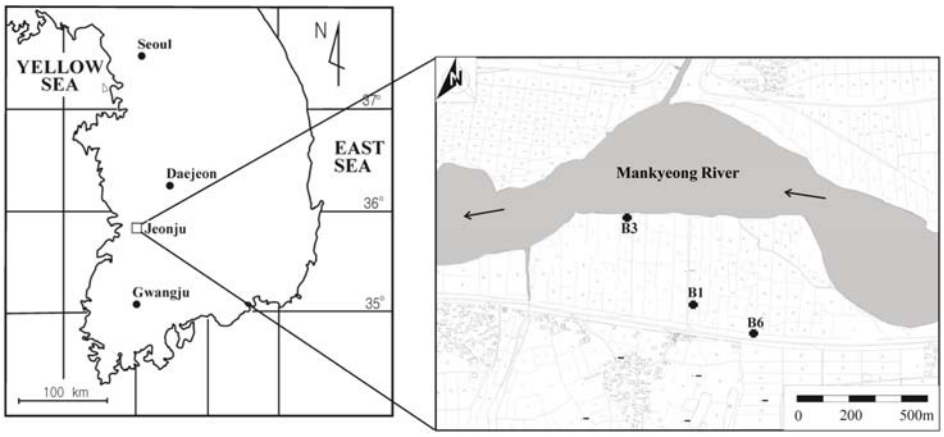

Fig. 1. Location of the three sampling sites on the alluvial aquifers of the Mankyeong River.

which is common in environments, by direct contact (Nevin and Lovley, 2000; Childers *et al.*, 2002) or by electron shuttle (Newman and Kolter, 2000; Turick *et al.*, 2002). A variety of other anaerobic microorganisms, such as the many sulfate reducing, methanogenic, and fermenting microorganisms, are known to reduce Fe(III) as an electron sink, but their growth is not supported by Fe(III) reduction (Coleman *et al.*, 1993; Lovley *et al.*, 1993; Bond and Lovley, 2002). The potential roles of the iron reduction process in mitigating organic pollution in alluvial riverine aquifer environments in the Mankyeong River area have been investigated previously (Choi *et al.*, 2007). However, the diversity and abundance of IRB have not yet been studied in these environments. The principal objective of this study was to investigate the abundance of potential IRB and analyze the environmental variables affecting the distribution of IRB in groundwaters of the alluvial aquifers. This study will help to understand and predict diversity, abundance, and activity of IRB, and their contribution to the biogeochemical cycles, in the alluvial aquifers.

Materials and Methods

Sampling site description, sample collection and characterization

For this study, multilevel samplers installed at the Mankyeong River in Jeonju, Republic of Korea (127°01′E, 35°53′N) (Fig. 1) were used. To install the sampler, a 10 cm diameter stainless casing was drilled into the sediment until fresh bedrock was encountered, a bundle of polyethylene tubes was placed into the casing, and then the casing was removed with the bundle left in the sediment as described by Kim *et al.* (2009). Each sampler had 5-6 sampling ports (Table 1). In July 2008, depth-specific groundwater samples were collected from wells of three alluvial aquifer sites (B1, B3, and B6). The Mankyeong River flows to the Yellow Sea and the study area had been influenced by sea water until a water gate was constructed 7 km downstream from the site in 1964. Agricultural activities including rice-field and dry-field farming are conducted in, and livestock waste waters

Table 1. The hydrogeochemical properties of groundwater collected from the multilevel monitoring wells at the B1, B3, and B6 sites. The abbreviations of environmental variables are described in 'Materials and Methods'. The sampling depth of the active iron-reducing zone used for analysis of IRB is highlighted in bold. The values are the average of duplicate measurements. ND, not detected.

Site	Depth (m)	Temp. (°C)	pH	DO (mg/L)	E_h (mV)	EC (μS/cm)	NPOC (mg/L)	nitrate (mg/L)	Mn (mg/L)	Fe(II) (mg/L)	sulfide (μg/L)	sulfate (mg/L)	As (μg/L)	Fe(II)/ sulfide	Predominant redox process[a]
B1	6	17	5.42	0.3	215	376	1.29	0.43	0.44	0.04	1	67.3	< 10	40	Mn(IV) reduction
	9[b]	18	5.76	0.2	152	420	6.46	ND	2.02	0.12	2	65.1	< 10	60	Fe(III) reduction
	12	18.1	6.13	0.5	2	459	1.38	ND	3.06	1.65	11	49.9	11	150	Fe(III) reduction
	18[b]	**17.9**	**6.42**	**0.2**	**-40**	**453**	**1.70**	**ND**	**3.17**	**11.99**	**4**	**37.0**	**14**	**2998**	**Fe(III) reduction**
	22	17.9	7.45	0.2	-58	708	1.43	0.19	0.21	1.21	5	12.6	< 10	242	Fe(III) reduction
B3	4	20.1	6.23	7.7	198	808	6.60	4.99	0.01	0.03	1	70.3	14	30	Oxic
	8	18.9	5.8	0.2	135	761	1.74	0.65	0.17	0.03	2	68.7	11	15	Mn(IV) reduction
	11[b]	17.5	5.45	0.2	193	512	1.34	2.17	1.35	0	3	62.6	12	0	Mn(IV) reduction
	14	17.4	6.88	0.2	26	758	1.13	ND	0.22	0.45	3	57.1	10	150	Fe(III) reduction
	16[b]	**17.7**	**6.97**	**0.2**	**-46**	**750**	**1.13**	**ND**	**0.17**	**0.23**	**3**	**56.7**	< 10	**77**	**Fe(III) reduction**
	19[b]	17.5	7.13	0.2	-120	829	0.97	ND	0.13	1.18	3	57.1	< 10	393	Fe(III) reduction
B6	**9[b]**	**15.4**	**6.42**	**0.4**	**-105**	**460**	**2.46**	**ND**	**1.65**	**34.86**	**5**	**1.9**	**28**	**6972**	**Fe(III) reduction**
	11	15.6	6.87	0.2	-108	349	2.38	ND	0.76	45.15	9	2.4	38	5017	Fe(III) reduction
	14[b]	16.1	6.87	0.2	-87	349	1.03	ND	1.08	4.95	6	1.8	< 10	825	Fe(III) reduction
	21	16.5	6.92	0.4	-12	327	1.22	0.04	0.77	0.38	5	2.7	< 10	76	Fe(III) reduction
	27[b]	16.1	6.94	0.3	-28	327	1.35	ND	0.34	0.31	2	3.1	< 10	155	Fe(III) reduction

[a] Dominating redox processes based on criteria of McMahon and Chapelle (2008) and Chapelle *et al.* (2009)
[b] Depth used for real-time PCR

are frequently introduced into, this estuarine region. The river usually does not have much stream flow; however, during the summer monsoon, the river overflows and floods the study area. The composition of sediment in the study area has been investigated previously (Choi *et al.*, 2008). The three study sites (B1, B3, and B6) were selected based on previous observations on iron reduction and As contamination (Choi *et al.*, 2007; Kim *et al.*, 2009).

Temperature, pH, dissolved oxygen (DO), electrical conductivity (EC), and redox potential (E_h) of each sample was measured anaerobically with an overflow chamber using a multimeter CPD-65N (Istek, Korea). Groundwater samples were collected using peristaltic pumps, in duplicate, in 2-L glass bottles and sealed with butyl rubber stoppers after the temperature, pH, DO, EC, and E_h were stabilized. All groundwater samples were kept in sterile bottles at 4°C until analyzed. Groundwater samples were filtered through a 0.2 μm membrane filter, and subsamples for cation analysis were acidified to pH<2 in the field.

The concentrations of sulfate and nitrate were measured by ion-exchange chromatography (ICS-1500, Dionex). Fe and Mn were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES; Optima 4300DU, Perkin Elmer). Low levels of Mn were detected by ICP mass spectrometry (ICPMS; X-7 model, Thermo Elemental). Concentrations of As were measured using ICP-AES (ULTIMA 2C, Jobin-Yvon) (detection limit, 10 μg/L). The sulfide ion was quantified by the methylene blue method (Lindsay and Baedecker, 1988). Dissolved organic carbon (DOC) in groundwater was measured as non-purgeable organic carbon (NPOC) using a total organic carbon analyzer (TOC-VCPH, Shimadzu, Japan). Water samples for DOC were passed through glass fiber filters (GF/F, 0.7 μm, Whatman) and acidified by concentrated H_2SO_4 before analysis. All the presented results are the average of the duplicate samples.

Extraction of genomic DNA

One liter of groundwater from each sample was pre-filtered with a 1 μm glass fiber filter (Adventec, Japan) and the flow-through was filtered with a 0.2 μm filter (Adventec) under vacuum (Milipore, USA). The 0.2 μm filter was placed in a sterile conical tube and stored at -70°C until analysis. Total genomic DNA was extracted from the 0.2 μm filter using the Power Soil™ DNA isolation kit (Mo Bio Laboratories, USA). From each depth of the sampling sites, two independent water samples were collected and used for extraction of DNA. Genomic DNA concentrations were measured with a NanoDrop® ND-1000 spectrometer (Nanodrop Technologies, USA).

Cloning, sequencing, and phylogenetic analysis of bacterial 16S rRNA gene

An active iron reduction zone, where high Fe(II) concentrations were observed by E_h transition, was selected for clone library analysis (see the Table 1). Duplicate DNA samples were independently amplified using *EF*-Taq DNA polymerase (Solgent, Korea), with primers 27F and 1492R to analyze general bacterial community profiles (Weisburg *et al.*, 1991). The temperature profile for the PCR reaction was as follows: 5 min at 95°C; 30 cycles of 30 sec at 95°C, 30 sec at 55°C, 45 sec at 72°C; 10 min at 72°C; hold at 4°C. Duplicate PCR products were combined and purified using a PCR purification Kit (Solgent), ligated using the T&A Cloning Vector Kit (Real Biotech Corporation, Taiwan), and transformed into *Escherichia coli* DH5α in accordance with the manufacturer's instructions. Transformants were transferred to 96-well plates containing Luria broth with 100 μg/ml of ampicillin, grown overnight at 37°C, and stored at -70°C prior to screening.

Transformants were PCR-screened to select the clones with the correct insert size of approximately 1.5 kb using the M13 universal primer set, M13F (5′-GTT TCC CAG TCA CGA C-3′) and M13R (5′-TCA CAC AGG AAA CAG CTA TGA C-3′). PCR was conducted under the following conditions: 5 min at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 45 sec at 72°C; 7 min at 72°C; hold at 10°C. Clones with the correct insert size were randomly selected to evaluate the genetic diversity of each sample. The positive PCR products were purified using a PCR Purification Kit (Cosmo Gentech, Korea) and sequenced using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) coupled with an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems). Chimeric sequences were analyzed using Bellerophon (http://greengenes.lbl.gov/) and MALLARD v1.02 (http://www.cf.ac.uk/biosi/research/biosoft) software (Ashelford *et al.*, 2006). A total of 542 high-quality partial (~800 bp) sequences derived from aquifer samples were used for phylogenetic analysis.

The phylotype of the 16S rRNA gene was classified as a separate group when the sequences differed by 3% (Park *et al.*, 2008). A sequence from each phylotype was selected as a representative sequence and deposited into the GenBank database under the accession numbers HM228597–HM228870. Related taxa obtained from the GenBank and RDP database were aligned using the CLUSTALX program (Thompson *et al.*, 1997). Evolutionary distances were calculated with a Kimura two-parameter model (Kimura, 1983). The phylogenetic tree was constructed based on the neighbor-joining method by the MEGA 3 program (Kumar *et al.*, 2004) with bootstrap values based on 1,000 replications (Felsenstein, 1985).

Rarefaction analysis and estimation of bacterial diversity

The estimation of species richness, diversity index, and rarefaction curve in each clone library was determined on the basis of the bacterial 16S rRNA gene sequences using PAST (http://folk.uio.no/ohammer/past) and SPADE v2.1 software (Chao and Shen, 2005). The percentage of coverage was calculated via Good's method with the following formula: $[1-(n/N)]\times100$, where n is the number of phylotypes in a sample represented by one clone (singletons) and N is the total number of sequences in the sample (Good, 1953).

Quantification of IRB

Real-time quantitative PCR (qPCR) was conducted to determine the 16S rRNA gene copy number using the Mini-Opticon real-time PCR detection system (Bio-Rad Laboratories, USA) with built-in Opticon Monitor version 3.1

Table 2. Primers used for quantitative real-time PCR of IRB

Target	Primer name	Sequence (5′-3′)	Annealing temperature (°C)	Amplicon size (bp)	Reference
Bacteria	518F	CCA GCA GCC GCG GTA AT	55	285	Muyzer *et al.* (1993)
	786R	CTA CCA GGG TAT CTAATC			Baker *et al.* (2003)
Geobacteraceae	Geo564F	AAG CGT TGT TCG GAW TTA T	60	312	Cummings *et al.* (2003)
	Geo840R	GGC ACT GCA GGG GTC AAT A			
Geothrix spp.	Gx.182F	AGA CCT TCG GCT GGG ATG CT	60	323	Snoeyenbos-West *et al.* (2000)
	Gx.472R	AGG TAC CGT CAA GTA ACA SS			
Rhodoferax spp.	Rho219F[a]	CGA TTG GAG CGG CCG ATA T	60	219F and 830R : 609 471F and 830R : 357	This study
	Rho471F[a]	GGG CTA ATG ACG GTA CCG TA			
	Rho830R[a]	CCA GTT GAC ATC GTT TAG GG			
Anaeromyxobacter spp.	60F	CGA GAA AGC CCG CAA GGG T	55	401	Petrie *et al.* (2003)
	461R	ATT CGT CCC TCG CGA CAG T			
Clostridium spp. (B1_9; Identified in this study)	Clos166F[a]	GGA TTA ATA CCT AAT AAC	55	327	This study
	Close492R[a]	AGT TAG CCG GGG CTT CCT CCC			
Shewanella spp.	Sw.783-F	AAA GAC TGA CGC TCA KGC A	55	518	Snoeyenbos-West *et al.* (2000)
	Sw.1245-R	TTY GCA ACC CTC TGT ACT			

[a] Primer numbering relates to *E. coli* position complementary to the 5′ end of the primer.

software (Bio-Rad Laboratories). The genomic DNA used for 16S rRNA gene clone library construction was also used for these real-time PCR experiments. Information regarding the PCR amplification primers used for the 16S rRNA genes is provided in Table 2. Thermal cycling parameters for qPCR were as follows: 15 min at 95°C; 40 cycles of 10 sec at 95°C, 20 sec at annealing temperature, and 20 sec at 72°C. The annealing temperature was shown in Table 2. The specificity for qPCR was confirmed via melting curve analysis, PCR product size, and DNA sequencing. Copy numbers were calculated with an external standard curve that describes the relationship between a known copy number of genes and the cycle threshold (Ct) value as described previously (Sung *et al.*, 2006; Park *et al.*, 2008). Prior to real-time PCR, conventional PCR amplification was performed to test the specificities of the primers and the purity of community DNA templates with the conditions of denaturation of DNA (15 sec at 94°C), annealing of primers (30 sec at annealing temperature), and elongation (30 sec at 72°C) in 45 cycles with different series of dilutions of community DNA templates. This result showed that the primers were specific and community DNA did not inhibit PCR amplification.

Statistical analysis

In order to assess the relationship between distribution of potential IRB and the environmental parameters, detrended correspondence analysis (DCA) and canonical correspondence analysis (CCA) were conducted using CANOCO v. 4.5 software (Biometris, Netherlands). Abundance of IRB was obtained from real-time PCR data. The analysis was performed largely without data transformation to calculate the inter-sample distance. A Monte Carlo permutation test based on 499 random unrestricted permutations was performed to determine the significance of the relationship between manually selected environmental variables and abundance of IRB ($p<0.05$). The resulting ordination biplot approximated the weighted average of IRB groups with regard to each of the environmental variables as indicated by arrows. Length of each arrow indicates the relative importance of environmental variables, whereas an angle between arrows and an axis indicates the degree of correlation (Ter Braak, 1987; Jongman *et al.*, 1995).

Results

Site characteristics and identification of redox processes

The hydrogeochemical properties of the groundwater collected from multilevel monitoring wells at three study sites (B1, B3, and B6) are described in Table 1. The shallowest sampling depth at each site indicates the level of the saturated zone. Thus, site B6 had the deepest vadose zone. Overall, the values of non-purgeable organic carbon (NPOC) were higher in the shallow samples than in those of deep samples. The B3 site is closer to the Mankyeong river and the average EC value of the B3 site (736 μS/cm) was higher than that of the B1 site (483 μS/cm) or the B6 site (362 μS/cm). This elevated EC value is considered to be caused by remnant seawater. The pH value, which is known to be influenced by redox processes such as denitrification, Fe(III) reduction, and sulfate reduction (Massmann *et al.*, 2004; Kim *et al.*, 2008), increased with increasing depth.

Groundwater conditions were generally found to be anoxic, with low concentrations of dissolved oxygen and nitrate (Table 1), thereby indicating that a highly reduced condition predominates in the aquifer. It was highly anoxic even at the top of the saturated zone at the B6 (9 m) site. The dominating redox process at each site was estimated from the concentrations of redox-sensitive species, using the criteria of McMahon and Chapelle (2008). Fe(II) concentrations at B1 and B6 were generally higher than 0.1 mg/L and reached as high as 45 mg/L, whereas B3 showed weak iron reduction signatures. Fe(II) concentration in B1 (at depths of 12 m, 18 m, and 22 m) and B6 samples (all depths) exceeded the drinking water standard (0.3 mg/L) of the World Health Organization (WHO) (Choi *et al.*, 2008). Our data suggest that Fe(III) oxides at the B1 and B6 aquifers act as the prin-

cipal electron acceptors for microbial respiration, whereas Mn(IV) reduction predominates at the B1 and B3 aquifers above the iron reduction zones (Table 1). Sulfate concentrations were high in the aquifers' surface environment and distinctly reduced in the deeper parts of the aquifers (Table 1). The B6 site showed significantly low sulfate concentrations throughout the depth range. To determine the dominance between iron and sulfate reduction processes in the sampling sites, previously established criteria for Fe(II)/ sulfide mass ratios (Chapelle *et al.*, 2009) were used, revealing that iron reduction predominated over sulfate reduction at all anoxic depths. It is assumed that an Fe(II)/sulfide mass ratio >10 indicates a predominance of iron reduction processes over sulfate reduction processes. These geochemical profiles were reproducible and remarkably similar to the previous reports (Choi *et al.*, 2007).

In the B1 and B6 aquifers, the highest As concentration was observed in the layers with active iron reduction. Interestingly, As was also detected in the upper layers of the B3 aquifer, even though the concentration of Fe(II) was low at all depths (Table 1).

Bacterial communities in the iron-reducing zone

To compare the bacterial communities of three active iron reduction zones [B1 (18 m), B3 (16 m) and B6 (9 m) sites], where high Fe(II) concentrations were observed with E_h transition (see Table 1), 16S rRNA gene clone libraries were prepared and 542 clones randomly selected from clone libraries for the three zones were sequenced: 55 sequences from B1 (18 m), 225 from B3 (16 m), and 262 from the B6 (9 m) site. A total of 264 unique phylotypes

Table 3. Estimation of relative clone abundance and diversity of bacterial 16S rRNA gene sequences obtained from iron-reducing zones. Diversity was estimated using operational taxonomic units defined as having ≥97% sequence identity.

	Site		
	B1 (18 m)	B3 (16 m)	B6 (9 m)
Clones	55	225 (55[a])	262 (55[a])
Phylotypes	19	119 (40[a])	134 (46[a])
Singletons	8	77 (27[a])	83 (37[a])
Chao1 estimated richness	29.7	260.2 (73.1[a])	252.8 (122.1[a])
Chao1 standard deviation	10.3	46.1 (17.2[a])	35.8 (36.7[a])
Shannon's index for diversity	2.6	4.4 (3.6[a])	4.6 (3.8[a])
Simpson's index for diversity (Inverse)	0.90	0.98 (0.97[a])	0.98 (0.98[a])
Evenness	0.72	0.69 (0.92[a])	0.71 (0.95[a])
Fisher's diversity	10.28	102.4 (66.0[a])	110.0 (132.4[a])
% Coverage	85.6	65.8 (50.9[a])	68.3 (32.7[a])
Phylogenetic group (% of total)			
Acidobacteria	ND	3.6	1.9
Bacteroidetes	18.2	6.2	4.2
BRC1	ND	ND	0.4
Chlorobi	ND	1.8	ND
Chloroflexi	1.8	7.6	1.9
Firmicutes	10.9	7.6	2.3
Gemmatimonadetes	5.5	1.8	ND
Nitrospira	5.5	3.1	14.9
OD1	ND	ND	0.8
OP3	1.8	2.2	3.4
OP11	ND	0.4	0.8
Planctomycetes	ND	1.8	1.2
Proteobacteria	45.5	44.5	43.9
α-Proteobacteria	7.3	5.8	8.4
β-Proteobacteria	29.1	23.6	13.7
γ-Proteobacteria	5.5	2.2	4.6
δ-Proteobacteria	3.6	12.9	17.2
SP	ND	0.4	ND
Spirochaetes	ND	0.9	1.2
Termite group 1	3.6	0.4	5.0
TG3	ND	0.4	1.9
TM7	ND	ND	1.5
Verrucomicrobia	ND	2.7	4.2
WS3	ND	0.4	ND
Unclassified bacteria	7.3	14.2	10.7

ND, not detected.
[a] Calculated based on 55 clones

were identified based on a cut-off value of 97% nucleotide sequence identity. As a result, 19, 119, and 134 phylotypes were retrieved from the B1 (18 m), B3 (16 m), and B6 (9 m) site, respectively. Bacterial communities at the three sites showed significantly different characteristics (Table 3 and Supplementary data Fig. S1). The B1 (18 m) and B6 (9 m) sites shared one phylotype, while the B3 (16 m) and B6 (9 m) sites shared seven phylotypes. The percentage of singletons in each clone library was 14.5%, 34.2%, and 31.7% for B1 (18 m), B3 (16 m), and B6 (9 m) site, respectively. The clone library coverage ranged from 65.8% to 85.5% of estimated richness (Table 3). Analyses of rarefaction (data not shown), species richness, and diversity demonstrate a higher bacterial diversity at the B3 (16 m) and B6 (9 m) sites, as compared to the B1 (18 m) site (Table 3).

Sequence analysis of clone libraries from the three sites identified representatives of various phyla (Table 3). Clones of the *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Nitrospira*, OP3, *Proteobacteria*, and Termite group I were found for all three sites. The phylogenetic tree based on the bacterial 16S rRNA gene sequences is shown in Supplementary data Fig. S1. Three dominant clones from the B1 (18 m) iron reduction zone are represented by B1_2, B1_5, and B1_9 (18.2%, 16.4%, and 10.9% of total clones in the B1 clone library, respectively). The B1_2 clone is a member of the *Rhodocyclaceae* in the *Betaproteobacteria* and is related to the environmental clones detected in wastewater and iron-manganese nodules (He *et al.*, 2008). The clone B1_5 is affiliated with environmental clones of *Bacteroidetes* detected in heavy metal-contaminated sediments and suboxic freshwater pond sediments (Abulencia *et al.*, 2006). The B1_9 clone is associated with *Clostridium aminobutyricum* (Collins *et al.*, 1994) within the *Firmicutes*. Among the clones from the iron reduction zone at the B3 (16 m) site, B3_11 is a representative of the major clones (10.6% of total clones). This clone shows a 99% sequence identity with *Rhodoferax ferrireducens*, which is a facultative anaerobic iron-reducing bacterium (Finneran *et al.*, 2003). Clones B6_3 and B6_17 are representatives of dominant clones from the B6 (9 m) site, comprising 5.7% and 5.3% of total clones, respectively.

Table 4. Summary of clones related to IRB, SRB, denitrifying bacteria, and clostridia

Site (depth)	Taxonomic group	Clone	Abundance	Nearest cultured and uncultured relatives[a] (% identity, GenBank Accession no.)
B1 (18 m)	*Rhodocyclaceae*	2	10/55	*Georgfuchsia toluolica* (95.6, EF219370); Uncultured bacterium JH-WH250 (96.4, EF492892)
	Syntrophaceae	57	1/55	*Desulfobacca acetoxidans* (88.0, CP002629); Uncultured *Desulfobacca* sp. (92.9, EF613372)
	Clostridiales	9	6/55	*Clostridium aminobutyricum* (93.2, X76161); Uncultured bacterium TSNIR002_E08 (96.2, AB487236)
B3 (16 m)	*Comamonadaceae*	11	24/226	*Rhodoferax ferrireducens* (99.1, AF435948); Uncultured bacterium KuyT-water-76 (99.4, EU263785)
	Desulfobacteraceae	13	2/226	*Desulfobacula toluolica* (92.5, X70953); Uncultured *Desulfobacterium* sp. (93.6, AB189380)
		24	2/226	*Desulfonema magnum* (93.2, U45989); Uncultured sulfate reducing bacterium BznS327 (94.3, EU047539)
		273	1/226	*Desulfobacterium indolicum* (89.0, AJ237607); Uncultured *Desulfobacteraceae* bacteriumTDNP_Wbc97_93_1_235 (90.9, FJ517135)
	Desulfobulbaceae	203	2/226	*Desulfobulbus propionicus* (88.7, CP002364); Uncultured *Desulfobulbus* sp. (90.0, AB188774)
	Desulfovibrionaceae	218	1/226	*Desulfovibrio idahonensis* (98.7, AJ582755); *Desulfovibrio* sp. (99.8 , AF193026)
	Syntrophaceae	204	1/226	*Desulfobacca acetoxidans* (88.0, CP002629); *Desulfobacca* sp. (92.5, EF613372)
	Rhodocyclaceae	1	6/226	*Sterolibacterium denitrificans*(93.9, NR_025450); Uncultured beta proteobacterium B-AG46 (97.7, AY622253)
		313	1/226	*Propionivibrio limicola* (97.2, AJ307983); Uncultured beta proteobacterium MVP-58 (98.4, DQ676380)
	Clostridiales	29	7/226	*Acidaminobacter hydrogenoformans* (91.9, AF016691); Uncultured bacterium Ebpr14 (97.9, AF255644)
B6 (9 m)	*Geobacteraceae*	6	1/262	*Geobacter psychrophilus* (97.3, AY653548); Uncultured *Geobacter* sp. D12_30 (98.9, EU266834)
		306	3/262	*Geobacter pelophilus* (94.0, NR_026077); Uncultured bacterium oze05B17 (97.5, AB504915)
		359	1/262	*Geobacter chapelleii* (95.0, NR_025982); *Geobacter* sp. CdA-3 (95.9, Y19191)
	Desulfobacteraceae	57	2/262	*Desulfobacterium indolicum* (91.8, AJ237607); *Desulfobacteraceae* bacterium enrichment culture clone MS_HS_U8 (96.4, HQ400842)
		313	2/262	*Desulfobacula toluolica* (98.7, X70953); Uncultured delta proteobacterium 70mos_0d_F12 (97.4, GQ261767)
		405	4/262	*Desulfurivibrio alkaliphilus* (89.6, CP001940); Uncultured bacterium GOUTA17 (98.5, AY050586)
	Syntrophaceae	85	2/262	*Desulfobacca acetoxidans* (89.7, CP002629); *Desulfobacca* sp. (96.1, EF613372)
		411	2/262	*Desulfobacca acetoxidans* (88.9, CP002629); *Desulfobacca* sp. (91.8, EF613372)
	Syntrophobacteraceae	22	1/262	*Desulfacinum infernum* (91.9, L27426); Uncultured bacterium LT-SB-B118 (97.7, FJ755777)
		303	1/262	*Syntrophobacter pfennigii* (94.5, X82875); Uncultured delta proteobacterium RSg13-37 (94.3, AB603820)
	Clostridiales	77	1/262	*Anaerovorax odorimutans* (93.6, AJ251215); Uncultured *Firmicutes* bacterium MVP-25 (97.9, DQ676443)

[a] Based on BLAST analysis using the GenBank database.
[a] Nearest cultured and uncultured relatives with less than 90% sequence identity are not shown.

Table 5. Quantification of 16S rRNA genes from total bacteria and a group of IRB atselected depths of the sampling sites using real-time PCR

Site	Depth	*Bacteria*	*Geobacteraceae*	*Rhodoferax* spp. (219F)	*Rhodoferax* spp. (471F)	*Geothrix* spp.	*Anaeromyxobacter* spp.	*Clostridium* spp.
B1	9 m	2.3×10^{6}	1.2×10^{4}	2.0×10^{3}	-	-	-	-
	18 m	5.2×10^{6}	4.9×10^{4}	1.2×10^{5}	9.6×10^{4}	2.1×10^{2}	1.9×10^{2}	2.1×10^{4}
B3	11 m	1.0×10^{6}	6.5×10^{3}	1.3×10^{3}	-	-	4.4×10^{2}	-
	16 m	1.2×10^{6}	5.0×10^{4}	2.6×10^{5}	2.3×10^{5}	2.9×10^{3}	4.3×10^{1}	-
	19 m	2.8×10^{6}	2.8×10^{4}	1.9×10^{4}	-	1.5×10^{5}	-	-
B6	9 m	3.6×10^{6}	1.9×10^{5}	4.3×10^{3}	4.1×10^{3}	4.1×10^{1}	2.1×10^{3}	-
	14 m	7.3×10^{6}	1.4×10^{3}	5.4×10^{4}	3.4×10^{2}	3.1×10^{3}	9.9×10^{3}	-
	27 m	9.4×10^{6}	1.5×10^{5}	5.2×10^{4}	-	2.5×10^{5}	3.9×10^{2}	-

The B6_3 clone is closely related to *Variovorax* spp. (Scholten and Stams, 2000) and clones found during the anaerobic digestion of sludge (Watanabe *et al.*, 1999). Clone B6_17 shows 98.3% identity with *Achromobacter* sp. AO22, a heavy metal-tolerant strain found within a lead-contaminated industrial site (Ng *et al.*, 2009).

Bacterial clones related to iron reduction

Most of these iron-reducing microorganisms are able to utilize a broad array of electron acceptors (Coates *et al.*, 1999; Finneran *et al.*, 2003; Nevin *et al.*, 2005). Table 4 summarizes potential anaerobic bacterial clones associated with respiration and fermentation processes. Typical IRB were not recovered from the B1 (18 m) iron reduction zone clone library, but a clone related to sulfate-reducing bacteria (SRB) of the genus *Desulfobacca* was found. Interestingly, one of the major clones found in the iron reduction zone at the B1 site was closely related to *Clostridium* spp. (10.9%), which have the ability to reduce iron regardless of their growth (Weber *et al.*, 2006; Lin *et al.*, 2007). In the clone library of the iron reduction zone at the B3 (16 m) site, 24 clones representing 10.6% of the B3 library were closely related to a well-known iron-reducing bacterium, *Rhodoferax ferrireducens*, with 99% 16S rRNA gene identity. In addition to *R. ferrireducens*-like clones, the following SRB-related clones were also detected forthe B3 (16 m) site: *Desulfobacteraceae* (B3_13, B3_24, and B3_273), *Desulfobulbaceae* (B3_203), *Desulfobacca* (B3_204), and *Desulfovibrio* (B3_218). In the clone library of the iron reduction zone at the B6 (9 m) site, four clones (B6_6, B6_306, B6_349, and B6_359) were affiliated with *Geobacter* spp., which are known as typical IRB (Lovley *et al.*, 2004; Nevin *et al.*, 2005). Eight clones in the B6 (9 m) site clone library are associated with the following SRB; *Desulfobulbaceae* (B6_405 and B6_114), *Desulfobacteraceae* (B6_57 and B6_313), *Desulfobacca* (B6_85 and B6_411), and *Desulforhabdus* (B6_22 and B6_303). SRB clones occupy 4.0% and 5.3% of the total clones for the B3 (16 m) and B6 (9 m) site libraries, respectively. Clones belonging to *Rhodocyclaceae* within the *Betaproteobacteria* were detected in the B1 and B3 clone libraries. Clones or isolates related to the family *Rhodocyclaceae* have been reported to be related to Fe(III) reduction (Cummings *et al.*, 1999; Porsch *et al.*, 2009; Hori *et al.*, 2010).

Quantification of typical IRB groups

The 16S rRNA gene-based clone library analysis demonstrated that clones affiliated with *R. ferrireducens* and *Geobacteraceae* were present at the B3 (16 m) and B6 (9 m) site, respectively. We need to be cautious in determining diversity and abundance of typical known IRB groups, because of possible PCR amplification bias during the construction of the clone libraries (Acinas *et al.*, 2005) and the low coverage for clone library analysis. Real-time PCR was used for quantification of IRB in the three sampling sites. The 16S rRNA gene copy numbers of total bacteria and IRB are presented in Table 5. The *Shewanella* spp. were not detected in samples from any of our aquifer sites. Also, *Shewanella* spp. were not detected in the iron reduction zones of other aquifer environments (Snoeyenbos-West *et al.*, 2000; Röling *et al.*, 2001; Stein *et al.*, 2001). Sequences of *Anaeromyxobacter* spp. was detected with low copy number for all three sites (0.002–0.8% of total 16S rRNA genes). In agreement with the clone library analysis, *Rhodoferax* spp. were dominant at the B3 (16 m) site representing 21.3% with primer set1 [Rho219F and Rho830R] and 19.2% with primer set2 [Rho471F and Rho830R]. At the B1 (18 m) site, *Rhodoferax* spp. were also dominant. *Geobacteraceae* were abundant at the B6 (9 m) site representing 6.1% of total bacterial 16S rRNA gene copies, while they occupied 2.3% of total clones in the clone library. *Geobacteraceae* were also abundant at the B6 (27 m) site. *Clostridium* spp. (B1_9) were only detected at the B1 (18 m) site (0.4%), although abundant (10.9%) in the clone library.

Correlation between IRB and hydrogeochemical factors

CCA was conducted to evaluate the correlation between environmental variables and IRB abundance (Fig. 2 and Supplementary data Table S1). The high IRB group-environment variable correlations in axis 1 and axis 2 indicate that IRB abundances were well correlated with the environmental variables. Monte-Carlo significance tests of all axes revealed that the environmental parameters explained a significant amount of within-data variation ($p<0.05$). Axis 1 was correlated closely with sulfate concentration, whereas axis 2 was correlated highly with pH and sulfide concentration. This analysis shows that As and Fe(II) concentrations were also important environmental variables. When the arrows for Fe(II) and As concentrations were extended and perpendiculars were dropped to these axis from six IRB, *Geobacteraceae* could be inferred to have the highest weighted average with respect to Fe(II) and As concentrations [i.e., they largely occur in the Fe(II)- and As-rich samples]. IRB distributions also differ along the following environmental variables: NPOC, pH, sulfate, and sulfide concentrations.

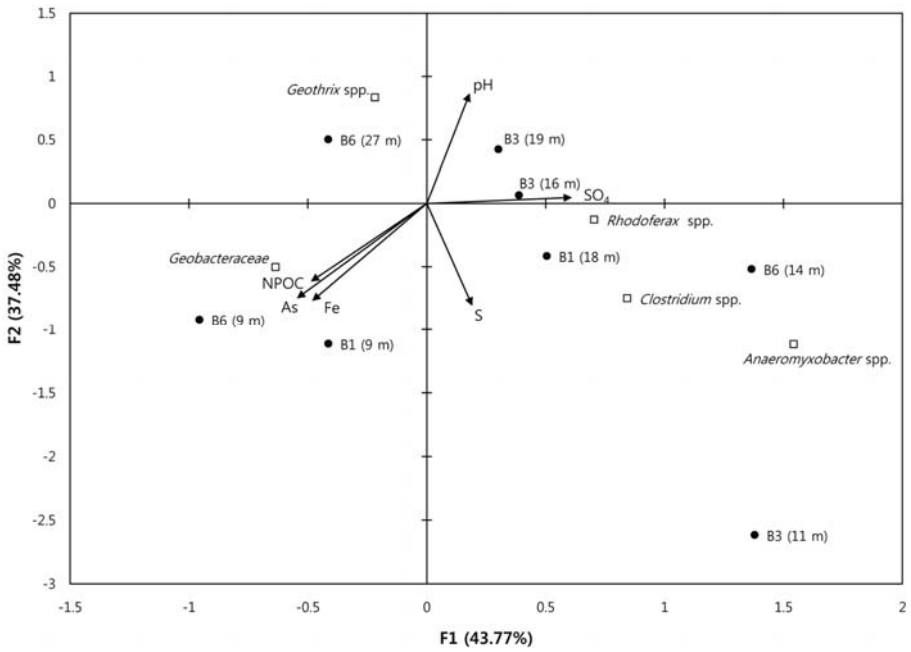

Fig. 2. Canonical correspondence analysis ordination showing IRB based on real-time PCR and environmental variables (arrows). The environmental variables shown are: SO_4, sulfate concentration; Fe, Fe(II) concentration; S, sulfide concentration; As, As concentration; NPOC, and pH. IRB are indicated as open squares. Analyzed sites are indicated as closed circles.

Discussion

Nitrate, iron, manganese, and sulfate reduction are commonly detected in aquifer environments. Iron reduction is assumed to be a dominant anaerobic redox process at most of the depths in the aquifers studied, based on a high Fe(II)/sulfide concentration ratio (see Table 1). The depth profiles shown by the overall bacterial communities (and IRB) varied significantly (Supplementary data Fig. S1 and Fig. 2), although the sampling sites are located fairly close to one another (see Fig. 1). This indicates that subsurface conditions affecting the IRB community might be highly variable in the riverine aquifers of the Mankyeong river area.

The aquifer at the B6 site was covered by layers of silt (ca. 5 m depth), which have generally low water permeability. Thus, layers of silt may limit the penetration of oxygen and agricultural chemicals such as nitrate and sulfate from the ground surface (Kim *et al.*, 2008, 2009) into the groundwater. The combination of a lack of other electron acceptors (nitrate and sulfate), and a relatively high amount of organic materials contained in the silts, may cause a higher iron reduction at the B6 site than at the other study sites. At the B6 (9 m) site, *Geobacter* spp. were identified as possible IRB due to the detection of several *Geobacter* clones (2.3%) (Supplementary data Fig. S1), and this is consistent with the results of quantitative PCR (Table 5). CCA results based on quantitative PCR supported the observation that *Geobacter* spp. were dominant at the B6 (9 m) site and highly correlated with iron reduction (and As release). *Geobacter* spp. have been intensively studied as model IRB for bioremediation in petroleum- or landfill leachate-polluted aquifers (Snoeyenbos-West *et al.*, 2000; Röling *et al.*, 2001; Lin *et al.*, 2005).

The aquifer at the B3 site is covered with water-permeable layers of (silty) sand (Kim *et al.*, 2008, 2009). This geological property could decrease the activity of iron (or sulfate) reduction in the aquifers of the B3 site. The possible role of *R. ferrireducens*-like bacteria as IRB could be suggested only for the B3 (16 m) site, in keeping with the dominance of clones associated with *R. ferrireducens* (Table 4) and the qPCR results (Table 5). However, the overall Fe(II) concentration at the B3 (16 m) site was relatively lower than those of the B1 and B6 sites, suggesting low iron reduction activity at the B3 site. Furthermore, it was difficult to accurately determine the specific depth with the highest Fe(II) concentration at the B3 site (Table 1). For this reason, *R. ferrireducens*-like bacteria might not be actively involved in iron reduction but flourish using other electron accepting processes, possibly by denitrification. The following evidence supports this suggestion: 1) *R. ferrireducens* is capable of reducing oxygen, soluble Fe(III), nitrate, and Mn(IV) oxide, but not insoluble iron (Finneran *et al.*, 2003); 2) high concentrations of nitrate were observed in the upper layers of the B3 site, which were reduced in the deeper parts of the aquifer (Table 1), as observed previously (Choi *et al.*, 2007); 3) CCA indicated that the distribution of *R. ferrireducens* was not well correlated with Fe(II) concentration but rather was correlated with sulfate concentration.

It is intriguing that 10.9% of clones in the B1 (18 m) site clone library were affiliated with *Clostridium* spp. which have often been observed at sites with iron-reducing environments (Lin *et al.*, 2005; Porsch *et al.*, 2009). *Clostridium* spp. are capable of using Fe(III) as an electron sink without coupling with growth (Lin *et al.*, 2005; Weber *et al.*, 2006). In contrast, quantitative analysis showed that the count of *Clostridium* spp. (0.4%) related to *Clostridium* sp. (B1_9) was much lower than that found by clone library analysis. Although *R. ferrireducens* was found to be abundant in the B1 (18 m) DNA sample by real time PCR analysis, it was not well correlated with Fe(II) concentration (see Fig. 2). Thus, possible IRB candidates could not be unambiguously determined at the B1 (18 m) site. Phylogenetic studies of

IRB have shown a widespread occurrence of iron reduction capability in prokaryotes (Weber *et al.*, 2006). Many clones belonging to SRB, fermenting bacteria, and *Rhodocyclaceae*, which could be related to Fe(III) reduction were detected in our clone libraries. For example, many SRB can reduce Fe(III), but most of them cannot conserve energy to support their growth, whereas some SRB such as *Desulfovibrio*, *Desulfobulbus*, and *Desulfomonas* spp. are believed to be relevant to the oxidation of hydrogen and organic matter coupled with Fe(III) reduction (Coleman *et al.*, 1993; Lovley *et al.*, 1993). Furthermore, since the majority of our clone sequences were only distantly related to their cultivated relatives, the possibility that some of these novel bacterial lineages may be involved in iron reduction, especially at the B1 site, cannot be dismissed. In this study, domain *Archaea*, in which methanogens are also suggested to be iron-reducing microorganisms, were not included in the analysis (Bond and Lovley, 2002).

Arsenic concentration was highest at the B6 site and appeared well correlated with Fe(II) concentration. Groundwater was not available above the depth of the highest Fe(II) concentration (9 m) at the B6 site. High organic materials contained in the silts above the B6 site may be supplied as electron donors for active iron reduction (Harvey *et al.*, 2002; McArthur *et al.*, 2004; Papacostas *et al.*, 2008; Quicksall *et al.*, 2008) and could be one of the main reasons for the high As concentration. The hydrogeochemical properties of the B6 site are characterized by high Fe(II), lower E_h and sulfate, which are similar with those of As-containing ground waters in West Bengal and Bangladesh (Polya *et al.*, 2003). Fe(II) and As concentrations were also observed at the B1 site, but their concentrations were lower than those at the B6 site as observed previously at this location (Kim *et al.*, 2009). This result supports the potential correlation between Fe(II) concentration (i.e., activity of IRB) and As contamination in this area (Kim *et al.*, 2009), as previously observed in other countries (Islam *et al.*, 2004). In the case of the B3 site, the origin of As is not clear from this study. Since the Fe(II) level is low at all depths, and most of the As is detected in the upper layers, the As might not be related to iron reduction activity at the B3 site.

From the molecular analysis of IRB, potential IRB in the riverine aquifers of the Mankyeong River could be proposed. Further correlation analysis for IRB distribution and environmental variables indicated that distribution of potential IRB was highly variable, which might reflect environmental heterogeneity in the aquifers. Further investigations on the activity-based identification of potential IRB need to be conducted for better understanding of the process of iron reduction in the riverine aquifers of the Mankyeong River.

Acknowledgements

This work (2010-0014384) was supported by Mid-career Researcher Program through an NRF (National Research Foundation) grant funded by MEST (Ministry of Education, Science and Technology).

References

Abulencia, C.B., Wyborski, D.L., Garcia, J.A., Podar, M., Chen, W., Chang, S.H., Chang, H.W., Watson, D., Brodie, E.L., Hazen, T.C., and *et al.* 2006. Environmental whole-genome amplification to access microbial populations in contaminated sediments. *Appl. Environ. Microbiol.* **72**, 3291–3301.

Acinas, S.G., Sarma-Rupavtarm, R., Klepac-Ceraj, V., and Polz, M.F. 2005. PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Appl. Environ. Microbiol.* **71**, 8966–8969.

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., and Weightman, A.J. 2006. New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl. Environ. Microbiol.* **72**, 5734–5741.

Baker, G.C., Smith, J.J., and Cowan, D.A. 2003. Review and reanalysis of domain-specific 16S primers. *J. Microbiol. Methods* **55**, 541–555.

Bond, D.R. and Lovley, D.R. 2002. Reduction of Fe(III) oxide by methanogens in the presence and absence of extracellular quinones. *Environ. Microbiol.* **4**, 115–124.

Bourg, A.C.M. and Bertin, C. 1993. Biogeochemical processes during the infiltration of river water into an alluvial aquifer. *Environ. Sci. Technol.* **27**, 661–666.

Brown, C.J., Walter, D.A., and Colabufo, S. 1999. Iron in the aquifer system of suffolk county, New York, p. 10. USGS, New York, N.Y., USA.

Chao, A. and Shen, T.J. 2005. Program SPADE (Species Prediction and Diversity Estimation). v2.1. Program and User's Guide published at http://chao.stat.nthu.edu.tw.

Chapelle, F.H., Bradley, P.M., Thomas, M.A., and McMahon, P.B. 2009. Distinguishing iron-reducing from sulfate-reducing conditions. *Ground Water* **47**, 300–305.

Childers, S.E., Ciufo, S., and Lovley, D.R. 2002. *Geobacter metallireducens* accesses insoluble Fe(III) oxide by chemotaxis. *Nature* **416**, 767–769.

Choi, B.Y., Kim, H.J., Kim, K., Kim, S.H., Jeong, H.J., Park, E., and Yun, S.T. 2008. Evaluation of the processes affecting vertical water chemistry in an alluvial aquifer of Mankyeong Watershed, Korea, using multivariate statistical analyses. *Environ. Geol.* **54**, 335–345.

Choi, B.K., Koh, D.C., Ha, K., and Cheon, S.H. 2007. Effect of redox processes and solubility equilibria on the behavior of dissolved iron and manganese in groundwater from a riverine alluvial aquifer. *Econ. Environ. Geol.* **40**, 29–45.

Coates, J.D., Ellis, D.J., Gaw, C.V., and Lovley, D.R. 1999. *Geothrix fermentans* gen. nov., sp. nov., a novel Fe(III)-reducing bacterium from a hydrocarbon-contaminated aquifer. *Int. J. Syst. Bacteriol.* **49**, 1615–1622.

Coleman, M.L., Hedrick, D.B., Lovley, D.R., White, D.C., and Pye, K. 1993. Reduction of Fe(III) in sediments by sulphate-reducing bacteria. *Nature* **361**, 436–438.

Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H., and Farrow, J.A. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int. J. Syst. Bacteriol.* **44**, 812–826.

Cummings, D.E., Caccavo, F.Jr., Spring, S., and Rosenzweig, R.F. 1999. *Ferribacterium limneticum*, gen. nov., sp. nov., an Fe(III)-reducing microorganism isolated from mining-impacted freshwater lake sediments. *Arch. Microbiol.* **171**, 183–188.

Cummings, D.E., Snoeyenbos-West, O.L., Newby, D.T., Niggemyer, A.M., Lovley, D.R., Achenbach, L.A., and Rosenzweig, R.F. 2003. Diversity of *Geobacteraceae* species inhabiting metal-polluted freshwater lake sediments ascertained by 16S rDNA analyses. *Microb. Ecol.* **46**, 257–269.

Felsenstein, J. 1985. Confidence limit on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Finneran, K.T., Johnsen, C.V., and Lovley, D.R. 2003. *Rhodoferax ferrireducens* sp. nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). *Int. J. Syst. Evol. Microbiol.* **53**, 669–673.

Good, I.J. 1953. The population frequencies of species and the estimation of population parameters. *Biometrika* **40**, 237–264.

Harvey, C.F., Swartz, C.H., Badruzzaman, A.B., Keon-Blute, N., Yu, W., Ali, M.A., Jay, J., Beckie, R., Niedan, V., Brabander, D., and *et al.* 2002. Arsenic mobility and groundwater extraction in Bangladesh. *Science* **298**, 1602–1606.

He, J., Zhang, L., Jin, S., Zhu, Y., and Liu, F. 2008. Bacterial communities inside and surrounding soil iron-manganese nodules. *Geomicrobiol. J.* **25**, 14–24.

Hiscock, K.M. and Grischek, T. 2002. Attenuation of groundwater pollution by bank filtration. *J. Hydrol.* **266**, 139–144.

Hori, T., Muller, A., Igarashi, Y., Conrad, R., and Friedrich, M.W. 2010. Identification of iron-reducing microorganisms in anoxic rice paddy soil by ^{13}C-acetate probing. *ISME J.* **4**, 267–278.

Islam, F.S., Gault, A.G., Boothman, C., Polya, D.A., Charnock, J.M., Chatterjee, D., and Lloyd, J.R. 2004. Role of metal-reducing bacteria in arsenic release from Bengal delta sediments. *Nature* **430**, 68–71.

Jongman, R.H., Braak, C.J.F.t., and Van Tongeren, O.F.R. 1995. Data analysis in community and landscape ecology, p. xxi, 299, Cambridge University Press, Cambridge, UK.

Kelly, W.R. 1997. Heterogeneities in ground-water geochemistry in a sand aquifer beneath an irrigated field. *J. Hydrol.* **198**, 154–176.

Kim, K., Kim, H.J., Choi, B.Y., Kim, S.H., Park, K., Park, E., Koh, D.C., and Yun, S.T. 2008. Fe and Mn levels regulated by agricultural activities in alluvial groundwaters underneath a flooded paddy field. *Appl. Geochem.* **23**, 44–57.

Kim, K., Moon, J.T., Kim, S.H., and Ko, K.S. 2009. Importance of surface geologic condition in regulating as concentration of groundwater in the alluvial plain. *Chemosphere* **77**, 478–484.

Kimura, M. 1983. The neutral theory of molecular evolution. Cambridge University Press, Cambridge, UK.

Kumar, S., Tamura, K., and Nei, M. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform.* **5**, 150–163.

Lin, B., Braster, M., Röling, W.F.M., and van Breukelen, B.M. 2007. Iron-reducing microorganisms in a landfill leachate-polluted aquifer: complementing culture-independent information with enrichments and isolations. *Geomicrobiol. J.* **24**, 283–294.

Lin, B., Braster, M., van Breukelen, B.M., van Verseveld, H.W., Westerhoff, H.V., and Roling, W.F. 2005. *Geobacteraceae* community composition is related to hydrochemistry and biodegradation in an iron-reducing aquifer polluted by a neighboring landfill. *Appl. Environ. Microbiol.* **71**, 5983–5991.

Lindsay, S.S. and Baedecker, M.J. 1988. Determination of aqueous sulfide in contaminated and natural water using the methylene blue method, pp. 349–356. American Society for Testing and Materials, Philadelphia, USA.

Lovley, D.R. 1995. Bioremediation of organic and metal contaminants with dissimilatory metal reduction. *J. Ind. Microbiol.* **14**, 85–93.

Lovley, D.R., Holmes, D.E., and Nevin, K.P. 2004. Dissimilatory Fe(III) and Mn(IV) reduction. *Adv. Microb. Physiol.* **49**, 219–286.

Lovley, D.R., Roden, E.E., Philips, E.J.P., and Woodward, J.C. 1993. Enzymatic iron and uranium reduction by sulfate-reducing bacteria. *Marine Geobiolgy* **113**, 41–53.

Massmann, G., Pekdeger, A., and Merz, C. 2004. Redox processes in the Oderbruch polder groundwater flow system in Germany. *Appl. Geochem.* **19**, 863–886.

McArthur, J.M., Banerjee, D.M., Hudson-Edwards, K.A., Mishra, R., Purohit, R., Ravenscroft, P., Cronin, A., Howarth, R.J., Chatterjee, A., Talukder, T., and *et al.* 2004. Natural organic matter in sedimentary basins and its relation to arsenic in anoxic ground water: the example of West Bengal and its worldwide implications. *Appl. Geochem.* **19**, 1255–1293.

McMahon, P.B. and Chapelle, F.H. 2008. Redox processes and water quality of selected principal aquifer systems. *Ground Water* **46**, 259–271.

Min, J.H., Yun, S.T., Kim, K., Kim, H.S., and Kim, D.J. 2003. Geologic controls on the chemical behaviour of nitrate in riverside alluvial aquifer, Korea. *Hydrolog. Proc.* **17**, 1197–1211.

Muyzer, G., de Waal, E.C., and Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695–700.

Nevin, K.P., Holmes, D.E., Woodard, T.L., Hinlein, E.S., Ostendorf, D.W., and Lovley, D.R. 2005. *Geobacter bemidjiensis* sp. nov. and *Geobacter psychrophilus* sp. nov., two novel Fe(III)-reducing subsurface isolates. *Int. J. Syst. Evol. Microbiol.* **55**, 1667–1674.

Nevin, K.P. and Lovley, D.R. 2000. Lack of production of electron-shuttling compounds or solubilization of Fe(III) during reduction of insoluble Fe(III) oxide by *Geobacter metallireducens*. *Appl. Environ. Microbiol.* **66**, 2248–2251.

Newman, D.K. and Kolter, R. 2000. A role for excreted quinones in extracellular electron transfer. *Nature* **405**, 94–97.

Ng, S.P., Davis, B., Palombo, E.A., and Bhave, M. 2009. A Tn5051-like mer-containing transposon identified in a heavy metal tolerant strain *Achromobacter* sp. AO22. *BMC Res. Notes* **2**, 38.

Nickson, R.T., McArthur, J.M., Ravenscroft, P., Burgess, W.G., and Ahmed, K.M. 2000. Mechanism of arsenic release to groundwater, Bangladesh and West Bengal. *Appl. Geochem.* **15**, 403–413.

Norris, R.D. and Matthews, J.E. 1994. Handbook of bioremediation, p. xiii, p. 257 Lewis Publishers, Boca Raton, USA.

Papacostas, N.P., Bostick, B.C., Quicksall, A.N., Landis, J.D., and Sampson, M. 2008. Geomorphological controls on groundwater arsenic distribution in the Mekong River Delta, Cambodia. *Geology* **36**, 891–894.

Park, S.J., Park, B.J., and Rhee, S.K. 2008. Comparative analysis of archaeal 16S rRNA and *amoA* genes to estimate the abundance and diversity of ammonia-oxidizing archaea in marine sediments. *Extremophiles* **12**, 605–615.

Petrie, L., North, N.N., Dollhopf, S.L., Balkwill, D.L., and Kostka, J.E. 2003. Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium (VI). *Appl. Environ. Microbiol.* **69**, 7467–7479.

Polya, D.A., Gault, A.G., Bourne, N.J., Lythgoe, P.R., and Cooke, D.A. 2003. Coupled HPLC-ICP-MS analysis indicates highly hazardous concentrations of dissolved arsenic species in Cambodian groundwaters. Plasma Source Mass Spectrometry: Applications and Emerging Technologies. The Royal Society of Chemistry, Cambridge, UK.

Porsch, K., Meier, J., Kleinsteuber, S., and Wendt-Potthoff, K. 2009. Importance of different physiological groups of iron reducing microorganisms in an acidic mining lake remediation experiment. *Microb. Ecol.* **57**, 701–717.

Quicksall, A.N., Bostick, B.C., and Sampson, M. 2008. Linking organic matter deposition and iron mineral transformations to groundwater arsenic levels in the Mekong delta, Cambodia. *Appl. Geochem.* **23**, 3088–3098.

Röling, W.F.M., van Breukelen, B.M., Braster, M., Lin, B., and van Verseveld, H.W. 2001. Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer. *Appl. Environ. Microbiol.* **67**, 4619–4629.

Scholten, J.C. and Stams, A.J. 2000. Isolation and characterization of acetate-utilizing anaerobes from a freshwater sediment. *Microb. Ecol.* **40**, 292–299.

Snoeyenbos-West, O.L., Nevin, K.P., Anderson, R.T., and Lovley, D.R. 2000. Enrichment of *Geobacter* species in response to stimulation of Fe(III) reduction in sandy aquifer sediments. *Microb. Ecol.* **39**, 153–167.

Stein, L.Y., La Duc, M.T., Grundl, T.J., and Nealson, K.H. 2001. Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. *Environ. Microbiol.* **3**, 10–18.

Sung, Y., Ritalahti, K.M., Apkarian, R.P., and Loffler, F.E. 2006. Quantitative PCR confirms purity of strain GT, a novel trichloroethene-to-ethene-respiring *Dehalococcoides* isolate. *Appl. Environ. Microbiol.* **72**, 1980–1987.

Ter Braak, C.J.F. 1987. The analysis of vegetation-environment relationships by canonical correspondence analysis. *Vegetatio* **69**, 69–77.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882.

Tufenkji, N., Ryan, J.N., and Elimelech, M. 2002. The promise of bank filtration. *Environ. Sci. Technol.* **36**, 422A–428A.

Turick, C.E., Tisa, L.S., and Caccavo, F. 2002. Melanin production and use as a soluble electron shuttle for Fe(III) oxide reduction and as a terminal electron acceptor by *Shewanella algae* BrY. *Appl. Environ. Microbiol.* **68**, 2436–2444.

Watanabe, K., Teramoto, M., and Harayama, S. 1999. An outbreak of nonflocculating catabolic populations caused the breakdown of a phenol-digesting activated-sludge process. *Appl. Environ. Microbiol.* **65**, 2813–2819.

Weber, K.A., Achenbach, L.A., and Coates, J.D. 2006. Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nat. Rev. Microbiol.* **4**, 752–764.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol* **173**, 697–703.