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# Bioreduction of vanadium (V) in groundwater by autohydrogentrophic bacteria: Mechanisms and microorganisms

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## ABSTRACT

As one of the transition metals, vanadium (V) (V(V)) in trace amounts represents an essential element for normal cell growth, but becomes toxic when its concentration is above 1 mg/L. V(V) can alter cellular differentiation, gene expression, and other biochemical and metabolic phenomena. A feasible method to detoxify V(V) is to reduce it to V(IV), which precipitates and can be readily removed from the water. The bioreduction of V(V) in a contaminated groundwater was investigated using autohydrogentrophic bacteria and hydrogen gas as the electron donor. Compared with the previous organic donors, H<sub>2</sub> shows the advantages as an ideal electron donor, including nontoxicity and less production of excess biomass. V(V) was 95.5% removed by biochemical reduction when autohydrogentrophic bacteria and hydrogen were both present, and the reduced V(IV) precipitated, leading to total-V removal. Reduction kinetics could be described by a first-order model and were sensitive to pH and temperature, with the optimum ranges of pH 7.5–8.0 and 35–40°C, respectively. Phylogenetic analysis by clone library showed that the dominant species in the experiments with V(V) bioreduction belonged to the  $\beta$ -Proteobacteria. Previously known V(V)-reducing species were absent, suggesting that V(V) reduction was carried out by novel species. Their selective enrichment during V(V) bioreduction suggests that *Rhodocyclus*, a denitrifying bacterium, and *Clostridium*, a fermenter known to carry out metal reduction, were responsible for V(V) bioreduction.

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## Introduction

As one of the transition metals, vanadium (V) in trace amounts represents an essential element for normal cell growth, but becomes toxic when its concentration is above 1 mg/L (Patel

et al., 1990). The toxic effects of V are based on the structural similarity between vanadate and phosphate (Rehder, 2003). As a phosphorus analog, V inhibits the activity of phosphate-metabolizing enzymes (Stankiewicz et al., 1995). Vanadate has mutagenic, genotoxic, and cytotoxic effects,

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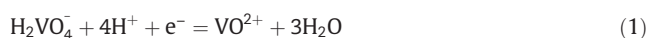


altering cellular differentiation, gene expression, and other biochemical and metabolic phenomena (Altamirano-Lozano, 1998).

In China, the V concentration in drinking water is limited to below 0.05 mg/L (Standard of China GB 5749-2006). However, as the main source of drinking water in many regions, the V concentration in groundwater can rise to high levels once V pollution occurs (Giammanco et al., 1996). Sources of V in groundwater are dissolution of V-rich rocks and waste streams from industrial processes (Wright and Belitz, 2010). Pentavalent V and tetravalent V are relatively stable in aqueous solution at neutral pH in the forms of  $\text{H}_2\text{VO}_4^-$  and  $\text{VO}_2^{2+}$ , respectively (Crans et al., 2004). V(IV) is less toxic (Patel et al., 1990) and much more readily precipitated (Ortiz-Bernad et al., 2004) than V(V). Ortiz-Bernad et al. (2004), using electron microprobe analysis, found that a V(IV) precipitate was mainly composed of V and P, suggesting that it was a vanadyl phosphate, such as the green mineral sincosite ( $\text{CaV}_2(\text{PO}_4)_2(\text{OH})_4 \cdot 3\text{H}_2\text{O}$ ). Thus, a feasible method to detoxify V(V) is to reduce it to V(IV), which precipitates and can be readily removed from the water.

According to Antipov et al. (2000), *Pseudomonas isachenkovii* has two kinds of nitrate reductases that can participate in V(V) reduction. Other bacteria found to reduce V(V) to V(IV) are *Shewanella oneidensis* (Carpentier et al., 2003), *Geobacter metallireducens* (Ortiz-Bernad et al., 2004), *Acidithiobacillus ferrooxidans* and *thiooxidans* (Bredberg et al., 2004), and *Rhodoferrax ferrireducens* in a microbial fuel cell (Li et al., 2009). One eukaryotic strain, *Saccharomyces cerevisiae*, was capable of reducing V(V) to vanadyl (Bisconti et al., 1997). A mesophilic (*Methanosarcina mazei*) methanogen and a thermophilic (*Methanothermobacter thermautotrophicus*) methanogen, belonging to archaeal, were reported as vanadium-reducing microbes (Zhang et al., 2014). All the reported microbial reductions of V(V) are in common that the bacteria are anaerobic, chemotrophic heterotrophs.

Autohydrogentrophic bacteria use hydrogen gas ( $\text{H}_2$ ) as their electron donor. Compared with the organic donors,  $\text{H}_2$  has advantages as an electron donor, including nontoxicity to environments, less production of excess biomass, and usually lower cost per electron equivalent (Rittmann et al., 2004). In addition, many oxidized contaminants can be reduced to less toxic or less mobile form by autohydrogentrophic microorganisms. For instance, nitrate can be reduced to  $\text{N}_2$  (Lee and Rittmann, 2002; Xia et al., 2013); perchlorate ( $\text{ClO}_4^-$ ) can be reduced to less toxic  $\text{Cl}^-$  and  $\text{H}_2\text{O}$  (Nerenberg and Rittmann, 2004); selenium can be reduced from selenate ( $\text{SeO}_4^{2-}$ ) to less mobile selenite ( $\text{Se}^{2-}$ ) or elemental selenium ( $\text{Se}^0$ ) (Chung et al., 2006); and chromium can be reduced from hexavalent chromate ( $\text{CrO}_4^{2-}$ ) to less toxic  $\text{Cr}^{3+}$ , which precipitates as  $\text{Cr}(\text{OH})_3$  and is removed from the water. Due to its chemical similarity, vanadate ( $\text{H}_2\text{VO}_4^-$ ) should be reduced similarly to chromate ( $\text{CrO}_4^{2-}$ ) (Gubanov et al., 1975). The stoichiometry of vanadate ( $\text{H}_2\text{VO}_4^-$ ) reduction with hydrogen as the electron donor is (Li et al., 2009; Nerenberg and Rittmann, 2004):



Here, we explored the bioreduction of V(V) by autohydrogentrophic bacteria. We investigated the effects of some key environmental factors – pH and temperature – on bioreduction, and we also assessed the microbial community structure by clone library and identified species potentially reducing V(V).

## 1. Materials and methods

### 1.1. Experimental setup

Serum bottles (250-mL total volume) were selected as reactors because they can be made gas-tight. The liquid volume of each bottle was 200 mL, and we added 180 mL feed medium and 20 mL inoculation sludge by sterilized syringes. Air in the top of the reactor was expelled out then  $\text{H}_2$  was injected via a syringe needle. The bottles were sealed with a rubber plug after  $\text{H}_2$  injection. Each bottle was covered with aluminum foil and incubated on a shaking table operated at 150 r/min. Liquid samples were taken by sterilized syringes. Each time after sampling, hydrogen gas was replenished to ensure that the bacteria had sufficient electron donor.

### 1.2. Medium

The composition of the culture medium was (mg/L):  $\text{KH}_2\text{PO}_4$  292,  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  663,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  128,  $\text{NaNO}_3$  60,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  1,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  1,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.013,  $\text{H}_3\text{BO}_3$  0.038,  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  0.001,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  0.004,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  0.004,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.025, and  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  0.001 (Chung et al., 2006). Phosphate buffer ( $\text{KH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$ ) was used to control the pH, since denitrification, sulfate reduction, and vanadate reduction add base to the aqueous phase (Crans et al., 2010; Lee and Rittmann, 2003). All media were purged with  $\text{N}_2$  gas to eliminate dissolved  $\text{O}_2$ .

### 1.3. Inoculum and V(V)-bioreduction experiments

The inoculum was obtained from the anoxic pond at Quyang Wastewater Treatment Plant (Shanghai, China). The main characteristics (average  $\pm$  standard deviations of triplicates) were: pH  $6.5 \pm 0.3$ , total solids (TS)  $2.84 \pm 0.21$  g/L, volatile solids (VS)  $2.23 \pm 0.23$  g/L, total suspended solids (TSS)  $2.68 \pm 0.18$  g/L, and volatile suspended solids (VSS)  $2.13 \pm 0.32$  g/L. Inoculated sludge was firstly enriched with medium containing nitrate (10 mg  $\text{NO}_3^-$ -N/L) and sulfate (50 mg  $\text{SO}_4^{2-}$ /L). The nitrate and sulfate were completely removed within 3 days. Then the mixed gas was exhausted and the reactor was replenished with pure hydrogen. V(V) was added as the only acceptor. To ensure that all reactions were biologically mediated, two controls (without inoculum and without  $\text{H}_2$ ) were carried out in parallel: without inoculum and without  $\text{H}_2$ . Each parallel was carried out starting with V(V) of 2 mg/L, pH of 7.5, and a temperature of 35°C. Matrix experiments were conducted to study the effects of pH and temperature. To evaluate the effect of pH, the starting pH values were set to 6.0, 6.5, 7.0, 7.5, or 8.0, with starting V(V) concentration and temperature at 2 mg/L and 35°C, respectively. To study the

impact of temperature, the experiments were carried out at 15, 20, 25, 30, 35, and 40°C, with the initial V(V) concentration and pH value at 2 mg/L and 7.5, respectively.

#### 1.4. Sampling and analyses

For daily sampling, 1 mL suspension was taken to meet the minimum analysis requirements. All the liquid samples were filtered with a 0.22- $\mu$ m polyether sulfone membrane filter (Anpel Company, Shanghai, China) and kept in the refrigerator at 4°C. V(V) is soluble in basic and acidic solutions, while V(IV) is soluble in acid solutions and not oxidized to V(V) below pH 2.4 (Carpentier et al., 2003). Therefore, to preserve the V(V) and V(IV) concentrations, we adjusted the pH of samples for V analysis to below 2 with 5-N nitric acid. Samples for other analyses did not receive nitric acid. Analyses of  $\text{NO}_3^-$ -N,  $\text{NO}_2^-$ -N, and  $\text{SO}_4^{2-}$  were carried out by ion chromatography (ICS-1000, Dionex, USA) using an AS-20 column, an AG-20 pre-column, and a 150-mg/L injection loop (Xia et al., 2011). The different valence states of V were separated by a liquid chromatograph (LC) equipped with a CRC8 reversed-phase column (Agilent, 3  $\mu$ m diam. particles, 3 mm i.d.  $\times$  150 mm length) and determined by ICP-MS (Agilent Technologies 7700 Series). The starting and ending pH values were measured with a PHS-29A meter (HACH, USA).

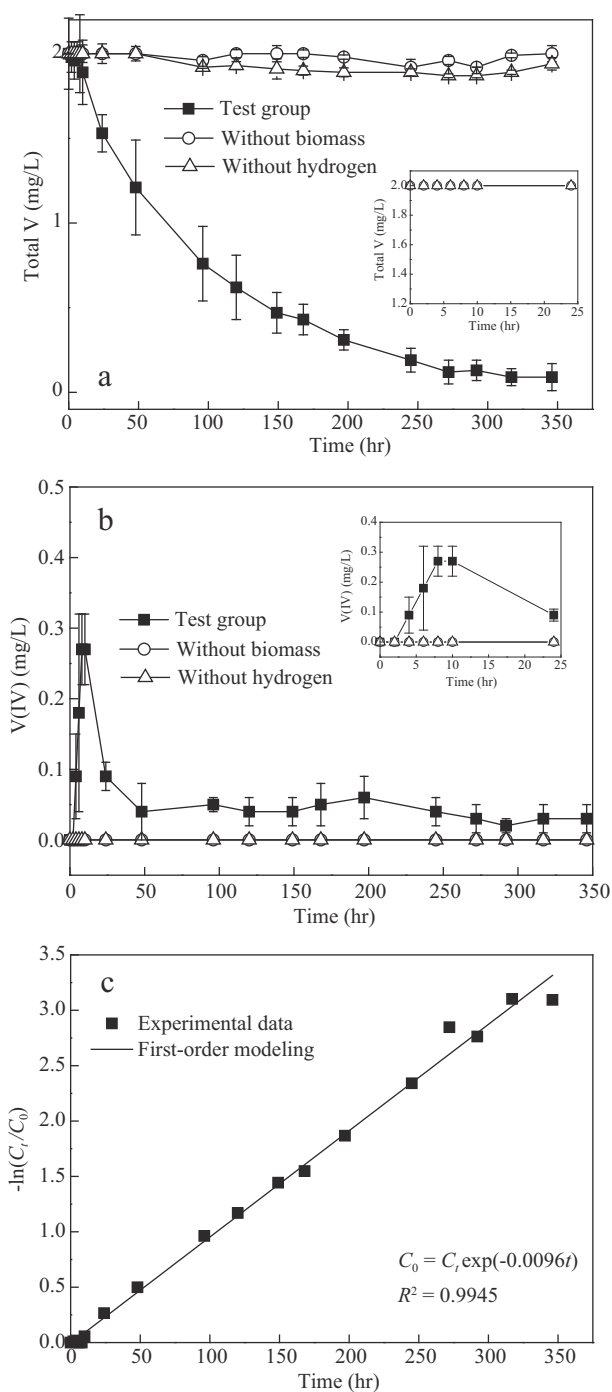
#### 1.5. 16S rRNA clone libraries

Three 16S rRNA clone libraries were constructed to identify potentially important species for V(V)-bioreduction: the inoculum sludge (inoculum), sludge enriched with nitrate and sulfate (NS-bioreduction) and the V(V)-selected biomass (V(V)-bioreduction) on day 12, when V(V) reduction was almost finished. For each sample, we removed 10 mL of suspension with a sterilized syringe, centrifuged the samples for 20 min at 12,000 g, decanted the supernatant, resuspended the pellet in PBS buffer ( $\text{Na}_2\text{HPO}_4$ ;  $\text{KH}_2\text{PO}_4$ ; NaCl; KCl), and immediately froze and stored the cells at  $-70^\circ\text{C}$  (Xia et al., 2010). For DNA extractions, the samples were thawed and put into a Fast DNA Spin Kit (MP Biomedical, LLC, France) following the manufacturer's protocol. We amplified the extracted DNA with the bacterial universal primers 27f [5'-AGAGTTTGATCCTGGCTCAG-3'] and 1492r [5'-GGTTACCTTGTTACGACTT-3'] and purified it with a QIAquick PCR purification kit (QIAGEN) (Duan et al., 2009). For 16S rDNA gene cloning, we inserted the purified PCR amplicons into a cloning vector. The individual PCR amplicons in each vector were cloned via the growth of the host cells on an ampicillin-supplemented LB medium. When the vectors containing PCR products were isolated, we randomly selected for sequencing 120 for inoculum and NS-bioreduction and 150 for V(V)-bioreduction (BGI, Shanghai, China); 109, 100 and 132 clones gave successful results, respectively. We constructed a phylogenetic tree using the neighbor-joining algorithm in MEGA5 software (Tamura et al., 2011). The 16S rRNA gene sequences from this study have been deposited in the National Institutes of Health genetic sequence database (GenBank) under accession numbers GU257488 to GU257893.

## 2. Results and discussion

### 2.1. Vanadium (V) bioreduction

Fig. 1 summarizes the results of V(V)-reduction experiments, as well as the controls. In the abiotic control (no inoculum), the concentration of V(V) did not change. In the control



**Fig. 1 – Concentrations of (a) total soluble V and (b) V(IV) with starting V(V) of 2 mg V/L, pH of 7.5, and a temperature of 35°C; (c) first-order fit of the V(V) removal results. The  $k_1$  value is  $0.0096 \text{ hr}^{-1}$ .**

without  $H_2$  ( $N_2$  replacing  $H_2$ ), the removal percentage of total soluble vanadium was only 3% and the V(IV) concentration was almost zero. When  $H_2$  and inoculum were both present, V(V)-reduction occurred *versus* time. As shown in Fig. 1a, the concentration of total soluble V decreased from 2 to 1.89 mg/L by 10 hr after V(V) addition, and the residual vanadium was 1.62 mg/L V(V) and 0.27 mg/L V(IV). The concentration of total soluble vanadium continued to decline, but detected V(IV) remained at a low concentration (Fig. 1b), leading to the removal of V(V). By day 12, the average removal of total V stabilized at 95.5%.

The results were fit with a first-order model, as shown in Fig. 1c. The linearized form of first order equation is:

$$\ln (C_t/C_0) = -k_1 t \quad (3)$$

where,  $C_0$  (mg/L) and  $C_t$  (mg/L) are the concentrations at the initial condition and at time  $t$  (hr), respectively, and  $k_1$  is the first-order rate coefficient. The estimated  $k_1$  was  $0.0096 \text{ hr}^{-1}$ .

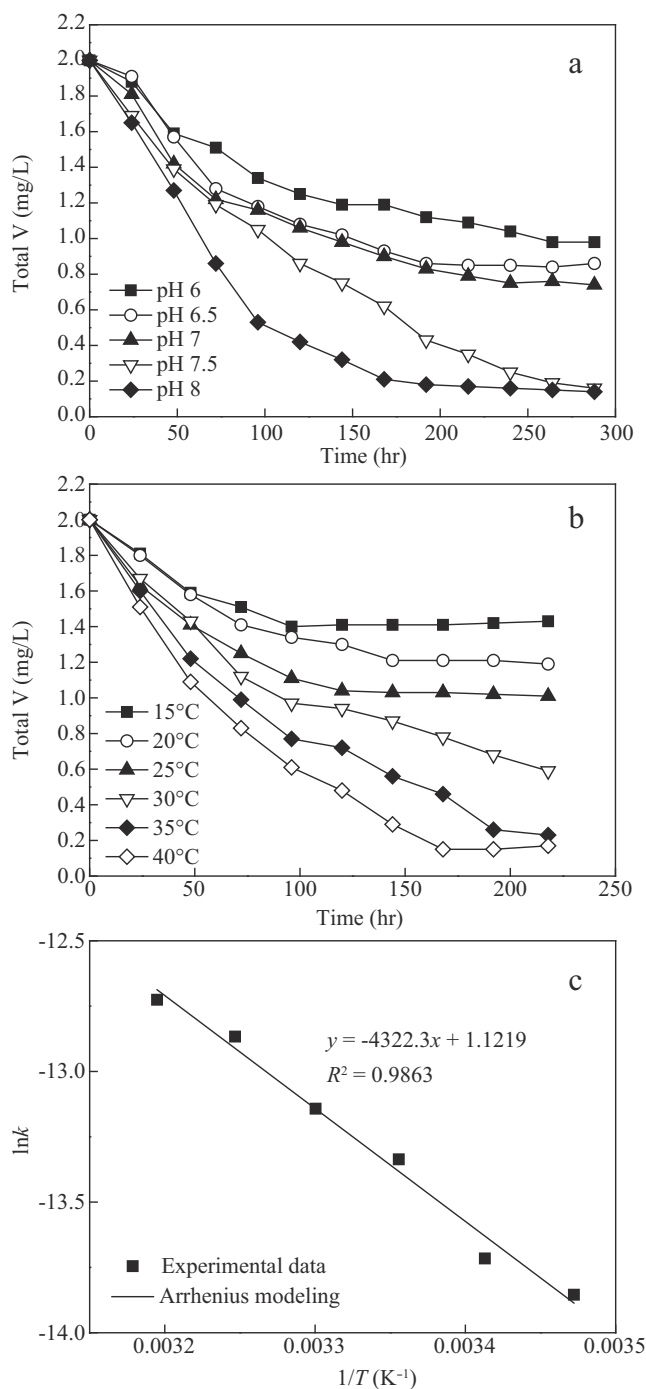
The results of V(V)-reduction experiments, as well as the controls demonstrate that V(V)-reduction by autohydrogentrophic bacteria is a bioreduction process. The approximately stable V(V) concentration in the abiotic control (no inoculum) and the control without  $H_2$  ( $N_2$  replaced  $H_2$ ) indicate that chemical reduction of V(V) by  $H_2$  was infeasible and that endogenous respiration and adsorption to biomass caused minimal reduction of V(V), respectively. The undetected V(IV) in the no- $H_2$  control also suggests that V(V) was not reduced without an added electron donor. Bioreduction of V(V) occurred when  $H_2$  and autohydrogentrophic inoculum were present simultaneously. The detected V(IV) clearly indicated V(V) reduction to V(IV). Then, the detected V(IV) decreased and remained at a low concentration, suggesting that V(IV) was precipitated and removed by filtration.

## 2.2. Effect of pH value and temperature

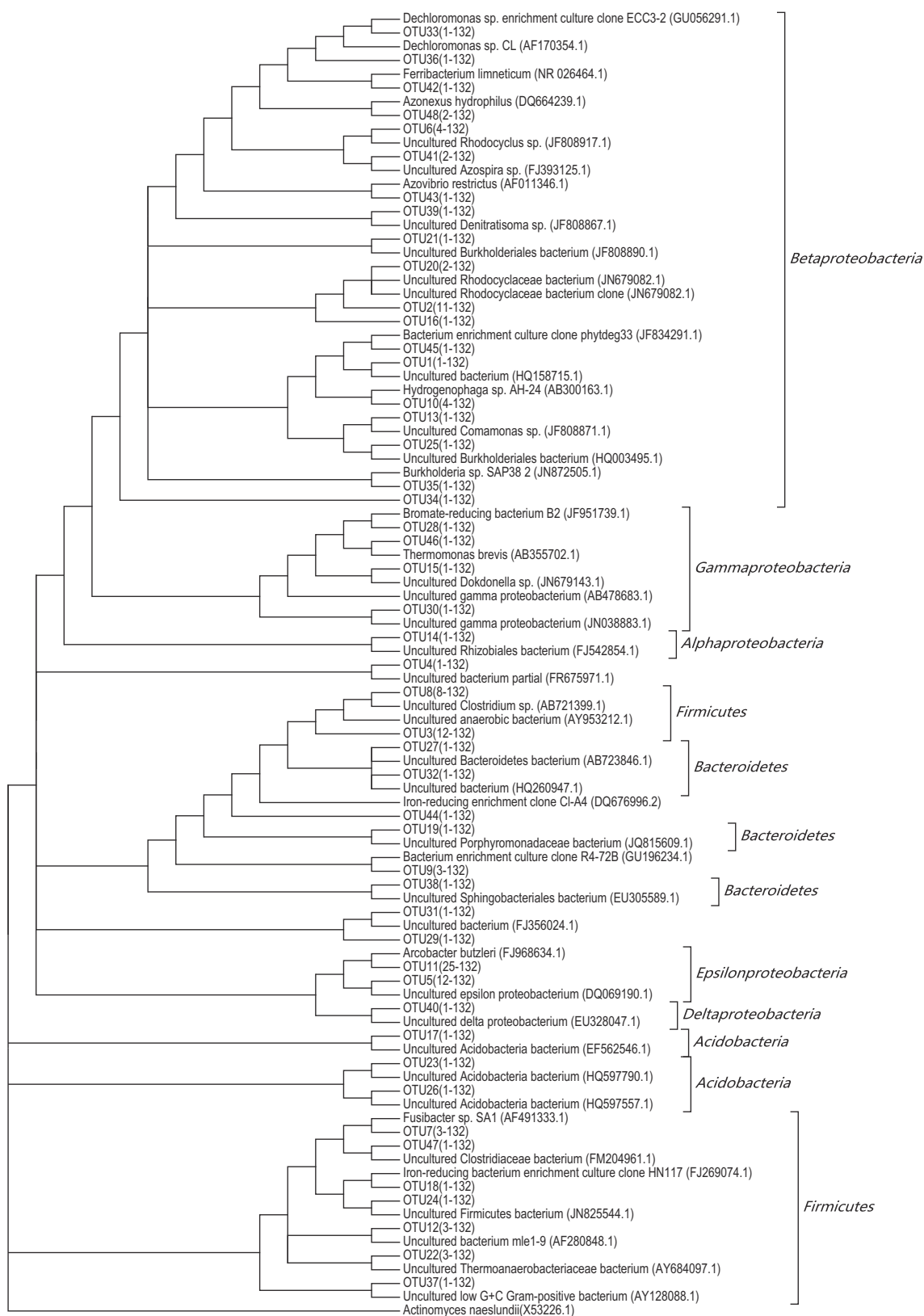
The results of the experiments evaluating the effect of pH are shown in Fig. 2. The pH range was set between 6–8 due to the restrictions of the buffering capacity. Because the ending pH was only slightly higher ( $<0.2$  pH units) than the starting one, the effects from pH change can be ignored. As shown in Fig. 2a, the ending total soluble V concentration decreased as pH increased from 6.0 to 8.0. The biggest impact of pH was between pH values of 7.0 and 7.5, pH = 8.0 gave faster removal from 50 to 150 hr, and pH  $\geq 7.5$  gave more than 90% total-V loss. The optimum pH was identified as 7.5–8.0 based on the experimental results. This trend is similar to Li et al. (2009), who found an optimal pH of 7.5 with heterotrophic bacteria. The benefit from slightly alkaline conditions may be beneficial, because denitrification and sulfate reduction add base that can cause a pH increase.

The influence of temperature on V(V)-reduction is presented in Fig. 2b. As is expected, higher temperature led to faster reduction in the range of 15–40°C. 40°C and 35°C achieved 91.5% and 88.5% removal percentages in 225 hr, respectively. Since the temperature for groundwater is scarcely above 40 °C, experiments under higher temperature were not carried out. Similar to the trends with lower pH (Fig. 1a), total soluble V plateaued for 15, 20, and 25 °C.

The kinetic results were fitted to a first-order kinetic model to obtain  $k_1$  values (Fig. 2b); we used only the results before plateauing. We then fit the  $k_1$  results with the Arrhenius



**Fig. 2 – (a) Concentration of total soluble V with starting V(V) 2 mg V/L, different pH values, and a temperature of 35°C; (b) Concentration of total soluble V with starting V(V) 2 mg V/L, pH value 7.5, and different temperatures; (c) Experimental and Arrhenius modeling results for the temperature influence (panel b). The  $E_a$  value is 36 kJ/mol.**



**Fig. 3 – Phylogenetic relationships among all bacteria (identified by OTU) determined with the clone library from the V(V)-reduction experiment.**

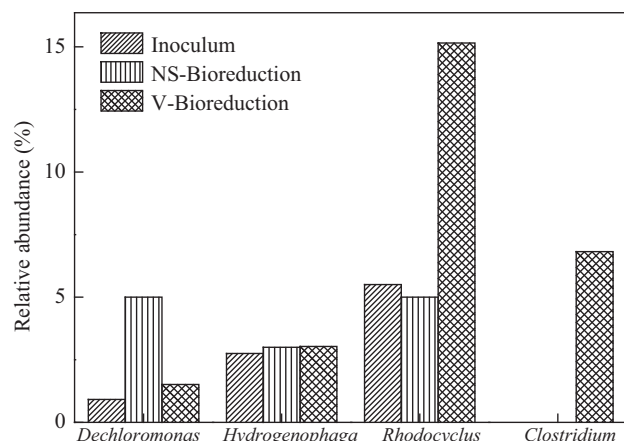
model. The linearized form of Arrhenius equation is:

$$\ln k = -E_a/RT + \ln A$$

where,  $E_a$  (J/mol) is the activation energy, and  $T$  (K) is the absolute temperature. The Arrhenius fit in Fig. 2c quantifies how V(V)-reduction was sensitive to temperature. The  $E_a$

(4)





**Fig. 4 – Relative proportions of the predominant bacterial genera in *Acidobacterium* inoculum, NS-bioreduction, and V-bioreduction.**

value is 36 kJ/mol, which is a moderately low value for aqueous-solution reactions (Stumm and Morgan, 1981), one consistent with biochemical catalysis.

### 2.3. Phylogenetic analysis

The clones for each sample could be grouped into 37 OTUs (inoculum), 40 OTUs (NS-bioreduction), and 48 OTUs (V(V)-bioreduction) on the basis of more than 97% sequence similarity within an OTU. Fig. 3 shows the phylogenetic tree of the 48 OTUs in V(V)-bioreduction. Prominent OTUs in V(V)-bioreduction include  $\beta$ -Proteobacteria (28.8%),  $\epsilon$ -Proteobacteria (28.0%), Firmicutes (24.2%), and  $\gamma$ -Proteobacteria (3.0%). Many known  $H_2$ -oxidizers were present, such as *Rhodocyclus*, *Hydrogenophaga*, and *Dechloromonas* (Van Ginkel et al., 2010; Zhang et al., 2009). *Rhodocyclus* was reported to do denitrification (Smith et al., 1994, 2005); *Dechloromonas* can reduce sulfate, nitrate, and nitrite (Horn et al., 2005); and *Hydrogenophaga* is an autotrophic denitrifier (Kämpfer et al., 2005). The presence of nitrate and sulfate reducers is expected, because the inoculum was enriched with both acceptors present.

Previously known V(V)-reducing species were absent (Antipov et al., 2000; Bredberg et al., 2004; Carpentier et al., 2003; Li et al., 2009; Ortiz-Bernad et al., 2004), suggesting that V(V) bioreduction was carried out by novel strains of V(V) reducers, which may include some of the sulfate- and/or nitrate-reducing bacteria in the inoculum.

Fig. 4 shows the relative proportions of the predominant bacterial genera in the three samples. The genera that became especially dominant in V(V)-bioreduction were *Rhodocyclus* and *Clostridium*, because their abundance increased significantly compared with the inoculum and NS-bioreduction.

This selective dominance in V-bioreduction suggests that *Rhodocyclus*, a denitrifying bacterium (Smith et al., 1994, 2005) and *Clostridium*, a fermenter (Rutter, 1970) may have been responsible for V(V) bioreduction.

*Rhodocyclus* is a purple non-sulfur photosynthetic bacterium, with the capacity to grow chemoautotrophically based on

$H_2$  oxidation with either oxygen or nitrate as the electron acceptor (Smith et al., 1994), although nothing is reported on V(IV) reduction. Strains of the phototrophic bacteria previously referred to as “*Rhodocyclus gelatinosus*-like (RGL)” were studied in comparison with *Rhodocyclus* species by Hiraishi et al. (1991). DNA hybridization studies showed that the RGL strain was closely related to the other strains, but exhibited low levels of the homology to the other *Rhodocyclus* species. Thus, the RGL group was established as a new taxon of the purple non-sulfur bacteria: *Rhodoferrax* (Hiraishi et al., 1991). According to Li et al. (2009), *Rhodoferrax* can reduce V(V) to V(IV), which lends support to that a *Rhodocyclus* strain might also carry out V(V)-reduction.

*Clostridium*, which was normally classified as a fermenter, was reported to solubilize ferric iron in hematite, goethite, and ferrites and to solubilize Mn(IV) in pyrolusite by enzymatic reduction and the oxides of cadmium, copper, lead, and zinc related to the production of organic acid metabolites (Francis and Dodge, 1990, 1988). According to Francis et al. (1994), U(VI) was reduced to U(IV) by the growing and resting *Clostridium* due to the enzymatic action. Thus, physiological characterization of *Clostridium* supports that it could be active in V(V) reduction.

*Dechloromonas* and *Hydrogenophaga* were important in V(V)-bioreduction, but their relative abundances were similar to NS-bioreduction, suggesting that *Dechloromonas* and *Hydrogenophaga* were not specially associated with V(V)-reduction.

### 3. Conclusions

This study investigated the bioreduction of V(V) in groundwater by autohydrogentrophic bacteria. Microbial V(V) reduction led to about 95.5% removal in 14 days. Detected V(IV) indicated that V(V) was reduced to V(VI) and then precipitated in the reactor. Phylogenetic analysis by clone library showed that the predominant OTUs were within the Proteobacteria in the V(V)-reducing community. Previously known V(V)-reducing species were absent, suggesting that V(V) reduction was carried out by novel species. *Rhodocyclus* and *Clostridium* became selectively dominant when V(V) was reduced, suggesting that one or both was responsible for V(V) reduction.

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