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1 **Synchronous microbial vanadium (V) reduction and denitrification**

2 **in groundwater using hydrogen as the sole electron donor**

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ABSTRACT

Groundwater co-contaminated by vanadium (V) (V(V)) and nitrate requires efficient remediation to prevent adverse environmental impacts. However, little is known about simultaneous bio-reductions of V(V) and nitrate supported by gaseous electron donors in aquifers. This study is among the first to examine microbial V(V) reduction and denitrification with hydrogen as the sole electron donor. V(V) removal efficiency of $91.0 \pm 3.2\%$ was achieved in test bioreactors within 7 d, with synchronous, complete removal of nitrate. V(V) was reduced to V(IV), which precipitated naturally under near-neutral conditions, and nitrate tended to be converted to nitrogen, both of which processes helped to purify the groundwater. Volatile fatty acids (VFAs) were produced from hydrogen oxidation. High-throughput 16S rRNA gene sequencing and metagenomic analyses revealed the evolutionary behavior of microbial communities and functional genes. The genera *Dechloromonas* and *Hydrogenophaga* promoted bio-reductions of V(V) and nitrate directly coupled to hydrogen oxidation. Enriched *Geobacter* and denitrifiers also indicated synergistic mechanism, with VFAs acting as organic carbon sources for heterotrophically functional bacteria while reducing V(V) and nitrate. These findings are likely to be useful in revealing biogeochemical fates of V(V) and nitrate in aquifer and developing technology for removing them simultaneously from groundwater.

Keywords: Vanadium (V); Hydrogen; Nitrate; Bio-reduction; Denitrification

1. Introduction

Vanadium is a transition metal prevalent in the Earth's crust and is extensively used in modern technologies (Naeem et al., 2007; Sturini et al., 2013; Cao et al., 2017). Geological weathering and discharges from industrial processes lead to the presence of vanadium in groundwater (Chen and Liu, 2017). In the U.S., substantial vanadium contamination has been recorded at 283 superfund sites, with vanadium concentration reaching 100 μM in an aquifer at a site near Rifle, Colorado (Yelton et al., 2013), far exceeding the 0.2 μg/L minimum reporting level proposed by the U.S. Environmental Protection Agency. In China, groundwater at Panzhihua, famous for its vanadium titanomagnetite resources and vanadium production, also contains very high concentrations of vanadium, up to 0.2 mg/L (Liu et al., 2017). Vanadium is moderately poisonous, with its toxicity increasing with valence state and solubility (Ortiz-Bernad et al., 2004; Zhang et al., 2010). Vanadium (V) (V(V)) is the most toxic and mobile form, whereas vanadium (IV) (V(IV)) is less toxic and insoluble at near-neutral pH (Safavi et al., 2000; Wang and Ren, 2014). Over recent decades, nitrate pollution of groundwater has become a serious issue worldwide owing to the increasing use of nitrogenous fertilizers, and discharges of domestic and industrial wastewater (Zhang et al., 2014a; Xie et al., 2018). Excess nitrate in drinking water poses health risks, including gastric problems in adults, decreased functioning of the thyroid gland, and multiple sclerosis (Cai et al., 2015; Zhai et al., 2016). Groundwater co-contaminated by V(V) and nitrate is increasingly commonplace, such as in the Gulf Coast aquifer of Texas, with concentrations of both contaminants exceeding

65   established maximum levels or health risk limits (Glenn and James Lester, 2010).

66   Reduction of V(V) to V (IV) is the approach usually taken to remove vanadium

67   from contaminated groundwater (Zhang et al., 2015). Physical and chemical methods

68   are commonly used for V(V) removal (Reul et al., 1999), but their cost-effectiveness

69   is questionable, and secondary pollution may also occur. Microbial V(V) reduction is

70   increasingly recognized as a promising future strategy for remediation of V(V)

71   contaminated groundwater, due to its simplicity, sustainability, and low cost (Lovley

72   and Coates, 1997; Yelton et al., 2013). Various microorganisms, such as *Geobacter*

73   *metallireducens*, *Shewanella oneidensis*, and *methanogens*, which naturally occur in

74   groundwater, are capable of reducing V(V) to V(IV) (Liu et al., 2016). Most known

75   species are heterotrophic and consume organic carbon; however available organics

76   decrease with increasing depth in the subsurface geological environment (Singh et al.,

77   2015). Supplementary addition of soluble organics can increase remediation costs and

78   bring about potentially secondary pollution. Aquifer clogging may also take place due

79   to higher biomass production of heterotrophs (Li et al., 2010). Thereby autotrophic

80   bioremediation with inorganic electron donors is of particular importance.

81   Furthermore, aquifers may contain dissolved hydrogen due to the hydration and

82   oxidation of rocks and minerals (Miller et al., 2017). As an electron donor, hydrogen

83   possesses several advantages in that it is inexpensive, widely available, and non-toxic

84   (Rittmann et al., 2004). As a result hydrogen has been successfully employed to

85   support bio-reductions of contaminants in groundwater, including chromate (Singh et

86   al., 2015), arsenate (Chung et al., 2006), selenate (Van Ginkel et al., 2011),

87 perchlorate and nitrate (Zhao et al., 2013). To date, limited studies have focused on

88 hydrogen-based microbial V(V) reduction (Xu et al., 2015), and the interactions

89 between V(V) and other co-contaminants in bio-reduction processes remain unknown.

90   Herein, we investigate experimentally the bioremediation of V(V) and nitrate

91 co-contaminated groundwater with hydrogen as the sole electron donor. The paper

92 aims to explore the simultaneous removal of V(V) and nitrate which often occur

93 together in groundwater, and to reveal dynamics of microbial communities, dominant

94 species, and functional genes.

95

96 **2. Materials and methods**

97 *2.1. Experimental setup and operation*

98   Eight cubic bioreactors were employed, made of plexiglass with total volume of

99 280 mL and covered with aluminum foil. Two holes were located in the top of the

100 reactor, one for replacing the culture medium and for sampling, and the other for

101 hydrogen injection. Each bioreactor was filled with synthetic groundwater containing

102 the following mineral salts per L: $CaCl_2$ 0.2464 g, $MgCl_2 \cdot 6H_2O$ 1.0572 g, NaCl

103 0.4459 g, KCl 0.0283 g, $NaHCO_3$ 0.504 g, and KH_2PO_4 0.0299 g. V(V) was provided

104 in the form of $NaVO_3$ at a prescribed concentration. Each bioreactor was inoculated

105 with 20 mL anaerobic sludge extracted from an upflow anaerobic sludge blanket

106 reactor used to treat high strength wastewater (Beijing YanJing Brewery Co. Ltd,

107 China). The sludge shared the similar microbial community structure with

108 groundwater microbes in vanadium contaminated aquifer (Cao et al., 2017). They

109   were divided into four groups: BR-V-N comprising medium containing both 1 mM

110   V(V) and 1 mM nitrate with hydrogen donor; BR-V consisting solely of 1 mM V(V),

111   again with hydrogen donor; and BR-N consisting solely of 1 mM nitrate, with

112   hydrogen donor; and a control group BR comprising medium containing both 1 mM

113   V(V) and 1 mM nitrate without hydrogen donor. In all cases, except BR, air in the

114   headspace of all bioreactors (30 mL) was first expelled, and then hydrogen was

115   injected via a syringe, after which the bioreactors were sealed with a rubber plug. A

116   further two reactors without inoculation but with hydrogen in the headspace were

117   assigned as AR, and filled with same medium as BR-V-N.

118   The inoculated bioreactors were first incubated for 3 months, refreshing the

119   aqueous solution every 7 days. Soluble organics existing in the inocula originally

120   were almost depleted after cultivation. After that the feasibility of simultaneous

121   removal of V(V) and nitrate was evaluated by employing hydrogen as the sole

122   electron donor in three consecutive operating cycles (each lasting 7 d), and comparing

123   the resulting concentrations against those in the control tests. Consumption of

124   hydrogen and generation of gaseous products during this process were examined by

125   collecting samples from the headspace into airbags for measurement. Soluble and

126   solid products were also analyzed. A parameter study was then undertaken whereby

127   the influence of key operating factors on hydrogen-supported V(V) reduction and

128   denitrification was examined for different initial nitrate concentrations (0.5 mM, 1

129   mM and 1.5 mM) with fixed 1 mM V(V), and different V(V) loadings (0.5 mM, 1

130   mM and 1.5 mM) with fixed mole ratio of V(V) and nitrate (1:1). Liquid samples

131   were acquired by sterilized syringes at selected time intervals, and the samples filtered

132   immediately through a 0.22-μm polyether sulphone membrane filter for analysis.

133   Each time after sampling, hydrogen was replenished through a needle connecting with

134   hydrogen cylinder at the rate of 100 mL/min for 30 min to supply sufficient electron

135   donor. Then another 3 months accumulation was conducted for BR-V-N for

136   high-throughput sequencing analysis. For each sample, microbial community analysis

137   was carried out in triplicate to confirm the reproducibility. The two reactors in each

138   group were operated under identical conditions, and the mean results recorded. All

139   experiments were conducted at room temperature (22 ± 2 $^{\circ}$C).

140   *2.2. Analytical methods*

141   The presence of V(V) was analyzed using spectrophotometry (Zhang et al.,

142   2012). Total V was analyzed using inductively coupled plasma-mass spectrometry

143   (ICP-MS, Thermo Fisher X series, Germany). Nitrate, nitrite and ammonium were

144   monitored using a spectrophotometer (DR6000, HACH, the USA). Total organic

145   carbon (TOC) was measured by Multi N/C 3000 TOC analyzer (Analytik Jena AG,

146   Germany). Gas chromatograph (Agilent, 4890, J&W Scientific, USA) was employed

147   to analyze gases, including hydrogen and nitrogen by means of a thermal conductivity

148   detector, and volatile fatty acids (VFAs) using a flame ionization detector. Precipitates

149   that appeared during the operation were collected through centrifugation at 10,000

150   rpm and analyzed using X-ray photoelectron spectroscopy (XPS) (XSAM-800, Kratos,

151   UK).

2.3. Microbiological analysis

Biomass in BR-V-N and the originally inoculated sludge underwent ultrasonic pretreatment, and their total genomic DNA was extracted using a FastDNA® SPIN Kit (Qiagen, CA, the USA), following manufacturer's instructions. Then the above DNA was amplified with PCR primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). A mixture of amplicons was used for high-throughput 16S rRNA gene and metagenomics analyses using MiSeq (Illumina, USA), performed by Shanghai Majorbio Technology (Shanghai, China). Raw data were submitted to the public National Center for Biotechnology Information (NCBI) database with accession numbers: SRP096812 and SRP120206. Operational taxonomic units (OTUs) were clustered from sequences by setting a 0.03 distance limit (equivalent to 97% similarity). Rarefaction curves and alpha diversity indexes were obtained using Mothur (version v.1.30.1). Phylogenetic affiliations and metagenomic results were analyzed using the RDP Classifier by comparison with the silva (SSU115) 16S rRNA database and Kyoto Encyclopedia of Genes and Genomes (KEGG) database, following previous studies (Lai et al., 2016).

3. Results and discussion

3.1. Hydrogen-supported V(V) and nitrate removal

Both V(V) and nitrate gradually decreased in BR-V-N over three consecutive operating cycles (Fig. 1a), during which simultaneous removal of V(V) and nitrate

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173   took place, with a V(V) removal efficiency of $91.0 \pm 3.2\%$ ($p < 0.05$), and complete

174   removal of nitrate within 7 d operation. The average removal rates were 124.7 ± 2.6

175    $\mu M/d$ for V(V) and 173.6 ± 3.9 $\mu M/d$ for nitrate ($p < 0.05$) (Fig. 1b), with pseudo

176   first-order rate constants of 0.2601 d^{-1} and 0.7252 d^{-1} (Supplementary information (SI),

177   Table S1). To the authors' knowledge, this is the first report concerning the

178   bio-reduction of V(V) and nitrate as co-contaminants, using hydrogen as the sole

179   electron donor. The present research appears to be an improvement over existing

180   studies to hydrogen-based V(V) bio-reductions in that the V(V) removal efficiency in

181   350 h was 95.5% for initial 2 mg/L V(V) and the calculated removal rate was only

182   0.005 mg/L·h by autohydrogentrophic bacteria (Xu et al., 2015), whereas 30 d was

183   required to reduce 2 mM V(V) by mesophilic and thermophilic methanogens with

184   H_2/CO_2 (80:20 gas mix) (Zhang et al., 2014b). The obtained V(V) removal rate was

185   lower than that of 24.2 ± 1.7 $\mu M/h$ in the presence of nitrate and acetate due to

186   differences in electron donors and carbon sources (Liu et al., 2017). V(V) was

187   removed more slowly than nitrate in BR-V-N because of its higher toxicity to

188   microbes (Liu et al., 2017).

189   Compared with removals of V(V) in BR-V and nitrate in BR-N (Fig. 1a), both

190   V(V) and nitrate bio-reductions were suppressed in BR-V-N, due to the competitive

191   consumption of electron donor. This phenomenon differed from result obtained using

192   soluble organic carbon sources, where the bio-reduction of V(V) was accelerated by

193   addition of chromium (VI), which is more toxic (Wang et al., 2017). Nitrate was

194   removed relatively more slowly in the present study due to the discontinuous supply

195   of hydrogen (Wu et al., 2018). Furthermore, concentrations of V(V) and nitrate

196   remained almost unchanged in AR (SI Fig. S1), which proved that V(V) and nitrate

197   removal in BR-V-N was biologically mediated. Hardly any V(V) was removed in BR,

198   with slight removal of nitrate (SI Fig. S1), implying that hydrogen played a key role

199   by providing electrons for microbes to detoxify V(V) and nitrate.

200   *3.2. Reaction products identification and process elucidation*

201   Gradually decreasing total V was observed in a typical operating cycle (7 d) in

202   BR-V-N (Fig. 2a), with appearance of blue precipitates, indicating the less mobile

203   characteristic of the generated products. V 2p spectra of XPS were recorded for the

204   precipitates (Fig. 2b). The sub-band with a peak at the binding energy of 515.9 eV

205   was identified as V(IV) (Zhao et al., 2016; Zhang et al., 2018), providing direct

206   evidence that V(V) was bio-reduced to less toxic V(IV). *In situ*, V(IV) can precipitate

207   naturally in near-neutral conditions in the form of $VO(OH)_2$ and/or vanadyl phosphate

208     $[CaV_2(PO_4)_2(OH)_4 \cdot 3H_2O]$ (Qiu et al., 2017). Peaks corresponding to V(V) were also

209   observed, most likely due to the re-oxidation of generated V(IV) during the collection

210   and testing of precipitates (Zhang et al., 2009a). As hydrogen was consumed, its

211   content declined during successive sampling intervals (Fig. 2a). The hydrogen-based

212   V(V) bio-reduction could therefore be elucidated by the following stoichiometric

213   equation,

214     $HVO_4^{2-} + 14.5H_2 + NO_3^- + 5HCO_3^- + 8H^+ \rightarrow VO(OH)_{2(s)} + C_5H_7NO_2 + 17H_2O$ (1)
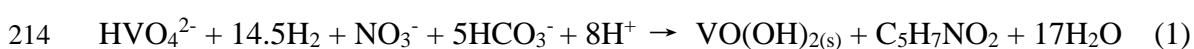
215   Meanwhile, nitrite and ammonium initially accumulated in BR-V-N (Fig. 2c),

216   indicating the presence of multiple metabolic pathways, including denitrification and

217   dissimilatory nitrate reduction to ammonium (Tong et al., 2013). Both nitrite and

218   ammonium gradually disappeared, perhaps due to the occurrence of anammox

219   activities. Nitrogen accumulated in the headspace of the reactor during operation (Fig.

220   2a), and so hydrogen-based denitrification could be expressed as follows,

221     $NO_3^- + 3H_2 + 0.23HCO_3^- + 1.23H^+ \rightarrow 0.48N_2 + 0.046C_5H_7O_2N + 3.63H_2O$ (2)
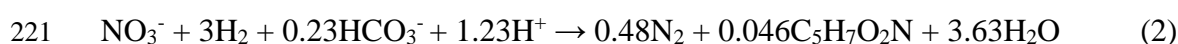
222   Mass balance and electron transfer were investigated based on the above

223   equations. During a typical operating cycle (7 d), 11.36 ± 0.04 mmol electrons were

224   released from hydrogen oxidation. The theoretically required amounts of electrons for

225   removal of V(V) and nitrate were 0.25 ± 0.01 mmol and 1.40 ± 0.01 mmol ($p < 0.05$),

226   along with the detected terminal reduction products. Hence, $2.20 \pm 0.03\%$ and $12.32 \pm$

227   0.07% ($p < 0.05$) of released electrons were consumed for V(V) respiration and

228   denitrification. The remaining electrons contributed to microbial growth and

229   proliferation. It should be noted that the utilization of hydrogen could be further

230   improved by diffusing hydrogen through the gas transfer membrane (Wu et al., 2018).

231   When practical bioremediation was conducted, the insufficiency of hydrogen could be

232   compensated by supplying hydrogen continuously through sustainable hydrogen

233   production, such as via bioelectrochemical systems (Zou and He, 2018).

234   Detoxification of V(V) and nitrate could be realized by bioaugmentation through

235   stimulating indigenous microorganisms in aquifer by hydrogen. According to Equ. (2),

236   the theoretical nitrogen yield was 0.13 ± 0.02 mmol, while the monitored nitrogen

237   was 0.11 ± 0.03 mmol in 7 d operation; this was probably due to the generation of

other nitrogen-containing intermediates and assimilations (Zhang et al., 2014a).

Hydrogen was found in aquifers with concentration around 15 nM (Chapelle et al., 1996), thus results from this study could be helpful to reveal the biogeochemical fates of V(V) and nitrate in groundwater. Lower biomass yields were produced in hydrogen-based bioprocess (Wu et al., 2017), thus possible clogging of aquifer could be prevented when it was practically applied.

Residual VFAs were also detected during the operation of BR-V-N (Fig. 2d), with average concentration of 9.84 ± 1.56 mg/L in a 7 d operating cycle ($p < 0.05$), comparable with result from methane-based biological bromate reduction (Luo et al., 2017). These metabolites might be derived from hydrogen oxidation and bicarbonate reduction by autotrophic microbes with the function of hydrogenases encoded by genes such as *hhyL* gene (Khdhiri et al., 2015). They could be consumed by heterotrophic microbes for V(V) and nitrate bio-reductions (Lai et al., 2016). Unlike acetate, which was the main form of residual VFAs under quasi-anaerobic condition (Luo et al., 2018), valeric species were the dominant components in our study, probably due to the immediate consumption of other species after their synthesis by heterotrophic microbes. For example, microorganisms were favor to using acetate as carbon source during V(V) bio-reduction (Liu et al., 2016). Less VFAs were accumulated with average TOC concentration in a typical operating cycle of 1.26 ± 0.11 mg/L ($p < 0.01$), which was much lower than original TOC concentration in natural groundwater (around 10 mg/L) (Zhang et al., 2017a). This result suggested that the VFAs residue could hardly cause significant fluctuations of organics

concentration when this hydrogen-based process was performed.

261 *3.3. Influences of operating factors*

262 Both V(V) and nitrate in BR-V-N decreased progressively with time, with lower

263 values observed at any given time for lower initial nitrate concentrations (Fig. 3a).

264 Removal efficiencies of V(V) reached 99.2 ± 0.9%, 91.0 ± 3.2% and 78.6 ± 4.2%

265 after 7 d for initial nitrate concentrations of 0.5 mM, 1 mM and 1.5 mM ($p < 0.05$).

266 The corresponding pseudo first-order rate constants were 0.3561 d^{-1}, 0.2603 d^{-1} and

267 0.1921 d^{-1} (SI Fig. S2a, Table S2). Nitrate was removed more quickly than V(V)

268 under each test condition (Fig. 3b), with pseudo first-order rate constants of 0.9391 d^{-1},

269 0.7245 d^{-1} and 0.6035 d^{-1}. In practice, V(V) and nitrate compete with respect to

270 electron donor consumption, whereas nitrate is less toxic to microbes than V(V).

271 Larger amounts of nitrate are commonly present in actual aquifers (Degnan et al.,

272 2016), and so the interaction of nitrate with V(V) should be considered whenever

273 practical bioremediation is conducted with hydrogen as the sole electron donor.

274 Both V(V) and nitrate were progressively removed with time under different

275 V(V) loadings (Fig. 3c). Here V(V) removal efficiencies decreased as the V(V)

276 loading increased, ranging from complete removal at a V(V) loading of 0.5 mM to

277 71.2 ± 1.3% at 1.5 mM V(V) loading ($p < 0.05$), with corresponding pseudo

278 first-order rate constants of 1.5372 d^{-1} to 0.1882 d^{-1} (SI Fig. S2b, Table S2). Nitrate

279 removal was faster than V(V) (Fig. 3d), with pseudo first-order rate constants of

280 1.6095 d^{-1}, 0.7252 d^{-1} and 0.5661 d^{-1} at V(V) loadings of 0.5 mM, 1 mM and 1.5 mM,

281 respectively. V(V) and nitrate vary in aquifers (Schlesinger et al., 2017; Nolan and

282 Weber, 2015) and so the present results suggest that hydrogen-based bio-reductions of

283 V(V) and nitrate can be feasible over a large range of V(V) loadings.

3.4. Microbial communities and mechanisms

285 Fewer OTUs were detected in the biomass from BR-V-N, compared with

286 inoculated sludge (SI Fig. S3). This implies that the microbial richness decreased

287 significantly due to the presence of V(V) and nitrate, which was also reflected by the

288 Chao1 and Ace indexes (SI Table S3). The lower Shannon index and higher Simpson

289 index suggested lower diversity and evenness in BR-V-N, indicating that the microbes

290 were highly selected. Moreover, the microbial abundance within the hydrogen-fed

291 autotrophic bioreactor was lower than that in heterotrophic systems for V(V)

292 bio-reductions (Liu et al., 2016), most likely because of the limited metabolic

293 pathways.

294 Evolutions of different microbial communities were observed in BR-V-N at class

295 level (Fig. 4a). *Spirochaetes* increased dramatically from $1.72 \pm 0.02\%$ in the inocula

296 to $47.9 \pm 7.2\%$ and become dominant in BR-V-N ($p < 0.05$). Microbes in this class are

297 capable of fermenting carbohydrates into simple organic acids (Sun et al., 2010), a

298 process closely akin to nitrate reduction (Xu et al., 2017). *Bacteroidetes* BD2-2,

299 *Betaproteobacteria*, *Actinobacteria* and *Mollicutes* were also noticeably enriched after

300 cultivation in BR-V-N. Notably, *Betaproteobacteria* and *Actinobacteria* species are

301 associated with dissimilatory metal reductions and denitrification (Hao et al., 2016;

302 Cesarano et al., 2017).

303 Functional species were found at genus level in BR-V-N (Fig. 4b). Primary

304 examples included the genera *Dechloromonas* and *Hydrogenophaga* which are

305 reported to be autohydrogenotrophic denitrifiers (Zhang et al., 2009b; Zhang et al.,

306 2017b) and *Geobacter* which promotes the reduction of V(V) (Ortiz-Bernad et al.,

307 2004). Enriched *Nitrospira*, *Ideonella* and *Methyloversatilis* took part in nitrogen

308 cycling (Shi et al., 2017; Pepe-Ranney et al., 2015; Baytshtok et al., 2009). The

309 gathered denitrifiers could be also responsible for V(V) removal given that V(V)

310 bio-reductions shared similar reductases with denitrification (Xu et al., 2015).

311 Interestingly, Fe(III) and sulfate-reducing bacteria also accumulated, including

312 *Sphaerochaeta* and *Desulfuromonas* (Ritalahti et al., 2012).

313 Metagenomic analysis with functional genes and encoding proteins summarized

314 into eight catalogs revealed the microbial dynamics at molecular level (Fig. 4c).

315 Details were listed in SI Table S4 and Table S5. Metal reduction genes and their

316 corresponding proteins increased significantly in BR-V-N, confirming the resultant

317 V(V) bio-reductions through pathways presented in SI Table S6. Similar tendencies

318 were found in denitrification genes and the corresponding enzymes, consistent with

319 the detected denitrifiers. NADP genes related to hydrogen metabolism bloomed due to

320 the presence of hydrogen. Although genes related to phosphate transporters decreased,

321 their corresponding proteins were enriched, which favored V(V) removal, noting that

322 ABC transporters have been reported to be linked to selenate bio-reduction (Lai et al.,

323 2016). Notably, genes responsible for the synthesis of electron shuttles (flavodoxin,

324 ubiquinone, cytochrome, etc) also increased, facilitating electron transfers from

325 electron donor (hydrogen) to electron acceptors (V(V) and nitrate) (Kranz-Finger et

326 al., 2018).

327   Statistical studies revealed correlations between representative accumulated

328 functional genes and microbes (Fig. 4d). Genera *Dechloromonas*, *Ideonella* and

329 *Methyloversatilis* showed strongly positive relationships with genes involved in

330 metabolic pathways, microbial metabolism in diverse environments, biosynthesis of

331 secondary metabolites, and nitrogen metabolism such as *sdhA*, *nifJ*, *gltD* and *narG*

332 (Guardia et al., 2018). Genera *Hydrogenophaga* and *Geobacter* were linked with the

333 *chrA* gene, which participated in metal reductions (Shaw and Dussan, 2018). Genus

334 *Desulfuromonas* was positively related to *phsA* and *cysH* genes, which took part in the

335 removal of nitrite and metal (Haveman et al., 2004; Saltikov and Newman, 2003).

336   The mechanisms of hydrogen-based V(V) bio-reduction and denitrification were

337 now summarized. Both direct and synergistic routes were proposed (Fig. 5). Detection

338 of *Dechloromonas* and *Hydrogenophaga* proved that hydrogen oxidation could be

339 coupled directly to nitrate removal. V(V) bio-reduction could also be performed by

340 these species given that their membrane-bound nitrate reductases had been implicated

341 in V(V) respiration (Xu et al., 2015). Through synergy, hydrogen was consumed by

342 hydrogen-utilizing bacteria such as *Bacillus* to synthesize VFAs (Chubukov and Sauer,

343 2014), after which the generated VFAs served as electron donors and carbon sources

344 for the heterotrophic V(V)-reducing bacterium *Geobacter* involved in V(V)

345 bio-reduction, and denitrifiers for nitrate removal. Functional genes such as *gltD*,

346 *chrA* and *narG* encoded corresponding enzymes that conducted simultaneous V(V)

347 bio-reduction and denitrification coupled to hydrogen oxidation.

4. Conclusions

In this study, co-contaminants V(V) and nitrate in groundwater were handled in anaerobic bioreactors. The major conclusions and outcomes are:

- Synchronous V(V) bio-reduction and denitrification can be realized with hydrogen as the sole electron donor.
- V(V) removal efficiency of 91.0 ± 3.2% was achieved in a typical operating cycle (7 d), along with complete removal of nitrate.
- V(IV) formed as the reduction product of V(V), and nitrate was reduced to nitrogen.
- The dynamics of microbial communities and functional genes provide evidence for both direct and synergetic mechanisms.

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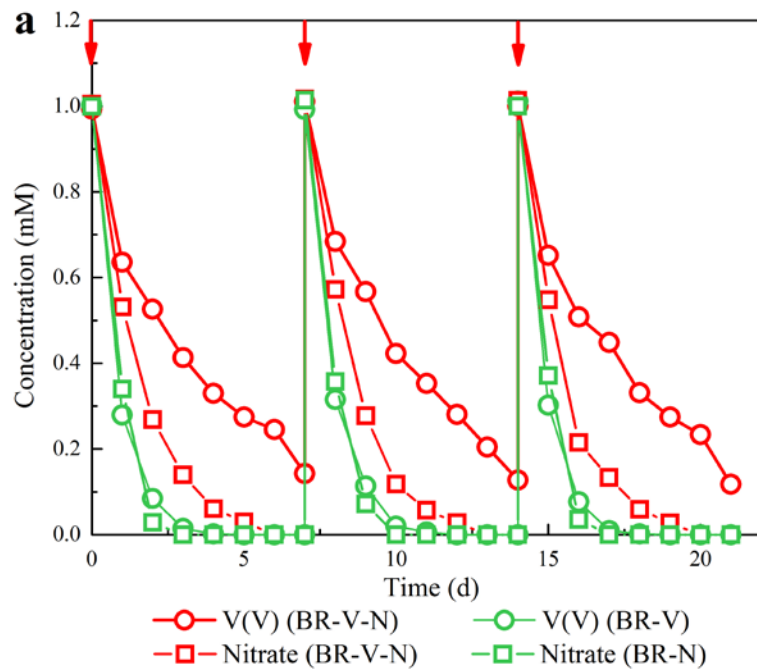
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Figure captions.

Fig. 1. Temporal variations in V(V) and nitrate in hydrogen-based bioreactors. (a) time histories of V(V) and nitrate concentrations during three consecutive operating cycles; (b) pseudo first-order kinetic plots for a typical operating cycle (7 d). Red arrows indicate the replacement of synthetic groundwater. C_0 (μM) and C_t (μM) are the concentrations of V(V) or nitrate at initial condition and at time t (d), respectively.

Fig. 2. Reaction products during the operation of BR-V-N. (a) variation in total V, hydrogen, and nitrogen with time; (b) XPS spectra of V 2p for the generated precipitates; (c) variation in nitrite and ammonium with time; (d) average concentrations of residual VFAs.

Fig. 3. Parameter study of operating factors affecting the performance of BR-V-N. (a) V(V) and (b) nitrate removal time histories for different initial nitrate concentrations; (c) V(V) and (d) nitrate bio-reduction time histories for different V(V) loadings.

Fig. 4. Microbial community compositions and functional genes revealed by sequencing of inoculated sludge and BR-V-N. (a) class-level abundance; (b) functional bacterial genera; (c) relative abundance of functional genes and proteins; (d) heatmap of representative functional genes based on bacterial genera.

Fig. 5. Proposed mechanisms of V(V) bio-reduction and nitrate removal with hydrogen as the sole electron donor under anaerobic conditions.

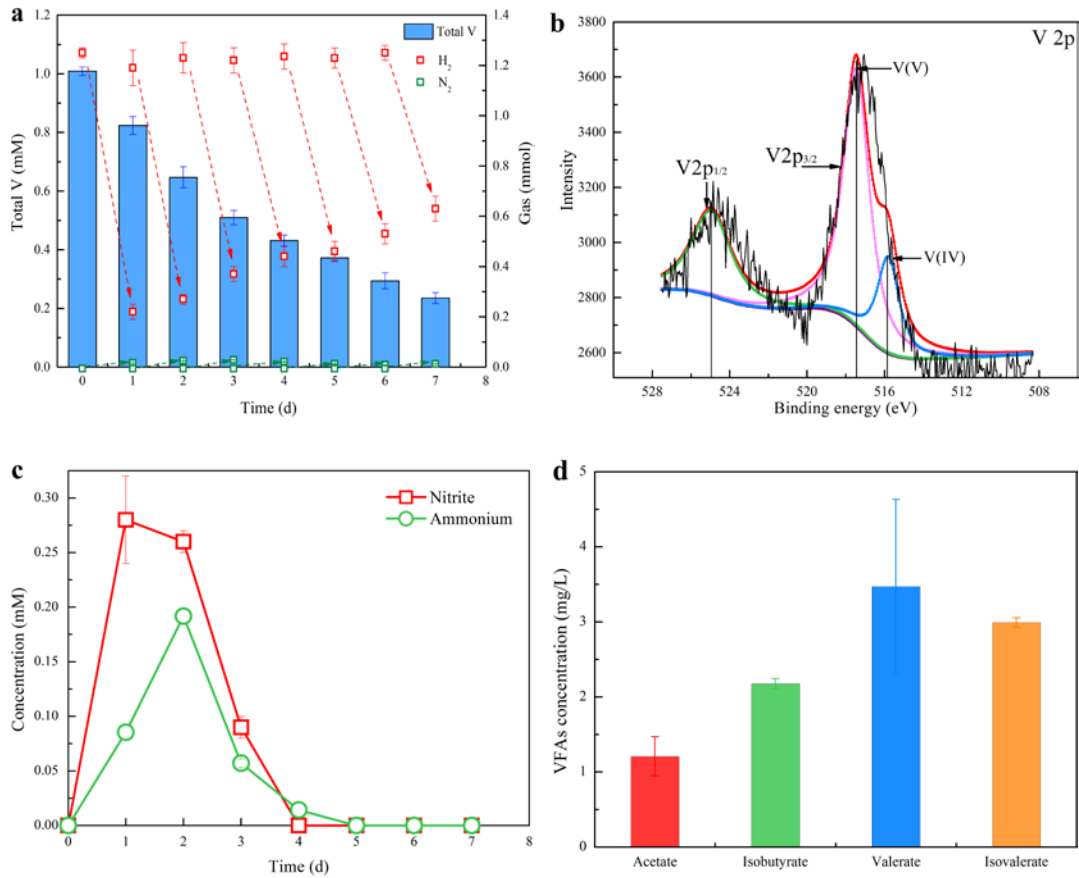**Fig. 1.** Temporal variations in V(V) and nitrate in hydrogen-based bioreactors. (a) time histories of V(V) and nitrate concentrations during three consecutive operating cycles; (b) pseudo first-order kinetic plots for a typical operating cycle (7 d). Red arrows indicate the replacement of synthetic groundwater. C_0 (μM) and C_t (μM) are the concentrations of V(V) or nitrate at initial condition and at time t (d), respectively.
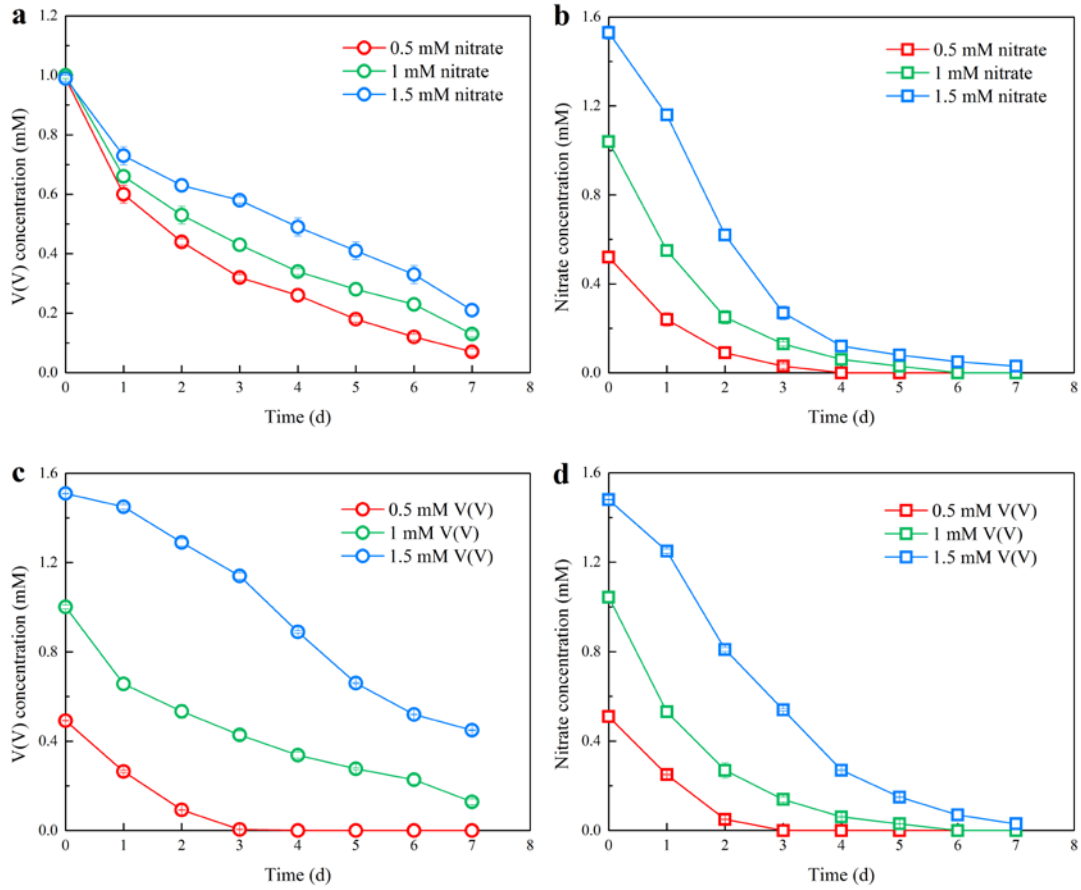
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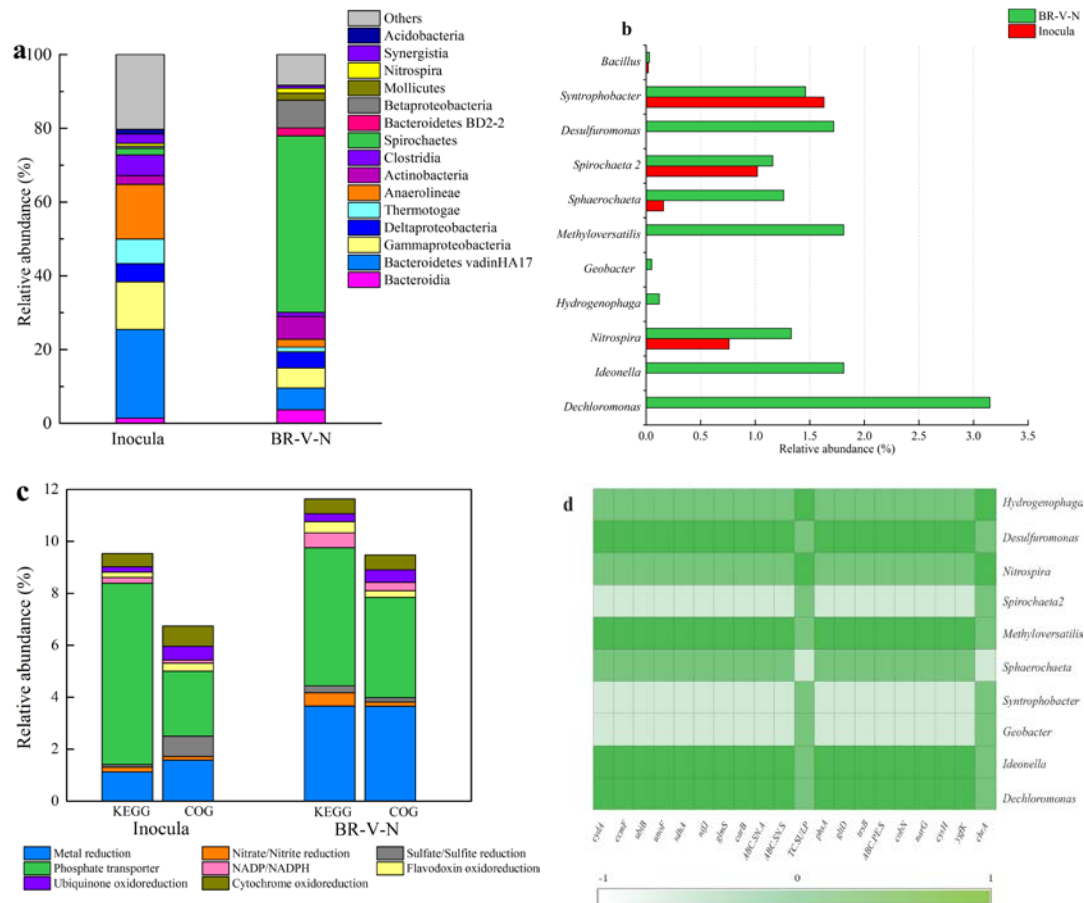
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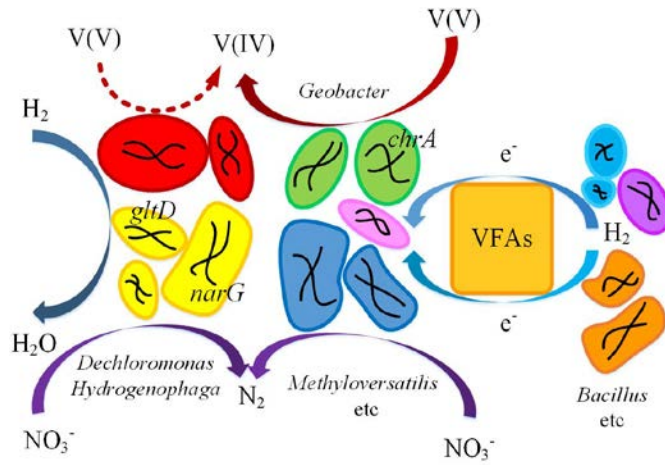(c) V(V) and (d) nitrate bio-reduction time histories for different V(V) loadings.

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