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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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23 **ABSTRACT**

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

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

43 **1. Introduction**

44 Vanadium is a transition metal prevalent in the Earth's crust and is extensively
45 used in modern technologies (Naeem et al., 2007; Sturini et al., 2013; Cao et al.,
46 2017). Geological weathering and discharges from industrial processes lead to the
47 presence of vanadium in groundwater (Chen and Liu, 2017). In the U.S., substantial
48 vanadium contamination has been recorded at 283 superfund sites, with vanadium
49 concentration reaching 100 μM in an aquifer at a site near Rifle, Colorado (Yelton et
50 al., 2013), far exceeding the 0.2 $\mu\text{g/L}$ minimum reporting level proposed by the U.S.
51 Environmental Protection Agency. In China, groundwater at Panzhihua, famous for its
52 vanadium titanomagnetite resources and vanadium production, also contains very
53 high concentrations of vanadium, up to 0.2 mg/L (Liu et al., 2017). Vanadium is
54 moderately poisonous, with its toxicity increasing with valence state and solubility
55 (Ortiz-Bernad et al., 2004; Zhang et al., 2010). Vanadium (V) (V(V)) is the most toxic
56 and mobile form, whereas vanadium (IV) (V(IV)) is less toxic and insoluble at
57 near-neutral pH (Safavi et al., 2000; Wang and Ren, 2014). Over recent decades,
58 nitrate pollution of groundwater has become a serious issue worldwide owing to the
59 increasing use of nitrogenous fertilizers, and discharges of domestic and industrial
60 wastewater (Zhang et al., 2014a; Xie et al., 2018). Excess nitrate in drinking water
61 poses health risks, including gastric problems in adults, decreased functioning of the
62 thyroid gland, and multiple sclerosis (Cai et al., 2015; Zhai et al., 2016). Groundwater
63 co-contaminated by V(V) and nitrate is increasingly commonplace, such as in the
64 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: CaCl₂ 0.2464 g, MgCl₂·6H₂O 1.0572 g, NaCl
103 0.4459 g, KCl 0.0283 g, NaHCO₃ 0.504 g, and KH₂PO₄ 0.0299 g. V(V) was provided
104 in the form of NaVO₃ 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- μ m 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 °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).

152 *2.3. Microbiological analysis*

153 Biomass in BR-V-N and the originally inoculated sludge underwent ultrasonic
154 pretreatment, and their total genomic DNA was extracted using a FastDNA[®] SPIN Kit
155 (Qiagen, CA, the USA), following manufacturer's instructions. Then the above DNA
156 was amplified with PCR primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and
157 806R (5'-GGACTACHVGGGTWTCTAAT-3'). A mixture of amplicons was used for
158 high-throughput 16S rRNA gene and metagenomics analyses using MiSeq (Illumina,
159 USA), performed by Shanghai Majorbio Technology (Shanghai, China). Raw data
160 were submitted to the public National Center for Biotechnology Information (NCBI)
161 database with accession numbers: SRP096812 and SRP120206. Operational
162 taxonomic units (OTUs) were clustered from sequences by setting a 0.03 distance
163 limit (equivalent to 97% similarity). Rarefaction curves and alpha diversity indexes
164 were obtained using Mothur (version v.1.30.1). Phylogenetic affiliations and
165 metagenomic results were analyzed using the RDP Classifier by comparison with the
166 silva (SSU115) 16S rRNA database and Kyoto Encyclopedia of Genes and Genomes
167 (KEGG) database, following previous studies (Lai et al., 2016).

168

169 **3. Results and discussion**

170 *3.1. Hydrogen-supported V(V) and nitrate removal*

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

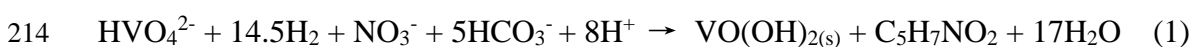
173 took place, with a V(V) removal efficiency of $91.0 \pm 3.2\%$ ($p < 0.05$), and complete
174 removal of nitrate within 7 d operation. The average removal rates were 124.7 ± 2.6
175 $\mu\text{M/d}$ for V(V) and $173.6 \pm 3.9 \mu\text{M/d}$ for nitrate ($p < 0.05$) (Fig. 1b), with pseudo
176 first-order 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 $24.2 \pm 1.7 \mu\text{M/h}$ 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.

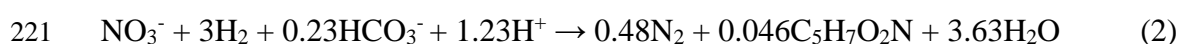
200 3.2. Reaction products identification and process elucidation

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 [CaV₂(PO₄)₂(OH)₄·3H₂O] (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,



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,



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

238 other nitrogen-containing intermediates and assimilations (Zhang et al., 2014a).
239 Hydrogen was found in aquifers with concentration around 15 nM (Chapelle et al.,
240 1996), thus results from this study could be helpful to reveal the biogeochemical fates
241 of V(V) and nitrate in groundwater. Lower biomass yields were produced in
242 hydrogen-based bioprocess (Wu et al., 2017), thus possible clogging of aquifer could
243 be prevented when it was practically applied.

244 Residual VFAs were also detected during the operation of BR-V-N (Fig. 2d),
245 with average concentration of 9.84 ± 1.56 mg/L in a 7 d operating cycle ($p < 0.05$),
246 comparable with result from methane-based biological bromate reduction (Luo et al.,
247 2017). These metabolites might be derived from hydrogen oxidation and bicarbonate
248 reduction by autotrophic microbes with the function of hydrogenases encoded by
249 genes such as *hhyL* gene (Khdhiri et al., 2015). They could be consumed by
250 heterotrophic microbes for V(V) and nitrate bio-reductions (Lai et al., 2016). Unlike
251 acetate, which was the main form of residual VFAs under quasi-anaerobic condition
252 (Luo et al., 2018), valeric species were the dominant components in our study,
253 probably due to the immediate consumption of other species after their synthesis by
254 heterotrophic microbes. For example, microorganisms were favor to using acetate as
255 carbon source during V(V) bio-reduction (Liu et al., 2016). Less VFAs were
256 accumulated with average TOC concentration in a typical operating cycle of $1.26 \pm$
257 0.11 mg/L ($p < 0.01$), which was much lower than original TOC concentration in
258 natural groundwater (around 10 mg/L) (Zhang et al., 2017a). This result suggested
259 that the VFAs residue could hardly cause significant fluctuations of organics

260 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 $99.2 \pm 0.9\%$, $91.0 \pm 3.2\%$ and $78.6 \pm 4.2\%$
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 $71.2 \pm 1.3\%$ 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.

284 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.

348

349 **4. Conclusions**

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

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

360

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566 **Figure captions.**

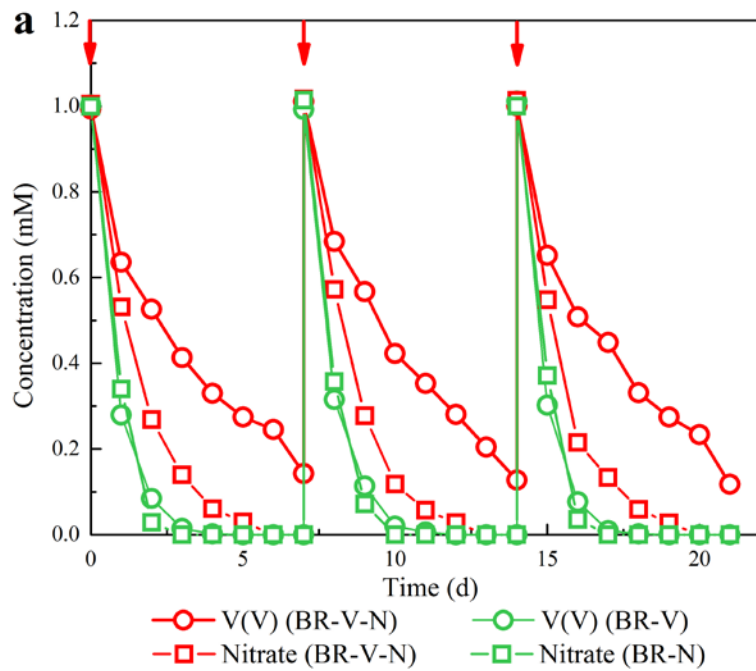
567 **Fig. 1.** Temporal variations in V(V) and nitrate in hydrogen-based bioreactors. (a)
568 time histories of V(V) and nitrate concentrations during three consecutive operating
569 cycles; (b) pseudo first-order kinetic plots for a typical operating cycle (7 d). Red
570 arrows indicate the replacement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

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

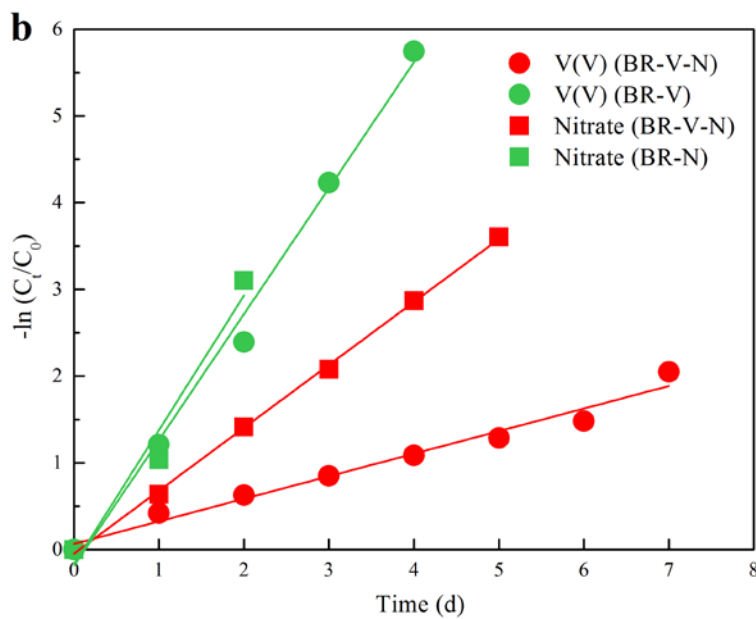
576 **Fig. 3.** Parameter study of operating factors affecting the performance of BR-V-N. (a)
577 V(V) and (b) nitrate removal time histories for different initial nitrate concentrations;
578 (c) V(V) and (d) nitrate bio-reduction time histories for different V(V) loadings.

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

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

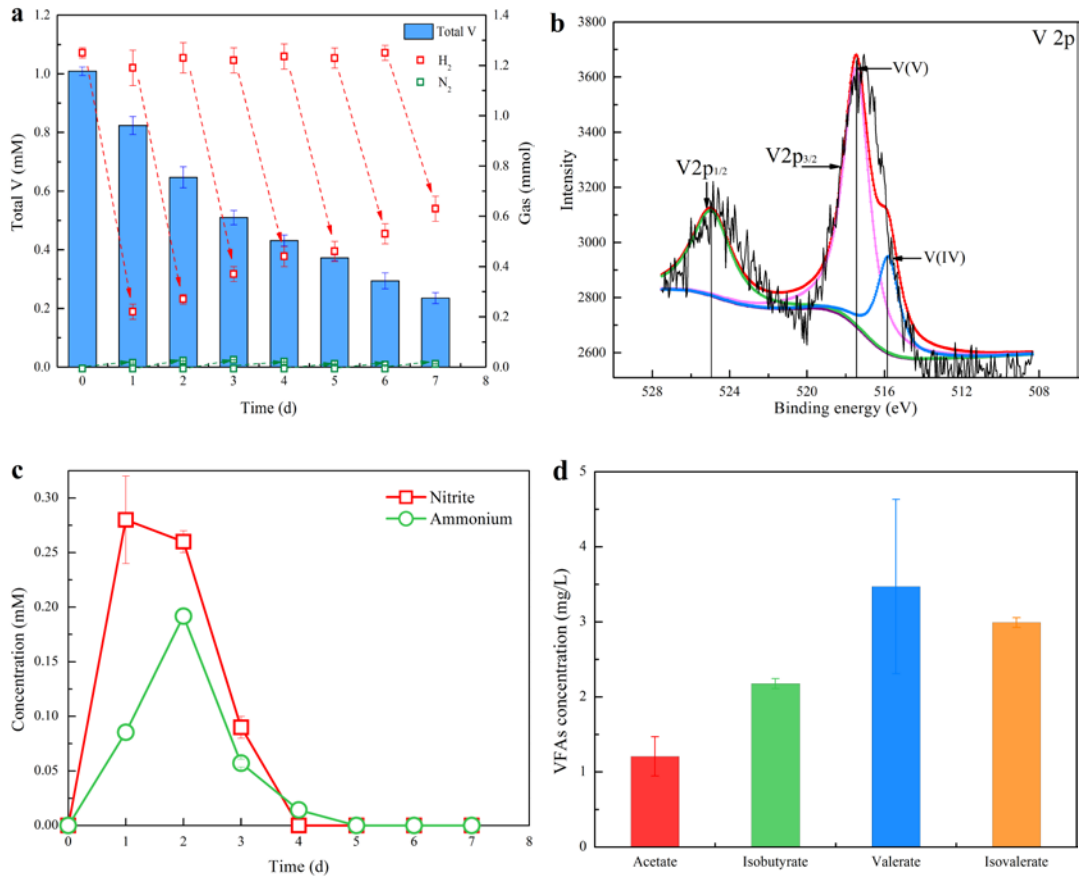


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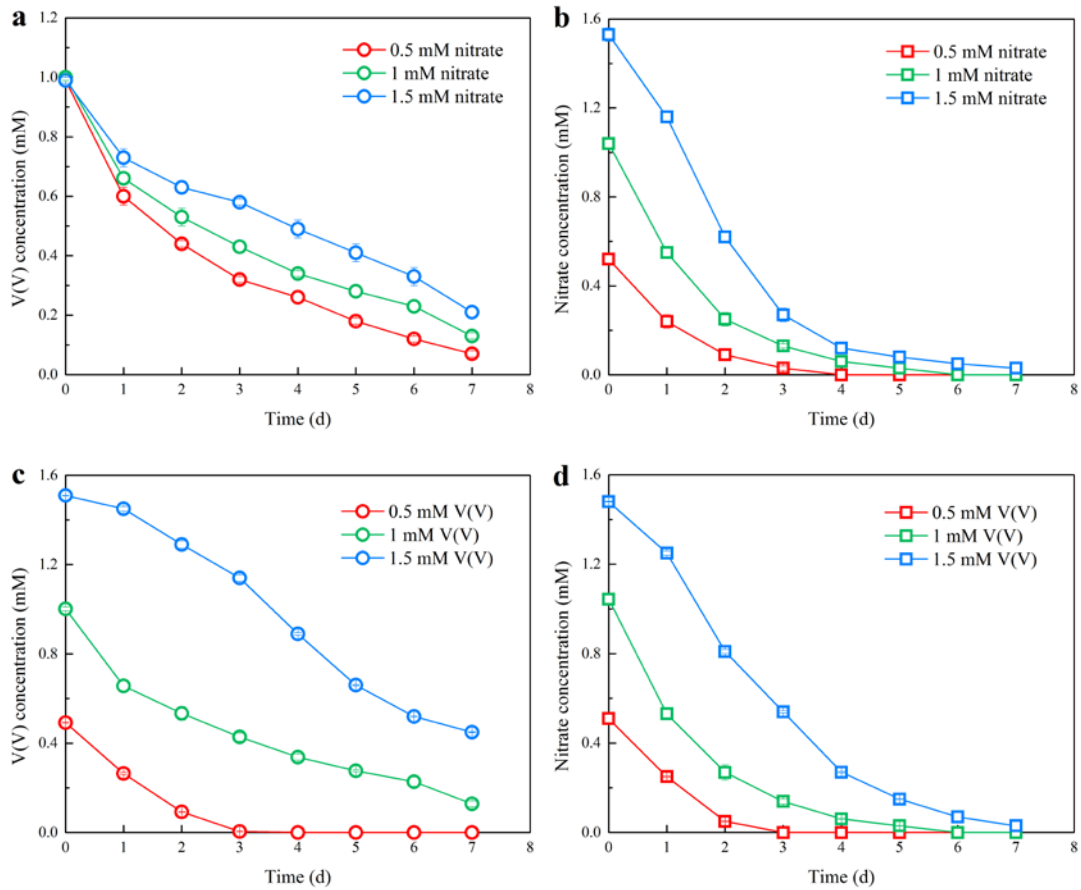


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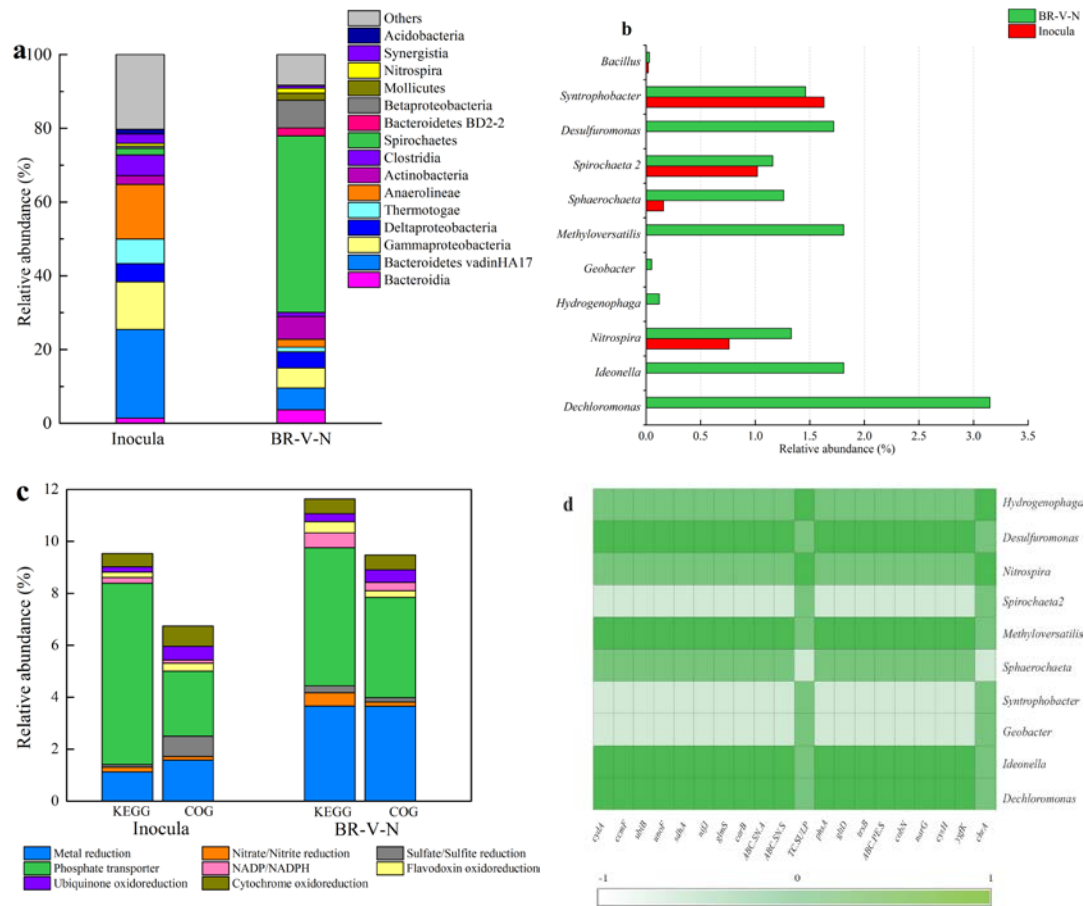
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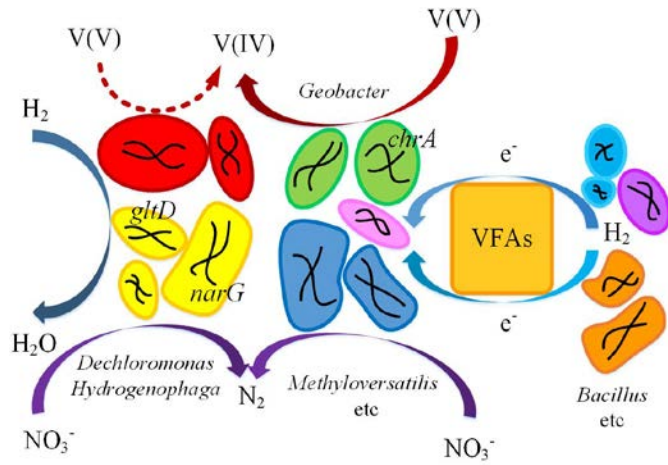
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