Anaerobic dissolution rates of U(IV)-oxide by abiotic and nitrate-dependent bacterial pathways

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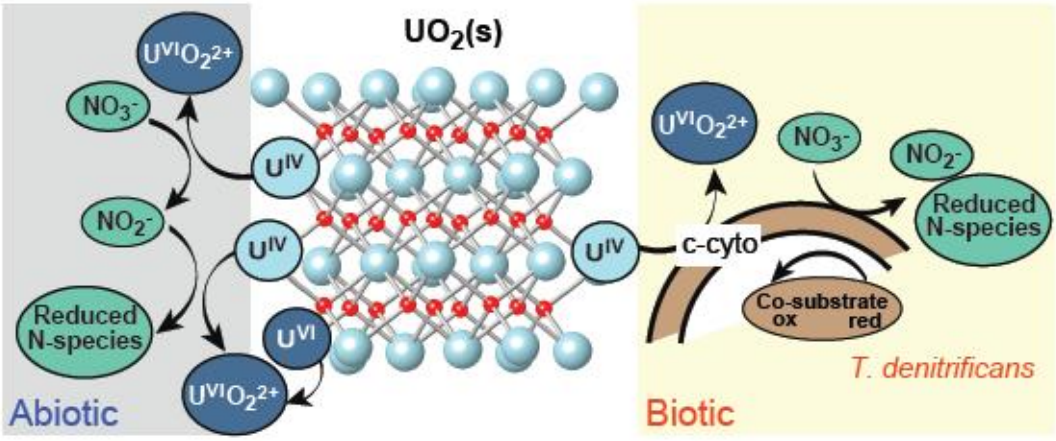
UO2(s)
UVIO22+
NO3-
NO2-
Reduced N-species
UIV
UIV
UIV
UVI
UVIO22+
Abiotic
UVIO22+
NO3-
NO2-
Reduced N-species
c-cyto
Co-substrate
ox
red
T. denitrificans
Biotic

ABSTRACT

The long-term stability of U(IV) solid phases in anaerobic aquifers depends upon their reactivity in the presence of oxidizing chemical species and microbial catalysts. We performed flow-through column experiments under anaerobic conditions to investigate the mechanisms and dissolution rates of biogenic, non-crystalline UO_2(s) by chemical oxidants (nitrate, nitrite), or by *Thiobacillus denitrificans*, a widespread, denitrifying, chemolithoautotrophic model bacterium. Dissolution rates of UO_2(s) with dissolved nitrite were approximately 5 to 10 times higher than with nitrate alone. In the presence of wild-type *T. denitrificans* and nitrate, UO_2(s) dissolution rates were similar to those of abiotic experiments with nitrite (from $1.15 \cdot 10^{-14}$ to $4.94 \cdot 10^{-13}$ mol m^{-2} s^{-1}). Experiments with a *T. dentrificans* mutant strain defective in U(IV) oxidation supported microbially mediated U(IV) oxidation. X-ray absorption spectroscopy (XAS) analysis of post-reaction solids showed the presence of mononuclear U(VI) species rather than as a solid phase. At steady state U release, kinetic and spectroscopic results suggest detachment of oxidized U(VI) from the UO_2(s) surface as the rate-determining step rather than electron transfer or ion diffusion. Under anaerobic conditions, production of nitrite by nitrate-reducing microorganisms and enzymatically catalyzed, nitrate-dependent U(IV) oxidation are likely dual processes by which reduced U solids may be oxidized and mobilized in the aqueous phase.

Keywords: biogenic UO_2(s), anaerobic U(IV) dissolution rates, nitrate, nitrite, *Thiobacillus denitrificans*

INTRODUCTION

Uranium (U) groundwater contamination is a worldwide environmental concern. Stimulated *in situ* bioreduction of dissolved U(VI) and precipitation as a poorly soluble U(IV) oxide (nominally $UO_2(s)$) has received considerable attention as a remediation approach for immobilizing U in aquifers under moderately reducing conditions (known as reductive immobilization)[1,2,3,4,5]. Nanoparticulate uraninite ($UO_{2+x}(s)$)[6,7] and non-crystalline disordered U(IV) (monomeric and polymeric U(IV)-oxides)[8,9,10] have been reported as the product of microbially reduced U(IV). The presence of multiple U(IV) species has been reported in field sediments[5,11,12,13] and laboratory experiments[14]. Some of these studies proposed that uraninite was the end product of abiotic reduction of U(VI)[5,12], which, in complex systems such as natural sediments, may occur concomitantly with biological U(VI) reduction[12], whereas others suggested precipitation of non-crystalline U(IV) species and transformation to nanocrystalline uraninite with aging.[14,15] Microbially mediated U(IV) oxidation under anaerobic conditions may remobilize these U(IV) forms, and thus render long-term reductive immobilization less effective.[16,17,18,19,20,21] Therefore, identifying mechanisms and quantifying rates of U release from re-oxidation and dissolution of $UO_2(s)$ and related U(IV) solid-phase contaminants are important for understanding potential U mobility in the environment.

Abiotic oxidation of $UO_2(s)$ by molecular O_2 proceeds rapidly and oxidation rates have been investigated under a variety of conditions, including variable pO_2, pH, and dissolved inorganic carbon and cations[22,23]. Dissolution rates of $UO_2(s)$ produced by microbial reduction (biogenic $UO_{2+x}(s)$) under anaerobic and aerobic conditions were found to be within experimental error of those of chemically synthesized $UO_2(s)$ in carbonate-free systems in one study[22]. Another study proposed that chemogenic uraninite was more resistant to oxidation than forms of biogenic U(IV)-oxides in the presence of dissolved oxygen[23]. Monomeric forms of U(IV) have been found to be less stable and more susceptible to oxidation than biogenic uraninite under

anoxic condtions[22]. However, Cerrato et al.[23] suggested that exposure of biogenic U(IV) solids to oxidants and transport processes that control exposure and U release are more important than the actual form of the solid, given relatively small differences observed in the oxidation rate constants of biogenic uraninite and monomeric U(IV) in the presence of oxygen.

Anaerobic oxidation of U(IV) solids in the presence of dissolved nitrate (NO_3^-) and its reduction products is of particular concern in subsurface U.S. Department of Energy sites and other sites worldwide where U and nitrate are found as co-contaminants.[17,24,25,26,27,28,29,30] In anaerobic systems, the abiotic oxidation of UO_2(s) coupled to nitrate reduction in the absence of iron was observed to be slow[31]. In the presence of nitrate-reducing and denitrifying microorganisms, enzymatic reduction of nitrate may produce nitrite (NO_2^-), nitrous oxide (N_2O), or nitric oxide (NO) species that may serve as abiotic oxidants for UO_2(s) and co-occur with direct enzymatic oxidation of U(IV) by anaerobic, nitrate-reducing bacteria.

Thiobacillus denitrificans is a widely distributed and well-characterized obligate chemolithoautotroph with a diverse metabolism[32], capable of anaerobic, nitrate-dependent U(IV) oxidation at circumneutral pH, as well as nitrate-dependent Fe(II) oxidation and coupled oxidation of inorganic sulfur compounds, under either aerobic or denitrifying conditions.[18,32,33,34,35,36,37] Beller[18] found that *T. denitrificans* was capable of anaerobic, nitrate-dependent oxidative dissolution of synthetic and biogenic U(IV) oxides in batch laboratory experiments; comparable U(IV) oxidation was not observed in killed controls or in no-nitrate controls with live cells. The role of denitrification intermediates (NO_2^-, NO, N_2O) was not explicitly investigated in that study, although nitrite was not detected (detection limit, 10 μM) throughout the U(IV) experiment or in no-U(IV) controls that consumed 13 mM nitrate in 2 h. Biochemical studies of *T. denitrificans* insertion mutants[38] strongly suggest that, at least for this bacterium, direct enzymatic U(IV) oxidation under denitrifying conditions may have primacy over secondary abiotic U(IV) oxidation by denitrification intermediates such as nitrite. A

mutant strain with a defective *Tbd_0187* gene (*Tbd_0187*::*kan*, also used in the present study) had only 47% of wild-type U(IV) oxidation activity and 64% of wild-type denitrification activity. However, when this mutant was complemented with an intact copy of the mutated gene (in *trans*, on a plasmid), the U(IV) oxidation activity was restored to 130% of wild-type levels but denitrification activity was not improved (55% of wild-type)[38]. These results suggest that the presence of the intact *c*-type cytochrome encoded by the *Tbd_0187* gene was more important for U(IV) oxidation than the level of denitrification activity.

Although previous studies examined the dissolution of synthetic and biogenic U(IV) oxide phases under a variety of conditions, quantification of reaction rates and comparisons between different studies are challenging due to differences in experimental approach as well as in reactant material[39,40,41,42]. Comparisons between abiotic rates measured under sterile conditions and those measured in the presence of U(IV)-oxidizing microorganisms are often complicated by different experimental conditions needed to stimulate or maintain microbial activity. This study differs from prior investigations in its direct comparison of U dissolution in the absence and presence of a U(IV)-oxidizing model bacterium, *T. denitrificans*, and the addition of dissolved nitrate, nitrite, or both using relatively simple reactant solutions (i.e., buffered pH and carbonate-free solutions). Furthermore, our experiments employed flow through porous medium (ground quartz) with a low solution volume in a strictly anaerobic environment, rather than a batch or tank system in which the solution:solid ratio is high. This flow-through system provided a more realistic water:solid ratio with the advantage of removing dissolved species that may influence reaction rate or solubility of mineral phases[43], and thus better simulated the dynamic porewater flow regime of saturated subsurface environments.

MATERIALS AND METHODS

Biogenic UO_2(s) synthesis. Multiple batches of U(IV)-oxide (hereafter referred to as UO_2(s)) were produced by reduction of dissolved U(VI) by *Shewanella oneidensis* strain MR-1

following published methods.[6,44] *S. oneidensis* was grown in lysogeny broth (LB) under aerobic conditions. Cells were separated by centrifugation and washed with a buffer containing 30 mM $NaHCO_3$, 20 mM PIPES, and 20 mM lactate (pH 6.3) under anaerobic conditions (10% H_2, 90% Ar headspace). Solids were resuspended in the same solution amended with 1500 μM of uranyl acetate. After U(VI) reduction and precipitation of UO_2(s), solid particles were cleaned by resuspending the solids in 1 M NaOH for 12 h. Solids were then centrifuged and resuspended in anaerobic hexane for 12 h to separate UO_2(s) from biomass. Finally, the solids were centrifuged and stored under anaerobic conditions in a 1 M $NaHCO_3$ solution. All cultures and subsequent experiments were maintained under strict anaerobic and sterile conditions.

Dissolution experiments. Dissolution of UO_2(s) in column flow-through experiments in the absence or presence of *T. denitrificans* was performed under anaerobic conditions at room temperature (25±1 ºC) in an anaerobic glove box (Type A, Coy Laboratory Products, Inc., Grass Lake, MI). The entire experimental set-up (input and output solutions, peristaltic pump, flow-through reactors, and tubing) was enclosed in the glove box (25±1 ºC) and purged with a gas composition of 90% N_2 and 10% H_2. Ultrapure water (18.2 MΩ·cm, Milli-Q Plus, Millipore) used to prepare the input solutions was boiled before use to remove dissolved oxygen, cooled in the anaerobic glove box, and left for at least 24 h before performing experiments. Oxygen partial pressure in the glove box was continuously monitored with a gas analyzer (Model 10, Coy Laboratory Products) and read 0 ppm during the experiments. All material (tubes, quartz, solutions, columns, etc.) was sterilized before each experiment and equilibrated in the anaerobic glove box before use.

Flow-through column experiments were carried out by packing a mixture of UO_2(s) with ground natural quartz (~200-500 μm from Unimin, Inc. and treated to clean surface impurities) into either 1-mL (0.8-cm ID x 2-cm length) or 5-mL (1.3-cm ID x 4.5-cm length) polypropylene chromatography columns (SUPELCO) and protected from light exposure. The final mixtures

contained average concentrations of 570 mg U/kg of quartz to 170 mg U/kg of quartz for the 1-mL and 5-mL columns, respectively. Influent solutions were filtered with a 0.2-µm polycarbonate filter (Millipore) at both the inlet and outlet ends of the column. Flow was directed upward in the experiments to minimize bubbles (e.g., N_2 from denitrification) and was controlled with an HPLC pump (Ismatec C.P. 78001-02), which had a variation of less than 3%. Influent flow rates were gravimetrically determined. Influent dissolved oxygen and pH were measured periodically by electrode (Orion 081010MD for dissolved oxygen and Thermo Scientific Orion 9102DJWP for pH). In selected experiments, effluent pH was monitored in line with a Thermo-Scientific Orion 9102DJWP pH electrode.

For abiotic dissolution experiments, quartz and UO_2(s) were mixed under anaerobic conditions, then 1 mL of 10 mM NaCl (pH 7) was added to the mixture and packed into columns. For biotic experiments, *T. denitrificans* cells were harvested anaerobically by centrifugation in sealed polycarbonate bottles, washed once under anaerobic conditions, and resuspended in anaerobic 10 mM NaCl (pH 7). The optical density at 600 nm (OD_{600}) of this solution was adjusted to 2 (ca. 1.8 x 10^9 cells$\cdot$ mL^{-1}) by diluting with the same NaCl solution, 1 mL was added to the mixture of quartz and UO_2(s), and the slurry was immediately loaded into the columns (details of the cultivation and incubation of *T. denitrificans* can be found in the Supporting Information). In order to confirm that the process was enzymatically mediated, in addition to wild-type *T. denitrificans*, a mutant *T. denitrificans* strain that was ~50% defective in U(IV) oxidation activity (relative to wild-type) was used that had an insertion mutation in the *Tbd_0187* gene, which encodes a *c*-type cytochrome shown to be involved in anaerobic U(IV) oxidation[38].

Influent solutions for dissolution experiments were prepared by mixing reagents (ACS-grade or better $NaNO_3$, $NaNO_2$, NaCl, and MOPS as a pH buffer) with the deoxygenated ultrapure water. Solutions were made with variable nitrate, nitrite, and chloride concentrations

149 to maintain an approximately constant ionic strength (Table S1). The pH was adjusted to ~7 by
150 adding NaOH (2 M) (Table S1). Solids were reacted with influent solutions (Table S1) for ~2-
151 5 days and column effluent was collected using a multi-channel fraction collector
152 (Spectra/Chrom® CF-1).

153 **Uranium dissolution rates.** The UO_2(s) dissolution rate, R, (mol m^{-2} s^{-1}), was calculated
154 according to the following expression (Eq. 1):[41,45]

155 $$R = \frac{[U]_{ss} \times Q}{V \times A \times [UO_{2(s)}]} \qquad \text{Eq. 1}$$

156 where, assuming that the U concentration in the input solution is zero and the U released is due
157 to UO_2(s) dissolution, $[U]_{ss}$ is the concentration (mol U $\cdot L^{-1}$) in the effluent solution at steady-
158 state, A is the specific surface area ($m^2 \cdot g^{-1}$), V is the estimated pore volume (L), [UO_2(s)] is
159 the UO_2(s) solid concentration in the reactor ($g \cdot L^{-1}$), and Q is the volumetric flow rate through
160 the system ($L \cdot s^{-1}$) (Table S2). The $[U]_{ss}$ was the average concentration of 20 and 100 or more
161 pore volumes eluted for the 5-mL and 1-mL columns, respectively, over which the effluent U
162 concentration varied ≤15%. The error associated with the dissolution rate was estimated by
163 propagation of the uncertainty in each of the terms in Eq. 1 (Table S2) and ranged from 6-27%.
164 The estimated error was dominated by the uncertainty of the mass concentration of U in the
165 steady-state ($[U]_{ss} \leq 15\%$) and the uncertainty of the mass measurements (2-24%).

166 **Analytical methods.** Detailed information about aqueous and solid analytical methods are
167 presented in the Supporting Information. Briefly, column effluent solutions were analyzed for
168 total U by ICP-MS (Agilent 7500cs); the detection limit was 4.2 x 10^{-5} µM (10 ppt) and
169 analytical precision was < ±5%. We attempted to measure U(VI) speciation by kinetic
170 phosphorescence analysis (KPA), but concentrations were below detection after dilution to
171 reduce quenching from dissolved ions. Nitrate and nitrite were determined
172 spectrophotometrically with a Cary-300-Bio spectrophotometer. UO_2(s) was characterized by
173 X-ray diffraction (XRD) on a PANalytical X'pert Pro diffractometer. Scanning electron

174 microscopy (SEM) and TEM images were obtained using a FEI Quanta 200 ESEM and a JEOL-
175 JEM-2010 HRTEM, respectively. The surface area was measured using 7-point N_2 adsorption
176 isotherms with a Micrometrics Tri-Star 3000 surface area analyzer. Uranium L_{III}-edge X-ray
177 absorption spectroscopy (XAS) data, including X-ray absorption near edge structure (XANES)
178 and extended X-ray absorption fine structure (EXAFS) spectra, were collected at the Stanford
179 Synchrotron Radiation Lightsource (SSRL, California, USA) beamlines 11-2 and 4-1.

180 **RESULTS**

181 **Characterization of unreacted biogenic UO_2(s).** Powder XRD patterns of biogenic UO_2(s)
182 showed weak reflections that matched the major reflections of crystalline UO_2(s), but with
183 significant broadening, lower amplitude, and lower signal-to-noise ratio (Figure S1). These
184 features are similar to those of biogenic UO_2(s) produced in other studies and are attributed to
185 small particle size, low crystallinity, and/or structural disorder[6,46]. The surface area of freeze-
186 dried UO_2(s) was measured by the N_2-BET method on two batches of UO_2(s) (Table S1), with
187 a mean surface area of 72.81±0.69 m^2g^{-1}. Examination of unreacted UO_2(s) particles by SEM
188 showed particle aggregates of up to several micrometers in diameter (Fig. S2a, S2b). Lattice
189 fringes of particles observed by TEM images indicated individual particles of 2-4 nm in
190 diameter (Fig. S2c), consistent with other studies of biogenic UO_2(s) produced by the method
191 described above[41]. Results from XRD and TEM, and from XAS analysis (discussed below),
192 support the interpretation that the majority of the biogenic starting material was mostly
193 nanoparticulate UO_2(s).

194 **Flow-through column abiotic experiments.** The abiotic dissolution of UO_2(s) was examined
195 in the presence of nitrate and/or nitrite (0-20 mM) in buffered solutions at circumneutral pH
196 (7.0-7.3). Total U concentrations in column effluent normalized to the total molar mass of
197 UO_2(s) added to the column in the abiotic experiments are shown in Figure 1. At the beginning
198 of all the experiments, an initial peak of effluent U was observed, which is a common

199 phenomenon in flow-through experiments that is attributed to labile U(VI) on the UO_2 surface[47]
200 or to the passage of colloidal U through the filter membrane during the first flush[41]. Therefore,
201 the dissolution rate was calculated once a steady state in effluent U concentration was attained.
202 The results display differences in the total amount of U released, and therefore, in the amount
203 of U dissolved, depending on the influent composition (Figure 1, Table S2). In the absence of
204 an oxidant, the effluent U in replicate columns with anaerobic NaCl solutions buffered at pH
205 ~7 remained low and relatively constant (Figure 1a). The calculated average U dissolution rate
206 ($1.05 \cdot 10^{-14}$ mol m^{-2} s^{-1}) is similar to the lowest rate reported by Ulrich et al.[41] for biogenically
207 precipitated UO_2(s) (Table 1). The addition of 20 mM nitrate to the influent solution resulted in
208 an increase in the average dissolution rate ($3.02 \cdot 10^{-14}$ mol m^{-2} s^{-1}) compared to that of
209 experiments in the absence of an oxidant (Figure 1b). In contrast, when the columns were
210 supplied with nitrite, the mass-normalized U effluent concentrations and calculated steady-state
211 dissolution rates were higher than in the presence of nitrate alone (Figure 1, Table S2). The
212 dissolution rates with mixtures of nitrate and nitrite were similar to those obtained with nitrite
213 only, which is consistent with the expected stronger effect of nitrite as an oxidant in abiotic
214 UO_2(s) dissolution compared to nitrate (Figure 1d-f, Figure S3, Table S2).

215 **Flow-through column experiments in the presence of *T. denitrificans*.** Dissolution of UO_2(s)
216 in the presence of *T. denitrificans* and dissolved nitrate (± nitrite) was compared to abiotic rate
217 experiments. Replicate experiments always included an abiotic control column run
218 simultaneously with the same influent solution as the biotic columns (Table S2). In most
219 experiments, columns with *T. denitrificans* showed rates of UO_2(s) dissolution of about one
220 order-of-magnitude faster than those of the simultaneous abiotic control experiment (Figure 2,
221 Table S2). However, in a few simultaneous replicate columns, UO_2(s) dissolution rates were
222 lower and closer to those of abiotic controls (e.g. B1-R2, B2-R3, and B4-R1, Table S2) and
223 nitrite concentrations (Figure S4) were below detection limit (B1-R2), slightly lower (B2-R3)

than the biotic replicates, or followed the same trend as the control experiment (B4-R1), which could indicate low or no enzymatic oxidation. Overall, UO_2(s) dissolution rates were more variable in experiments with *T. denitrificans* than in abiotic experiments, and a relatively constant effluent U concentration was sometimes not observed until near the end of the experiment. Nitrite concentrations measured in the effluent solutions of experiments with *T. denitrificans* with nitrate (but no added nitrite) were higher (~20-160 μM) than in abiotic nitrate control experiments (~5-10 μM), demonstrating microbially accelerated conversion of nitrate to nitrite (Figure S4). Furthermore, in two control column experiments with *T. denitrificans* and quartz but no UO_2(s), solutions with influent nitrate concentrations of 1 or 10 mM produced effluent nitrite concentrations near the limit of detection (0.42 μM) (data not shown). Overall, abiotic dissolution rates measured in experiments with added nitrite were similar to rates observed in experiments with *T. denitrificans* and added nitrate (Table 1).

Column experiments were performed with a mutant strain of *T. denitrificans* that was previously shown to be ~50% defective in U(IV) oxidation.[38] Results showed a decrease in the average UO_2(s) dissolution rate of 44% compared to that of experiments with the wild-type strain. The decrease in dissolution rate was smaller (34% rather than 44%) if effluent U concentrations were compared with those of simultaneous abiotic control columns with the same influent nitrate concentration (10 mM) (e.g., controls B3b-CT6 and B5-CT8 in Figure 2c and 2e, respectively) (Table 1, Figure 2). The differences in rate exceeded the estimated propagated error for these experiments (Table 1). This result supports the role of enzymatic mediation by *T. denitrificans* in the U(IV) oxidation process (Figure 2).

X-ray absorption spectroscopy of unreacted and reacted UO_2(s). Because of the low concentrations of U and the presence of quartz in the reacted columns (which added background noise), XAS data were limited to mostly XANES spectra, with usable EXAFS spectra obtained for only three reacted samples. XANES spectra of UO_2(s) samples and reference compounds at

249 the U L_{III}-edge are shown in Figure S5. The absorption maximum for U(IV) is found at 17,176
250 eV, which is ~2.7 eV lower in energy than the maximum for U(VI). U(VI)-oxide compounds
251 show a shoulder at approximately 17,191 eV, which has previously been shown to result from
252 multiple scattering resonances of the linear uranyl ion structure, specifically due to the short U-
253 O_{ax} bonds[48,49]. Best fits of linear combinations of unreacted UO_2(s) and schoepite
254 [$(UO_2)_8O_2(OH)_{12}$·$12(H_2O)$] as a U(VI) reference compound showed that spectra were
255 dominated by unreacted material (72-100% ±10% in normalized spectra) with a small fraction
256 of U(VI) present in the solids (Figure S5, Table 2). Experiments performed with nitrite had the
257 highest uranyl fraction among the abiotic samples analyzed by XAS, although results were
258 variable among all columns. For columns reacted in the presence of *T. denitrificans*, the highest
259 U(VI) fractions [28% U(VI) in B1(R2) and 26% U(VI) in B3b(R2)] were in experiments with
260 nitrate (4 or 10 mM, respectively) that were run for longer duration (cumulative fluid volumes
261 of 647 and 776 mL, respectively; Table S2), although fit results were again variable.
262 Quantitative fits of EXAFS spectra of two unreacted UO_2(s) samples (Unr-1 and Unr-3)
263 indicated a local atomic environment around U consistent with the UO_2(s) structure[6] (Figure 3,
264 Table S3). As noted in prior studies, atomic backscattering amplitudes in EXAFS of biogenic
265 nanoparticulate UO_2(s) tend to be lower than in crystalline U compounds, particularly for atoms
266 beyond the first bonded shell of O atoms, due to small particle size, local disorder, and in some
267 cases a component of monomeric U(IV) species.[50,6,8,51,9,23] Backscattering from U-U shells at
268 interatomic distances of ~ 5.46 and 6.73 Å were not significant in the EXAFS spectrum of Unr-
269 1 (Figure 3), which may indicate a small difference in particle size or crystallinity, and/or a
270 variable fraction of monomeric U(IV), a common product of enzymatic U(VI) reduction in the
271 natural environment[52,53,54] and laboratory studies[55], in addition to nanoparticulate
272 UO_2(s).[50,8,51,23] In reacted column samples, XANES analysis indicated a low fraction of U(VI)
273 (< 30%), so the three EXAFS spectra were examined for evidence of backscattering from near-

neighbor axial oxygen atoms (O_{ax}) in the uranyl group (UO_2^{2+}) at interatomic distances from ~ 1.74 to 1.83 Å (Table S4), in addition to O atoms at distances indicative of unreacted UO_2(s) (2.30-2.35 Å). Quantitative EXAFS fits of reacted samples A2 (abiotic with 20 mM nitrite) and B2 (with *T. denitrificans* and 5 mM nitrate) showed no evidence for O_{ax} backscattering or U-O backscattering at distances other than expected for U-O in UO_2(s). Fit results were similar to those found for the U-O and U-U shells for Unr-1 and Unr-3 (Table S3). In the EXAFS fit of reacted sample A9(R2) (abiotic with 18 mM nitrate + 10 mM nitrite), weak backscattering from O_{ax} (at 1.77 Å) was permissible although backscattering at distances characteristic of U(VI) and equatorial O atoms (U-O_{eq}), which span a range of distances, could not be distinguished in the fit from O atoms in UO_2(s) (Figure 3, Table S3). This column was reacted at a high nitrate + nitrite concentration (28 mM) and with a higher cumulative pore volume than abiotic column A2 (285 vs. 220 mL). Overall, the XAS results showed that U in reacted columns mostly resembled unreacted UO_2(s), but suggested evidence for a minor fraction of U(VI), with slightly more U(VI) at high oxidant concentrations and longer reaction times. The lack of molecular-scale evidence for interatomic scattering indicative of uranyl oxyhydroxide minerals suggests the formation mostly of U(VI) surface complexes. If U(VI)-oxide solid phases were present, their fraction was below detection by XAS.

DISCUSSION

Abiotic and biotic rates of anaerobic UO_2(s) dissolution. Dissolution rates of UO_2(s) at steady-state U release as a function of nitrate and nitrite concentration, and in the absence and presence of *T. denitrificans*, are compiled in Figure 4. In the absence of an oxidant, the average UO_2(s) dissolution rate ($1.05 \cdot 10^{-14}$ mol m^{-2} s^{-1}) was similar to the slowest rates published previously for bio-UO_2(s) at neutral pH in carbonate-free solutions ($1.07 \cdot 10^{-14}$ to $3.42 \cdot 10^{-13}$ mol m^{-2} s^{-1})[41] (Table 1). Abiotic dissolution of UO_2(s) by nitrate alone was relatively slow ($2.51 \cdot 10^{-14}$ to $4.61 \cdot 10^{-14}$ mol m^{-2} s^{-1}), although slightly faster than without addition of an oxidant, and not dependent on nitrate concentration (4-20 mM NO_3^-) (Figure 4). These rates, measured at pH

300 7.0-7.3, are approximately two orders of magnitude slower than the rate reported by Ulrich et
301 al.[41] for dissolution of synthetic UO_2(s) in the presence of 1 mM nitrate at pH 8 ($4.03 \cdot 10^{-12}$ mol
302 m^{-2} s^{-1}; Table 1). Ulrich et al.[41] obtained slower dissolution rates for bio-UO_2 than for synthetic
303 UO_2 solids (Table 1), although these differences were estimated to be within their experimental
304 error. With the addition of nitrite as an oxidant (with or without nitrate), UO_2(s) dissolution
305 rates were about 5 to 10 times higher than with nitrate alone, with the exception of one
306 experiment at low nitrite and nitrate concentrations (Figure 4, Figure S3, Table 1). Prior studies
307 have pointed out that nitrite, a product of nitrate reduction, is more reactive than nitrate and
308 could abiotically oxidize U(IV).[16,17] To our knowledge, however, no anaerobic UO_2(s)
309 dissolution rates in the presence of nitrite under flow conditions have been reported previously.
310 In the absence of microbial activity, our experiments show the ability of nitrite to accelerate
311 UO_2(s) dissolution compared with nitrate, although there was not a strong dependence on
312 influent nitrite concentration over the range used in our experiments (0.08-20 mM; Figure S3).
313 Rates of UO_2(s) dissolution in the presence of *T. denitrificans* with nitrate (and with 0.08 mM
314 nitrite in one set of experiments) were generally higher than those for abiotic experiments with
315 nitrate-only, but similar to the range observed for abiotic experiments with nitrite (Figure 4).
316 Experiments with a mutant strain of *T. denitrificans* that is partially defective in U(IV)
317 oxidation[38] showed a decrease in average dissolution rate compared to rates from experiments
318 with wild-type *T. denitrificans* and the same concentration of influent nitrate, providing
319 evidence of enzymatically catalyzed U(IV) oxidation by *T. denitrificans*. Effluent U
320 concentrations in biotic experiments ranged from ~1-40 nanomolar, compared to effluent nitrite
321 of ~5-150 micromolar from the reduction of nitrate, with no apparent correlation between
322 effluent U and nitrite concentration, which was variable among biotic experiments (Figure S4).
323 Therefore, U(IV) alone could not have served as the dominant electron donor for nitrate
324 reduction by *T. denitrificans*. These observations support previously reported batch studies of

325 UO_2(s) oxidation by *T. denitrificans* indicating that nitrate reduction is coupled to a primary
326 electron donor other than U(IV) (e.g., H_2 from the glove box atmosphere).[18] In fact, nitrate-
327 dependent U(IV) oxidation catalyzed by *T. denitrificans* has been reported to have a co-
328 metabolic dependence on H_2 oxidation.[18]

329 **Mechanisms of anaerobic UO_2(s) dissolution.** Under anaerobic conditions in the absence of
330 oxidants, radiolysis products such as H_2O_2 and O_2 from alpha decay of U reacting with water
331 molecules can oxidize U(IV) to produce surface species of U(V) or U(VI) [22,41,56,57,58,59]. In early
332 studies, Bruno et al.[39] assumed that detachment of a hydroxo-U(IV) species ($U(OH)_X^{(4-x)+}$) was
333 the rate-determining step in thin-film, flow-through dissolution experiments of synthetic UO_2(s)
334 under reducing conditions. In our experiments, U(IV) oxidation attributed to radiolysis should
335 be relatively small given the short time-scale (minutes to hours), but is probably not negligible.
336 Total U concentration, rather than U species, was measured in effluent solutions, and therefore
337 we do not have direct knowledge of U aqueous speciation. In the absence of an oxidant
338 (experiments with NaCl solution), it is possible that U release from UO_2(s) surfaces was a
339 combination of dissolved U(IV) and U(VI) species from reaction with oxidants, such as H_2O_2
340 from the radiolysis of water.

341 In anaerobic systems, the abiotic oxidation of UO_2(s) coupled to nitrate reduction is
342 slow[16,18]. Slightly faster UO_2(s) dissolution rates in the presence of dissolved nitrate compared
343 to experiments with NaCl only may be explained by adsorption of nitrate and direct oxidation
344 of UO_2(s), or radiolysis of nitrate to produce nitrite or other reactive NO_x species[60] at the UO_2(s)
345 surface and acceleration of U(IV) oxidation and U(V) or U(VI) release. Both nitrate and
346 chloride form weak complexes in solution with U(IV) of similar thermodynamic affinity (Table
347 S5), and thus their sorption behavior on UO_2(s) is probably similar. Dissolution experiments
348 showed no dependence on nitrate concentration, and XANES analysis of one abiotic sample
349 (A5) reacted with 20 mM nitrate (and no nitrite or NaCl) showed only U(IV) in the solids after

reaction. These observations indicate that direct U oxidation by nitrate, or radiolysis to nitrite (or other reduced N species) followed by U oxidation, is a weak effect. Aqueous complexes of U(IV) with nitrate or chloride are orders-of-magnitude weaker than U(IV)-hydroxo complexes (Table S5), and the latter are expected to be the stable U(IV) species detaching from the UO_2(s) surface in the absence of U oxidation[39]. Slightly faster UO_2(s) dissolution rates with nitrate compared to chloride at similar concentrations are indirect evidence of surface oxidative dissolution because, if detachment of U from the surface through formation of U(IV)-hydroxo complexes were rate-determining, then observed rates in the presence of either chloride or nitrate at constant pH should overlap. If nitrate adsorption accelerates surface oxidation of U(IV) to U(VI) by either of the mechanisms described above, then detachment of uranyl-hydroxo complexes would be rate-determining, and rates would be higher with more surface oxidation. This mechanism relies on surface enhancement of UO_2(s) dissolution, since the concentration of nitrate (1-20 mM) would be too low for radiolysis reactions to be important in bulk solution.[60] The lack of dependence of the dissolution rate on nitrate concentration is consistent with a surface mechanism that involves nitrate adsorption and detachment of an oxidized U product, perhaps in addition to a U(IV) species, but it is a relatively small effect overall (Figure 4).

Nitrite is a more effective oxidant of U(IV) than nitrate, with dissolution rates in abiotic experiments about 5 to 10 times faster than with nitrate, and similar to average rates measured in biotic experiments (Figure 4). In abiotic experiments with nitrite (± nitrate) or biotic experiments with nitrate, bulk XANES analysis indicated that a low fraction of total U (3-19%) was retained in the column as oxidized U(VI), with higher fractions (up to 32%) observed for experiments of longer duration. These results are consistent with the presence of some adsorbed or surface U(VI), or perhaps some U(V) that is not detectable by XANES[22]. EXAFS analysis showed the lack of formation of an oxidized U(VI) phase (e.g., schoepite) as a surface coating,

375 which was proposed in other studies of UO_2(s) dissolution[22,61], probably due to the short time
376 scale of our experiments (3-5 days) and relatively slow rates of oxidation, or to the mobilization
377 of desorbed U(VI) species in our flow-through columns. The presence of surface U(VI) in
378 similar proportions in both biotic and abiotic experiments, and the observation that dissolution
379 rates are independent of nitrite concentration, support the hypothesis that the rate-determining
380 step at steady-state release is detachment of oxidized U(VI) from the UO_2(s) surface rather than
381 electron transfer or ion diffusion.

382 In *T. denitrificans,* two membrane-associated, *c*-type cytochromes that are involved in
383 anaerobic U(IV) oxidation were previously identified by Beller et al.[38] The similarity between
384 abiotic experiments with nitrite and biotic experiments with nitrate suggests a close interplay
385 of molecular and enzymatic electron transfer among N and U species. In experiments with *T.*
386 *denitrificans*, it is likely that U(IV) oxidation takes place *via* both the membrane-associated *c*-
387 type cytochrome (enzymatic) pathway previously described[38], and as a secondary abiotic
388 reaction with denitrification intermediates (e.g., nitrite, nitric oxide, nitrous oxide), with the
389 relative proportions of each pathway varying during the experiments. Steady-state rates were
390 estimated from U effluent concentrations towards the end of the experiments after formation of
391 a fraction of U(VI) in the columns (detected by XAS), presumably as a surface species. The
392 observed biotic and nitrite-catalyzed rates in this study are similar to dissolution rates measured
393 for the uranyl oxyhydroxide minerals Na-compreignacite ($R = 3.71 \cdot 10^{-13}$ mol m^{-2} s^{-1}; $\log R = -$
394 12.43) and K-compreignacite ($R = 1.28 \cdot 10^{-13}$ mol m^{-2} s^{-1}; $\log R = -12.89$) at pH 6.6-6.8 and low
395 dissolved carbonate concentrations using a similar experimental set-up of flow-through, quartz-
396 packed columns[62]. In that study, the authors noted that despite ion exchange during the
397 experiments, mineral dissolution rates were relatively insensitive to the interlayer cation
398 identity because the molecular-scale rate-determining step was the detachment of the uranyl
399 group from the mineral surface[63,64,65]. The similarity in observed rates may indicate that uranyl

detachment from the surface is a common controlling mechanism when a steady-state surface concentration of oxidized U is achieved.

Environmental Implications. Reduced U forms, including crystalline uraninite and amorphous U(IV)-oxides, are susceptible to reoxidation[5,20,66,14] and pose challenges to long-term stability of U(IV) in aquifer sites remediated by reductive immobilization. This is mainly due to groundwater influxes continuously introducing U(IV)-oxidizing species (e.g., O_2, NO_3^-, and NO_2^-). Of these species, one of the most relevant for contaminated subsurface environments at nuclear sites is nitrate, a common co-contaminant with uranium at U.S. Department of Energy (DOE) sites[16,24,67]. Nitrate inputs to aquifers have been observed to threaten U(IV) stability, not only at nuclear sites, but also in areas unaffected by nuclear activities[28,29,30]. Although O_2 is a stronger oxidant[22,68,69] , this study showed that oxidative dissolution of reduced U under anaerobic conditions is accelerated substantially by the addition of dissolved nitrite, or by a denitrifying chemolithoautotroph, *T. denitrificans,* that is pervasive in aquifers and soils, together with nitrate (log rates from -13.94 to -12.31 mol m^{-2} s^{-1}). *T. denitrificans* or closely related species are prominent members of microbial communities at some uranium-contaminated sites[18,70,71,37]. In the environment, nitrite is a reactive, transient species that is produced primarily by microbial reduction of nitrate. In anaerobic aquifers with relatively high nitrate concentrations, the production of nitrite by nitrate-reducing microorganisms, together with enzymatically catalyzed, nitrate-dependent U(IV) oxidation, are likely dual processes by which reduced U solids may be oxidized and mobilized in the aqueous phase. Steady-state dissolution rates appear to be controlled by U(VI) detachment and release to solution as the rate-determining step once surface-oxidized U(VI) species form on UO_2(s) particle surfaces. Initial dissolution rates, which were not considered in this study and may involve release of both U(IV) and U(VI) species, are more likely to be controlled by electron-transfer processes involving microbial enzymatic oxidation of U(IV) and reduction of nitrate,

and by surface adsorption, complexation, and U(IV) oxidation[22,41]. If a steady-state concentration of surface-oxidized U species develops, the overall rate of U release to solution may be similar to that of uranyl oxyhydroxide minerals, and thus would be strongly controlled by pH and dissolved carbonate concentration[62]. In natural groundwater, the presence of dissolved inorganic carbon, an ubiquitous component of groundwater, would promote the removal of surface U(VI) by formation of uranyl-carbonate complexes[22,62], increasing U mobility. Our findings improve our understanding of U(IV) oxidation and remobilization processes in nitrate-contaminated aquifers, and will contribute to predictive modeling and strategies for mitigating U mobilization.

ASSOCIATED CONTENT

Supporting Information

Supporting information is available free of charge via the Internet at http://pubs.acs.org.

Methods; Figure S1, XRD biogenic UO_2(s); Figure S2, SEM and TEM images of biogenic UO_2(s); Figure S3, Log UO_2(s) dissolution rate vs. influent nitrite concentration; Figure S4, nitrite effluent concentrations in the experiments performed in the presence of *T. denitrificans* and nitrate; Figure S5, Uranium L_{III} XANES spectra and LCF of the first derivative of spectra; Table S1, experimental conditions for dissolution experiments and solid analyses performed before and after reaction; Table S2, experimental conditions and UO_2(s) dissolution rates based on steady-state values; Table S3, uranium L_{III} EXAFS fit results of biogenic UO_2(s) spectra; Table S4, uranium L_{III} EXAFS fit results of reference compound spectra; Table S5, stability constants for selected U(IV) complexes.

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Notes

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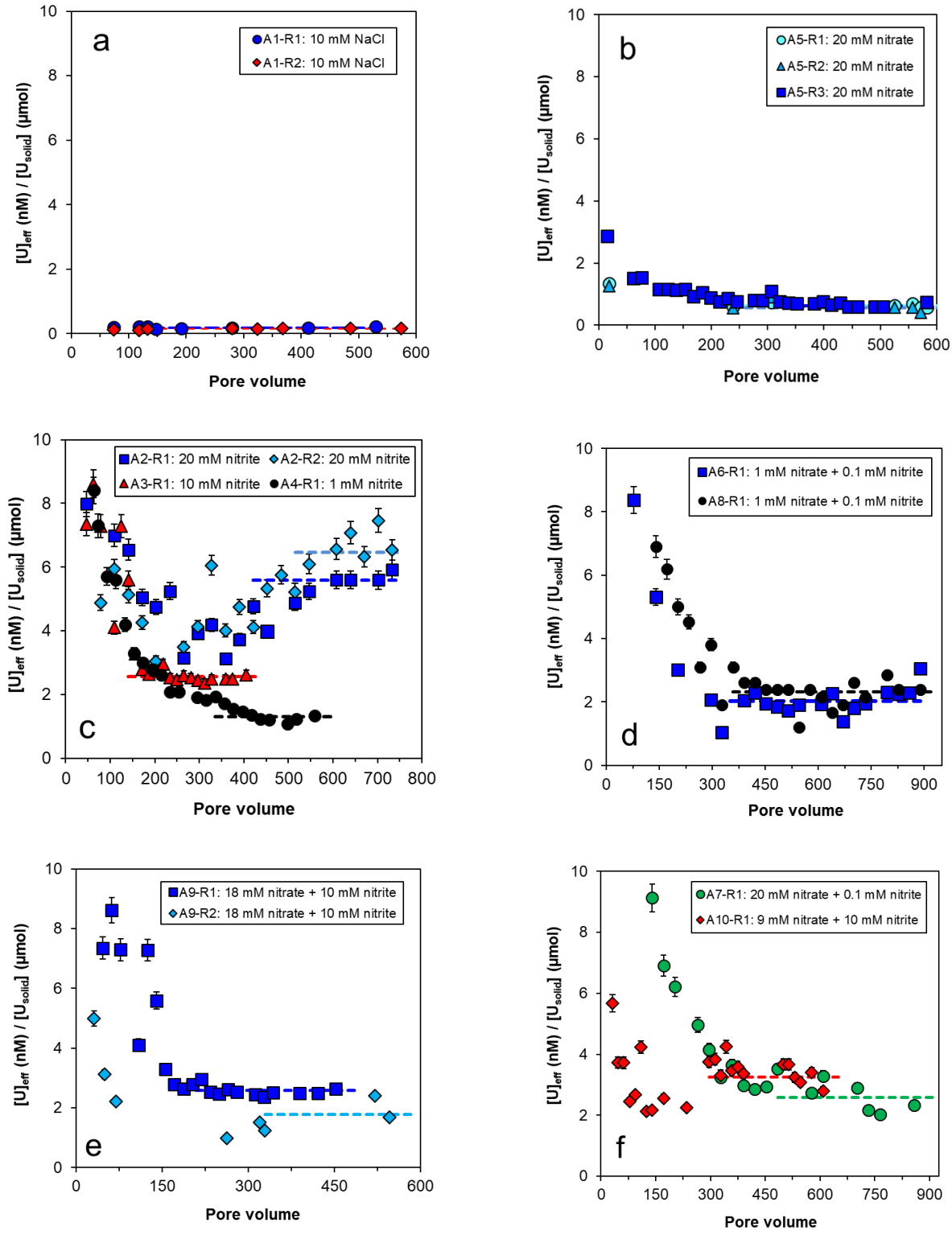

715 **Figure 1.** Uranium released in column experiments normalized by the initial U mass as a
716 function of pore volume (a) without oxidant; (b) with NO_3^-; (c) with NO_2^-; and (d-f) with a
717 mixture of NO_3^- and NO_2^-. Error bars correspond to the analytical uncertainty in measured U
718 concentration. Dashed lines represent the steady-state values for each experiment used to
719 calculate U dissolution rate. Initial flush-out of high U is not shown.

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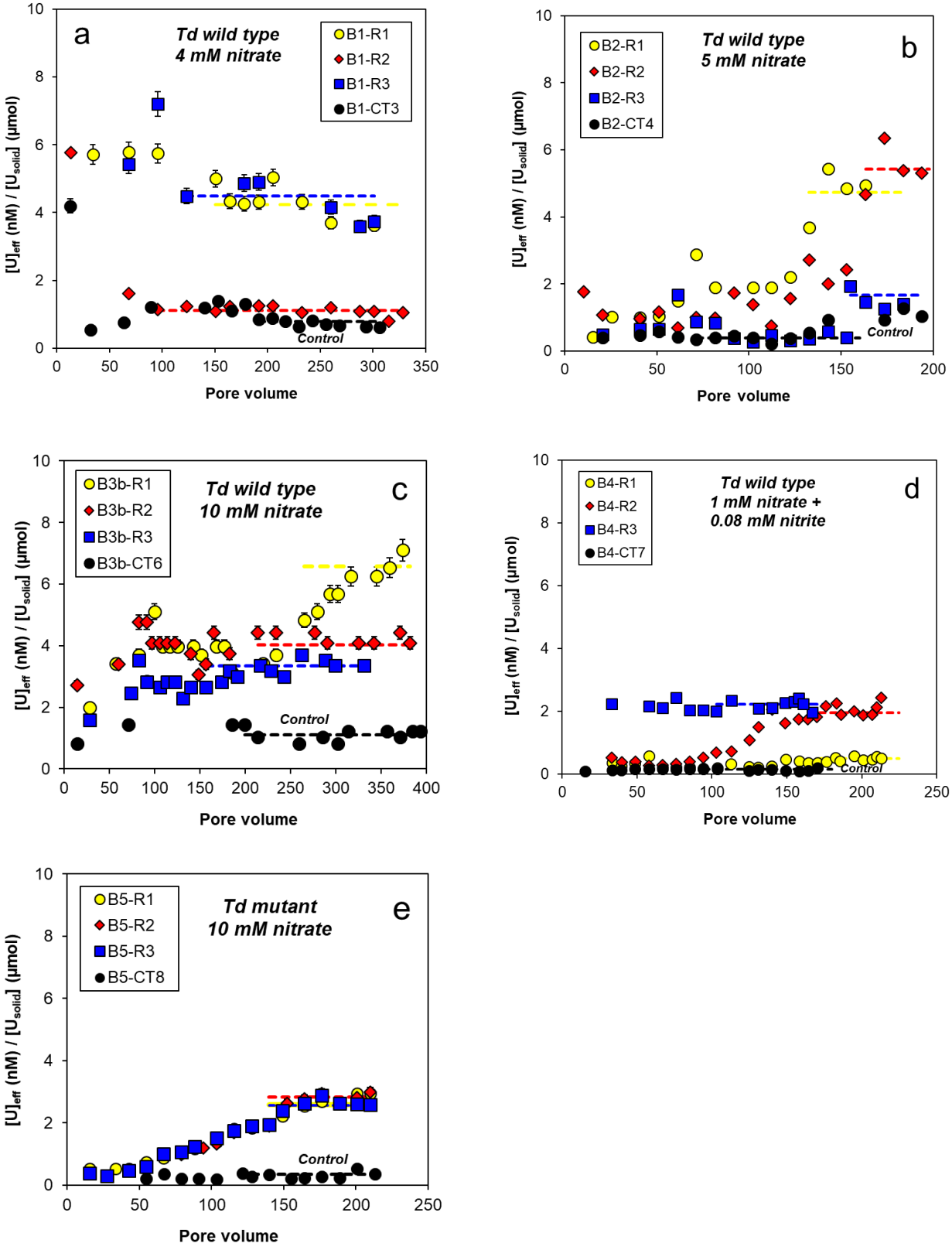
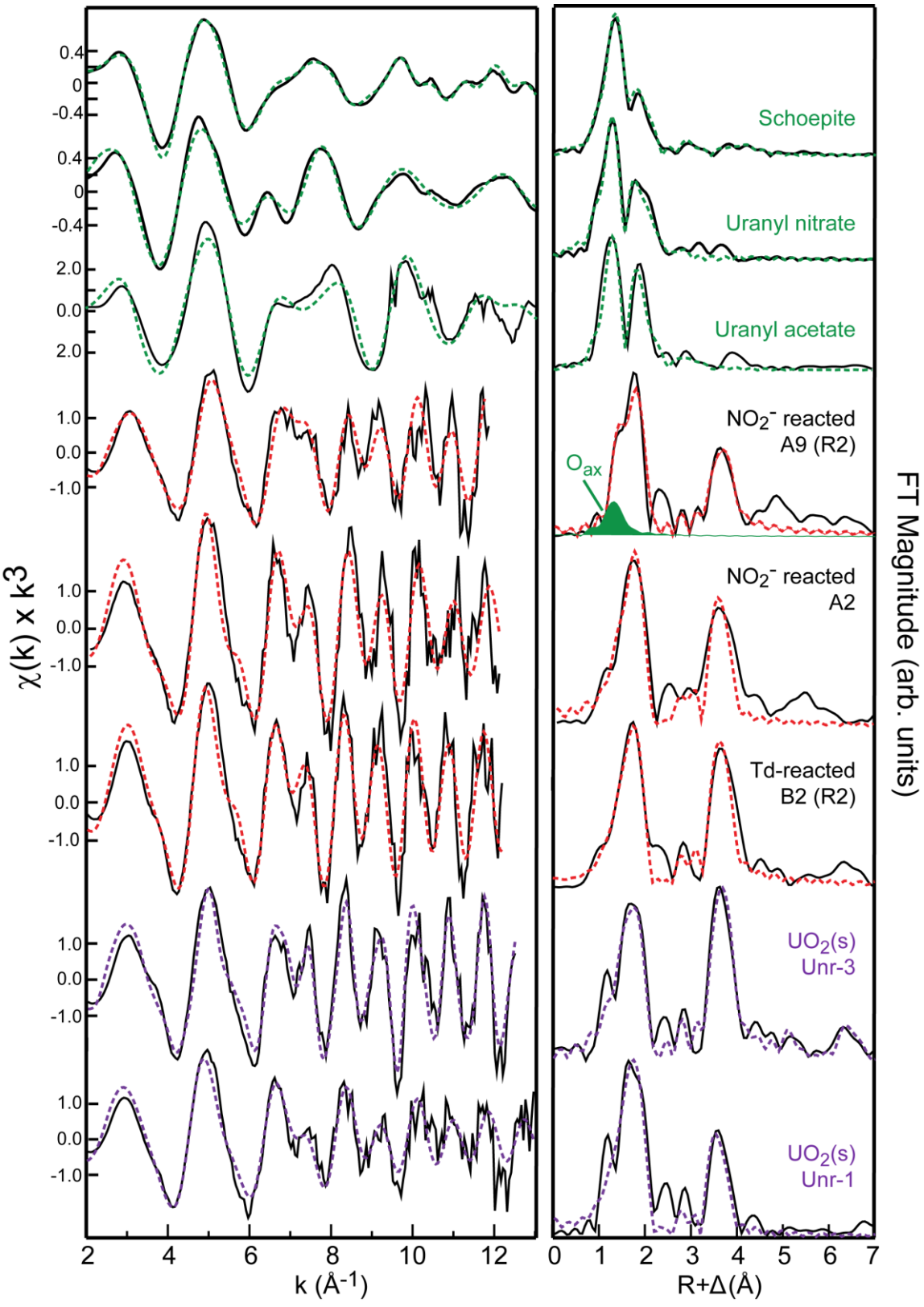


721 **Figure 2.** Uranium released in column experiments normalized by the initial U mass as a function
722 of pore volume in the presence of wild-type *T. denitrificans* and (a) 4 mM NO_3^-; (b) 5 mM NO_3^- (c)
723 10 mM NO_3^-; (d) 1 mM NO_3^- and 0.08 mM NO_2^-; and (e) 10 mM NO_3^- and a *T. denitrificans* mutant
724 strain (*Tbd_0187*::*kan*) ~50% defective in U(IV) oxidation compared to simultaneous abiotic control
725 experiments without *T. denitrificans* (black circles). Error bars correspond to the analytical

uncertainty in measured U concentration. Dashed lines represent the steady-state values for each experiment used to calculate U dissolution rate. Initial flush-out of high U is not shown.



729

730 **Figure 3.** Uranium L_{III} EXAFS spectra and Fourier Transforms (FT) of unreacted UO_2(s) samples
731 (Unr-1, Unr-3), reacted samples (B2(R2), A2, A9(R2)), and U(VI) reference compounds (uranyl
732 acetate, uranyl nitrate, schoepite). Overlapping dashed lines represent the non-linear least-squares
733 shell-by-shell fits (numerical fit results shown in Tables S4 and S5).

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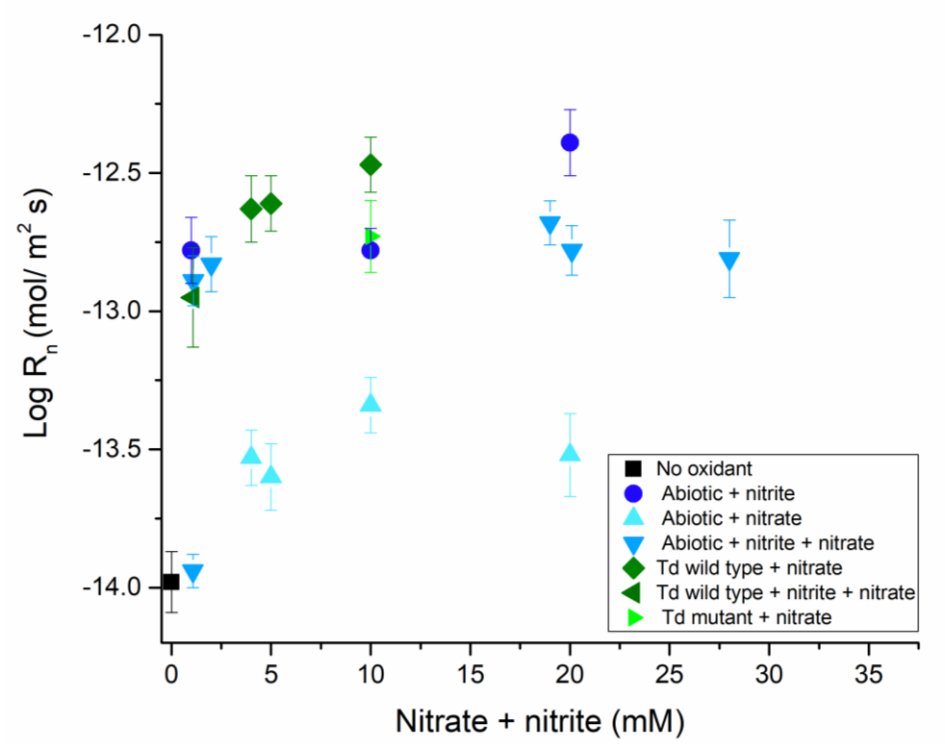

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736

737 **Figure 4.** Log UO_2(s) dissolution rate vs. oxidant concentration (no oxidant, black squares;
738 NO_2^-, NO_3^-, or a mixture of NO_3^- and NO_2^-) in the absence (abiotic experiments, blue symbols)
739 or presence of *T. denitrificans* (Td wild type and Td mutant, green symbols). Error was
740 estimated by propagation of experimental uncertainties and dominated by the variation in
741 effluent U concentration at steady state ($[U]_{ss} \leq 15\%$), and the uncertainty of the U mass
742 measurements (0.5-25%).

Table 1. Experiment type, influent solutions, and UO_2(s) dissolution rates obtained in this work and previous studies under anaerobic conditions.

UO_2(s)	type of experiment	pH	influent electrolyte	Rate (mol m^{-2} s^{-1})	log Rate	experiment	replicates	reference
bio-UO_2	flow-through column	7.0-7.3	10 mM NaCl	$1.05 \cdot 10^{-14} \pm 2.67 \cdot 10^{-15}$	-13.98±0.11	A1	R1, R2	*This work*
			abiotic, 1 mM NO_2^-	$1.66 \cdot 10^{-13} \pm 3.81 \cdot 10^{-14}$	-12.78±0.10	A4	R1	
			abiotic, 10 mM NO_2^-	$1.64 \cdot 10^{-13} \pm 2.98 \cdot 10^{-14}$	-12.78±0.08	A3	R1	
			abiotic, 20 mM NO_2^-	$4.11 \cdot 10^{-13} \pm 1.11 \cdot 10^{-13}$	-12.39±0.12	A2	R1, R2	
			abiotic, 1 mM NO_3^- + 0.1 mM NO_2^-	$1.30 \cdot 10^{-13} \pm 2.61 \cdot 10^{-14}$	-12.89±0.09	A6	R1	
			abiotic, 1 mM NO_3^- + 1 mM NO_2^-	$1.49 \cdot 10^{-13} \pm 3.33 \cdot 10^{-14}$	-12.83±0.10	A8	R1	
			abiotic, 20 mM NO_3^- + 0.1 mM NO_2^-	$1.65 \cdot 10^{-13} \pm 2.62 \cdot 10^{-14}$	-12.78±0.09	A7	R1	
			abiotic, 9 mM NO_3^- + 10 mM NO_2^-	$2.09 \cdot 10^{-13} \pm 4.09 \cdot 10^{-14}$	-12.67±0.08	A10	R1	
			abiotic, 18 mM NO_3^- + 10 mM NO_2^-	$1.55 \cdot 10^{-13} \pm 4.71 \cdot 10^{-14}$	-12.81±0.14	A9	R1, R2	
			abiotic, 1 mM NO_3^- + 0.08 mM NO_2^-	$1.15 \cdot 10^{-14} \pm 1.60 \cdot 10^{-15}$	-13.94±0.06	B4	CT7	
			abiotic, 4 mM NO_3^-	$2.93 \cdot 10^{-14} \pm 6.70 \cdot 10^{-15}$	-13.53±0.10	B1	CT3	
			abiotic, 5 mM NO_3^-	$2.51 \cdot 10^{-14} \pm 6.68 \cdot 10^{-15}$	-13.60±0.12	B2	CT4	
			abiotic, 10 mM NO_3^-	$4.61 \cdot 10^{-14} \pm 1.40 \cdot 10^{-14}$	-13.34±0.13	B3a, B3b, B5	CT5, CT6, CT8	
			abiotic, 20 mM NO_3^-	$3.02 \cdot 10^{-14} \pm 1.03 \cdot 10^{-14}$	-13.52±0.15	A5	R1, R2, R3	
			Td + 1 mM NO_3^- + 0.08 NO_2^-	$1.12 \cdot 10^{-13} \pm 5.63 \cdot 10^{-14}$	-12.95±0.18	B4	R1, R2, R3	
			Td + 4 mM NO_3^-	$2.35 \cdot 10^{-13} \pm 8.06 \cdot 10^{-14}$	-12.63±0.12	B1	R1, R2, R3	
			Td + 5 mM NO_3^-	$2.44 \cdot 10^{-13} \pm 7.10 \cdot 10^{-14}$	-12.61±0.10	B2	R1, R2, R3	
			Td + 10 mM NO_3^-	$3.41 \cdot 10^{-13} \pm 8.05 \cdot 10^{-14}$	-12.47±0.10	B3b	R1, R2, R3	
			Td mutant + 10 mM NO_3^-	$1.91 \cdot 10^{-13} \pm 5.50 \cdot 10^{-14}$	-12.72±0.13	B5	R1, R2, R3	
synthetic UO_2[a]	flow-through	7-11	8 mM $NaClO_4$	$1.90 \cdot 10^{-12}$	-11.72±0.02			Bruno et al.[39]
synthetic UO_2	flow stirred tank reactor	7.50	10 mM $NaClO_4$	$7.50 \cdot 10^{-13}$	-12.12±0.02			Frazier et al.[42]
bio-UO_2	flow-through	7.60	1 mM HEPES	$9.85 \cdot 10^{-14}$	-13.01			Ulrich et al.[41]
		7.25	1 mM HEPES	$3.42 \cdot 10^{-13}$	-12.47			
		7.67	1 mM HEPES	$1.07 \cdot 10^{-14}$	-13.97			
synthetic UO_2		8.00	1 mM NaNO3, TAPS	$4.03 \cdot 10^{-12}$	-11.39			
		7.70	1 mM HEPES	$5.51 \cdot 10^{-13}$	-12.26			
		7.25	1 mM HEPES	$2.80 \cdot 10^{-13}$	-12.55			
		7.00	-	$2.15 \cdot 10^{-12}$	-11.72±0.24 [b]			

Td: Thiobacillus denitrificans wild type; *Td mutant: Thiobacillus denitrificans* mutant

[a] a thin film was used

[b] calculated at pH 7 from the kinetic rate law obtained by Ulrich et al.[41] under reducing conditions for pH range 3-8

Table 2. Linear combination fit (LCF) results for U L_{III} XANES for a control experiment without oxidant (CT0), and for biogenic UO_2(s) samples after dissolution experiments under abiotic (*A* samples) conditions and in the presence of *T. denitrificans* (*B* samples). End-members used for LCF were biogenic UO_2(s) (unreacted sample Unr-1) for U(IV) and schoepite ($(UO_2)_8O_2(OH)_{12}\cdot 12(H_2O)$) for U(VI).

Sample	Oxidant	Model fit (%)			Goodness-of-fit [a]	Normalized fit (%) [b]	
		U(IV)	*U(VI)*	*Total*		*U(IV)*	*U(VI)*
CT0 [c]	no oxidant	100	0	100	0.002	100	0
A2	20 mM NO_2	77	26	103	0.004	75	25
A5	20 mM NO_3	98	0	98	0.004	100	0
A6	1 mM NO_3 + 0.1 mM NO_2	88	9	98	0.002	90	10
A8	1 mM NO_3 + 1 mM NO_2	89	13	102	0.002	88	12
A9(R2)	18 mM NO_3 + 10 mM NO_2	85	15	100	0.001	85	15
B1 (R1)	4 mM NO_3	76	13	89	0.007	85	15
B1 (R2)	4 mM NO_3	75	29	103	0.018	72	28
B2 (R1)	5 mM NO_3	77	17	94	0.003	82	18
B2 (R2)	5 mM NO_3	78	15	93	0.006	83	17
B3b (R1)	10 mM NO_3	88	9	97	0.004	91	9
B3b (R2)	10 mM NO_3	73	26	98	0.009	74	26

[a] R factor = $\Sigma(\text{data-fit})^2/\Sigma(\text{data})^2$

[b] The percentage of U components is normalized to a sum of 100%.

[c] Batch control experiment with biogenic UO_2(s) and 10 mM sodium acetate (no oxidant).