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Title:

Bioremediation of highly toxic arsenic via carbon-fiber-assisted indirect As(III) oxidation by moderately-thermophilic, acidophilic Fe-oxidizing bacteria [Rivised]

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Abstract

Objective To enable removal of highly toxic As(III) from acidic waters by inducing indirect microbial As(III) oxidation by Fe-oxidizing bacteria via carbon-assisted redox-coupling between As(III) oxidation and Fe^{3+} reduction.

Results Carbon-fiber (CF) was shown to function as an electron-mediator to catalyze chemical (abiotic) redox-coupling between As(III) oxidation and Fe^{3+} reduction. Accordingly, by taking advantage of Fe^{3+} regeneration by Fe-oxidizing bacteria, it was possible to promote oxidative removal of As(III) as ferric arsenate at moderate temperature. This reaction can be of use under the situation where a high-temperature treatment is not immediately available. Arsenic once concentrated as ferric arsenate on carbon-fibers can be collected to undergo phase-transformation to crystalline scorodite as the next re-solubilization/re-crystallization step at a higher temperature (70°C).

Conclusions While extremely acidophilic Fe-oxidizing bacteria are widely found in nature, the As-oxidizing counterparts, especially those grown on moderately-thermophilic and mesophilic temperatures, are hardly known. In this regard, the finding of this study could make a possible introduction of the semi-passive, low-temperature As-treatment using readily available Fe-oxidizing bacteria.

Introduction

Arsenic (As) is the 20th most abundant element in the Earth's crust and is often found as sulfide minerals such as enargite (Cu_3AsS_4), tennantite ($Cu_{12}As_4S_{13}$) and arsenopyrite (FeAsS) (Ehrlich and Newman 2009; Mandal and Suzuki 2002). Since As is dissolved in water as As(III) or As(V) and the former more toxic/mobile than the latter (especially at acidic pH), As(III) is generally first oxidized to As(V) prior to immobilization (Cullen and Reimer 1989; Matschullat 2000). Mining-impacted highly-acidic waters often contain As(III) and its effective remediation approach needs to be developed.

Mineralization of soluble As into scorodite ($Fe^{III}As^{V}O_4 \cdot 2H_2O$) is considered to be one of the ideal approaches for long-term As storage due to the mineral's stability and low Fe demand (Langmuir et al. 2006; Riveros et al. 2001). Chemical scorodite synthesis is generally effective for concentrated As solutions (~hundreds of millimolar) using higher temperatures (mostly 95-160°C) either by hydrothermal methods (e.g. Monhemius and Swash 1999; Demopoulos et al. 1995) or by atmospheric methods (e.g. Filippou and Demopoulos 1997; Singhania et al. 2006; Fujita et al. 2009). On the other hand, use of microbiological reaction enables scorodite crystallization even under thermodynamically less feasible, milder conditions (e.g., lower temperatures, lower As concentrations) (Gonzalez-Contreras 2010, 2012; Okibe et al. 2013, 2014, 2017, 2018; Tanaka and Okibe 2018): e.g. By using the thermophilic, extremely acidophilic archaeon *Acidianus brierleyi* at 70°C, oxidation of As(III) (in the range of 3.3 – 20 mM) and Fe^{2+} were simultaneously progressed to form biogenic scorodite without addition of chemical oxidants. However, under situations where As(III)-contaminated waters cannot be immediately processed at this level of temperature (~70°C), a necessity arises to look for As-oxidizing acidophiles of lower growth temperatures. Among those autotrophic and heterotrophic As-oxidizers reported so far, the majority are neutrophilic bacteria with a few studies on acid-tolerant species such as *Thiomonas* spp. (Cavalca et al. 2013) and unknown acidophilic strains (Nakazawa and Hareyama 2007). Nonetheless, to our knowledge, there is yet no detailed study available on extremely-acidophilic As-oxidizers with moderately-thermophilic or mesophilic growth temperatures, which exhibit robust As(III) oxidation at the concentration of several millimolar.

To overcome unavailability of such useful microbes, this study investigated the possibility to indirectly induce As(III) oxidation via Fe^{2+} oxidation by readily available Fe-oxidizing acidophiles. As an electron-mediating catalytic material, carbon-fiber (CF) was studied. Carbon-fibers contain at least 90% carbon and are generally produced by pyrolysis of an

appropriate precursor, predominantly from polyacrylonitrile. While the use of CF in aircraft and automotive industries are well-known (Minus and Kumar 2005), it is worth investigating their potential applicability in water treatment, owing to their high physical strength and conductivity, as a durable electron-mediating redox catalyst.

Materials and methods

Microorganism

Three moderately-thermophilic, acidophilic Fe-oxidizing strains were used: *Acidimicrobium ferrooxidans* ICP^T (DSM 10331), *Sulfobacillus sibiricus* N1^T (DSM 17363), and *Sulfobacillus thermotolerans* Kr1^T (DSM 17362). The three strains were maintained and pre-grown aerobically at 45°C in heterotrophic basal salts (HBS) media (per liter; 450 mg $(NH_4)_2SO_4$, 50 mg KCl, 50 mg KH_2PO_4, 500 mg $MgSO_4 \cdot 7H_2O$, 14 mg $Ca(NO_3)_2 \cdot 4H_2O$, 142 mg Na_2SO_4: pH 2.0 with H_2SO_4) containing 0.02% yeast extract.

As(III) tolerance during heterotrophic growth (on yeast extract) of Fe-oxidizing strains

Each Fe-oxidizing strain (*Am. ferrooxidans*, *Sb. sibiricus* or *Sb. thermotolerans*) was inoculated (at 1.0 x 10^7 cells/ml) in 100 ml of HBS media (pH 2.0) containing 0.02% yeast extract (in 300 ml flasks). As(III) was added (as $NaAsO_2$) to the media at 0, 1.3, 2.6, 6.5 or 13 mM. The flasks were incubated shaken at 100 rpm, 45°C. Samples were regularly taken to monitor cell density (using bacterial counting chamber). The experiments were conducted in duplicate flasks.

Evaluation of As-oxidizing ability of Fe-oxidizing strains

Each Fe-oxidizing strain (*Am. ferrooxidans*, *Sb. sibiricus* or *Sb. thermotolerans*) was pre-grown, harvested at the late-exponential phase, washed, and re-suspended (at 1.0 x 10^8 cells/ml in order to ease the detection of As(III) oxidation) in 200 ml of fresh HBS medium (pH 2.0) containing 0.02% yeast extract, 6.5 mM As(III), with or without 9 mM Fe^{2+} (as $FeSO_4 \cdot 7H_2O$) (in 500 ml flasks). The flasks were incubated shaken at 100 rpm, 45°C. Samples were regularly taken to monitor cell density (using bacterial counting chamber), pH, Eh (vs. SHE) and concentrations of As(III) (Nano-Band Explorer stripping voltammetry; GL Sciences), Fe(II) (*o*-phenanthroline method), As_{total} and Fe_{total} (ICP-OES; PerkinElmer Optima8300). The experiments were conducted in duplicate flasks.

Evaluation of chemical (abiotic) Fe- and As-oxidizing capability of CF

Milled CF (CFMP-30X, Nippon Polymer; 1% (w/v)) was added into 200 ml of HBS medium (pH 2.0) containing 0.02% yeast extract (in 500 ml flasks).

Cell-free controls: Either 9 mM Fe^{2+}, 9 mM Fe^{3+}, 6.5 mM As(III), 6.5 mM As(V) or 9 mM Fe^{2+} plus 6.5 mM As(III) was added to each flask to test the chemical oxidation/absorption effect of CF on individual ions.

Microbial cultures: Either 9 mM Fe^{2+}, 6.5 mM As(III) or 9 mM Fe^{2+} plus 6.5 mM As(III) was added to each flask to test the indirect CF-assisted As(III) oxidation via microbial Fe^{2+} oxidation. In Fe^{2+}-containing media, *Am. ferrooxidans* cells were inoculated at 1.0 x 10^7 cells/ml. In Fe^{2+}-free media, cells were inoculated at 1.0 x 10^8 cells/ml, as no growth was expected during incubation.

Solid analysis

Precipitates on the CF surface were collected and freeze-dried overnight for scanning electron microscope observation (SEM; KEYENCE VE-9800; Vacuum Device MSP-1S for Au-Pd sputtering) and X-ray diffraction analysis (XRD; Rigaku Ultima IV; CuKα 20 mA, 40 kV). To quantify the elemental composition of the precipitates, the sample was embedded into resin, polished and measured by SEM-EDX (HITACHI SU-70; 6.0 kV; 30 sec for each point) using the Phi-Rho-Z method (Packwood and Brown 1981). The sample was also mixed with boron nitride to form tablets, to perform X-Ray near edge structure (XANES) analysis. The Fe K-edge XANES spectra (fluorescence mode; 6800–7200 eV) and As K-edge XANES spectra (fluorescence mode; 11600–12000 eV) at SAGA-LS (1.4 GeV, 75.6 m), using standards of $FeSO_4 \cdot 7H_2O$ (Wako chemicals; No. 7782-63-0), Fe_2O_3 (Wako chemicals; No. 1309-37-1), $NaAsO_2$ (Wako chemicals; No. 7784-46-5), KH_2AsO_4 (Wako chemicals; No. 7784-41-0), chemically synthesized $FeAsO_4 \cdot 2H_2O$ (Tanaka and Okibe 2018) and bacterial cells.

Phase transformation of amorphous As-precipitates into crystalline scorodite

Carbon-fibers were repeatedly used according to the reaction shown in Fig. 3 (Fe^{2+} + As(III) with cells; 45°C) until 77 mg-Fe and 68 mg-As (per g-CF) were accumulated as amorphous Fe^{III}-As^{V} precipitates. In order to dissolve this precipitate to produce more

129 concentrated As- and Fe-containing solution (to facilitate scorodite mineralization), 1.4 g (dry-
130 weight) of the CF-precipitate mixture was put into 40 mL acidic water (pH 1.0 with H_2SO_4) in
131 100 mL flasks, incubated shaken at 100 rpm at ambient temperature (the complete dissolution of
132 the precipitate would give 41 mM Fe and 27 mM As in the 40 mL solution). At 14 hours, CFs
133 were removed from the leachate by filtration and scorodite-seeds were fed at 3% (w/v) instead.
134 The leachate was then further incubated at 70°C. In the control experiment, CF was not removed
135 at 14 h.
136
137
138 **Results and discussion**
139 **As(III) tolerance of Fe-oxidizing strains during heterotrophic (Fe^{2+}-free) growth.**
140 The highest cell densities were recorded with the heterotrophic Fe-oxidizer *Am.*
141 *ferrooxidans* (Clark and Norris 1996), compared to the other two heterotrophic Fe-/S-oxidizing
142 *Sulfobacillus* spp. (Melamud et al. 2003; Bogdanova et al., 2006): Its growth was not much
143 affected by the presence of up to 2.6 mM As(III), but was significantly inhibited by 6.5 mM
144 As(III) (Fig. 1a). In the case of *Sb. sibiricus*, 6.5 mM As(III) completely inhibited its growth (Fig.
145 1b). *Sb. thermotolerans* showed relatively robust growth in the presence of elevated
146 concentrations of As(III), even at 13 mM As(III) (Fig. 1c). Nonetheless, the growth of *Am.*
147 *ferrooxidans* at 6.5 mM As(III) was still comparable (Fig. 1a) to the other two *Sulfobacillus* spp.
148 without As(III) (Fig. 1b, c). Some acidophiles were shown to possess the arsenic resistance
149 mechanism encoded by the *ars* operon (Dopson et al. 2003) and its presence was also suggested
150 in *Am. ferrooxidans* based on the complete genome sequence (Clum et al. 2009).
151 **[Fig. 1 inserted here]**
152
153 **Evaluation of As-oxidizing ability in Fe-oxidizing strains**
154 In order to ease the detection of microbial As(III) oxidation activity, the initial cell
155 density was set 10-times higher in this test (at 1.0×10^8 cells/ml) (Fig. 2). In Fe^{2+}-free media, no
156 further cell growth was observed (Fig. 2e) and no obvious microbial As(III) oxidation was noted
157 (Fig. 2d). In Fe^{2+}-containing media, although rapid microbial Fe^{2+} oxidation (Fig. 2a) resulted in
158 further cell growth (Fig. 2c), no As(III) oxidation was detected (Fig. 2b). Unlike some thermo-
159 acidophilic Fe-oxidizing archaea (e.g., *Ac. brierleyi* and some *Sulfolobus* spp.; Okibe et al. 2014;

Vega-Hernandez et al. 2019) the obvious As(III) oxidase activity was detected in neither of moderate-thermophiles tested in this study (at least under the conditions tested): i.e., in addition to the three Fe-oxidizing strains mentioned, no apparent As-oxidizing activity was observed in others including *Ferroplasma acidiphilum* Y (DSM 12658), *Acidiplasma* Fv-Ap and *Acidithiobacillus caldus* KU (DSM8584) (data not shown). So far in majority of cases, As(III) oxidase activity is known in neutrophiles (Cavalca et al. 2013), while there are a few cases with acid-tolerant species such as *Thiomonas* sp. and *Acidiphilium* sp. (Battaglia-Brunet et al. 2011; Wakao et al. 1994). However, when *Th. cuprina* Hö5 (DSM 5495T) and *Ap. multivorum* AIU301 (JCM 8867) were tested under highly-acidic condition (pH 2.0), their growth and As(III) oxidation were completely inhibited (Okibe et al. unpublished data). With regard to extreme acidophiles, neither mesophilic Fe-oxidizer *At. ferrooxidans* nor moderately-thermophilic mixed cultures were shown to exhibit As(III) oxidation (Barrett et al. 1993). Overall, the results from previous and present studies indicate that the existence of As-oxidizing ability in moderately-thermophilic and mesophilic acidophiles is yet largely unknown.

Hence, although no direct microbial As(III) oxidation was possible using these moderate thermophiles, *Am. ferrooxidans* was selected for further studies owing to its robust growth and Fe-oxidizing ability in acidic As(III) solution. The observation that a mere generation of Fe^{3+} did not induce As(III) oxidation indicates the presence of a kinetic restriction in the reaction shown in Eq. 1. Although Eq. 1 is thermodynamically favored according to the standard redox potentials shown in Eqs. 2-3, such a kinetic restriction needs to be overcome by the addition of an electron-mediating catalyst. Kamde et al. (2018) reported that As(III) oxidation was induced via microbial Fe^{2+} oxidation (by *Acidothiobacillus ferrooxidans*) without using such a catalyst: In their study, however, a highly excessive amount of Fe^{2+} (2 g/L; 36 mM) was added to trigger oxidation of a small amount of As(III) (5 mg/L; 0.07 mM), with the final product of jarosite and schwertmannite. Nonetheless, since our study targets almost equivalent molar amounts of As and Fe, the necessity of an effective electron-mediator is emphasized.

$$H_3As^{III}O_3 + 2Fe^{3+} + H_2O \rightarrow H_2As^{V}O_4^- + 2Fe^{2+} + 3H^+ \quad \text{[Eq. 1]}$$

$$Fe^{3+} + e^- \rightarrow Fe^{2+} \quad (E_0' = 771\ \text{mV}) \quad \text{[Eq. 2]}$$

$$H_3As^{V}O_4 + 2H^+ + 2e^- \rightarrow H_3As^{III}O_3 + H_2O \quad (E_0' = 560\ \text{mV}) \quad \text{[Eq. 3]}$$

[Fig. 2 inserted here]

Carbon-fiber-assisted As(III) oxidation coupled with Fe^{3+} reduction in microbial cultures

In cell-free controls:

When only Fe^{2+} ions were initially added to CF, nearly a half were oxidized to Fe^{3+} by day 1 (Fig. 3a), suggesting that CF naturally possesses chemical catalytic effect for Fe^{2+} oxidation (using atmospheric O_2 as e-donor). While it was not evident from the Fe_{total} concentration profile (Fig. 3b), a tiny portion of the resultant Fe^{3+} ions (but not original Fe^{2+} ions) were found to be adsorbed onto the CF surface (Fig. 3b), according to the XANES analysis (Fig. 4a-4). No precipitate deposition on the CF surface was noted by SEM observation (data not shown). While when only As(III) ions were initially added to CF, CF displayed neither obvious chemical As-oxidizing effect (Fig. 3c) nor As adsorption effect (Fig. 3d). In fact, the XANES analysis did not detect the presence of As on the CF surface (Fig. 4b-5).

When Fe^{2+} and As(III) ions were simultaneously added to CF, nearly a half of Fe^{2+} ions were chemically oxidized by day 1 (Fig. 1a). Although not clearly seen from the Fe_{total} concentration change (Fig. 3b), an aliquot of generated Fe^{3+} were immobilized onto the CF surface based on the XANES analysis (Fig. 4a-6). Under this condition, no major changes in the concentration of As(III) and As_{total} were noticed from Fig. 3c and 3d, respectively. A separate experiment showed that As(V) ions do not absorb onto the CF surface without Fe addition (data not shown). Therefore, it was suggested from the XANES result that a small fraction of As(III) was oxidized to As(V) and co-precipitated with Fe^{3+} on the CF surface (Fig. 4b-6), while the formation of precipitates was not visible by SEM (data not shown). Lastly, when Fe was initially added as Fe^{3+} together with As(III), Fe^{2+} ions appeared and gradually increased (Fig. 3a), coincided with a gradual decrease in the As(III) concentration (Fig. 3c). This indicates that As(III) oxidation was facilitated on the CF surface via chemical redox-coupling with Fe^{3+} reduction. A similar observation was made by Barrett et al. (1993), wherein a kinetic restriction of As(III) oxidation coupled with Fe^{3+} reduction was overcome by the presence of semi-conductive pyrite mineral. Oxidation of 3.3 mM As(III) to As(V) (Fig. 3c) was accompanied with "apparent" reduction of 3 mM Fe^{3+} to Fe^{2+} (Fig. 3a), although theoretically, 6.6 mM of Fe^{3+} needs to be reduced to Fe^{2+} (according to Eq. 1). This difference indicates the occurrence of Fe-cycling (i.e., chemical Fe^{2+} oxidation by atmospheric O_2 [Eq. 4] and Fe^{3+} reduction coupled with As(III) oxidation [Eq. 1]) in the system. Generation of As(V) in the presence of Fe^{3+} allowed removal of

222 approx. 2 mM Fe (22%; Fig. 3b) and 1.5 mM As (23%; Fig. 3d) as Fe^{III}-As^{V} precipitates (Fig. 4a-
223 6; Fig.4b-6) as visualized by SEM (Fig. 5e).
224
225 $4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$ (abiotic) [Eq. 4]
226
227 In microbial cultures:
228 When only Fe^{2+} ions were initially added to CF, oxidation of Fe^{2+} was promptly
229 completed by day 1 accompanied by cell growth (Fig. 3e), after which Fe^{3+} predominated the Fe
230 species owing to microbial activity (Fig. 3a). This led to the removal of about 60% of Fe by day
231 8 (Fig. 3b) on the CF surface in the form of Fe^{III}-precipitates (Fig. 4a-1; Fig. 5b). When only
232 As(III) ions were initially added to CF, about 40% of As(III) was unexpectedly oxidized to As(V)
233 (Fig. 3c). Since neither microbial As-oxidizing ability of *Am. ferrooxidans* (Fig. 2) nor the
234 chemical (abiotic) catalytic activity of CF for As(III) oxidation was clearly noted (cell-free
235 controls in Fig. 3), it was not expected that As(III) is oxidized to As(V) on the CF surface in the
236 presence of cells. It can be hypothesized that *Am. ferrooxidans* originally possesses a negligible
237 extent of As-oxidizing activity (possibly via a negligible amount of carryover Fe^{3+} on the cell
238 surface, but not clearly detectable under the conditions tested), which can be amplified and
239 visualized by the presence of CF. However, this hypothesis must be validated by further detailed
240 experiments. Although not clearly visible from the As_{total} concentration change (Fig. 3d), an
241 aliquot of the resultant As(V) ions were shown to be adsorbed onto the CF surface according to
242 the XANES result (Fig. 4b-2).
243 When Fe^{2+} and As(III) ions were simultaneously added, Fe^{2+} oxidation was rapidly
244 completed by day 1 (Fig. 3a). As(III) oxidation readily proceeded via Fe^{3+} reduction (Fig. 3c),
245 owing to continuous Fe^{3+} regeneration by microbes (Fig. 3a) according to Eq. 5. As a result, about
246 70% Fe (6.5 mM; Fig. 3b) and 60% As (4 mM; Fig. 3d) were effectively removed by day 8 in the
247 form of Fe^{III}-As^{V} precipitates (Fig. 4a-3; b-3) as observed by SEM (Fig. 5c). The precipitate was
248 amorphous (by XRD analysis) with the average elemental composition of As: Fe: O = 1: 1.8 : 5.4
249 (Fig. 6). In our parallel tests started with double amount of Fe^{2+} (18 mM) and As(III) (13 mM),
250 both ions were completely oxidized on the 1% CF surface (data not shown). Therefore, the use of
251 1% CF in unlikely the rate-limiting factor in this study. Rather, the absolute amount of Fe^{3+} may
252 affect the effectiveness of the coupling reaction. However, further investigations are needed to

clarify this. When Fe^{3+}, instead of Fe^{2+}, was initially added together with As(III), the trends in Fe^{2+}/Fe_{total} and As(III)/As_{total} concentrations were identical to those observed in the cell-free counterpart. The initial planktonic-cell count was dramatically decreased (Fig. 3e), likely due to the lack of energy source as well as cell attachment/encrustation on CF via formation of Fe^{III}-As^{V} precipitates (Fig. 5d). Since the initial cell number was unchanged when cells were simply mixed with CF without the addition of Fe and As (data not shown), it is suggested that deposition of amorphous Fe^{III}-As^{V} precipitates has a role to 'glue' cells onto the CF surface. The later slight recovery of planktonic cell counts (after day 3; Fig. 3e) may be due to a gradual supply of Fe^{2+} to the cells.

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \qquad \text{(biotic)} \qquad \text{[Eq. 5]}$$

The above results can be summarized as follows:

(i) Regardless of the presence of Fe^{3+}, no As(III) oxidation was observed in catalyst-free bulk solution (neither abiotic nor biotic).

(ii) The effect of CF catalyst on individual ion (Fe^{2+} or As(III)) was different: Abiotic Fe^{2+} oxidation was catalyzed but to a limited extent. Abiotic As(III) oxidation, on the other hand, was not observed.

(iii) In solutions containing both Fe^{3+} and As(III), abiotic As(III) oxidation proceeded via Fe^{3+} reduction on the CF catalyst. This led to the As removal but to a limited extent. The presence of Fe-oxidizing cells unaffected this trend.

(iv) Continuous regeneration of Fe^{3+} by Fe-oxidizing cells were essential to facilitate As(III) oxidation on the CF catalyst. This enabled effective As removal as Fe^{III}-As^{V} precipitate.

[Fig. 3, 4, 5, 6 inserted here]

Phase transformation of amorphous As-precipitates into crystalline scorodite

Carbon-fibers coated with amorphous Fe^{III}-As^{V} precipitates (Fig. 7c-1) were transferred into a smaller volume of acidic water (pH 1.0) and incubated at ambient temperature to temporarily resolubilize Fe^{3+} and As(V) to produce the 4-times concentrated solution. The theoretical peak concentration (upon complete solubilization) of 41 mM Fe and 27 mM As were

284 expected, but were not visible in Fig. 7a and b, respectively, due to the simultaneous occurrence
285 of re-solubilization and re-crystallization. When CFs were replaced with scorodite-seeds and
286 incubation temperature increased to 70°C at 14 h, the final As removal was 91% at 170 h.
287 Chemically-synthesized scorodite-seed crystals (Fig. 7c-2) became evenly coated with newly
288 generated secondary layers (Fig. 7c-4). In the control test, CFs were not pulled out at 14 h (no
289 seeds added) but the incubation temperature raised to 70°C. In this case, Fe^{3+} and As(V) quickly
290 re-crystallized onto the CF surface to form riziform particles similar to biogenic scorodite
291 particles observed in our previous studies (Okibe et al. 2014). In either case, the products were
292 identified as scorodite as shown in Fig. 8. During the phase transformation from amorphous
293 precipitate to crystalline scorodite, the ratio of Fe/As was reduced from 1.8 (according to the
294 EPMA analysis; Fig. 6) to 1.3 (calculated from liquid analysis data; Fig. 7ab).
295 A preliminary investigation on CF-assisted As(III) oxidation (at 45°C) by Fukano et al.
296 (2015), was followed by studies employing the concept of this carbon-assisted indirect microbial
297 As(III) oxidation using activated carbon (AC) both at a lower (45°C; Hotta et al. 2017) and a
298 higher (65°C; Vega-Hernandez et al. 2019) temperature. Amorphous Fe^{3+}/As(V)-precipitates
299 generally showed high affinity to the carbon surface as observed with CFs in this study (Fig. 5c;
300 Fig.6; Fig. 7c-1) and also with ACs (Okibe et al. unpublished data). Due to the structural
301 differences between CF and AC (i.e., the latter with higher porosity and surface area; Manocha
302 2003), amorphous Fe^{3+}/As(V)-precipitates were difficult to re-solubilize from ACs (okibe et al.,
303 unpublished data). Therefore, in the case that carbon-free scorodite minerals are to be produced
304 for the sake of waste volume reduction (as well as carbon materials are to be recycled), the use of
305 CF rather than AC may be more favorable. Also, due to their high physical strength (Minus and
306 Kumar 2005), deformation of CFs was not noticed by SEM observation after days of vigorous
307 shaking, while ACs seemed to be less physically resistant.
308 In conclusion, based on the overall findings, the following As-removal procedure can
309 be proposed (Fig. 9): Under the situations where the on-site high-temperature scorodite
310 production is not immediately feasible, or direct scorodite crystallization is infeasible due to the
311 low As(III)-contamination level on site, the first As-removal can be realized as amorphous Fe^{III}-
312 As^{V} precipitates, using a combination of electron-mediating carbon material and locally available
313 Fe-oxidizing bacteria. This step can be done at moderate temperatures (also at mesophilic
314 temperatures; Okibe et al. unpublished data) as a semi-passive, first on-site treatment. For safer

315 As storage as crystalline scorodite, the CF-precipitate mixture can be transported for the further
316 re-solubilization, concentration and re-crystallization process at higher temperatures (~70°C).
317 **[Fig. 7, 8, 9 inserted here]**
318
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444

445

446 **Figure Legends**

447

448 **Fig. 1** Inhibitory effect of As(III) on heterotrophic (Fe^{2+}-free) growth of *Am. ferrooxidans* (a), *Sb.*
449 *sibiricus* (b) and *Sb. thermotolerans* (c). Initial conditions: [As(III)] = 0 mM (×), 1.3 mM (○), 2.6
450 mM (▲), 6.5 mM (◆) or 13 mM (-); 1.0 x 10^7 cells/ml. Data points are mean values from
451 duplicate cultures.

452

453 **Fig. 2** Evaluation of As-oxidizing ability in Fe-oxidizing strains (● *Am. ferrooxidans*; × *Sb.*
454 *sibiricus*, △ *Sb. thermotolerans*; − cell-free control). Changes in the Fe^{2+} concentration (a),
455 As(III) concentration (b, d) and cell density (c, e) were monitored. Initial conditions: [As(III)] =
456 6.5 mM; 1.0 x 10^8 cells/ml; [Fe^{2+}] = 9 mM (a-c) or [Fe^{2+}] = 0 mM (d, e). Data points are mean
457 values from duplicate cultures.

458

459 **Fig. 3** Carbon-fiber-assisted As(III) oxidation/immobilization induced by microbial Fe^{2+}
460 oxidation. Changes in the concentration of Fe^{2+} (a), Fe_{total} (b), As(III) (c), As_{total} (d) and cell density
461 (e) are shown. Carbon-fiber was added at 1% (w/v) in all cases. Cell-free controls (broken lines):
462 Fe^{2+} (−), As(III) (△), Fe^{2+} plus As(III) (○), Fe^{3+} plus As(III) (□). Microbial cultures (solid lines):
463 Fe^{2+} (□), As(III) (▲), Fe^{2+} plus As(III) (●), Fe^{3+} plus As(III) (■). *Am. ferrooxidans* cells were
464 inoculated at 1.0 x 10^7 cells/ml if media initially contained Fe^{2+}. Otherwise, cells were inoculated
465 at 1.0 x 10^8 cells/ml, as no cell growth was expected in the absence of Fe^{2+}. Data points are mean
466 values from duplicate cultures.

467

468 **Fig. 4** Normalized XANES spectra at the Fe K-edge (a) and As K-edge (b) for CF samples
469 collected after the reaction with respective ions (with or without cells) shown in Fig. 3. Standards:
470 $Fe^{II}SO_4 \cdot 7H_2O$ (a); $Fe^{III}_2O_3$ (a); $NaAs^{III}O_2$ (b), $KH_2As^{V}O_4$ (b), chemically synthesized
471 $FeAsO_4 \cdot 2H_2O$ and bacterial cells.

472

473 **Fig. 5** SEM images of CF before (a) and after (b-e) the As(III) oxidation/immobilization reaction
474 shown in Fig. 3. Microbial cultures: (b) Fe^{2+} + As(III), (c) Fe^{2+} only, (d) Fe^{3+} + As(III).

475 Cell-free control: (e) Fe^{3+} + As(III).

476

477 **Fig. 6** Backscattered electron image of a CF section covered with Fe^{III}-As^{V} precipitates

478 Cross points 1–3 indicate the beam spot positions for quantitative analysis.

479

480 **Fig. 7** Dissolution of amorphous Fe^{3+}/As(V)-precipitate on CF and its phase transformation into

481 crystalline scorodite. Changes in Fe_{total} (a) and As_{total} (b) concentrations and SEM morphological

482 observation (c) are shown. The arrow on the graph (a) indicates the time 14 h and temperature

483 shift from ambient to 70°C. At 14 h, CFs were pulled-out from the solution and instead, 3%

484 scorodite-seeds were fed (▲). In control, CFs were left in the solution and no seeds were added

485 (×). The SEM images were taken at 0 h (c-1), 14 h (c-2), and 170 h (c-3, 4), corresponding to

486 the timing indicated on graphs (a) and (b).

487

488 **Fig. 8** XRD patterns of the final As-precipitates shown in Fig. 7c-3 (a) and Fig. 7c-4 (b). Grey

489 vertical lines are assigned to scorodite (JCPDS 37-0468). The broad peak at around 20-30° (b)

490 derives from CF.

491

492 **Fig. 9** Proposed flowsheet of carbon-assisted As-remediation process. Fe-oxidizing ability of

493 indigenous acidophilic microbes can be combined with electron-mediating carbon material to

494 induce As(III) oxidation in acidic conditions. On-site semi-passive As-removal can be possible

495 as amorphous Fe^{3+}/As(V)-precipitates at lower temperatures as the first step. These precipitates

496 can be further processed off-site (by acid re-solubilization, concentration and re-crystallization)

497 for safer As disposal as scorodite ($Fe^{III}As^{V}O_4 \cdot 2H_2O$).