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Article

Microbial reduction of Fe(III)-bearing solids recovered from hydraulic fracturing flowback water: Implications for wastewater treatment

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Abstract

Hydraulic fracturing generates large volumes of flowback and produced water, composed of complex mixtures of organic and inorganic constituents. The solids associated with these fluids are Fe-rich and can contain toxic organics, heavy metals and naturally occurring radioactive materials (NORMs). Despite this, only a few studies have analysed their composition and there is a lack of understanding about their interactions with microbial communities and their long-term fate in the environment. In this study, we analysed the solids associated with flowback water derived from a hydraulically fractured well in the Bowland Shale, UK. We also investigated the microbial reduction of these Fe(III)-rich materials under anaerobic conditions using anthraquinone-2.6-disulfonate (AQDS) as an electron shuttle and identified the resulting bioreduced mineral phases. XRD characterization indicated that the solids contained akaganeite (β-FeOOH, Cl) and Ba-bearing celestine ($SrSO_4$). These Fe(III)-containing solids served as an electron acceptor for *Shewanella frigidimarina* and a flowback-derived Fe(III)-reducing enrichment culture. The bioreduced Fe(II)-bearing mineral phase was identified as ankerite [$Ca(Fe,Mg,Mn)(CO_3)_2$]; however, the presence of amorphous mineral phases is not ruled out. Microbial community composition was analysed using 16S rRNA gene sequencing. Amplicon sequence variants (ASVs) most closely related to *Chromohalobacter*, *Caminicella* and putative Fe(III)-reducing genera were dominant across treatments. Our findings highlight the potential of these Fe(III)-bearing sludges to be harnessed for the development of wastewater treatment strategies; for example, coupling the oxidation of toxic organics with Fe(III) reduction through either the introduction of microbial inocula or biostimulation of the native microbial communities. Furthermore, microbial processing can also be optimized to transform the Fe(III) sludges into denser materials, which are easier to handle and can immobilize toxic metals, thereby reducing the toxicity of this waste.

Keywords: Bowland Shale; waste solids; bioreduction; flowback water; hydraulic fracturing

Introduction

The extraction of natural gas from unconventional reservoirs through hydraulic fracturing generates large volumes of flowback and produced fluids (Sun *et al.*, 2019). These are comprised of a complex mixture of organic and inorganic solutes, which can originate from the injected fluids, the shale itself, and the interactions between these two sources (Ferrer and Thurman, 2015). Most of the research on flowback and produced waters has focused on the characterization of their soluble fraction, highlighting temporal and geographical patterns of the organic and inorganic compounds (Akob *et al.*, 2015; Butkovskyi *et al.*, 2017; Cluff *et al.*, 2014; Lester *et al.*, 2015; Oetjen *et al.*, 2018; Rosenblum *et al.*, 2017). Some of the organics detected in the solution have raised concerns due to their mobility and toxicity; for example, benzene and polycyclic aromatic hydrocarbons (PAHs), including naphthalene and

phenanthrene (Luek and Gonsior, 2017). Furthermore, the inorganic soluble fractions can also pose toxicity challenges as they may contain heavy metals, such as As, Pb, Cu, Cd, Hg and Zn (Estrada and Bhamidimarri, 2016), and naturally occurring radioactive materials (NORMs), mostly Ra-226 (Torres *et al.*, 2018).

The solids, or 'sludge', associated with the large volumes of flowback and produced waters can generate waste-management issues and toxicity risks. However, the sludges have received little attention, with only a few studies addressing their composition and toxicity. The limited examples include the characterization of solids associated with the flowback fluids from the Duvernay formation, Canada, which consisted of two distinct mineral phases, an amorphous silica-enriched Fe(III) oxyhydroxide and a baryte–celestine solid solution (Flynn *et al.*, 2019). An analysis of solids from the Marcellus shale, US, revealed that most of the Ra-226 was distributed in the labile fraction of the solids (Ouyang *et al.*, 2019). Moreover, it has been noted that the solids account for 50% of PAHs loads in flowback and produced fluids (He *et al.*, 2017). A study of the microbial community associated with sludge recovered from produced water impoundments in the Sichuan shale, China, identified high-diversity communities dominated

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by the family *Rhodobacteraceae* and genus *Erythrobacter*, and these communities were able to degrade a range of organic compounds in aerobic bioreactors (Zhou *et al.,* 2022). These findings suggest that the solids derived from flowback and produced waters should not be overlooked in wastewater management strategies. Despite this, the analysis of these solid phases has been mostly limited to characterization of sludges derived from North American shales (Flynn *et al.,* 2019; Ouyang *et al.,* 2019). As such, there remains a knowledge gap regarding the composition of solids from geographically distinct formations, their interactions with microbial communities and their potential to be upcycled in wastewater treatment processes; for example, the conversion of amorphous Fe(III) minerals to more crystalline magnetically recoverable nanomaterials (*e.g.* magnetite) (Lloyd *et al.,* 2020; Lovley *et al.,* 1987).

Microbial Fe(III) reduction can have a significant impact on the fate of both organic and inorganic contaminants in flowback and produced fluids. Metal-reducing bacteria use Fe(III) minerals as an electron acceptor coupled to the oxidation of organic compounds (and hydrogen) as electron donors (Lloyd, 2003, Lovley, 1987). The produced Fe(II) can either accumulate in solution under anoxic conditions, adsorb to surfaces of minerals or become incorporated into new Fe(II)-bearing minerals such as magnetite or siderite (Lloyd, 2003). Previous work has highlighted the applications of biomagnetite to remediate toxic metals such as Cr(VI) and Tc(VIII) (Cutting *et al.,* 2010) and organics (Watts *et al.,* 2017). Furthermore, the potential to leverage Fe(III)-waste minerals for the production of commercially valuable materials has been investigated using iron-rich precipitates from mine tailings, which were bioreduced using *Geobacter sulfurreducens* into Fe(II)-bearing nanoparticles that are easier to handle than highly amorphous Fe(III) 'gel' suspensions, and can be recovered for re-use by magnetic separation (Joshi *et al.,* 2018). There is, therefore, the potential to harness microbial Fe(III) reduction to aid the management and remediation of environmentally harmful flowback fluids from hydraulic fracturing for natural gas.

In this work, we characterized the composition and mineralogy of solids associated with the flowback water from an exploratory shale gas well in the Bowland Shale, UK. Additionally, we investigated the suitability of these materials to serve as electron acceptors for microbial Fe(III) reduction using both a facultatively anaerobic halophilic bacterium (*Shewanella frigidimarina*) and a flowback water-derived Fe(III)-reducing enrichment culture. The resulting bioreduced phases were identified and the microbial community composition was profiled using 16S rRNA gene sequencing. To the best of our knowledge, we report the first characterization of flowback water-associated Fe(III)-rich solids from outside North American shale systems and uniquely explore their potential to support microbial Fe(III) bioreduction. Insights about these otherwise overlooked waste materials and their interactions with microbial communities can support the development of wastewater strategies for the management of hydraulic fracturing flowback and produced fluids.

Methods

Study site and sampling

The Bowland Shale in the north of England, UK, has significant natural gas potential (Gross *et al.,* 2015), with an estimated natural gas in place of around 140 trillion cubic feet (Tcf)

(Whitelaw *et al.,* 2019) to 1329 Tcf (Andrews, 2013). Although there is now a moratorium on hydraulic fracturing for shale gas extraction in response to enhanced seismic activity, two exploratory wells were fractured in the Bowland Formation between 2018 and 2019. Flowback samples were obtained from one of these wells which targeted the Lower Bowland Shale, hereinafter designated Bowland-2 (see Hernandez-Becerra *et al.,* 2023, for further operational details). Well operators collected two types of samples from the water storage tanks after flowback; inoculum source samples (5) and a mineral-recovery sample (Samples description in supplementary material S.1). Samples for microbial culturing were collected in 500 mL sterile Nalgene bottles filled to capacity to minimize headspace. The sample used for suspended solids recovery (~1.5 L) was collected in polyethylene canisters. Due to site access limitations, the samples were stored on-site for weeks before transportation. Throughout this duration, they were maintained at 4°C to preserve their integrity prior to transfer to the laboratories at the University of Manchester, UK. Upon arrival, inoculum samples were transferred into sterile serum vials flushed with N_2 and stored in the dark at room temperature. Samples for mineral recovery were stored in the dark at 4°C prior to downstream analyses.

Recovery of suspended solids from flowback water

The suspended solids were recovered from the flowback water by centrifuging at 4000 RPM (2670 x *g*) in a BOECO C-28A centrifuge for 20 minutes. The supernatant was discarded, while the pellets were transferred into a sterile serum bottle. This concentrated sludge, which served as the electron acceptor in the Fe(III) bioreduction experiments, was flushed with N_2 and stored at 4°C. The concentration of total bioavailable Fe and Fe(II) of the sludge were determined using the ferrozine assay (Stookey, 1970; Viollier *et al.,* 2000). Briefly, homogeneous aliquots of the slurry were taken anoxically using an N_2 flushed syringe. Fe(II) was quantified by adding 0.1 mL of the aliquot to 4.9 mL of 0.5 M HCl, digested for 1 h, after which the absorbance was measured at 562 nm using a Mettler-Toledo UV5 UV–vis spectrometer. Subsequently, total bioavailable Fe was determined by reducing the digestate with 0.2 mL hydroxylamine hydrochloride (6.25 M) for 1 h and measuring the absorbance as previously described.

Microbial Fe(III)-reducing enrichments

Microbial Fe(III)-reducing enrichments were initiated in 30 mL serum bottles containing 25 mL of modified freshwater medium (MFM). The medium contained (in g/L deionized water) $NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4·H_2O$ (0.06), KCl (0.2), NaCl (35), vitamin solution (10 mL) and trace elements solution (10 mL). The vitamin solution contained (in mg/L deionized water) biotin (2.0), folic acid (2.0), pyridoxine-HCl (10.0), riboflavin (5.0), thiamine (5.0), nicotinic acid (5.0), pantothenic acid (5.0), vitamin B-12 (0.1), p-aminobenzoic acid (5.0) and thioctic acid (5.0).The trace elements solution was prepared with (in mg/L deionized water) nitrilotriacetic acid (1.5), $MgSO_4$(3.0), $MnSO_4·H_2O$ (0.5), NaCl (1.0), $FeSO_4·7H_2O$ (0.1), $CaCl_2·2H_2O$ (0.1), $CoCl_2·6H_2O$ (0.1), $ZnCl_2$(0.13), $CuSO_4·5H_2O$ (0.01), $AlK(SO_4)2·12H_2O$ (0.01), H_3BO_3(0.01), $NaMoO_4$(0.025), $NiCl_2·6H_2O$ (0.024) and $NaWO_4·2H_2O$ (0.025). The medium was dispensed into serum bottles, capped with butyl rubber stoppers and aluminium crimp seals, and subsequently flushed with N_2:CO_2 (80:20) and autoclaved at 121°C for 20 minutes. An electron acceptor, electron donors and electron shuttle were added

from sterile anoxic stocks as follows: ferrihydrite sludge ~30 mM, synthesized following the method described elsewhere (Lovley and Phillips, 1986), sodium lactate (~5 mM), sodium acetate (~5 mM) and anthraquinone-2, 6-disulfonate (AQDS) (10 μM). The pH was adjusted to the range 7–7.3. The medium was inoculated with a 10% vol/vol mixture containing equal parts of each flowback-inoculum sample (further inoculum sample description in S1). Cultures were incubated in the dark at 20°C and the production of Fe(II) was measured periodically with the ferrozine assay (Stookey, 1970; Viollier *et al.,* 2000), as previously described. Positive enrichment cultures (showing an increase of >5 mM Fe(II)) were subcultured by transferring 10% vol/vol of the cultures into fresh MFM three subsequent times. In preparation for the Fe(III) bioreduction assay, a highly enriched culture was grown in the same basal medium, substituting ferrihydrite for Fe(III)-citrate (30 mM).

Fe(III) bioreduction assay

Microbial Fe(III) reduction of solids recovered from flowback water was assessed using two sources of inoculum: (1) a pure culture of *S. frigidimarina* (strain NCIMB 400) and (2) an Fe(III)-reducing enrichment culture described above. *S. frigidimarina* was grown anaerobically in MFM with 25 mM sodium lactate as the electron donor and 40 mM sodium fumarate as the electron acceptor. The culture was incubated at 20°C in the dark for 48 hours.

The bioreduction assay was deployed in 30 mL serum bottles containing MFM amended with flowback solids to a final concentration of ~10 mM of bioavailable Fe(III). Treatments were based on the type of inoculum added and electron shuttle amendment, as follows: uninoculated control (uninoculated to assess the extent of Fe(III)-bioreduction by native microorganisms); *S. frigidimarina*; *S. frigidimarina* supplemented with AQDS, and Fe(III)-reducing enrichment supplemented with AQDS. Inoculations were carried out with 10% v/v of the respective culture. The cells were not washed prior to inoculation, which may have resulted in the carryover of electron donors or acceptors from the growth medium into the bioreduction assay. All the treatments were set up in triplicate, with the exception of the Fe(III)-reducing enrichment supplemented with AQDS treatment, which was set up in duplicate due to insufficient hydraulic fracturing solid waste material. Electron donors and electron shuttles were amended from anaerobic sterile stocks as described in Table 1. All serum vials contained media to a final volume of 20 mL. The experimental bottles were incubated in the dark at 20°C for one month and monitored weekly for Fe(II) production and total bioavailable Fe using the ferrozine assay (Stookey, 1970; Viollier *et al.,* 2000), as previously described.

Samples were taken at the beginning and end time points of the experiment for geochemical analyses (3 mL) and microbial community characterization (1 mL). Samples for microbial community characterization were stored at −80°C until downstream DNA extraction and 16S rRNA gene sequencing analyses. At the final time point, a slurry sample (3 mL) was taken to characterize the mineral phase of the bioreduced solids.

Solid and aqueous phase geochemical characterization

The bulk elemental composition of the solid and aqueous phases was determined by coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500cx. Samples were first centrifuged at 4000 rpm (2670 x *g*) in a BOECO C-28A centrifuge. The pellets were then acid

Table 1. Description of type and concentration of electron donors, electron shuttle and inoculum in each treatment.

Treatment	Electron donor	Electron shuttle	Inoculum
1. Uninoculated control	Lactate 10 mM	–	–
2. *S. frigidimarina*	Lactate 10 mM	–	*S. frigidimarina* (10% v/v)
3. *S. frigidimarina* supplemented with AQDS	Lactate 10 mM	AQDS 10 μM	*S. frigidimarina* (10% v/v)
4. Enrichment supplemented with AQDS	Lactate 10 mM + acetate 10 mM	AQDS 10 μM	Fe(III)-reducing enrichment (10% v/v)

extracted for analysis of solid phase composition (see below), with the supernatant used to quantify aqueous metal concentrations.

Pellets were freeze-dried, weighed, and transferred into PTFE tubes; 1 mL of 70% HNO_3 was added to digest the solids, and the tubes were then transferred to a sand bath heated to 60°C overnight. Digests were left to cool and diluted with 25 mL of DIW; 10 mL of the solution was filtered with a syringe and a 45 μm filter and analysed with ICP-MS. For aqueous phase analysis by ICP-MS, 100 μl of the supernatant was diluted with 9.9 mL of 2% HNO_3.

Mineral characterization

Samples for mineral characterization were washed with N_2 flushed deionized water to remove Na and Cl, and dried in the anoxic chamber. Dried samples were transferred to silicon wafers for X-ray diffraction (XRD) analysis or mounted on adhesive carbon tape on aluminium tabs for scanning electron microscopy (SEM) imaging and EDS elemental analysis.

XRD was carried out on a Bruker D2 Phaser diffractometer. The X-ray generator was set to 30 kV and 10 mA, with a $CuK_{\alpha1}$ source (wavelength of 1.5406 Å). Samples were scanned from 5-70°2θ, with a step size of 0.04° and a count time of 0.5 s per step. The patterns were evaluated using Diffrac. EVA which compares experimental data to standards from the International Centre for Diffraction Data Database. The morphology of the bioreduced solids was assessed using an FEI Quanta 650 FEG ESEM with a 15 kV beam in a high vacuum mode.

DNA extraction and 16S rRNA gene sequencing

DNA was extracted from microcosm slurries and recovered solids (prior to bioreduction) with a DNeasy PowerLyzer PowerSoil kit (Qiagen, Hilden, Germany). Extracted DNA was quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA) using the high sensitivity double stranded DNA (dsDNA) assay following the protocols provided by the manufacturer. The V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primer set 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso *et al.,* 2011). The PCR reaction was set up as follows: initial denaturation step at 95°C for 2 min, 36 cycles of melting (95°C, 30 s), annealing (58°C, 30 s) and extension (72°C, 2 min), followed by a final extension at 72°C for 5 min. Amplified DNA was sequenced with the Illumina MiSeq platform (Kozich *et al.,* 2013). QIIME2 version 2021.4 (Bolyen *et al.,* 2019) was used for sequencing processing.

Briefly, DADA2 was used for denoising and amplicon sequence variant (ASVs) assignments (Callahan *et al.,* 2015). Taxonomy was assigned with the *q2-feature classifier* plugin (Bokulich *et al.,* 2018) using the classify-sklearn naïve Bayes taxonomy classifier (Pedregosa *et al.,* 2011) against the Silva v138 99% reference sequence database (Bolyen *et al.,* 2019; Quast *et al.,* 2013). ASVs classified as chloroplast and mitochondria were flagged as potential artefacts and removed. Contaminant sequences identified in extraction, PCR, and sequencing controls were removed manually.

Results and discussion

Characterization of solids associated with flowback water derived from the Bowland Shale

Solids recovered from flowback fluids derived from the Bowland-2 exploratory well were characterized. These fluids were collected from storage tanks after flowback and stored for weeks following their recovery. We note that samples collected at a gas-water separator from the adjacent Bowland-1 well did not appear to contain solids upon collection, with solid phases appearing only in samples exposed to oxygen in the days and weeks following collection (see Hernandez-Becerra *et al.,* 2023 for details of the 'Bowland-1' well). We interpret this observation as an indication of high dissolved Fe(II) in anoxic production fluids, which oxidized following exposure to the atmosphere following collection and

storage at the surface, in agreement with prior reports in Duvernay shale production fluids (Flynn *et al.,* 2019). We therefore assume that solids in Bowland-2 fluids similarly formed during storage. Preliminary XRD analysis of the solids recovered from Bowland-2 flowback water identified halite, quartz, celestine and baryte as the dominant crystalline phases (Supplementary S.1). These minerals have been identified previously in solids suspended in production fluids derived from the Marcellus shale (Ouyang *et al.,* 2019). The relatively high concentration of halite skewed the identification of less dominant mineral phases, but further XRD analysis of washed solids detected akaganeite (Fig. 2a). Akaganeite (β-FeOOH, Cl) is a chloride-containing Fe(III) oxyhydroxide that occurs in saline environments (Cornell and Schwertmann, 2003), which can be reduced by Fe(III)-reducing bacteria, such as members of the genera *Shewanella* (Lee *et al.,* 2003; Roh *et al.,* 2003) and *Geobacter* (Cutting *et al.,* 2009). Electron shuttles, including AQDS, can enhance this reductive process (Cutting *et al.,* 2009).

Microbial reduction of Fe(III)-bearing flowback solids

Fe(III)-rich solids associated with flowback and produced waters derived from hydraulic fracturing operations can possess significant toxicity levels (He *et al.,* 2017), and given their potentially diffuse 'gel-like' properties they can pose challenges for water reuse and treatment. Understanding how microorganisms might interact with these diffuse and hard-to-handle solids can provide useful insights
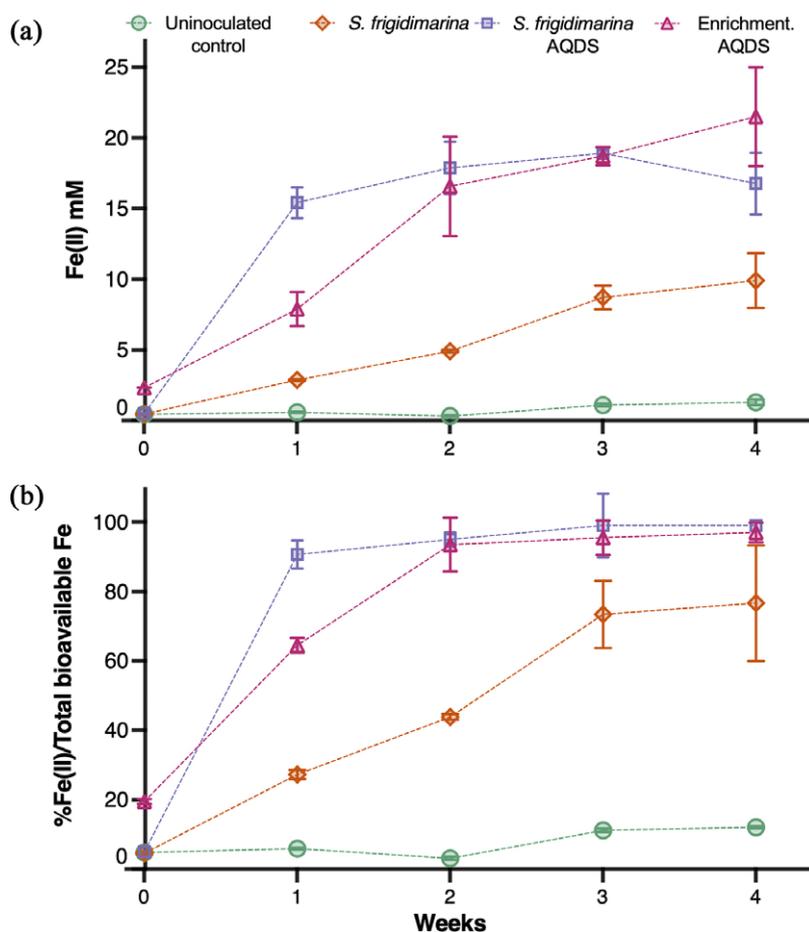
Figure 1. Microbial Fe(III) reduction of solids recovered from flowback water. (a) Mean of Fe(II) concentration and (b) mean of Fe(II) normalized to the total mean Fe bioavailable in the uninoculated control *S. frigidimarina*, *S. frigidimarina* supplemented with AQDS and Fe(III)-reducing enrichment supplemented with AQDS. Error bars represent standard deviation.
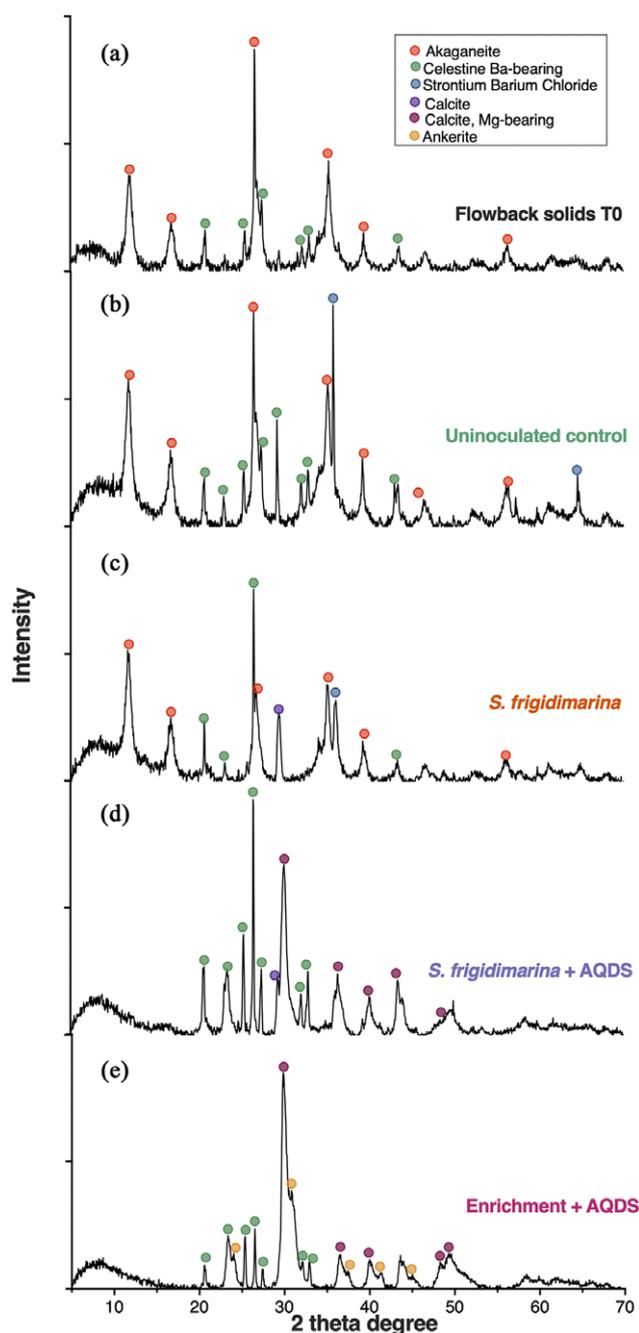
Figure 2. XRD patterns of mineral phases in the starting flow-back solids and in the corresponding bioreduced materials. Materials were subjected to washing with 18Ω de-ionized water to remove halite.

for the design of water treatment strategies, potentially revalorizing them as magnetically recoverable, easier-to-process materials (Joshi *et al.,* 2018). Here, we recovered the flowback water sludge to determine its suitability as an electron acceptor for Fe(III)-reducing bacteria. Both a pure culture type strain of *Shewanella frigidimarina* and a flowback water-derived Fe(III)-reducing enrichment culture were tested. A combination of solid phase characterization techniques was employed to characterize bioreduced Fe(II)-bearing mineral phases formed.

Fe(II) production was monitored on a weekly basis and these values were normalized to the total concentration of bioavailable Fe. Bioreduction results are summarized in Fig. 1. Fe(II) was

produced in all the inoculated treatments (both with *S. frigidimarina* and the Fe(III)-reducing enrichment). Treatments also amended with an electron shuttle (AQDS) showed more rapid rates of Fe(III) reduction (Fig. 1a). After seven days of incubation, the Fe(II) concentration in the *S. frigidimarina* treatment supplemented with AQDS increased from 0.5 mmol/L of slurry to 15 mmol/L of slurry and remained stable, peaking at 18 mmol/L after three weeks. Similarly, the concentration of Fe(II) in the enrichment culture supplemented with AQDS increased from 2 mmol/L of slurry to 7 mmol/L slurry within a week of incubation and reached 21 mmol/L of slurry by week four. The slightly higher initial concentration of Fe(II) in the enrichment treatment probably reflects a carryover from the growth medium. By contrast, the treatment with no added electron shuttle increased from 0.4 mmol/L of slurry to 2 mmol/L of slurry Fe(II) after the first week of incubation and to 9 mmol/L of slurry Fe(II) by four weeks. Fe(II) levels remained around 1 mmol/L of slurry throughout the experiment in uninoculated controls. However, a slight increase of Fe(II) from 0.4 m mmol/L of slurry at the beginning of the experiment to 1.2 mmol/L of slurry after four weeks of incubation was identified (Fig. 1a). This low level of reduction might be indicative of the activity of Fe(III) reducers in the sludge. AQDS is a humic analogue known to enhance the extent and rate of Fe(III) bioreduction (Cutting *et al.,* 2009; Fredrickson *et al.,* 1998; Lloyd *et al.,* 1999; Lovley *et al.,* 1996). Thus, higher reduction rates in the AQDS amended treatments were expected. The normalized values of Fe(II)/total bioavailable Fe(III) showed similar ratios in the treatments supplemented with AQDS (above 96%), while the ratio in the vessels without the added electron shuttle was around 70% (Fig. 1b).

The bioreduced mineral phases recovered after four weeks of incubation were characterized by XRD. The XRD patterns identified barium (Ba)-bearing celestine in all the treatments (Fig. 2), which was also identified in the starting material. Ba-bearing celestine ($SrSO_4$) is a common secondary mineral derived from the oxidation of pyrite or fracturing additives combined with Ba (mainly used in the drilling muds or originating from the formation) and Sr from the shale brines (Esteves *et al.,* 2022). Moreover, similar phases have been reported in solids from the Duvernay (Flynn *et al.,* 2019) and Marcellus shale formations (Ouyang *et al.,* 2019).

Regarding Fe-containing mineral phases, akaganeite was detected in the uninoculated control and in the *S. frigidimarina* treatment (Fig. 2b,c), confirming minimal Fe(III) reduction in the former and suggesting incomplete microbial Fe(III) reduction in the latter. Ankerite [$Ca(Mg,Fe^{2+},Mn)(CO_3)_2$] was the sole Fe(II)-bearing mineral identified in these experiments and was observed in the treatment inoculated with the Fe(III)-reducing enrichment culture amended with AQDS (Fig. 2e). The formation of bioreduced Fe(II) minerals is influenced by many factors, including the concentration of cations and anions in solution, headspace composition, pH and bacterial growth conditions (Lee *et al.,* 2003; Roh *et al.,* 2003). For instance, *Shewanella* strains grown under an $H_2:CO_2$ headspace can bioreduce synthetic akaganeite forming siderite ($FeCO_3$); while the formation of magnetite (Fe_3O_4) is favoured under an N_2 headspace using lactate and formate as electron donors (Roh *et al.,* 2003). Similarly, medium composition influences the bioreduction end products, as amendments of Ca (10 mM) (Roden *et al.,* 2002) have promoted the formation of ankerite while a high concentration $NaHCO_3$ buffer in the media leads to increased siderite precipitation (Roh *et al.,* 2003). The Lower Bowland Shale is a carbonate-dominated formation (Newport *et al.,* 2018), as evidenced by the Ca concentrations in the experimental treatments of 30 mM in the aqueous phase, consistent with the formation of

ankerite. Despite the high ratio of Fe(II)/ total bioavailable Fe detected in the *S. frigidimarina* supplemented with AQDS treatment, XRD analysis did not identify highly crystalline Fe(II)-bearing minerals, for example magnetite or siderite. By contrast, Mg-bearing calcite was detected (Fig. 2d). The lack of crystalline Fe(II) minerals together with the production of Fe(II) in the treatments inoculated with *S. frigidimarina*, could suggest the accumulation of soluble and sorbed Fe(II) or the formation of poorly ordered or amorphous bioreduced mineral phases, which are not detected with XRD.

SEM with EDS analysis was used to explore the morphology and elemental compositions of any post-reduction minerals formed. Acicular celestine Ba-bearing crystals were found in all the samples (Fig. 3). The akaganeite in the uninoculated control and *S. frigidimarina* treatments presented a granular particulate structure (Figs 3a and b). Ovoidal and spheroidal ankerite particles were found in the enrichment culture treatment (Fig. 3e). Interestingly, minerals

of similar morphologies were observed in the *S. frigidimarina* cultures supplemented with AQDS (Fig. 3c), even though ankerite was not confirmed with XRD; only a calcite Mg-bearing phase was identified. Ankerite is the Fe analogue of dolomite CaMg(CO$_3$)$_2$, in which Fe(II) substitutes for Mg (Lippmann 1973); the threshold to be considered ankerite is over 50 mol.% Fe in the Mg site. The chemical characterization of the solid phase by ICP-MS (Fig. 4) showed a higher concentration of Fe in the treatment inoculated with the Fe(III)-reducing enrichment (3127 ppm) compared with the one inoculated with *S. frigidimarina* and supplemented with AQDS (1937 ppm). Fe concentrations in the aqueous phase both in the *Shewanella* and *Shewanella* supplemented with AQDS treatments started with an average concentration of 0.1 ppm Fe, which increased to 227 ppm and 409 ppm, respectively. In the enrichment treatment, the Fe in the aqueous phase increased from 124 ppm to 266 ppm (Fig. 4). This overall increase of Fe concentration across inoculated
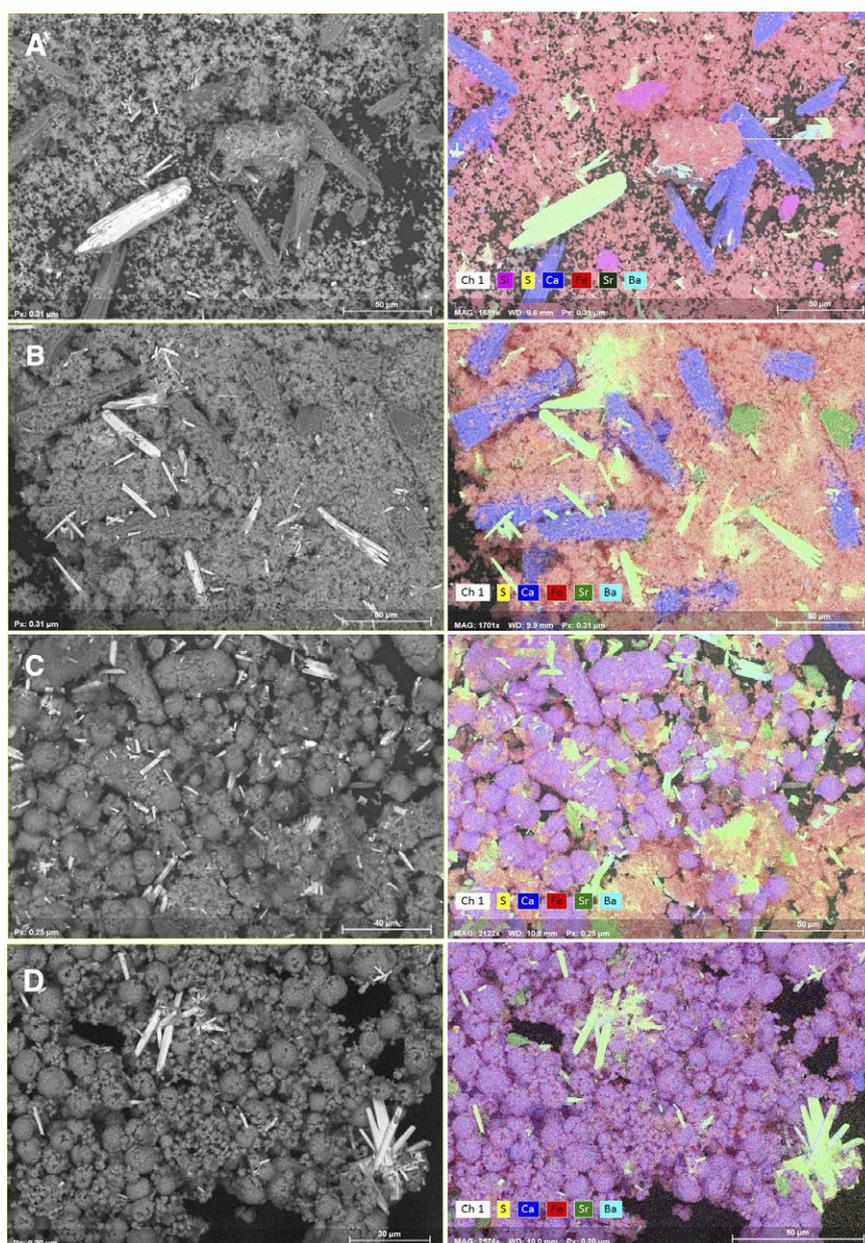
Figure 3. SEM micrographs and corresponding EDS elemental mapping showing the morphology of the mineral phases formed after microbial reduction. (a) uninoculated control, (b) *S. frigidimarina*, (c) *S. frigidimarina* supplemented with AQDS and (d) Fe(III)-reducing enrichment supplemented AQDS. Elemental mapping; silicon (pink), sulfur (yellow), calcium (dark blue), iron (red), strontium (green) and barium (light blue). Materials with mixed composition of Ca and Fe are shown in purple. Materials containing strontium, sulfur and barium are a light green colour.
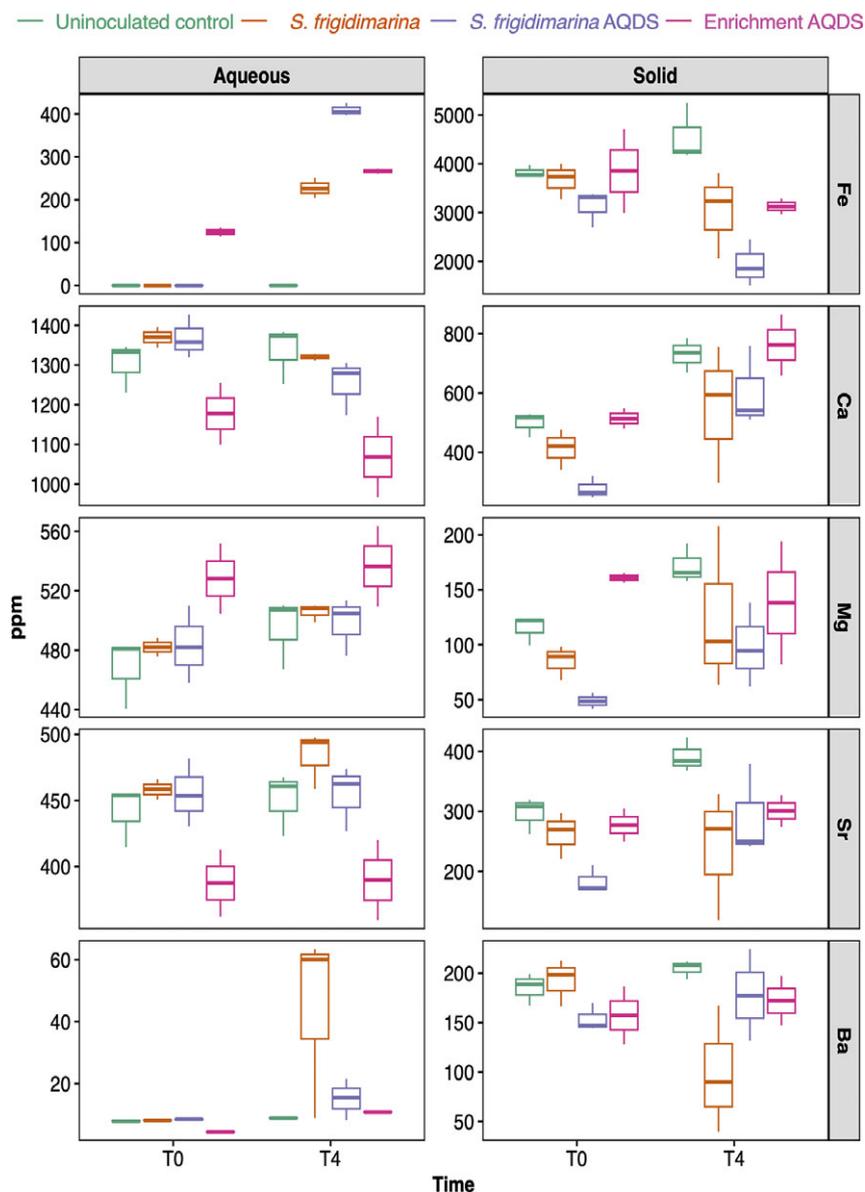
Figure 4. Major elements concentrations (ppm) in the aqueous and solid phases. Box plots show differences in element concentrations between the start (T0) and the end (T4) of the experiment. Distinct colours reflect treatments: uninoculated control (green), *S. frigidimarina* (orange), *S. frigidimarina* supplemented with AQDS (purple) and Fe(III)-reducing enrichment supplemented with AQDS (pink).

treatments suggests the production of soluble Fe(II) resulting from the sludge bioreduction.

Taken together, the differences in bioreduction rates, bulk mineral compositions and surface morphology across treatments suggest that Fe(III) bioreduction resulted in a mineral transformation sequence. In all inoculated treatments, the Fe(III) solids associated with flowback water, which included akaganeite, were progressively bioreduced, releasing soluble Fe(II). In the enrichment treatment, the Fe(II) substituted for Mg in calcite Mg-bearing minerals, resulting in the formation of ankerite. By contrast, in the treatments inoculated with *Shewanella*, the bioreduction resulted in the accumulation of soluble and sorbed Fe(II) or amorphous minerals.

Elemental analysis revealed a slight increase of Ba concentration in the aqueous phase in both treatments amended with AQDS, rising from 8.4 ppm to 15 ppm in the *Shewanella* treatment and from 4.4 ppm to 10.8 ppm in the enrichment culture treatment. A more pronounced increase was observed in the *Shewanella* treatment without an added electron shuttle, as Ba concentrations in the

aqueous phase increased from 8.1 ppm to 55.7 ppm, while the concentration in the solid phase decreased from 192 ppm to 98 ppm, suggesting Ba release during dissolution (Fig. 4). This observation is consistent with previous research showing that microbial Fe(III) reduction can induce the release Ba into solution (Landa *et al.*, 1991). Despite the changes in Ba concentration, Ba-bearing celestine was consistently identified across treatments, suggesting that this phase may resist leaching under Fe(III)-bioreduction conditions.

The microbial community composition of solids associated with flowback water, the Fe(III)-reducing enrichment, culture inoculum and sludges resulting from the bioreduction treatments, were profiled using 16S rRNA gene sequencing, with 15 ASVs dominating the microbiota (Fig. 5). The solids associated with flowback fluids, which were used as the electron acceptor in the experiments, hosted a diverse community with most of the sequences assigned to the genera *Chromohalobacter* (32%) and *Aeromonas* (10%). The Fe(III)-reducing enrichment used as an inoculum had a high
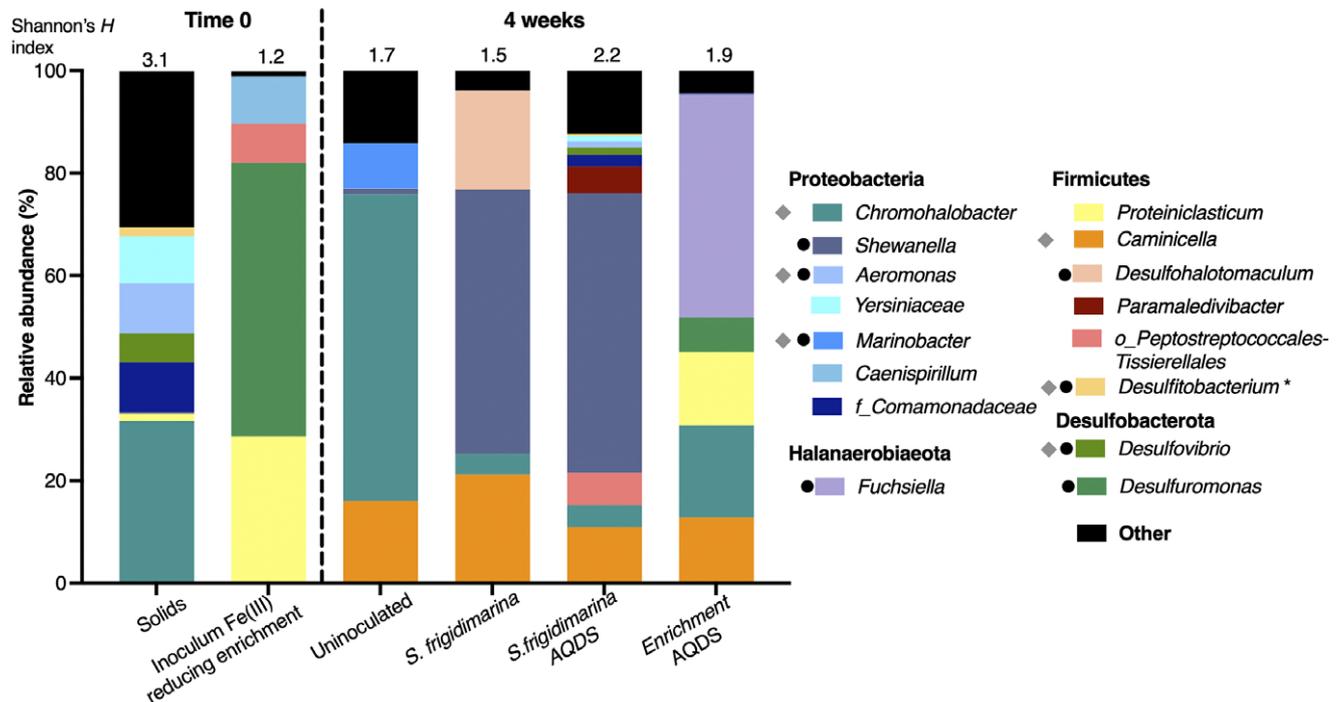
Figure 5. Bacterial community composition in the flowback solids, enrichment inoculum (T0) and endpoint post-reduction samples (four weeks) based on 16S rRNA gene sequencing. Taxa displayed at genus level or next highest resolved phylogeny. All genera that represent ≥ 5% relative abundance from any sample are listed in the bar plot, the rest are grouped as 'Other'. Putative Fe(III)-reducing (●) and hydrocarbon-degrading bacteria (◆) are annotated. Asterisk(*) denotes genus with ≤ 5% relative abundance.

abundance of sequences affiliated with *Desulforomonas* (53%). The uninoculated control was dominated by ASVs assigned to *Chromohalobacter* (60%) and *Caminicella* (16%). As expected, treatments inoculated with *S. frigidimarina* were dominated by sequences assigned to this genus (over 50% relative abundance). Most of the sequences obtained from the treatment inoculated with the Fe(III)-reducing enrichment were closely affiliated with *Fuschiella* (43%). Thus, the 16S rRNA sequencing analysis revealed differences in microbial community composition across experimental treatments. These compositional variations are significant, as distinct microbial species exhibit particular Fe(III) bioreduction capabilities. For instance, species within the genus *Shewanella* have shown variability in the rate and extent of lepidocrocite bioreduction, which might influence the morphology of the resulting green rust mineral phases (O'Loughlin *et al.*, 2007). In addition to the composition of the microbial communities, the amount of biomass can influence the resulting bioreduced phases. For example, *Geobacter sulfurreducens* has been shown to form distinct Fe(II) mineral phases depending on biomass concentration. At lower concentrations (0.015 to 0.05 OD_{600}), goethite (α-FeOOH) was the dominant phase, while magnetite formed at an intermediate range (0.2 to 2 OD_{600}) and siderite was observed at higher concentrations (4 OD_{600}) (Byrne *et al.*, 2011). In our bioreduction assay, microbial loads were not controlled, and this may have influenced the production of specific mineral phases.

Sequences affiliated with *Chromohalobacter* and *Caminicella* species were noted across all treatments (Fig. 5). *Chromohalobacter* sequences had a relative abundance ranging from 3.9% in the *S. frigidimarina* treatment to 60% in the uninoculated control. Members of this genus are moderately halophilic chemoorganotrophic bacteria (Ventosa *et al.*, 1989), which can precipitate carbonate minerals, such as magnesian calcite and 'protodolomite', and can also form clusters of spheroidal bioliths (Rivadeneyra *et al.*, 2006).

Moreover, *Chromohalobacter* species can degrade aromatic hydrocarbons via the *ortho*-cleavage of the β-ketoadipate pathway (Erdogmus *et al.*, 2015). The relative abundance of sequences assigned to *Caminicella* varied from 10% to 21% in the *S. frigidimarina* treatments amended and unamended with the electron shuttle, respectively. Little is known about this genus; the only isolated species *Caminicella sporogenesis* is a thermophilic heterotrophic anaerobe recovered from a hydrothermal vent (Alain *et al.*, 2002). Metagenome-assembled genomes obtained from a hot oil reservoir showed that *C. sporogenes* has a glycyl radical enzyme that may be used for hydrocarbon metabolism via alkene activation through the addition of fumarate (Christman *et al.*, 2020). *Chromohalobacter* and *Caminicella* sequences have been identified in flowback and produced water from hydraulically fractured shales (Davis *et al.*, 2012; Harris *et al.*, 2018, Hernandez-Becerra, 2023); however, they do not usually dominate. This suggests a differentiation between the microbial community in the solid and aqueous phases of flowback fluids derived from hydraulic fracturing. Furthermore, their functional potential for hydrocarbon degradation could enable them to utilize these compounds as an energy source and persist in fractured shale systems.

Half of the dominant ASVs (7) identified in all samples (solids, Fe(III)-reducing enrichment culture and inoculum and experimental treatments) were most closely related to putative Fe(III)-reducing bacterial genera. Their abundance across samples, from highest to lower, was in the order: *Shewanella* > *Desulfuromonas* > *Fuchsiella* > *Desulfohalotomaculum* > *Aeromonas* > *Marinobacter* > *Desulfovibrio* (Bale *et al.*, 1997; Coates *et al.*, 1995; Handley *et al.*, 2009; Myers and Nealson, 1990; Ventura *et al.*, 2015; Yang *et al.*, 2016; Zhilina *et al.*, 2015). Although a pure strain of *S. frigidimarina* was used as the inoculum for some of the experimental treatments (and was detected at high relative abundance in these treatments, as

expected), it must be noted that sequences most closely related to the genus *Shewanella* were identified across all experiments, indicating they are associated with the solids in the flowback fluids from this well, and proliferated under our cultivation conditions. This is consistent with our earlier findings of flowback fluid microbial composition from Bowland Shale wells, in which *Shewanella* was a dominant ASV (Hernandez-Becerra et al., 2023).

Implications for hydraulic fracturing flowback and produced water management

Hydraulic fracturing generates large volumes of flowback and produced water, around 1700 to 14,300 m^3/year per well during its first decade of operation (Kondash et al., 2017). Following their collection, these fluids generate a significant volume of solids. Conventionally, flowback and produced waters have been disposed of through seepage or sealed pits (Silva et al., 2017), and the solids are discarded in landfills. The fate of these materials in the environment has not been studied in detail, nor has the potential for microbial processes to alter them during wastewater treatment options. Given the high content of bioavailable Fe(III) in these solids, their potential alterations via Fe(III)-reducing bacteria is a clear knowledge gap that could inform the development of microbial processing strategies to detoxify the materials or convert them to more easily managed materials safe for re-use or safe disposal.

The organic compounds in these production fluids are of particular concern due to their toxicity, mobility and persistence (Rogers et al., 2015). Among these are aromatic and halogenated compounds (Butkovskyi et al., 2017). The metabolic versatility of Fe(III)-reducing bacteria allows them to utilize a wide range of organic substrates as electron donors, including aromatic compounds (Lovley et al., 1989). As such, their metabolism could be harnessed in wastewater treatment strategies. For example, Azam and Finneran (2013) demonstrated that various ferric amendments increased the mineralization of carbon compounds in septic wastewater, such as low-molecular-weight organic acids, carbohydrate monomers and polymers, and lipids. In the context of hydraulic fracturing wastewater, coupling the bioreduction of Fe(III)-bearing waste sludge with the oxidation of hydrocarbons, such as BTEX, might reduce the toxicity of these fluids. Some Fe(III)-reducing bacteria metabolize these hydrocarbons completely to CO_2. For instance, *Desulfitobacterium aromaticivorans* can oxidize toluene and xylene (Kunapuli et al., 2010); sequences closely related to this genus were identified in the solids at the beginning of the experiment. Our microbial community profiling analysis also detected sequences most closely related to putative hydrocarbon-degrading bacteria, such as *Marinobacter* (Gauthier et al., 1992), *Desulfovibrio* (Qian et al., 2021), *Aeromonas* (Nie et al., 2016) and *Chromohalobacter* (Erdogmus et al., 2015) (Fig. 5). Furthermore, hydrocarbon biodegradation can occur via syntrophic interactions between members of the microbial community (Kleinsteuber et al., 2012). This could also occur in our system where hydrocarbon-degrading bacteria might catabolize hydrocarbons to H_2 or acetate, which then could be used as electron donors for Fe(III) reduction.

As for the inorganic constituents of flowback and produced fluids, including toxic metals and NORMs, their fate can also be influenced by the microbial reduction of the Fe(III)-bearing solids. Distinct Fe(II) mineral phases can be formed depending on the water chemistry, including magnetite, vivianite and siderite (Vaughan and Lloyd, 2011). The remediation potential of these

biogenic minerals has been explored; for example, magnetite can reduce and immobilize toxic metals such as Cr(VI) to Cr(III) (Cutting et al., 2010) and Sr can be incorporated into siderite thereby preventing its migration (Roden et al., 2002). Biomagnetite was not identified in our bioreduction assay but the process could be optimized by modifying experimental conditions such as pH and salinity to enhance Fe(II) sorption (and magnetite formation). Elevated ionic strength in high salinity experiments can inhibit the production of sorption-driven minerals, including biomagnetite (Dong et al., 2020). Our bioreduction assay was conducted in a medium containing 3.5% NaCl, a salinity level probably unfavourable for magnetite formation. Further research could explore lower salinity levels that may facilitate Fe(II) sorption and magnetite production.

In our system, bioreduction of Fe(III)-solids and the formation of ankerite can be influenced by (1) the Ca-rich geochemistry of the fluids, which is determined by the formation lithology, and promotes the formation of ankerite instead of siderite, and (2) the high abundance of *Chromohalobacter* ASVs, a known genus able to induce carbonate precipitation (Rivadeneyra et al., 2006). Furthermore, Ra-226 in flowback fluids can readily co-precipitate with Ba and Sr, forming baryte and celestine (Zhang et al., 2014). Our data indicate that even under enhanced Fe(III)-reducing activities, Ba and Sr (and probably Ra-226) remain immobilized in such materials supporting the hypothesis that this mineral phase is resistant to leaching (Ouyang et al., 2019).

Conclusions

Here, we characterized the mineral content and the bioreduction potential of Fe(III)-bearing phases associated with flowback water from the hydraulically fractured Bowland Shale. Mineral phases in the starting materials we studied included akaganeite and celestine Ba-bearing minerals. We have shown that these materials contain bioavailable Fe(III), which can be respired by Fe(III)-reducing bacteria, resulting in the formation of mineral phases including ankerite, although we cannot rule out the formation of other amorphous mineral phases not detected by XRD. Finally, our data suggest that native flowback water microbial communities have the ability to reduce these starting Fe(III) minerals in the presence of AQDS. This microbial reduction process can be further optimized to transform these amorphous waste materials into minerals exhibiting improved settling properties, including potentially magnetic characteristics. Such enhanced properties hold significant potential for wastewater management, as they facilitate the recovery and disposal of the treated materials (Joshi et al., 2018). Additionally, the reduced Fe(II) or mixed-valance (Fe(II/III)) minerals, such as biomagnetite, can be used to remediate a range of toxic metals, including Cr(VI) and U(VI), as these can potentially become incorporated into the newly formed mineral phases during the bioreduction process (Cutting et al., 2010; Telling et al., 2009).

Furthermore, these findings can underpin flowback and produced water management strategies addressing the removal of toxic organics, coupling the reduction of Fe(III)-rich solids to the oxidation of problematic hydrocarbons. However, further research is required to explore the potential of native microbial communities to degrade hydrocarbons in these systems. Metagenomic approaches can provide evidence of the presence of functional genes related to hydrocarbon degradation in the flowback and produced water microbial communities, and this is ongoing. Additional studies stimulating *in situ* communities with different electron shuttles to facilitate the Fe(III) bioreduction and assessing the

degradation of organics can help with the design strategies addressed to the removal of toxic organics.

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