Reduction of structural Fe(III) in nontronite by humic substances in the absence and presence of *Shewanella putrefaciens* and accompanying secondary mineralization

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Abstract

Studies have shown the electron shuttling role of humic substances (HS) in enhancing microbial reduction of solid-phase Fe(III), but it is unknown if native HS can reduce structural Fe(III) in clays and how their chemical properties affect this process and secondary mineralization. The objective of this study was to evaluate the role of natural HS, Leonardite humic acid (LHA), and Pahokee Peat humic acid (PPHA) in reducing structural Fe(III) in nontronite with or without Shewanella putrefaciens. The extent of Fe(III) reduction was determined with a wet chemical method. Electrochemical methods, spectroscopy, and mass spectrometry were used to determine the changes of HS electrochemical and molecular composition after bioreduction. X-ray diffraction and electron microscopy were used to observe mineralogical transformations. The results showed that natural HS not only served as an electron donor to abiotically reduce Fe(III) in nontronite but also served as an electron shuttle to enhance Fe(III) bioreduction by S. putrefaciens. In the presence of CN32 cells, both the rate and extent of Fe(III) reduction significantly increased. Between the two HS, PPHA was more effective. The final bioreduction extents were 12.2 and 17.8% with LHA and PPHA, respectively, in bicarbonate buffer. Interestingly, when CN32 cells were present, LHA and PPHA donated more electrons to NAu-2, suggesting that CN32 cells were able to make additional electrons of LHA and PPHA available to reduce structural Fe(III). Although LHA reduced less Fe(III), it induced more extensive mineral transformation. In contrast, PPHA reduced more Fe(III) but did not induce any mineralogical change. These contrasting behaviors between the two humic acids are ascribed to their differences in electron-donating capacity, reactive functional group distribution, and metal complexation capacity. A unique set of secondary minerals, including talc, illite, silica, albite, ilmenite, and ferrihvdrite formed as a result of reduction. The results highlight the importance of coupled C and Fe biogeochemical transformations and have implications for nutrient cycling and contaminant migration in the environment.

Keywords: LHA, mineralization, nontronite, PPHA, Shewanella putrefaciens

INTRODUCTION

Humic substances (HS) are an important component of organic matter in soils and sediments, which are often referred to as stable soil organic matter (Perdue et al. 1990). Studies have focused on adsorption behaviors of humic substances onto iron (oxyhydr) oxides and clay minerals (Chen et al. 2014; Coward et al. 2018, 2019; Eusterhues et al. 2008; Gouré-Doubi et al. 2018; Ha et al. 2008; Henneberry et al. 2012; Lv et al. 2016; Saidy et al. 2012; Sowers et al. 2019; Theng 1976; Vermeer et al. 1998; Zhang et al. 2012). In the 1990s, humic substances were demonstrated to shuttle electrons between microorganisms and Fe(III) oxide (Lovley et al. 1996). Since then, the role of HS as electron shuttle has been widely recognized in solid-phase Fe(III) reduction by microorganisms (Amstaetter et al. 2012; Fredrickson et al. 2000; Kappler et al. 2004; Liu et al. 2016; Lovley et al. 1998, 1999;

Shimizu et al. 2013). In these studies, organic compounds such as acetate and lactate typically serve as electron donors.

Although the electron shuttling role of natural HS is well established, it is still poorly understood whether they can serve as electron donor to reduce solid-phase Fe(III). Microbially reduced HS were able to reduce insoluble Fe oxides (Jiang and Kappler 2008). Other studies showed that even native HS (nonreduced) could reduce aqueous Fe³⁺ (Peretyazhko and Sposito 2006) and Fe(III) (oxyhydr)oxides (Bauer and Kappler 2009; Piepenbrock et al. 2014; Sundman et al. 2017), suggesting that native HS retain certain reduced functional groups under the ambient oxic condition. More recently, Stern et al. (2018) and Mejia et al. (2018) demonstrated that natural HS can play dual roles as electron donor and shuttle when coupled with bioreduction of ferrihydrite and hematite. The ability of native HS to reduce Fe oxides is related to their intrinsic electron-donating capacity (EDC) (Aeschbacher et al. 2012; Klüpfel et al. 2014). In these processes, humic sub-

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stances may undergo some changes in functional groups (Aeschbacher et al. 2012) and composition (Mejia et al. 2018).

Fe-bearing clay minerals are common in soils and sediments, and structural Fe(III) in clays can be reduced either microbially or chemically (Dong et al. 2009; Pentráková et al. 2013; Stucki and Kostka 2006). Despite common co-presence of clay minerals, HS, and microbes (Barré et al. 2014; Borden et al. 2010), the role of HS in clay redox reactions has not been well-studied. Most studies have used HS analog, anthraquinone-2,6-disulfonate (AQDS), as an electron shuttle to enhance bioreduction of structural Fe(III) in clay minerals (Dong et al. 2009). To date, only a few studies (Liu et al. 2016, 2017; Lovley et al. 1998) have used natural HS as electron shuttles to enhance Fe(III) bioreduction in clays. However, it is currently unknown if natural HS can donate electrons to structural Fe(III) in clay minerals. Because clay minerals are fundamentally different from Fe oxides in terms of both chemistry and structure, HS are expected to have different effects on electron transfer and mineral transformation. For example, reductive dissolution is a common consequence of Fe(III) oxide reduction (Weber et al. 2006), but clay minerals can undergo multiple redox cycles without much dissolution (Yang et al. 2012; Zhao et al. 2015). In this case, electron donation from HS to structural Fe(III) in clay minerals may be more difficult than Fe(III) (oxyhydr)oxides and may be directional (i.e., parallel to or perpendicular to the clay layers). It is not known how the EDC of HS affects their roles in electron donation and shuttling in clay bioreduction. Furthermore, the metal complexation properties of HS may differentially affect clay mineral and (oxyhydr)oxide transformations. In addition, it remains unclear if HS undergoes any electrochemical and molecular change as a result of electron donation.

The objective of this study was to determine the role of natural HS as electron donor when coupled with reduction of structural Fe(III) in an iron-rich smectite, nontronite (NAu-2), in the absence and presence of an iron-reducing bacterium, Shewanella putrefaciens CN32. Specifically, the goals of the study were to determine: (1) the roles of HS as electron donor/shuttle in affecting abiotic and biotic Fe(III) reduction in nontronite; (2) the relationship between such roles and the physical/chemical properties of HS (EDC, molecular composition, metal ion complexation); (3) mineralogical transformation as a result of clay-HS-microbe interactions. We hypothesize that the EDC of natural HS determines the rate and extent of Fe(III) bioreduction, but the metal ion complexation of HS controls their role in mineral transformation. Furthermore, HS can undergo compositional change as a result of redox reactions. Wet chemical methods were used to monitor Fe(III) reduction. Electrochemistry, spectroscopy, microscopy, and mass spectrometry methods were employed to characterize changes of HS composition and mineralogy. The results of this study provide important insights into coupled Fe-C biogeochemical transformations in the environment.

MATERIALS AND METHODS

Materials and experimental setup

Nontronite (NAu-2), an Fe-rich smectite, was purchased from the Source Clays Repository of the Clay Minerals Society. It was originally collected from Uley Mine in Southern Australia. The formula of this NAu-2 is $(Ca,Na,K)_{0.72}(Si_{7.55}Al_{0.45})$ (Fe_{3.83}Mg_{0.05})O₂₀(OH)₄ (Keeling et al. 2000). Total Fe content of NAu-2 is 23.4%, with 0.6% as Fe(II) (Jaisi et al. 2005), while Mg content is about 0.15% (Gates

et al. 2002). Fe(III) is distributed in both octahedral (92%) and tetrahedral sites (8%) (Gates et al. 2017; Jaisi et al. 2005). The NAu-2 sample was Na-saturated and size-separated as previously described (Jaisi et al. 2005). The size fraction of 0.02–0.5 μ m was collected, dried, and made into 5 g/L suspension in 30 mM sodium bicarbonate buffer (29.76 mM NaHCO₃ and 1.34 mM KCl, pH 7.0), followed by purging (N₂:CO₂ 80:20) and autoclaving.

Humic substances Leonardite humic acid (LHA, 1S104H) and Pahokee Peat humic acid (PPHA, 1S103H) were purchased from the International Humic Substances Society (IHSS) (Klüpfel et al. 2014; Rosario-Ortiz 2015). These substances are natural organic matter obtained by alkali extraction, and the names do not imply secondary synthesis or chemical recalcitrance (Kleber and Lehmann 2019; Lehmann and Kleber 2015). According to the IHSS website, the carbon contents of LHA and PPHA are 63.81 and 56.37%, respectively. Stock solutions (20 g/L) of LHA and PPHA were prepared by dissolving them in sodium bicarbonate buffer, followed by purging (N₂:CO₂ 80:20) and filter sterilization (0.22 μm).

CN32 was originally purified from a subsurface rock at Cerro Negro in New Mexico, U.S.A., and is routinely maintained in -80 °C freezer. For this study, CN32 cells were cultured in a tryptic soy broth (TSB) (BD Bacto, Franklin Lakes, New Jersey) medium to the exponential growth phase.

Nontronite and humic acid stock solutions were suspended in bicarbonate buffer and mixed in 30 mL serum bottles (suspension volume 8 mL) to achieve HS and NAu-2 concentrations of 2.5 g/L and 4.38 g/L [~18 mM Fe(III) conc.], respectively, with *Shewanella putrefaciens* CN32 as a mediator. However, natural concentrations of HS range from 0.1 to a few hundreds of mg C/L (Aiken et al. 1985), high concentrations of LHA and PPHA were used here to facilitate observation of their effects on Fe(III) reduction, similar to previous studies (Lovley et al. 1998, 1996). CN32 cells of the exponential growth phase were washed by repeated centrifugation (at 2000 g for 10 min) and resuspension in anoxic and sterile bicarbonate buffer and injected into the experimental bottles to achieve 1×10^8 cells/mL. For heat-killed microbial control, autoclaved (121 °C, 1 h) cells replaced live ones. All experimental treatments were performed in duplicate. The pH was checked to be 6.92–6.94 throughout the duration of the experiments.

To study the effects of HS concentration and medium type on Fe(III) reduction, another experiment was set up, in which two concentrations of HS (2.5 and 0.25 g/L) but the same NAu-2 concentration (4.38 g/L) were used in a growth medium. The growth medium contained NH₄Cl (4.67 mM), KH₂PO₄ (4.41 mM), KCl (1.34 mM), NaHCO₃ (29.76 mM), Wolfe's mineral solution (10 ml/L), and Wolfe's vitamin solution (10 ml/L). The composition of Wolfe's mineral solution includes nitrilotriacetic acid (1.5 g), MgSO4·7H2O (3.0 g), MnSO4·H2O (0.5 g), NaCl (1.0 g), FeSO4·7H2O (0.1 g), CoCl2·6H2O (0.1 g), CaCl2 (0.1 g), ZnSO4·7H2O (0.1 g), CuSO4·5H2O (0.01 g), AlK(SO4)2·12H2O (0.01 g), H3BO3 (0.01 g), and Na2MoO4·2H2O (0.01 g) in 1.0 L distilled water. Wolfe's vitamin solution contains biotin (2.0 mg), folic acid (2.0 mg), pyridoxine hydrochloride (10.0 mg), thiamine HCl (5.0 mg), riboflavin (5.0 mg), nicotinic acid (5.0 mg), calcium D-(+)-pantothenate (5.0 mg), vitamin B12 (0.1 mg), p-aminobenzoic acid (5.0 mg) and thioctic acid (5.0 mg) in 1.0 L distilled water. Cells were washed by repeated centrifugation (at 2000 g for 10 min) and resuspension in the growth medium and injected into the experimental bottles to achieve 1×10^8 cells/mL. Because both CN32 cells (using energy reserves) (Jiang and Kappler 2008; Klüpfel et al. 2014) and HS may be able to reduce Fe(III) in NAu-2, it is important to determine their electron-donating capacities so that electron balance may be calculated. If CN32 cells could donate electrons, they might transfer electrons to HS first and reduced HS, then donate electrons to Fe(III). Therefore, in a supplementary experiment, CN32 cells (1×108 cells/mL) were mixed with HS in the growth medium (2.5 or 0.25 g/L) for two days to allow the reduction of HS by CN32 cells. After removal of CN32 cells by filtration, NAu-2 (4.38 g/L) was added to reduced HS. By comparing the amount of Fe(II) produced by reduced and nonreduced HS, the amount of Fe(III) reduction that was contributed by CN32 cells was calculated.

Analytical methods

Time course production of total Fe(II) was measured to monitor the progress of Fe(III) reduction. Electron donating capacity (EDC) was measured for aqueous HS before and after the Fe(III) bioreduction under growth condition (Table 1) because more changes were expected in this experiment. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) was used to measure chemical changes of HS from both the buffer and growth medium experiments. Preliminary analyses revealed similar changes in solid phase between the two types of experiments. Therefore, all solid analyses were performed for the bicarbonate buffer bioreduction experiment

TABLE 1. The conditions of experiments and analytical methods used

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	Non-growth condition	Growth condition	
Medium	Bicarbonate buffer	Growth medium	
Bioreduction experiment	\checkmark	✓	
Supplementary experiment		\checkmark	
Production of Fe(II) over time	\checkmark	\checkmark	
EDC		\checkmark	
FT-ICR-MS	\checkmark	\checkmark	
Analysis of the solids (FTIR, ToF	\checkmark		

Notes: Bicarbonate buffer: NaHCO₃ (29.76 mM), KCl (1.34 mM), pH 7.0. Growth medium: NH₄Cl (4.67 mM), KH₂PO₄ (4.41 mM), KCl (1.34 mM), NaHCO₃ (29.76 mM), Wolfe's Mineral solution (10 ml/L) and Wolfe's vitamin solution (10 ml/L), pH 7.0.

under non-growth condition only (for the HS concentration of 2.5 g/L). Fourier transform infrared spectroscopy (FTIR) and time-of-flight secondary ion mass spectrometry (ToF-SIMS) were used to determine the changes of chemical/ molecular compositions of HS. X-ray diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) were used to observe mineralogical transformations after Fe(III) reduction.

Total Fe(II) and Fe(III) measurements

Production of Fe(II) over time was measured with the 1,10-phenanthroline method (Amonette and Templeton 1998). The only modification was that after the addition of 5% H₃BO₃ clay suspension was centrifuged at 12 000 g for 20 min to remove precipitated humic acids before absorbance measurement at 510 nm. Specifically, 0.2 mL clay suspension was first added to 0.48 mL H₂SO₄ (3.6 N). Hydrofluoric acid was then added to dissolve the clay suspension. 1,10-phenanthroline assay measures total Fe(II) (Boyanov et al. 2007). To measure total Fe, Fe(III) was first reduced to Fe(II) with hydroxylammonium chloride (Sigma), and then total Fe(II) was measured with the 1,10-phenanthroline assay. A negligible amount of Fe(II) in those experiments without NAu-2 might be sourced from the HS (Zaccone et al. 2007b).

Measurement of electron-donating capacity (EDC)

Clay-HS samples were centrifuged at 12000 g for 30 min to remove CN32 cells and NAu-2. The EDC values of aqueous HS samples were measured using a mediated electrochemical oxidation (MEO) method developed previously (Aeschbacher et al. 2010; Klüpfel et al. 2014). The measurements were performed on an electrochemical workstation (PGSTAT302N, Metrohm, Switzerland) inside an anaerobic chamber at room temperature. The electrochemical cell was filled with 80 mL PIPES buffer (10 mM), which was pre-purged with N₂ to remove oxygen. The Eh of the buffer solution was set at +0.7 V. After the current was stabilized, 100 μ L stock solution (10 μ M) of electron transfer mediator zwitterionic viologen 4,4'-bipyridinium-1,1'-bis(2-ethylsulfonate) (ZiV) was added to the cell with a microsyringe. After the oxidative current peak became stabilized, a certain amount (200–750 μ L) of aqueous sample was added to the cell with a microsyringe. The MEO current peaks were monitored and integrated to yield the EDC of the samples. All samples were analyzed in triplicates.

Fourier transform-ion cyclotron resonance-mass spectrometry (FT-ICR-MS)

Clay-HS samples were centrifuged at 12 000 g for 30 min. Supernatants were solid phase extracted (SPE) and eluted with methanol based on a previous study (Dittmar et al. 2008). Specifically, the supernatant samples were diluted to 5 mL total volume with nanopore/MilliQ (18Ω) H2O and acidified to pH 2 with 85% H3PO4 (~2 µL of 85% H₃PO₄ for 5 mL sample). Samples were loaded onto a cartridge after activation with 1 mL of methanol. Samples were allowed to elute through a column with a Bond Elut-PPL solid-phase extraction cartridge (Agilent, U.S.A.). Eluants were injected into a 12 T Fourier transform ion cyclotron resonance mass spectrometer (12 T FTICR MS) (Bruker SolariX). Negatively charged primary molecular ions were generated with a standard Bruker electron spray ionization (ESI) source. Samples were introduced to the primary ions through a syringe pump at a flow rate of 3.0 µL/min. Experimental parameters were set as follows: 3.5 kV spray shield voltage, 4.0 kV capillary voltage, and -320 V capillary column end voltage. Each sample was co-added with 144 scans and internally calibrated using -CH2 groups. A m/z range of 100-800 was collected with a mass measurement accuracy of < 1 ppm. Chemical formulas were assigned using in-house software Formularity (Tolić et al. 2017) following the Compound Identification Algorithm described by Kujawinski and Behn (2006), and modified by Minor et al. (2012) and further developed by Tolic et al. (2017) with about 84% assignment for LHA group and 89% assignment for PPHA group.

Statistical analysis was performed to assign molecular formulas for each sample based on the number of carbon (C#), hydrogen (H#), oxygen (O#), nitrogen (N#), and phosphorus (P#). The magnitude-weighted parameters, including DBE (double bond equivalent), DBE/O, DBE-O, and AI (aromatic index), were calculated. DBE represents the degree of unsaturation, and DBE/O ratios are inversely related to the possible number of C=O bonds (i.e., DBE per O) (Roth et al. 2013). Moreover, DBE-O is used to describe C=C unsaturation by omitting all possible C=O bonds. AI is used to describe C=C unsaturation of a compound. All magnitude-weighted parameters were calculated as the sum of all compounds of chemical information and relative intensity by the following formulas (Koch and Dittmar 2006; Sleighter and Hatcher 2008; Sleighter et al. 2010):

$$\begin{split} DBE &= \frac{1}{2} \cdot (2C + N + P - H + 2) \\ (DBE)_w &= \Sigma (DBE_n \cdot \mathcal{M}_n) \\ (DBE/O)_w &= \Sigma [(DBE/O)_n \cdot \mathcal{M}_n] \\ AI &= \Sigma [(1 + C - O - S - 0.5H)/(C - O - S - N - P)]n \cdot \mathcal{M}_n \end{split}$$

where w signifies a magnitude-weighted calculation, *n* signifies that the parameter is calculated for every assigned molecular formula (e.g., C#) represents the number of the specified atoms in the molecular formula, and M_n is the relative intensity of each formula.

Fourier transform infrared spectroscopy (FTIR)

FTIR was used to study structural changes of HS and NAu-2 after reduction. Clay-HS suspension was dried on a parafilm in an anaerobic chamber for 2 days to form a thin and flat pellet. After drying, the clay pellet was carefully removed from the parafilm. Pure NAu-2 and HS controls were measured in powder. Attenuated total internal reflection (ATR) infrared spectra were collected using a Perkin Elmer Spectrum One FTIR that is interfaced to a Harrick Split-Pea ATR accessory. A Ge internal reflection element (IRE) was used and a loading of 0.5 kg (0.5 kg on the 250 μ m diameter sampling area) pressure was applied to the sample to achieve a good contact. FTIR spectra were collected at a 4 cm⁻¹ resolution over a range of 4000–400 cm⁻¹. Each spectrum represented the average of 32 individual scans. Before every scan, a background air spectrum was taken, and subtracted from sample spectra automatically by the control software.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

ToF-SIMS was employed to determine any chemical changes on solid surface as a result of oxidation of HS when coupled with reduction of Fe(III) in NAu-2. Clay suspensions were centrifuged, and pellets were dried in an anaerobic chamber. Pellet samples were analyzed on a ToF-SIMS instrument with a bismuth liquid metal ion gun at 25 keV. Bi3+ was chosen as the primary ion by using appropriate mass filter settings. The pulse width of the primary ion was <1.0 ns. The target current was ~0.58 pA with a scanning area of 200 × 200 µm. ToF-SIMS spectra were obtained in both negative (extraction voltage +2.0 kV) and positive secondary ion modes (extraction voltage -2.0 kV) with three spot analyses per sample. The results were internally calibrated (negative with H-, F-, and CN-, and positive with H+, CH+, and Fe⁺) followed by principal component analysis (PCA) in Matlab (Ding et al. 2016; Hua et al. 2016). PCA is a statistical method used to find combinations of variables that characterize the most important trends in the data. PCA of ToF-SIMS data helps to find out the relations between molecular species contained in complex systems. Peaks in the mass range of m/z 0–400 acquired in negative mode were selected to perform PCA. All peaks were normalized to total ion signal intensity.

X-ray diffraction (XRD)

XRD was performed to study mineral phase changes after reduction. XRD samples were prepared by mounting clay suspension on a petrographic slide and dried in an anaerobic chamber (98% N₂, 2% H₂, Coy Laboratory Products, Grass Lake, Michigan). To differentiate expandable clays (nontronite NAu-2) from non-expandable clays, such as illite, XRD patterns were also obtained by placing dried samples overnight in an ethylene glycol desiccator to allow expansion of nontronite interlayers. To minimize air oxidation, samples were sealed with vacuum glue during XRD analysis. XRD patterns were collected using a Scintag X1 X-ray diffractometer with CuKa radiation, a fixed slit scintillation detector, and power of 1400 W (voltage, 40 kV, current, 35 mA). Scans were collected over a range of 2–35 °(20) with a step size of 0.01° and dwell time of 10 s per step. Mineral phases were identified using MDI Jade 7 software (Zhao et al. 2013).

Scanning electron microscope (SEM) observation

SEM observations were made to observe cell-mineral associations, and any mineralogical changes as a result of Fe(III) reduction. A small amount of clay suspension (0.2 mL in volume) was withdrawn from the experimental bottles inside an anaerobic chamber with a sterile syringe needle and mounted onto a glass cover slip. To promote adhesion of particles to the substrate the glass cover slip was pretreated with 0.1% poly-L-lysine for 1 h at room temperature. Multiple cover slips were stacked into a sample holder (vertically separated by wavy washers) and submerged in a fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.2) for 30 min, followed by rinsing with the same buffer twice and ddH2O twice. A series of dehydration steps was carried out followed by CO2 critical point drying with a Tousimis Samdri-780A Critical Point Dryer (CPD) (Dong et al. 2003). All cover slips were attached onto SEM aluminum stubs with double-sided sticky tape and gold-coated for 45 s (~10 nm coating) with a Denton Desk II sputter coater. The prepared samples were observed with a Zeiss Supra 35 SEM, which was equipped with a Bruker energy-dispersive spectroscopy (EDS) detector. The SEM was operated using 10 keV accelerating voltage and 8.5 mm working distance. EDS spectra were collected for 5 min to determine the chemical composition of various minerals. To obtain representative images, several tens of images were collected from 3-5 cover slips.

Transmission electron microscope (TEM) observation

To further observe mineralogical changes at a high spatial resolution, reduced samples were observed under TEM. Clay suspensions were washed with anoxic and sterilized bicarbonate buffer (pH 7.0) and diluted by a factor of 30. Diluted suspensions were pipetted onto 300 mesh copper grids, which were covered with carbon-coated nitrocellulose membrane support film. All samples were dried overnight in an anaerobic chamber. TEM observations and analyses were carried out on a JEOL JEM-2100 LaB₆ TEM-STEM at a 200 keV accelerating voltage. Bright-field TEM images and selected-area electron diffraction patterns (SAED) were captured with a Gatan Orius SC200D camera and processed with the Digital Micrograph software. EDS spectra were collected for 5 min with a Bruker AXS Microanalysis Quantax 200 with 4030 SDD detector. To obtain representative images, several tens of images were collected from 3–5 grids.

RESULTS

Reduction of Fe(III) in NAu-2 with HS as electron donor

In the presence of HS, CN32 reduced structural Fe(III) in nontronite under both non-growth (i.e., bicarbonate buffer, Fig. 1) and growth conditions (Figs. 2a and 2b). In bicarbonate buffer, a substantial amount of Fe(II) was measured at time zero with 2.5 g/L LHA and PPHA (Fig. 1), suggesting rapid abiotic reduction of Fe(III). The amount of Fe(II) produced only slightly increased over time. In the presence of CN32 cells, both the rate and extent significantly increased. Between the two HS, PPHA reduced more Fe(III) at a higher rate. The final bioreduction extents were 12.2 and 17.8% with LHA and PPHA, respectively.

In growth medium with a 2.5 g/L HS concentration, redox reactions also occurred among HS, CN32 cells, and NAu-2 (Figs. 2a and 2b). Both HS and CN32 cells were able to reduce Fe(III) in NAu-2. In the LHA+NAu-2 treatment, the amount of Fe(II) produced [~0.4 mmol Fe(II) produced per g LHA, Fig. 2a] was similar to that in bicarbonate buffer over the same time frame (8 days), but higher than the corresponding amount of EDC decrease (0.12 mmol e^{-/}g LHA, Fig. 2e). Unexpectedly, CN32 cells also reduced a substantial amount of Fe(III) (Fig. 2a). There was also a redox reaction between CN32 and LHA, which was determined by a supplementary experiment. CN32-reduced LHA reduced more Fe(III) than nonreduced LHA (Fig. 2c). In this case, the net amount of Fe(II) increase, i.e., ~0.4 mmol Fe(II) per g of LHA, was similar to the amount of corresponding amount of EDC increase (i.e., 0.5 mmol e^{-/}g, Fig. 2e). This result suggests that the electrons transferred from CN32 cells to LHA were subsequently



FIGURE 1. Time-course changes of concentration of total Fe(II) in the clay suspensions as measured by the 1,10-phenanthroline method. Panel (**a**) represents the LHA group and panel (**b**) represents the PPHA group in bicarbonate buffer. The error bars in A and B are from duplicate experiments.

used to reduce structural Fe(III) in NAu-2, therefore LHA did not retain any of the CN32-derived electrons. The total extent of Fe(III) reduction in the ternary treatment (CN32+LHA+NAu-2, 5.6 mM) was higher than the sum of those in the two binary treatments, i.e., in CN32+NAu-2 treatment (4 mM) and in LHA + NAu-2 treatment (1.1 mM), suggesting that addition of CN32 cells made more LHA available to reduce Fe(III). The amount of Fe(III) reduction contributed by LHA (1.6/2.5 = 0.6 mmol e/g LHA) was again higher than the corresponding amount of EDC decrease (i.e., 0.2 mmol e⁻/g LHA).

Relative to LHA, PPHA exhibited a higher reducing capacity than LHA (Fig. 2b), apparently because of its higher EDC value (Fig. 2e). In the PPHA+NAu-2 treatment, the amount of Fe(II) produced [1.1 mmol Fe(II) produced per g PPHA] was higher than the corresponding amount of EDC decrease (0.42 mmol/g PPHA, Fig. 2e). Again, pre-reduced PPHA by CN32 reduced more Fe(III) than nonreduced PPHA (Fig. 2d). The net amount of Fe(II) increase by pre-reduced PPHA relative to pristine PPHA, i.e., ~0.4 mmol Fe(II) per g of PPHA, was the same as the corresponding amount of EDC increase (i.e., 0.4 mmol/g, Fig. 2e). The extent of Fe(III) bioreduction in the ternary CN32+PPHA+NAu-2 treatment (7.1 mM) was slightly higher than the sum of those in the two binary treatments, i.e., in CN32+NAu-2 treatment (4.0 mM) and in PPHA+NAu-2



FIGURE 2. Time-course changes of concentration of total Fe(II) in the suspensions as measured by the 1,10-phenanthroline method and EDC values for samples in growth medium. Panels (**a** and **c**) represent the LHA group, and panels (**b** and **d**) represent the PPHA group. (**a** and **b**) Fe(II) production for various controls and treatments. (**c** and **d**) Comparisons in Fe(III) reduction by pre-reduced (by *S. putrefaciens* CN32) and native HS. (**e**) The changes in electron donating capacity (EDC) between HS controls and treated samples (2.5 g/L concentration). The error bars are from duplicate experiments, and those in **e** are from triplicate analyses. (Color online.)

treatment (2.8 mM), suggesting that addition of CN32 cells made more PPHA available to reduce Fe(III), similar to the LHA experiment. The amount of Fe(III) reduction contributed by PPHA (1.2 mmol/g PPHA) was higher than the amount of EDC decrease (i.e., 0.7 mmol/g PPHA).

When the concentrations of LHA and PPHA decreased to 0.25 g/L, abiotic reduction of Fe(III) by these HS was negligible (Figs. 2a and 2b). Interestingly, the extent of Fe(III) reduction in the ternary treatment (NAu-2+HS+CN32 cells) was higher than the sum of those from the two binary systems (NAu-2+HS and NAu-2+CN32 cells). For example, for both LHA and PPHA, the extent of Fe(III) reduction was ~5 mM, but the sum of those from the two binary treatments was only ~4 mM. This result suggests that more LHA and PPHA became available to reduce Fe(III) due to addition of CN32 cells. The amounts of CN32-enabled LHA and PPHA reduction capacities at 0.25 g/L (~1 mM) were higher

than those at 2.5 g/L (~0.3 mM), suggesting that CN32 cells were able to use more LHA and PPHA when electron donor/carbon sources were limited. Because of limited electron accepting capacities (EAC) of HS at 0.25 g/L, there was little difference in the rate and extent of Fe(III) reduction by pre-reduced and nonreduced HS (Figs. 2c and 2d).

Reduction-induced structural changes of LHA and PPHA revealed by FTIR

In the range of 1800–1100 cm⁻¹ (Fig. 3), the aromatic C=C stretching band of LHA and PPHA (1609 and 1617 cm⁻¹, respectively) (Reza et al. 2012) shifted to 1623 and 1633 cm⁻¹ (Fig. 3), respectively, after reaction with NAu-2. After reaction with both NAu-2 and CN32 cells, the intensities of these peaks decreased. Moreover, the C-O stretching motions in the phenol moiety of both PPHA and LHA (Sánchez-Cortés et al. 1998) (at 1214–1219 cm⁻¹) disappeared after Fe(III) reduction regardless of the absence or presence of CN32 cells, suggesting the involvement of the phenolic group of these HS in Fe(III) reduction.

Reduction-induced compositional changes of aqueous HS revealed by FT-ICR-MS

The van Krevelen diagrams for the samples in bicarbonate buffer showed little change after reaction with NAu-2 (Online Materials¹ Fig. OM1). However, the magnitude-weighted parameters, which have been successfully used in the studies of organic



FIGURE 3. Fourier transform infrared (FTIR) spectra in the wavenumber range of 1800–1100 cm⁻¹ showing N-H and R-COObending vibration bands. Pure LHA/PPHA/NAu-2 controls were superimposed on top of samples for comparison. LHA/PPHA peak identification was adopted from International Humic Substances Society (http://humic-substances.org/) and NAu-2 adopted from a previous study (Petruk et al. 1977). The numbers in the figure note the peak position. (Color online.)

	LHA Group			PPHA Group				
	LHA Control	NAu-2&LHA	NAu-2&LHA&CN32	% Change (w.r.t. control)	PPHA Control	NAu-2&PPHA	NAu-2&PPHA&CN32	% Change (w.r.t. control)
(DBE) _w	14.827	14.862	14.678	1	13.949	14.093	13.613	2
St.dev.	0.003	0.003	0.003		0.002	0.002	0.002	
(DBE/O) _w	1.520	1.526	1.494	2	1.384	1.368	1.337	3
St.dev.	0.000	0.000	0.000		0.000	0.000	0.000	
(DBE-O) _w	4.333	4.445	4.204	3	3.288	3.297	2.967	10
St.dev.	0.001	0.001	0.001		0.001	0.001	0.001	
(AI) _w	0.366	0.380	0.359	2	0.276	0.277	0.240	13
St.dev.	0.000	0.000	0.000		0.000	0.000	0.000	
Note: With r	regard to (wrt)							

TABLE 2. Magnitude-weighted parameters for all formulas for samples in growth medium at 2.5 g/L concentration

matter composition (Flerus et al. 2011; Koch et al. 2005; Roth et al. 2013), showed important changes. The overall changes of these parameters were more apparent for treatments in growth medium (Table 2) than in bicarbonate buffer (Online Materials¹ Table OM1) and in the presence of PPHA than in the presence of LHA. (Table 1 and Online Materials¹ Table OM1). These results were consistent with the higher extents of Fe(III) reduction under growth condition and in the presence of PPHA (Fig. 2b). Interestingly, the DBE, DBE/O, DBE-O, and AI (aromatic index) parameters of both LHA and PPHA did not change from control to abiotically oxidized products but decreased upon addition of CN32 cells (by 1-15%). Previous studies have shown that changes in a few percent (2-13%) are considered significant (Roth et al. 2013; Seifert et al. 2016; Waggoner and Hatcher 2017). The DBE-O is used to describe C=C unsaturation by omitting all possible C=O bonds that are abundant in RCOOH functional group (D'Andrilli et al. 2010). The decrease in the DBE-O value with addition of CN32 indicates partial oxidation of LHA and PPHA that involve aromatic compounds (D'Andrilli et al. 2010). The decreased AI value suggests a compositional change from unsaturated aromatic compounds to saturated compounds, consistent with photochemically induced degradation of dissolved organic matter (Gonsior et al. 2009). Consistent with the EDCs and Fe(III) reduction extents (Figs. 1 and 2), these changes were more dramatic for PPHA than for LHA (Table 2).

Reduction-induced molecular changes of LHA and PPHA revealed by ToF-SIMS

As a complementary technique to FT-ICR-MS, ToF-SIMS is capable of detecting reduction-induced changes of chemical and molecular composition of sorbed HS and the underlying NAu-2. Relative to HS and NAu-2 mixtures, addition of heat-killed cells decreased PC2 scores (Fig. 4). The positive loadings of PC2 are mainly F-related clusters such as F-, F2-, CF3, and the negative loadings of PC2 are mainly N-rich organic clusters (Fig. 4c). A decrease of PC2 scores suggests that addition of dead cells increased microbially derived organic species. Live cell addition resulted in higher scores of PC1 (Fig. 4a). The positive loadings of PC1 are mostly N-rich organic species and POx. The negative loadings are SiO_x and hydrocarbon (from humic acids) related species (Fig. 4b). A positive PC1 shift indicates more microbially derived molecular clusters and less HS-derived clusters, suggesting that microbial Fe(III) reduction produced organic materials of microbial origin at the expense of HS. An increased score of PC1 also indicates less detection of mineral surface (Si-O surface), suggesting that more mineral surface might be covered by microbially derived organic species.

Reduction-induced mineralogical changes

Although LHA reduced a lower amount of Fe(III) than PPHA (Figs. 1 and 2), its effect on mineral transformation was more substantial (Fig. 5). After one-month incubation, while the NAu-2 control showed nontronite peaks only with no impurity minerals (Fig. 5a), consistent with the result of the Keeling et al. (2000) on the same size fraction ($<0.5 \mu$ m), several new peaks emerged in reduced samples in the presence of LHA (denoted with arrows in Fig. 5a). These new peaks emerged in the presence of LHA alone but became more intense after the addition of live CN32 cells. A prolonged incubation time, along with a comparison between XRD patterns of air-dried and ethylene glycolated samples allowed positive identification of these new peaks (Fig. 5b). The peak at 20 of 24° in the one-month sample (Fig. 5a), possibly a type of zeolite, disappeared after prolonged incubation (Fig. 5b),



FIGURE 4. Scores and loadings plot of the two principal component analysis of time-of-flight secondary-ion mass spectrometry (ToF-SIMS) spectra data collected in negative mode. (**a**) Spectral PCA scores plot of LHA (squares) and PPHA groups (circles). (**b**) Loadings plot of PC1 showing distributions of different ions in positive and negative values. (**c**) Loadings plot of PC2 showing distributions of different ions in positive and negative values.



FIGURE 5. X-ray diffraction patterns for NAu-2 control and HA-CN32 treated NAu-2 samples. (a) Leonardite humic acid (LHA) groups incubated for 1 month showing emergence of new peaks (denoted with arrows) in reduced samples. (b) XRD patterns for air-dried and ethylene glycolated Leonardite humic acid (LHA) groups that were incubated for 9 months, showing the presence of the two strongest peaks of talc (JCPDS no. 19-0770) and the strongest peak of albite (JCPDS no. 10-0393). (c) Pahokee Peat humic acid (PPHA) treatment groups showing no detectable difference in mineralogy among different samples.

suggesting its transient nature. Two small peaks at 20 of 9.45 and 28.59°, which remained after 9 month incubation, matched the two strongest lines of standard talc [d(001)] and d(003), Joint Committee on Powder Diffraction Standards (JCPDS) no. 19-0770, also see (Gunter et al. 2016; Kursun and Ulusoy 2006), among others]. A previous study similarly observed these two strongest lines of talc (Liu et al. 2016). One peak at 20 of 28°, which remained after prolonged incubation (Fig. 5b), matched the strongest line [d(002)] of disordered albite [JCPDS no. 10-0393, also see (Xu et al. 2017)]. Because the strongest peak was small, other weaker peaks were not visible [see for example Fig. 5a in Hupp and Donovan (2018)]. As expected, ethylene glycolation shifted all nontronite peaks to smaller 2θ (larger *d*-spacings), but the talc and albite peaks remained at the same positions. Secondary minerals in the presence of both LHA and CN32 cells (data not shown) were similar to those with the presence of LHA only. In contrast to LHA, no new peaks emerged after one-month incubation in the presence of PPHA (Fig. 5c).

SEM observations confirmed XRD data showing the formation of these new minerals in LHA-treated samples (Fig. 6). The original NAu-2 particles exhibited a flaky and wavy shape (Fig. 6a) with a typical nontronite composition but with a small Fe peak. A small Fe peak has been observed in multiple studies of NAu-2 (for example, Liu et al. 2016; Zhao et al. 2013). Absorption of characteristic X-ray of Fe by sample itself or Au coating could be a possible reason. After reduction, NAu-2 particles gained some K and Al, and lost Fe, yielding a composition similar to high-charge smectite or mixed-layer illite-smectite (Fig. 6b). Sapodilla-like silica oxide also formed (Fig. 6c), likely in an amorphous form, because quartz was not detected in XRD pattern (Fig. 5). Unexpectedly, ilmenite (FeTiO₃) was observed in the reduced sample, with $1.2 \times 3 \mu m$ in size (black arrow in Fig. 6c), in the same field of view as CN32 cells (white oval in Fig. 6c). The corresponding EDS spectrum showed O, Fe, and Ti, and Si. The Si peak was always present, apparently due to abundant silica available from reductive dissolution of NAu-2 (see silica oxide in Fig. 6c). Furthermore, albite [NaAlSi₃O₈] was observed (white arrow in Fig. 6d and black arrow Fig. 6e). Talc $[Mg_3Si_4O_{10}(OH)_2]$ was found in the same field of view as albite (white arrow Fig. 6e). Talc with a layered structure was also observed (Fig. 6f). A small amount of Fe was observed in talc, possibly forming a Mg-Fe solid solution (Agangi et al. 2018; Forbes 1969; Howe 2017). Al peak was absent in fibrous talc but present in layered talc. The common presence of albite and talc under SEM was consistent with XRD data (Fig. 5).

TEM observations of the reduced NAu-2-LHA sample confirmed XRD and SEM results. A talc particle of ~1.8 × 2.4 µm in size was observed under TEM (Fig. 7a). An enlarged TEM image showed subtle lattice fringes (Fig. 7b). The corresponding SAED pattern showed a pseudo-hexagonal electron diffraction pattern (Fig. 7c). The ideal composition of talc is Mg₃Si₄O₁₀(OH)₂, but the Fe content was quite high, apparently due to abundant Fe available from reductive dissolution of NAu-2. Fe can substitute Mg in trioctahedral sites in the talc structure to form a Mg-Fe solid solution (Forbes 1969). Ilmenite particles were also commonly observed with a typical size of ~1.4 × 1.2 µm (Fig. 8a). A high-resolution TEM image of ilmenite displayed 0.27 and 0.25 nm lattice fringes (Fig. 8b), corresponding to reflections



FIGURE 6. SEM images showing the formation of new minerals as a result of LHA and live cell treatment; (**a**) original NAu-2 sample with a typical nontronite composition; (**b**) an illite-smectite (I-S) particle showing a platy structure (white arrow) and CN32 cells (white oval). EDS composition of the I-S particle shows a small K peak. (**c**) Neoformed silica particle (white arrow) with Si and O peaks in the EDS spectrum and black arrow denotes ilmenite with an EDS spectrum showing O, Fe, and Ti. CN32 cells (white oval) are in the same field of view as neoformed silica and ilmenite particles. (**d**) A typical albite crystal with Na, Ca, Al, and Si as major elements. (**e**) Fibrous talc particles (white arrow) along with a plate-like albite particle. The talc exhibits a typical composition (e.g., Mg and Si with some Fe). (**f**) A talc particle showing a layered texture with a similar composition but with some Ca and Al. In all EDS spectra, the Au peak is from Au coating. The C peak should be derived from bicarbonate buffer and LHA.



FIGURE 7. Talc identification by TEM observation and EDS analysis of the LHA-NAu-2-CN32 sample. (a) A low-magnification TEM image of a talc particle displaying a layered structure with a size $\sim 1.8 \times 2.4 \,\mu$ m. (b) An enlarged TEM image displaying an edge of the particle in **a** (white square), showing lattice fringes; however, it was difficult to acquire a high-magnification lattice fringe image due to the large thickness. (c) SAED pattern of the corresponding area in **b** showing a pseudo-hexagonal electron diffraction pattern when taken along the [001] zone axis. (d) Corresponding EDS spectrum of talc in **a** showing a typical composition but with enriched Fe.



FIGURE 8. Authigenic ilmenite and ferrihydrite as observed under TEM and their corresponding EDS spectra. (a) A low-magnification TEM image showing sharp edges of an ilmenite particle. (b) A high-resolution TEM image of the squared area in **a**, showing 0.25 and 0.27 nm lattice fringes for ($\overline{120}$) and (104) reflections, respectively. (c) A corresponding EDS spectrum of the ilmenite particle of the squared area in **a**, showing a typical composition of Fe, Ti, and O. (d) A SAED pattern of ilmenite and ferrihydrite mixture. There appear to be three sets of SAED patterns superimposed onto one another: (1) the reflections of one single crystal of ferrihydrite (indexed, along the [$\overline{020}$] zone axis); (2) polycrystalline ferrihydrite; (3) ilmenite single-crystal patterns. 2 and 3 are not indexed for clarity. (e) A SAED pattern of polycrystalline ferrihydrite showing concentric rings. (f) An ilmenite single-crystal SAED pattern taken along the [$42\overline{11}$] zone axis.

of (104) and $(\overline{1}20)$, respectively. Even in thin edges of ilmenite, the corresponding EDS spectrum always contained a certain amount of Si (Fig. 8c). Under high resolution, there appeared to be another phase that was intimately mixed with ilmenite (Fig. 8d). To separate the two phases, a series of SAED patterns were obtained by slowly moving the sample stage. In doing so, SAED patterns for pure ilmenite and ferrihydrite were obtained (Figs. 8d-8f). Based on these end-members, the mixed SAED pattern was deconvolved into three sets: (1) one ferrihydrite crystal, taken along the $[0\overline{2}0]$ zone axis, based on Michel et al.(2007) (Fig. 8d); (2) a polycrystalline ferrihydrite (concentric rings) with d-spacings of 0.45, 0.26, 0.17, 0.15, and 0.13 nm (Fig. 8e); (3) an ilmenite single-crystal pattern taken along the $[42\overline{1}]$ zone axis (Fig. 8f). As expected, the SAED pattern of ilmenite displayed reflections (104) and $(\overline{120})$, consistent with the lattice fringe images (Fig. 8b).

DISCUSSION

HS as electron donor to reduce structural Fe(III) in nontronite

A few studies have investigated abiotic reduction of solidphase Fe(III) by HS. While an early study did not show reduction of poorly crystalline Fe(III) oxides by natural (nonreduced) HS (Lovley et al. 1996), subsequent studies showed some abiotic reduction of aqueous Fe^{3+} (Peretyazhko and Sposito 2006) and solid-phase Fe(III) by nonreduced LHA and/or PPHA (Bauer and Kappler 2009; Piepenbrock et al. 2014; Sundman et al. 2017). When a high concentration of LHA (2 g/L) was used, aqueous Fe^{3+} was reduced by nonreduced LHA (Peretyazhko and Sposito 2006). At a low-HS concentration (~0.3 g/L), nonreduced PPHA reduced small amounts of poorly crystalline ferrihydrite (Bauer and Kappler 2009; Piepenbrock et al. 2014). At 0.6 g/L, PPHA also reduced a negligible amount of Fe(III) in magnetite (Sundman et al. 2017). These studies collectively illustrate that intrinsically reduced functional groups present in native HS are able to reduce solid-phase Fe(III). However, the extent of reduction is limited, likely because of low-HS concentrations used in previous experiments.

Our study demonstrated that native LHA and PPHA significantly reduced structural Fe(III) in nontronite. Compared to Fe(III) oxides (reduction extents of 0.13–1.3%) (Bauer and Kappler 2009; Piepenbrock et al. 2014; Sundman et al. 2017), this study revealed much higher extents of Fe(III) reduction in nontronite NAu-2 (12.2 and 31.1% for 2.5 g/L LHA in bicarbonate and growth medium, respectively; 17.8 and 39.0% for 2.5 g/L PPHA in bicarbonate and growth medium, respectively). These much higher extents were likely because of a higher concentration of HS used (2.5 g/L in this work vs. 0.3–0.6 g/L in Fe-oxide studies). However, at a low concentration (0.25 g/L), abiotic reduction was negligible (Figs. 2a and 2b). Interestingly, when CN32 cells were present, LHA and PPHA donated more electrons to NAu-2. This result suggests that CN32 cells were able to make additional electrons of LHA and PPHA available to reduce more structural Fe(III) in NAu-2.

LHA and PPHA changes after electron donation to NAu-2

Although our measured EDCs of LHA and PPHA (0.86 and 0.95 mmol e- per gram C, respectively) fell within the previously reported ranges (1.9–2.4 and 1.0–1.8 mmol e- per gram C, respectively, Klüpfel et al. 2014; Zheng et al. 2019), the relative magnitude between LHA and PPHA was different. Nonetheless, our EDC data were consistent with the Fe(III) reduction extent, i.e., PPHA with a higher EDC reduced more Fe(III) (Figs. 1, 2a, and 2b). These data suggest that different batches of humic acids from the IHSS, even under the same name, may have different EDC values and reduction capacities. The following discussion is focused on the relative change of EDC.

A comparison between the amount of Fe(III) reduction (Figs. 2a and 2b) and the amount of EDC changes (Fig. 2e) suggests that the amount of Fe(III) reduction was in general higher than the corresponding amount of EDC decrease, i.e., excess amounts of Fe(III) were reduced relative to the amount of EDC decreased. Because EDC was measured for aqueous LHA and PPHA, and it is known that LHA and PPHA can sorb onto clay mineral surface (Ghosh et al. 2009; Liu et al. 2017; Wang and Xing 2005; Zhang et al. 2012), it is likely that aqueous HS may have only partially contributed to Fe(III) reduction. Sorbed HS may be important in reducing structural Fe(III) as well. Indeed, a previous study (Liu et al. 2017) observed significant amounts of Fe(III) reduction in NAu-2 by sorbed HS. Because mineral-sorbed and aqueous HS generally have different chemical composition and functional groups, due to sorptive fractionation of HS (Zhang et al. 2012), they are expected to have different Fe(III) reduction capacities (Liu et al. 2017; Sowers et al. 2019). Sorbed HS may be more readily donating electrons to structural Fe(III) in NAu-2, because of spatial proximity to the structural Fe(III). Therefore, more decrease in EDC is expected for sorbed HS, which would account for excess Fe(III) reduction relative to the measured EDC decrease of aqueous HS. Direct EDC measurement of sorbed HS was not possible because of their association with NAu-2.

Our FTIR, ToF-SIMS, and FT-ICR-MS data support this scenario in showing that sorbed HS exhibited significant compositional change. For example, our FTIR data revealed that, after LHA and PPHA donated electrons to Fe(III), their peaks at 1214–1219 cm⁻¹ disappeared (Fig. 3), suggesting that the phenolic moieties of sorbed HS were responsible for electron donation to Fe(III), consistent with a previous study (Aeschbacher et al. 2012). Furthermore, the positions of the aromatic C=C stretching bands of sorbed LHA and PPHA shifted after they donated electrons to Fe(III), but addition of CN32 cells only decreased their intensity. These changes were consistent with the reduction data, showing that most LHA and PPHA were oxidized abiotically by NAu-2, and addition of CN32 only slightly oxidized some LHA and PPHA (Figs. 2a and 2b).

In comparison to sorbed HS, aqueous LHA and PPHA did not show much change after abiotic reaction with NAu-2, but exhibited some change in the presence of CN32 cells (Table 2). These trends suggest that abiotic reduction of NAu-2 may have been accomplished by sorbed HS, perhaps because of their spatial proximity to the Fe(III) centers in the NAu-2 structure. Electron transfer from sorbed HS to structural Fe(III) in NAu-2 should have occurred fast [i.e., nearly instantaneous Fe(II) production at time zero, Figs. 1, 2a, and 2b]. In contrast, biotic reduction of NAu-2 may have been largely achieved by aqueous HS. In this case, electron transfer from aqueous HS and cells would have been slower (Figs. 1, 2a, and 2b, because of spatial distance and site blocking of NAu-2 surface by sorbed HS, similar to the site blocking of Fe-oxides by sorbed HS (Piepenbrock et al. 2011).

The observation that *S. putrefaciens* CN32 was able to use HS as electron donor was unexpected, because this strain is known to only utilize simple compounds, such as lactate and H_2 , as electron donors when coupled with reduction of structural Fe(III) in clay minerals (Dong et al. 2009; Zhao et al. 2013, 2015). However, LHA and PPHA are alkaline extracts of natural organic matter (Kleber and Lehmann 2019; Lehmann and Kleber 2015) and likely contain bioavailable electron donors such as amino acids (Sutton and Sposito 2005). Indeed, previous studies (Cao and Jiang 2019; Yang et al. 2016) showed that low molecular weight fractions of LHA and PPHA accounted for 0.4–2% of the total carbon content in these HS. Therefore, small molecules of HS may have been utilized by CN32.

S. putrefaciens CN32 as electron donor to reduce structural Fe(III) and HS

Our data demonstrated that this bacterium itself can serve as an electron donor to reduce Fe(III) and HS (Figs. 2a and 2b). A few studies have reported reducing the capacity of bacterial cells (Jiang and Kappler 2008; Klüpfel et al. 2014; Mejia et al. 2018). In one study (Jiang and Kappler 2008), Geobacter sulfurreducens reduced HS without any known electron donor. The authors concluded that either the cells stored electron equivalents or HA contained some electron donors such as amino acids (Sutton and Sposito 2005). Similarly, Klüpfel et al. (2014) observed a reduction of PPHA by S. oneidensis, and the authors ascribed this to electron donors present in either lactose mother liquor medium (Achal et al. 2009) or intracellular biomass. Mejia et al. (2018) observed ferrihydrite bioreduction by HS but without any known electron donor. Likewise, our bicarbonate buffer and growth medium did not contain any electron donor other than HS. Pre-reduced HS by CN32 cells reduced more Fe(III) relative to un-reduced HS, and the amount of enhancement was the same between LHA and PPHA (Figs. 2c and 2d). More importantly, CN32 cells reduced structural Fe(III) even without any HS. Therefore, in this case, energy reserve or secreted substances of CN32 cells more likely served as electron donors. Interestingly, this intracellular reducing capacity has been reported for an Fe(II) oxidizer Pseudogulbenkiania sp. strain 2002 (Weber et al. 2001; Zhao et al. 2013).

Secondary mineralization

Because of the presence of talc, ilmenite, and plagioclase in the >2 μ m size fraction of nontronite NAu-2 (Keeling et al. 2000), some of these mineral phases observed could have been inherited from the original NAu-2, despite the fact that the size fraction of 0.02-0.5 µm used in this study was free of any impurities (Keeling et al. 2000). Several lines of evidence suggest that talc and albite are newly formed from reductive dissolution of NAu-2 by HS and CN32 cells. First, the same size fraction of NAu-2 control (0.02-0.5 µm) did not show any of these impurities (Fig. 5a). Second, all XRD patterns in PPHA treatments did not show any new mineral formation (Fig. 5c). Third, time-course XRD data showed that talc and albite formed only at a later stage of reduction (4 and 12 days, respectively, data not shown), not at the beginning. Fourth, under both SEM and TEM talc and albite were commonly observed in reduced samples but were never observed in unreduced NAu-2 control. These HAand time-dependent formations of talc and albite suggest that they were newly formed, likely via a dissolution-precipitation mechanism (Liu et al. 2015, 2016). Although ilmenite was not present in the <0.5 µm size fraction of the original NAu-2 either (Keeling et al. 2000), its reduction-induced formation was less convincing because it was not detected by XRD in either unreduced or reduced samples (Fig. 5). SEM and TEM observations were not sufficient to suggest their exact origin because some ilmenite particles in the original NAu-2 may have escaped size fractionation and made into the <0.5 µm size fraction. However, the ilmenite composition consistently showed the presence of Si, likely because the aqueous solution was in excess of Si (as evidenced by silica oxide precipitation, Fig. 6c). In addition, the intimate association of ilmenite and ferrihydrite (Fig. 8) further suggests that ilmenite observed might not be entirely from the original NAu-2, because ferrihydrite was absent in the original NAu-2 (Keeling et al. 2000). In this case, a certain amount of Ti associated with HS (Zaccone et al. 2007a) can be a source of Ti for ilmenite formation.

Geochemical speciation modeling using Geochemist's Workbench (GWB standard 14, Aqueous Solutions LLC, Champaign, Illinois) did not show super-saturation of these minerals. This observation is not surprising considering that our system is heterogeneous due to the presence of minerals and HS. In fact, multiple studies have shown mineral nucleation and growth in under-saturated bulk solutions if organic materials and solid interfaces serve as nucleation templates to locally enrich ions (Deng et al. 2019; Hövelmann et al. 2018; Roberts et al. 2004). For example, Deng et al. (2019) showed barite formation on organic films, despite their bulk solution was undersaturated with respect to barite, likely because of local supersaturation near the organic molecules.

A comparison between LHA and PPHA demonstrated that the treatments with LHA produced more extensive mineralogical transformations, despite its lower EDC and lower extent of Fe(III) reduction (Figs. 1 and 2). The opposite effect was observed for PPHA, where PPHA reduced more Fe(III), but there was no secondary mineralization. A similar effect was observed in a comparative study between PPHA and AQDS (Liu et al. 2016), where PPHA reduced less Fe(III) in NAu-2 but produced more illite and talc. These results collectively suggest that the roles of HS in electron donation and mineral transformation are not coupled. The electron-donating capacity of HS should be related to the phenolic moieties of HS (Aeschbacher et al. 2012; Liu et al. 2017; Ratasuk and Nanny 2007). However, the role of HS in mineral transformation may be related to their metal complexation ability, which is positively correlated with aromatic carbon content (Fujii et al. 2014). Relative to PPHA, LHA has a higher aromatic carbon (58 vs. 47%), aromaticity (0.72 vs. 0.65), and C/H ratio (1.45 vs. 1.23) (Fujii et al. 2014), all of which may have accounted for its stronger role in catalyzing mineral transformation. Furthermore, this decoupling between Fe(III) reduction and mineral transformation suggests that electron transfer from HS to Fe(III) occurred largely in solid state before dissolution occurred. Otherwise, LHA would have reduced more Fe(III) because ligand- and HS-complexed aqueous Fe³⁺ is more reducible than structural Fe(III) in smectite (Kostka et al. 1999) and ferrihydrite (Amstaetter et al. 2012; Nevin and Lovley 2002). Mineralogical transformation likely occurred after Fe(III) reduction by LHA, because reduced NAu-2 is less stable than un-reduced NAu-2 due to structural dehydroxylation (Stucki et al. 1996).

In previous studies on abiotic reduction of Fe oxides by HS (Piepenbrock et al. 2014; Sundman et al. 2017), little mineral transformation occurred, likely because of low-HS concentrations used (0.3–0.6 g/L). In this study, with 2.5 g/L, secondary minerals were observed in both abiotic and biotic LHA treatments, but with apparently higher abundance in biotic treatment based on XRD peak intensity (Fig. 5a). Although talc, plagioclase, and illite have been observed individually in previous studies (Liu et al. 2015, 2016), this study observed their co-presence. Because Mg was not present in the bicarbonate buffer, we inferred that Mg in talc may have been derived from NAu-2 or LHA. An insufficient amount of Mg may explain the formation of Fe-containing talc (Figs. 6 and 7).

A two-step mechanism has been proposed previously to explain the HS-promoted tale formation (Liu et al. 2016). In the first step, HS was believed to enhance NAu-2 reduction and dissolution, and in the second step, the cations released from NAu-2 (such as Si and Fe) would combine with Mg to precipitate tale. In the presence of microbial cells and their metabolites, mineral precipitation may be facilitated due to the bacterial cell surface and/or metabolic products serving as templates. For example, microbial polysaccharides are believed to template the synthesis of secondary minerals (Chan et al. 2004), especially carboxyl groups that play an important role in mineral crystallization (Konhauser and Urrutia 1999). In our study, because Mg may be partially derived from LHA (Calvert et al. 1985; Zaccone et al. 2007a), HS may have served as an additional template for talc precipitation.

A small amount of ferrihydrite formation (Fig. 8) is unexpected because the overall experimental system was reducing. It may be possible that a small fraction of Fe(III) might be released from HS-induced dissolution of NAu-2, similar to the citrate-induced dissolution of NAu-2 (Liu et al. 2018), and released Fe(III) may form complexes with HS, thus stabilizing such complexed Fe(III) against bioreduction (Maldonado and Price 2001). Ferrihydrite is usually an unstable phase, and over time it should transform to more stable minerals such as goethite (Schwertmann and Murad 1983). However, the presence of HS may stabilize ferrihydrite, similar to the inhibition of aqueous Fe²⁺ induced transformation of ferrihydrite to crystalline Fe

oxides by coprecipitated organic matter (Chen et al. 2015). In this case, organic matter may block the reactive surface sites of ferrihydrite and thus retards its transformation to more stable Fe(III) oxide minerals.

IMPLICATIONS

Humic substances (HS) are a major organic constituent in peatlands and wetlands, which represent a vast global carbon reservoir (Davidson and Janssens 2006; Freeman et al. 2004; Rydin and Jeglum 2013). Our data demonstrated that natural HS can serve as electron donors to reduce structural Fe(III) in clays and promote secondary mineral formation. Although ecologically less relevant concentrations of HS were used in these experiments, given the fact that electron transfer from and to these substances may be reversible (Klüpfel et al. 2014), the accumulative effects of multiple redox cycles of HS may be substantial. Furthermore, in certain peatlands and permafrost (Gentsch et al. 2018; Kirk et al. 2015), organic carbon concentration can be as high as \geq 12–20%, which is in a similar range as those used in this study. In such environments, interactions between clay minerals and HS are expected to have an important consequence on Fe redox cycle and mineral transformations.

Our data demonstrated the neoformation of talc, albite, illite, and ferrihydrite at the expense of nontronite when humic acids are present in sufficient concentrations. During the dissolution process, nutrients and trace metals may be released into aqueous phase (Rogers and Bennett 2004), but mineral precipitation would resequester them (Donahoe 2004). Because clay minerals have higher surface area and adsorption capacity than secondary minerals, transformations of clays to secondary minerals would result in a decreased surface area and adsorption capacity, thus resulting in an overall release of nutrients, trace metals, and organic carbon. Therefore, investigation of the mutual interactions among clay minerals, organic matter, and microbes is important for understanding soil fertility, nutrient cycling, carbon cycle, and contaminant migration.

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Endnote:

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