**The Role of Fe(III) Bioreduction by Methanogens in the Preservation of Organic
Matter in Smectite**

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ABSTRACT

Organic matter in sediments is largely associated with clay minerals and can be preserved over geological time. However, microbial activity can possibly influence this association and release organic matter from clay minerals via reductive or oxidative dissolution of clay minerals. In this study, the relationship between bioreduction of structural Fe(III) in smectite and organic matter release from smectite structure was investigated. A model organic compound, 12-aminolauric acid (ALA) was intercalated into the interlayer region of an iron-rich smectite (nontronite, NAu-2). Two methanogens: mesophilic *Methanosarcina mazei* and thermophilic *Methanothermobacter thermautotrophicus* were selected to reduce structural Fe(III) in ALA-intercalated nontronite. As a comparison, sodium dithionite was used to chemically reduce structural Fe(III) in the same mineral. The results showed that the intercalation of ALA into the nontronite interlayer decreased both the rate and the extent of Fe(III) bioreduction. Furthermore, methanogenesis was more inhibited by the presence of intercalated ALA in the nontronite structure relative to pure nontronite. After the bioreduction, the intercalated ALA was partially released, and the extent of release was positively correlated with the extent of Fe(III) reduction. A low reduction extent of bioreduction (<30%) resulted in little ALA release, whereas a nearly complete chemical reduction by sodium dithionite released all intercalated ALA. SEM observations and aqueous chemistry data suggested that reductive dissolution was a main mechanism for the observed ALA release. Because naturally prevalent biological reduction is a slow process with a low reduction extent relative to chemical reduction, the

results of this study demonstrated that organic matter preserved within smectite structure should not be released by the mechanism of iron reduction.

Key words: methanogen, iron reduction, organic matter, intercalation, nontronite

1 INTRODUCTION

2 Organic matter burial in sediments results in a rise in the oxygen level in the
3 atmosphere, lowers the atmospheric CO₂ level, and thus decreases the temperature of the
4 Earth's surface (Beckmann et al., 2005; Rabalais et al., 2009; Negri et al., 2009; Jenkyns,
5 2003; Lutzow et al., 2006). In contrast, organic matter release from sediments and
6 subsequent oxidation consumes oxygen and increases the CO₂ level in the atmosphere
7 (Kennedy and Wagner, 2011). Organic matter preservation in sediments also has
8 important implications for petroleum generation (Bennett, et al., 2013).

9 Most organic matter in aquatic sediments is associated with minerals, especially fine-
10 grained clay minerals, due to their high surface area and strong adsorption capacity
11 (Mayer et al., 1985; Mayer, 1994; Keil et al., 1994; Ransom et al., 1998; Mayer and Xing,
12 2001; Arnarson and Keil, 2001; Kennedy et al., 2002; Galy et al., 2007). In clay-rich
13 sediments, organic matter abundance is found to positively correlate with smectite
14 abundance (Kennedy et al., 2002; Huguet, et al., 2008). This correlation suggests that the
15 expandable internal surface of smectite (i.e., interlayer region) can sequester large
16 amounts of organic matter (Kennedy and Wagner, 2011). Indeed, it has been suggested
17 that enhanced organic matter preservation in sediments is largely due to increased
18 smectite abundance in sedimentary record through geological time (Kennedy et al., 2006).
19 This organic matter preservation within expandable smectite has been postulated to lead
20 to subsequent cooling of the earth surface temperature and emergence and evolution of
21 macro-biota at the Precambrian/Cambrian boundary (Kennedy et al., 2006). One
22 assumption inherent in this model is that organic matter, once associated with smectite-
23 rich sediments, is physically protected against microbial oxidation. However, it is now

well-known that microbial activities can dissolve clay minerals via reduction of structural Fe(III); oxidation of structural Fe(II); or pH change (Weber et al., 2006; Dong et al., 2009). All these activities can possibly release organic matter from clay minerals, but these effects have not been directly examined.

Iron redox cycling represents a potential mechanism to release organic matter from clay minerals. Iron, as the fourth most abundant element in the earth crust, is ubiquitous in clay-rich soils, sediments, and sedimentary rocks (Stucki and Kostka, 2006; Dong et al., 2009; Dong, 2012). Structural Fe(III) in clay minerals can be reduced either chemically or biologically (Stucki and Kostka, 2006; Dong et al., 2009). Chemically, hydrazine and dithionite are common reductants for Fe(III) (Rozenon and Heller-Kallai, 1976; Stucki et al. 1984; Lee et al., 2006). Biologically, since the isolation of dissimilatory Fe(III) reducing bacteria (DIRB) in 1980s (Lovley and Philips, 1988; Myers and Nealson, 1988), numerous studies have demonstrated that a variety of anaerobic microorganisms are capable of iron reduction in iron-bearing clay minerals, including typical DIRB (Kostka et al., 1996; Lovley et al., 1998; Dong et al., 2003; Zhang et al., 2007a), sulfate-reducing bacteria (Li et al., 2004; Liu et al., 2012), and even methanogens (Liu et al., 2011a; Zhang et al., 2012). The reduction of structural iron in clay minerals can result in important changes in the physical and chemical properties of these minerals, such as layer charge, degree of swelling, and surface area (Stucki, 2011). Reductive dissolution of clay minerals can occur as a result of Fe(III) reduction, especially when the extent of reduction is greater than 30% (Jaisi et al., 2008; Dong et al., 2009), but the impact of the dissolution on the fate of clay-associated organic matter is unclear.

Methanogens are cosmopolitan archaea on both modern and ancient earth, and they

can utilize different types of organic matter to produce CH₄. Methanogens can co-exist with clay minerals in a variety of anoxic environments (Achnich et al., 1995; Van Bodegom and Stams, 1999; Liu et al., 2011a). Recent studies have revealed that certain types of methanogens can reductively dissolve some clay minerals and inhibit methane production (Liu et al., 2011a; Zhang et al., 2012; Zhang et al., 2013). This inhibition is likely due to diversion of electrons from methanogenesis to Fe(III) reduction (Bond and Lovley, 2002; Liu et al., 2011a; Zhang et al., 2012). These studies suggest that clay-bound Fe(III) as an alternative electron acceptor for methanogens may be an important factor in influencing organic carbon preservation in clay minerals.

The objective of this study was to investigate if organic matter could be released from clay minerals by either microbial or chemical reduction of structural Fe(III) in clay minerals. We conducted laboratory experiments to address the following questions: (i) can organic matter that is intercalated in expandable smectite be released by chemical or biological reduction of structural Fe(III)? If so, what are the extents and rates of organic matter release; (ii) does the presence of organic matter in the interlayer of smectite affect Fe(III) reduction by methanogens; (iii) what is the relationship between iron reduction, organic matter release, and methanogenesis; and (iv) what is the mechanism of organic matter release by methanogens. To address these questions, a model organic compound, 12-Aminolauric acid (ALA) was intercalated into the interlayer of an iron-rich smectite, nontronite (ALA-NAu-2). Two methanogens, mesophilic *Methanosarcina mazei* (optimal growth temperature of 37°C) and thermophilic *Methanothermobacter thermautotrophicus* (optimum growth temperature of 65°C), were selected to reduce structural Fe(III) in NAu-2 and ALA-NAu-2. Various geochemical and mineralogical methods were

employed to examine the reaction progress and to characterize mineralogical transformations. Our results demonstrated that the intercalated ALA in nontronite was largely protected from biological reduction of structural Fe(III), but chemical reduction by sodium dithionite resulted in a large amount of ALA release through reductive dissolution. The results of this study have important implications for the understanding of the relationship between the preservation of organic matter in clay minerals and iron biogeochemistry.

2. MATERIALS AND METHODS

2.1 Clay mineral preparation

Nontronite NAu-2 was purchased from the Source Clays Repository of the Clay Minerals Society (West Lafayette, IN) for its high iron content. Our previous study showed that Fe(III) bioreduction was positively correlated with Fe content (Bishop et al., 2011). Therefore, this iron-rich smectite represents an extreme case for testing the release of clay-associated organic matter. Previous studies have shown the following formula for NAu-2:

$(K_{0.01}Na_{0.30}Ca_{0.15})(Al_{0.55}Fe^{3+}_{3.27}Fe^{2+}_{0.06}Mg_{0.12})(Si_{7.57}Al_{0.15}Fe^{3+}_{0.28})O_{20}(OH)_4$ (Bishop et al., 2011). The bulk nontronite was manually ground and soaked in a 0.5 N NaCl solution overnight followed by centrifugation to separate the 0.02-0.5 μm fraction. The chloride anion was completely washed by double distilled water, and its complete removal was confirmed with $AgNO_3$. The BET surface area of the 0.02-0.5 μm fraction is 271 m^2/g (Bishop et al., 2011).

2.2 Intercalation of organic matter into nontronite

12-Aminolauric acid (ALA) [$\text{NH}_2(\text{CH}_2)_{11}\text{COOH}$] was used as a model organic compound. This compound has a similar structure as amino acid and was selected in this study for two reasons. First, the intercalation of ALA in smectite interlayer was more stable than amino acid because of its long chain length. There was no release of ALA even after several washes with hot water ($>80^\circ\text{C}$). This stability was an important consideration for the purpose of examining the effect of Fe(III) reduction on ALA release. Second, our selected methanogens could not use it as a carbon source so that again we could evaluate the effect of Fe(III) reduction on ALA release. ALA was commercially purchased from TCI AMERICA (CAS Number: 693-57-2).

Prior to ALA intercalation into the interlayer region of nontronite, 2g of the 0.02-0.5 μm fraction of NAu-2 was placed in a beaker and mechanically stirred in 200 mL deionized water for several hours until NAu-2 suspension was completely swollen. ALA (0.6 g, 2.8 mmol) was pre-treated with HCl (0.07 N; pH = 1.14, 40 mL) to form protonated ALA followed by addition to the NAu-2 suspension. After vigorously stirring for 30 min at 80°C , the resultant ALA-intercalated nontronite NAu-2 (ALA-NAu-2) was collected by centrifugation and washed thoroughly several times with deionized water. The whole procedure was operated under 80°C to ensure full dissolution of ALA in water. The intercalation of ALA into the nontronite interlayer was confirmed with X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, and total organic carbon (TOC) measurement. Total Fe and Fe(II) contents in NAu-2 and ALA-NAu-2 were measured with the 1,10 phenanthroline method ([Amonette and Templeton, 1998](#)).

2.3 Methanogen growth

Two methanogens, mesophilic *M. mazei* and thermophilic *M. thermautotrophicus* were kindly provided by Dr. Xiuzhu Dong (Institute of Microbiology, Chinese Academy of Sciences, Beijing, China). These strains are routinely cultured in a sulfate-free culture medium (revised from Zehnder and Wuhermann, 1977) under strictly anoxic condition. In brief, this medium consists of (per liter) 0.54 g KH_2PO_4 , 1.33 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 1.8 g yeast extract, 0.5 g tryptone, 0.5 g peptone, 4 g NaHCO_3 , 0.29 g NH_4Cl , 0.096 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0096 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.29 g NaCl , 1 mL vitamin solution (Kenealy and Zeikus, 1981), 1 mL trace mineral solution (Zehnder and Wuhermann, 1977), and 1 mL 0.1% resazurin solution (redox indicator). The medium was made anoxic in serum bottles (100 mL volume) by purging with O_2 -free N_2/CO_2 (80:20) mix gas and sterilized via autoclaving. Filter-sterilized methanol (final conc. 0.5%) was added as a substrate to support the growth of *M. mazei* (Boone and Mah, 1987). In the serum bottles with *M. thermautotrophicus*, filtered H_2/CO_2 (80:20) mix gas was injected into the headspace of the bottles until a pressure of 140 kPa was reached (Zhang et al., 2013). Before cell inoculation, all the serum bottles were stored at 65°C overnight to allow the sulfur functional group of yeast extract to react with any residual O_2 . The final pH of the medium was adjusted to 7.0 with 0.1 N HCl. *M. mazei* and *M. thermautotrophicus* were cultured in this sulfate-free medium at 37°C and 65°C, respectively. They were transferred three times before Fe(III) bioreduction experiments were initiated.

2.4 Biological reduction experiments

ALA-NAu-2 (final conc. = 5 g/L) was made into slurry with the sulfate-free

culture medium in serum bottles (total volume, 60 mL). Because both K and Al are the limiting factors in microbial conversion of smectite to illite (Zhang et al., 2007b), external sources of K and Al were needed to promote this reaction. The K concentration in the sulfate-free culture medium was already adequate (>20 mM). An external Al source (final conc. = 1 g/L) was provided in the form of amorphous $\text{Al}(\text{OH})_3 \cdot n\text{H}_2\text{O}$ to favor illite formation (Zhang et al., 2007b). The bottles were purged with N_2/CO_2 (80:20), and then sealed with thick butyl rubber stoppers (Chemglass, NJ) and aluminum crimps. After autoclaving, sterilized methanol (final conc. 0.5%) and H_2/CO_2 (80:20) gas mix were added as substrates into the bottles for *M. mazei* and *M. thermautotrophicus*, respectively, and all bottles were stored at 65°C overnight to achieve an anoxic condition. The amount of abiotic reduction of Fe(III) in ALA-NAu-2 by the sulfur functional group of yeast extract in control bottles was negligible in comparison with the amount of Fe(III) bioreduction (see below) and was subtracted from the measured Fe(II) concentration to quantify the overall Fe(III) bioreduction.

M. mazei and *M. thermautotrophicus* cells in their exponential growth phase were injected into the experimental bottles with a final concentration of 10^8 cells/mL based on acridine-orange direct counts (AODC). In selected experiments, 0.1 mM anthraquinone-2,6-disulfonate (AQDS), a humic acid analog, was supplied as an electron shuttle. There were three control groups in this experiment. Control A group was identical to the experimental bottles except that cells were replaced with an equal amount of culture medium (abiotic control), whereas Control B group was identical to the experimental bottles but without any clay minerals (methanogenesis control). Control C group was identical to the experimental bottles but with NAu-2 slurry instead of ALA-NAu-2

(bioreduction positive control). All treatments were performed in duplicates. All reagents and cultures were transferred using sterile needles and syringes. Experimental bottles with 20 mL slurry (40 mL headspace, total 60mL volume bottles) were incubated at 37°C for *M. mazei*, and 65°C for *M. thermautotrophicus*.

2.5 Chemical reduction ALA-NAu-2

To compare different mechanisms of ALA release from nontronite, we also performed chemical reduction of structural Fe(III) in NAu-2 and ALA-NAu-2 using the dithionite-citrate-bicarbonate (DCB) procedure (Stucki et al., 1984). Chemically reduced NAu-2 and ALA-NAu-2 was separated by centrifugation and washed four times with anoxic deionized water for subsequent characterization.

2.6 Analytical methods

2.6.1 Chemical analyses

In order to monitor the progress of biological and chemical Fe(III) reductions, total Fe(II) concentration was measured at selected times by the 1,10 phenanthroline method (Amonette and Templeton, 1998). To monitor the reductive dissolution of NAu-2 and ALA-NAu-2, aqueous concentrations of Fe, Al, and Si were measured at the end of the experiments. Aqueous suspension of 3 mL in volume was sampled and centrifuged inside an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI, USA) to collect supernatants followed by concentration measurements with inductively coupled plasma optical emission spectrophotometry (ICP-OES) (Agilent Technologies 700 Series). At the beginning and end of the experiments, pH values were measured inside a glove box with a pH meter (Accumet[®] AB15, Cole-Parmer).

2.4.2 CH_4 production

Time course CH_4 production was monitored to evaluate any effect of bioreduction on methanogenesis. At a given time point, 1 mL headspace gas was collected from the experimental bottles and analyzed for CH_4 amount with a Hewlett-Packard 6890 series gas chromatography system on a capillary column (GS GasPro, 30 m $\times$ 0.32 mm; J&W Scientific, Folsom, CA) that is connected to a flame ionization detector. Based on measured partial pressure of CH_4 in the headspace of experimental and control bottles, the total amount of CH_4 in both gas and liquid phases was calculated by the Ideal Gas Law and Henry's law using their respective Henry's constants (where K_H for CH_4 at 25 °C = 1.51×10^{-3} mol L⁻¹ atm⁻¹, [Schwarzenbach et al., 1995](#)).

2.4.3 Total organic carbon (TOC) measurement

In order to monitor the change of ALA content in ALA-NAu-2 throughout the Fe(III) reduction experiments, at selected time points, 2 mL of cell-clay suspension were taken from the experimental bottles inside an anaerobic glove box and washed with DI water three times to remove any interfering organic components from the medium (peptone, tryptone, and yeast extract). After drying, about 10 mg of dried ALA-NAu-2 was wrapped in tin capsule and analyzed with a Flash 2000 NC Soil Analyzer (CE Elantech) to determine TOC content.

2.4.4 XRD

Mineralogical changes before and after Fe(III) bioreduction were determined by

comparing XRD patterns of unreduced and reduced N_{Au}-2 and ALA-N_{Au}-2. Clay slurries were sampled with a syringe needle and smeared (Moore and Reynolds, 1997) onto petrographic slides and dried at 30°C overnight in a glove box. XRD patterns were collected on both untreated samples and those solvated with ethylene glycol (EG) vapor at 60°C overnight (Moore and Reynolds, 1997) to expand nontronite interlayers. The same slides were subsequently placed in a desiccator at room temperature for 24 hrs to allow further time for EG vapor to penetrate into the nontronite interlayer. XRD patterns were obtained immediately after saturation without significant exposure to air in a humidity-controlled laboratory. Powder XRD patterns were collected by a Scintag X-ray powder diffractometer using CuK α radiation, a fixed slit scintillation detector, and power of 1,400 W (40 kV, 35 mA). XRD slides were scanned in 0.02 two-theta steps with a count time of 2 s per step and a scanning range of 2° to 70° two-theta.

2.4.6 FTIR

FTIR spectroscopy was used to confirm intercalation of ALA into the expandable interlayer of nontronite N_{Au}-2 and to assess any structural changes of N_{Au}-2 and ALA-N_{Au}-2 after Fe(III) reduction. For this analysis, 200 mg of KBr and 2 mg of sample powder were weighed, mixed, and ground to fully disperse N_{Au}-2 and ALA-N_{Au}-2 samples in KBr. The samples were analyzed in the diffuse reflectance mode using a Perkin-Elmer Frontier Infrared Spectrometer. Fifty scans over the range 400–4000 cm⁻¹ with a spectral resolution of 4 cm⁻¹ were accumulated for each spectrum.

2.4.6 Scanning electron microscopy (SEM)

Mineralogical changes as a result of bioreduction of Fe(III) in N Au-2 and ALA-
N Au-2 were further characterized by using SEM. Clay suspension (0.5 ml) was mounted
on a 15 mm cover slip pretreated with poly-L-lysine for 15 min. The sample-coated cover
slip was sequentially dehydrated using varying proportions of ethanol followed by critical
point drying with a Tousimis Samdrol-780A Critical Point Dryer (CPD) (Dong et al.,
2003). The sample-coated cover slip was mounted on SEM stub via clear double-sided
sticky tape and coated with a Denton Desk II Gold Sputter Coater for SEM observations.
The samples were analyzed with a Zeiss Supra 35 VP SEM with Genesis 2000 X-ray
energy dispersive spectroscopy (SEM/EDS). The EDS provided a primary means for
mineralogical identification. The SEM was operated at an accelerating voltage of 3 to 20
kV. A short working distance (6-10 mm) and low beam current (30-40 μ A) were used to
achieve the best image resolution. A certain working distance (8.5 mm) and higher beam
current (50-70 μ A) were used for qualitative EDS.

3. RESULTS

3.1 Characterization of ALA-N Au-2

The amount of ALA intercalated into the N Au-2 interlayer was 8.5% (wt. %). The
total Fe content was 22% (wt. %) in the 0.02-0.5 μ m size fraction of N Au-2, of which 2%
was Fe(II); whereas the total Fe content decreased to 19% for the same size fraction of
ALA-N Au-2, of which 2% was Fe(II).

The increase in the d(001) spacing from 12.18 Å for N Au-2 to 16.65 Å for ALA-
N Au-2 confirmed that ALA was intercalated into the interlayer of the N Au-2 structure

(Fig. 1A). Intercalation of ALA into NAu-2 caused significant changes to the FTIR spectrum of NAu-2 (Fig. 1B) (Liu et al., 2011b). In comparison with ALA and NAu-2, ALA intercalation into the NAu-2 interlayer resulted in characteristic absorption bands in the 1700-1300 cm^{-1} and 3500-2500 cm^{-1} wave number regions (Fig. 1B). In particular, a broad band from 3500 to 3100 cm^{-1} was assigned to δNH_3^+ deformation, along with an intense band at 1629 cm^{-1} due to δNH_3^+ deformation and $\nu_{\text{as}}\text{COO}^-$ asymmetrical stretching from ALA (Brigatti et al., 1999; Barth, 2000; Liu et al., 2011b). The absorption band at 2918 cm^{-1} was assigned to νCH_2 stretching. Finally, vibration bands at 1406 and 1385 cm^{-1} were assigned to $\nu_s\text{COO}^-$ and δCH_2 , respectively (Barth, 2000).

3.3 Microbial reduction of Fe(III) in clay minerals

Both mesophilic and thermophilic methanogens were able to reduce structural Fe(III) in ALA-NAu-2 but with different rates and extents (Fig. 2). In general, the bioreduction experiments were complete in 4-10 days (depending on specific methanogen strain), but our experiments continued until 30 days to ensure the completion of bioreduction. There was a small amount of abiotic reduction of structural Fe(III) by the sulfur functional group of yeast extract (<1%). Thus, this amount, along with the initial amount of Fe(II) present in the unreduced NAu-2 and ALA-NAu-2, was subtracted from measured Fe(II) concentration when the final reduction extent was calculated. The pH in the experimental bottles ranged from 6.5 to 7.2 over the course of the experiments.

In the *M. mazei* experiments, the rate of Fe(III) bioreduction was rapid in the first 2 days but gradually decreased over time (Fig. 2A & B). Intercalation of ALA into the

NAu-2 interlayer decreased the initial rate of Fe(III) bioreduction. For example, the initial rate for ALA-NAu-2, calculated for the first 24 hrs, was 0.04 mM/hr without AQDS (open squares in Fig. 2A) and 0.10 mM/hr with AQDS (solid squares in Fig. 2A). In contrast, the initial rate for pure NAu-2, calculated over the same time duration, was 0.09 mM/hr and 0.16 mM/hr without and with AQDS amendments, respectively (Fig. 2B). Intercalation of ALA into the NAu-2 interlayer also lowered the final extent of bioreduction. In the absence of AQDS, the extent decreased from 21.6% for NAu-2 (open diamonds in Fig. 2B) to 16.8% for ALA-NAu-2 (open squares in Fig. 2A). With AQDS, the extent decreased from 28.1% for NAu-2 (solid diamonds in Fig. 2B) to 27.8% for ALA-NAu-2 (solid squares in Fig. 2A). Again AQDS stimulated Fe(III) reduction, consistent with previous research results (Liu et al., 2011a; Zhang et al., 2012).

Similar to the results with *M. mazei*, the rates of bioreduction by *M. thermautotrophicus* were moderately fast in the first 4 days for both the NAu-2 and ALA-NAu-2 experimental systems (Fig. 2C & D). After this initial time period, the bioreduction pattern differed between ALA-NAu-2 and NAu-2. For ALA-NAu-2, the rate of bioreduction decreased after day 4 and reached nearly zero after day 10 (Fig. 2C). However, the bioreduction rate for NAu-2 suddenly declined between day 4-7, increased afterwards, and finally reached zero after day 15 (Fig. 2D).

Similar to the mesophilic methanogen experiment, ALA intercalation into NAu-2 lowered initial bioreduction rate (in the first 24 hrs) by *M. thermautotrophicus*. In the absence of AQDS, the rate decreased from 0.03 mM/hr for NAu-2 to 0.02 mM/hr for ALA-NAu-2. In the presence of AQDS, the rate decreased from 0.06 mM/hr for NAu-2 to 0.03 mM/hr for ALA-NAu-2. The maximal extents of bioreduction by *M.*

thermautotrophicus were also lower for ALA-NAu-2 (only reaching 13.5% and 15.4% without and with AQDS, respectively, Fig. 2C) than those for NAu-2 (26.6% and 27.0%, Fig. 2D). However, unlike the *M. mazei* system, AQDS showed little enhancement of Fe(III) bioreduction by *M. thermautotrophicus*.

3.2 Reductive dissolution of NAu-2 and ALA-NAu-2 as a result of bioreduction

Aqueous Si and Fe concentrations in abiotic controls were negligible (data not shown), but in bioreduced, AQDS-amended NAu-2 and ALA-NAu-2 solutions, these concentrations increased with the increasing bioreduction extent (Fig. 3). The highest extent of reduction (83.0% and 91.0% for NAu-2 and ALA-NAu-2, respectively) was only achieved with chemical reduction (see below). In the non-AQDS amended experiments, similar correlations were observed between aqueous Si and Fe concentrations and reduction extent (data not shown).

3.3 TOC release from NAu-2 during bioreduction of Fe(III)

Time-course release of TOC from the NAu-2 interlayer exhibited a similar pattern for all experiments regardless of methanogen strain used. Because TOC content in pure NAu-2 was only 0.1-0.2% (data not shown, also from [Jaisi et al., 2005](#)), the measured TOC content of ~2% in the bioreduced NAu-2 should have come from the growth medium and the initial biomass that was added at the beginning of the experiments. Throughout the duration of the experiments with *M. mazei*, TOC content in these NAu-2 samples increased by 0.12% and 0.18% without and with AQDS amendments, respectively; whereas TOC content increased by 0.05% in the *M. thermautotrophicus*

experiments for both without- and with-AQDS treatments. These data suggest that the amount of cell growth over the experimental duration was insignificant.

As expected, the amount of TOC in ALA-NAu-2 was much higher (~8.5 wt%) than in NAu-2. In abiotic control group, the net decrease of TOC content in ALA-NAu-2 was <0.2 wt% by the end of the experiments (30 days, Fig. 4). In the presence of *M. mazei*, however, TOC content decreased by 1.7 wt% and 1.8 wt% without and with AQDS treatments, respectively. In comparison, higher amounts of TOC release were observed for the corresponding thermophilic methanogen experiments (e.g., 2.1 wt% and 2.3 wt% without and with AQDS, respectively). In particular, the TOC release patterns paralleled the Fe(III) bioreduction patterns (Fig. 2A & C; Fig. 4), e.g., TOC was released during the active bioreduction period. When bioreduction ceased, ALA release also stopped (after day 2 with *M. mazei*, and after day 7 with *M. thermautotrophicus*).

3.4 XRD

Although the bioreduction experiments were completed by 30 days, the samples were incubated under the same conditions for additional 70 days to promote any mineralogical transformations. At the end of 100-day incubation, however, no new mineral products were detected by XRD. The bioreduction of structural Fe(III) in ALA-NAu-2 by *M. mazei* resulted in a decrease of the d(001) spacing from 16.74 Å to 15.09 Å without AQDS. The AQDS amendment promoted a further decrease of the d(001) spacing, e.g., from 16.74 Å to 14.18 Å (Fig. 5A). With *M. thermautotrophicus*, the d(001) spacing decreased from 15.78 Å to 13.18 Å and 13.08 Å without and with AQDS, respectively (Fig. 5B). After the ethylene glycol treatment, the d(001) spacing of ALA-

NAu-2 was expanded to 17 Å for all samples (data not shown).

3.5 SEM observations

SEM images revealed that abiotic NAu-2 and ALA-NAu-2 controls displayed a similar flaky texture (Fig. 6A & B). EDS spectra showed a typical nontronite composition for both NAu-2 and ALA-NAu-2 but with a higher amount of carbon in ALA-NAu-2 (Fig. 6D & E). After the bioreduction, there were at least two types of clay textures in both the *M. mazei* and *M. thermautotrophicus* ALA-NAu-2 experimental systems: abundant rose-like flakes (Fig. 6C) and platy particles (Fig. 6G). In comparison with their abiotic controls, the rose-like bioreduced ALA-NAu-2 showed decreased Fe and C contents but with an increased K content (Fig. 6F). The elemental composition of most plate-shaped, bioreduced ALA-NAu-2 (Fig. 6I) exhibited a higher Al/Si ratio and a higher K content than in abiotic controls (Fig. 6E), but the Fe and C peak heights appeared to be similar to those in abiotic controls. A small amount of silica aggregates was observed in bioreduced ALA-NAu-2 (Figs. 6G and 6J).

3.6 Methane production

In the absence of clay minerals, there was a significant amount of methane production with time in both the *M. mazei* and *M. thermautotrophicus* experimental systems, similar to previous observations (van Bodegom et al., 2004; Liu et al., 2011a). *M. thermautotrophicus* growing on H₂/CO₂ produced slightly more methane than *M. mazei* growing on methanol (Fig. 7). However, the addition of NAu-2 into the *M. mazei* and *M. thermautotrophicus* experimental systems almost completely inhibited methane

production during active Fe(III) reduction periods (e.g., the first 5-7 days, Fig. 2). Methanogenesis resumed in all experimental groups after Fe(III) bioreduction ended. Intercalation of ALA into the nontronite interlayer resulted in lower amounts of methane production especially in *M. thermautotrophicus* systems (Fig. 7B).

3.7 FTIR

After the bioreduction of Fe(III) in ALA-NAu-2, the ALA-specific absorption bands did not change their positions but exhibited slightly lower intensities (Fig. 8A&B). Similarly, bioreduction resulted in lower intensities of NAu-2 –specific absorption bands in both bioreduced ALA-NAu-2 (Fig. 8C-E) and NAu-2 (Fig. 8F-H). In general, the IR spectrum of nontronite NAu-2 can be broken down into three main regions spanning from 4000 to 400 wavenumbers (cm^{-1}), i.e., 3800-3200, 1200-950, and 950-700 cm^{-1} (Morrison et al., 2013). The spectrum region from 3800 cm^{-1} to 3200 cm^{-1} is characterized by the $\nu\text{M-OH}$ stretching bands ($\text{M} = \text{Mg, Al or Fe}$ in the octahedral layer) (Fialips et al., 2002; Gates, 2005). In this study, the overall position of the OH-stretching band shifted from 3426 cm^{-1} to 3451 cm^{-1} after bioreduction of Fe(III) in ALA-NAu-2 (Fig. 8C), but this peak did not shift its position after bioreduction of NAu-2 (, e.g., stayed at 3438 cm^{-1} , Fig. 8F). The $\nu\text{Si-O}$ stretching region, representing tetrahedral layers, is present from 1200 cm^{-1} to 950 cm^{-1} and produces a very intense peak due to its structural dominance (Fialips et al., 2002; Gates, 2005). The position of the Si-O-stretching band shifted from 1042 cm^{-1} to 1032 cm^{-1} upon bioreduction of ALA-NAu-2 (Fig. 8D). This shift to a lower frequency is consistent with previous studies of bacterially reduced smectites (Lee et al., 2006). The final region of importance in IR spectra is the

δ M-OH deformation bands (M = Mg, Al or Fe) that ranges from 700 cm^{-1} to 950 cm^{-1} . These bands are characteristics of the vibrations of the octahedral cations. The principal features in the M₂-OH deformation region of ALA-NAu-2 (Fig. 8E) were strong bands at 785 cm^{-1} , 822 cm^{-1} , 848 cm^{-1} , and 875 cm^{-1} that could be assigned to FeMg-OH, FeFe-OH, FeFe-OH, and AlFe-OH, respectively (Fialips et al., 2002; Gates, 2005). The bioreduction of structural Fe(III) in ALA-NAu-2 resulted in decreased intensities of the 785 cm^{-1} , 822 cm^{-1} and 875 cm^{-1} bands, and loss of the 848 cm^{-1} band (Fig. 8E), similar to those in bioreduced NAu-2 (Fig. 8H).

3.8 Chemical reduction of Fe(III) in NAu-2 and ALA-NAu-2 by dithionite

The rates and maximal extents of chemical reduction of structural Fe(III) in NAu-2 and ALA-NAu-2 by the DCB procedure were much higher than those by the methanogens (83% and 91% for NAu-2, and ALA-NAu-2, respectively) with a nearly complete release of ALA (only ~0.38% remaining after Fe(III) reduction). Indeed, when ALA release was plotted against the extent of Fe(III) reduction for both biological and chemical reduction, a positive correlation resulted (Fig. 9).

Chemical reduction by DCB resulted in a decrease of d(001) spacing from 16.65 Å to 12.65 Å for both NAu-2 and ALA-NAu-2 (Fig. 5C). High aqueous Si and Fe concentrations in chemically reduced ALA-NAu-2 and NAu-2 (Fig. 3) suggested reductive dissolution of these minerals. Under SEM, there were abundant dissolution pits in ALA-NAu-2 forming a three-dimensional net-like texture (Fig. 6H), and these pits displayed various sizes that were different from those cell-sized pits typically formed after bioreduction of NAu-2 (Liu et al., 2011a; Zhang et al., 2012). DCB-reduced ALA-

NAu-2 exhibited a similar composition to that of the rose-like bio-reduced ALA-NAu-2 (i.e., absence of Fe and C contents) but without K (Fig. 6K).

FTIR data revealed that DCB reduction resulted in broad and low intensity ALA-specific peaks (1630 cm^{-1} , 2847 cm^{-1} , and 2918 cm^{-1}) relative to those in unreduced ALA-NAu-2, likely due to release of ALA from the NAu-2 interlayer (Fig. 8A & B). The overall position of the OH-stretching band shifted from 3426 cm^{-1} to 3451 cm^{-1} after DCB reduction of Fe(III) in ALA-NAu-2 (Fig. 8C), but this peak did not shift after DCB reduction of NAu-2 (Fig. 8F). The position of the Si-O-stretching band (Fig. 8D) shifted from 1042 cm^{-1} to 1024 cm^{-1} after DCB reduction of ALA-NAu-2, and from 1032 cm^{-1} to 1024 cm^{-1} after DCB reduction of NAu-2 (Fig. 8G). All peaks related to the M_2 -OH deformation (785 cm^{-1} , 822 cm^{-1} , 848 cm^{-1} , and 875 cm^{-1} , assigned to FeMg-OH, FeFe-OH, FeFe-OH, and AlFe-OH, respectively) in ALA-NAu-2 and NAu-2 became broader with lower intensities after DCB reduction (Fig. 8E&H). In comparison with bio-reduction of Fe(III) in ALA-NAu-2 and NAu-2, chemical reduction by DCB resulted in much lower intensities of all major bands (Fig. 8).

4. DISCUSSION

4.1 Effects of ALA intercalation on bio-reduction and methanogenesis

This research demonstrated that two methanogen strains could reduce structural Fe(III) in ALA-intercalated nontronite, but with lower bio-reduction extents, rates, and methane production relative to nontronite without ALA intercalation. These differences could be explained by changes of NAu-2 properties as a result of ALA intercalation. ALA is an amino acid analog that carries a carboxyl and an amino functional group, which

436 should be zwitterionic (Lilley, 1985). Protonated ALA may enter the N_{Au}-2 interlayer via
437 two mechanisms (Benincasa et al., 2000). First, protonated ALA can substitute for water
438 molecules in the nontronite interlayer such that N_{Au}-2 becomes hydrophobic (Chou et al.,
439 2005; Goebel et al., 2004; Jandl et al., 2004). This change from the hydrophilic to
440 hydrophobic nature makes the interlayer region unfavorable sites for water dispersion
441 (Chou et al., 2005). This water expulsion out of the interlayer and replacement by ALA
442 can hinder Fe(III) reduction, since the smectite interlayer region is important for electron
443 transfer process. Our previous results (Bishop et al., 2011; Liu et al., 2012; Zhang et al.,
444 2012) demonstrated that the interlayer region of clay minerals is involved in the electron
445 transfer process, based on the observations that expandable smectites usually exhibit
446 higher extents and rates of Fe(III) bioreduction by various bacteria than non-expandable
447 illite (otherwise, isostructural with smectite). Second, the protonated ALA molecule may
448 replace the exchangeable cations, such as Ca²⁺, Na⁺, K⁺, on both the external surfaces and
449 the interlayer positions of N_{Au}-2 (Alexander, 1999), which would form a coating of
450 organic matter on clay surfaces. This coating can block the sorption sites of clay minerals
451 (Onken and Traina, 1997; Shen and Jaffe, 2000; Charles et al., 2006). If the electron
452 transfer is perpendicular to the (001) layers (Dong et al., 2009), the intercalated ALA in
453 the interlayer region may block this electron transfer chain and result in lower extent and
454 rate of Fe(III) bioreduction, whereas surface-adsorbed ALA may have little or no effect.
455 If the electron transfer from methanol (*M. mazei*) and H₂ (*M. thermautotrophicus*) to the
456 octahedral Fe(III) of the nontronite structure is parallel to (001) layers (Dong et al., 2009;

Stucki et al., 2011), the sorbed ALA on NAu-2 external surfaces can hinder the electron transfer and subsequently result in lower extent and rate of Fe(III) bioreduction. In this case, the intercalated ALA would have little effect on this electron transfer mechanism. Therefore, the relative role of parallel vs. perpendicular electron transfer in Fe(III) bioreduction in iron-bearing clay minerals would determine the importance of surface-adsorbed vs. intercalated-ALA in adversely affecting Fe(III) bioreduction.

When AQDS was supplied as an electron shuttle, the ALA blocking effect was apparently alleviated for the *M. mazei* treatments because an equal amount of bioreduction was achieved between NAu-2 and ALA-NAu-2 (Fig. 2). The likely reason was that AQDS could easily enter the interlayer region and facilitate the electron transfer from cells to the octahedral Fe(III) in ALA-NAu-2. This observation is consistent with previous studies in showing that AQDS can significantly enhance the extent of Fe(III) reduction in nontronite by mesophilic methanogens (Liu et al., 2011a; Zhang et al., 2012).

In experiments with *M. thermautotrophicus* (at 65°C), however, AQDS did not alleviate the ALA blocking effect, and AQDS had a minimal effect on the final extent of bioreduction for both NAu-2 and ALA-NAu-2 (Fig. 2). A previous study (Zhang et al., 2013) also showed that AQDS did not enhance bioreduction of structural Fe(III) in NAu-2 by this thermophilic methanogen, likely because this strain might be able to produce its own electron shuttle. In this case, the ALA blocking effect may have been from adsorbed ALA only (on external surfaces of NAu-2). The melting temperature of ALA is about 80°C, so in the *M. thermautotrophicus* system (65°C), some protonated ALA may have

478 been partially melted, moved out of the nontronite interlayer and re-adsorbed onto the
479 negatively charged cell and clay surfaces. Such sorption of ALA on cell surfaces would
480 hinder microbial activity and might be partially responsible for observed lower extents of
481 Fe(III) bioreduction (Fig. 2C) relative to those achieved by the mesophilic methanogen
482 (Fig. 2A).

483 The observed inhibition of methanogenesis in the *M. thermautotrophicus*
484 experimental system further supported this explanation. There were two stages of Fe(III)
485 bioreduction in NAu-2 by *M. thermautotrophicus*, (Fig. 2D). According to our previous
486 research (Zhang et al., 2013), the first phase of bioreduction may have been responsible
487 for decreasing the reduction potential of the system so that methanogenesis could begin,
488 which would provide more energy to support cell growth. High cell growth in turn
489 promoted Fe(III) bioreduction at the second stage (Zhang et al., 2013). In contrast, such
490 two-stage bioreduction was not observed in ALA-NAu-2 with the same thermophilic
491 methanogen (Fig. 2C), and its Fe(III) bioreduction extent only reached 13.5-15.4%,
492 which was similar to the extent of Fe(III) bioreduction (15.6%) when methanogenesis was
493 fully inhibited by 10 mM 2-bromoethane sulfonate (Zhang et al., 2013). These data
494 therefore suggest that ALA may have coated cell surfaces and decreased microbial
495 activity. Methanogenesis is a membrane-bound electron transfer system (Deppenmeier,
496 2004). Therefore, a cell surface coating of ALA may have inactivated *M.*
497 *thermautotrophicus* cells, inhibited methanogenesis (Fig. 7B), and lowered bioreduction
498 (Fig. 2C).

The observed inhibition of methanogenesis in ALA-NAu-2 with *M. mazei* was different from that by *M. thermautotrophicus*. Methanogenesis did not begin until bioreduction ceased due to the diversion of electrons from CO₂ to Fe(III) during this stage (Liu et al., 2011a; Zhang et al., 2012). In our results, methane production in the ALA-NAu-2 treatment began later than in the NAu-2 treatment, likely because of the slower bioreduction rate in the ALA-NAu-2 treatment, but both ALA-NAu-2 and NAu-2 ultimately produced a similar amount of methane by the end of the experiments (Fig. 7A). One likely reason was that at 37°C (the optimal growth temperature for *M. mazei*), protonated ALA should have resided within the NAu-2 interlayer and would not be available for sorption onto cell surfaces. So cell inactivation was not likely in this case and the ultimate extent of methanogenesis was similar between ALA-NAu-2 and NAu-2 (Fig. 7A).

4.2 ALA release by Fe(III) reduction

Significant amounts of organic matter in sediments are usually associated with mineral matrix, especially clay minerals, and can be physically protected against biological oxidation (Baldock and Skjemstad, 2000). Although still subject to much debate, sorption of organic matter to mineral surface is the most likely mechanism for the observed physical protection of organic matter against oxidation in experimental investigations (Mayer et al., 1985; Keil et al., 1994; Mayer, 1994). Recently, Lalonde et al. (2012) developed a dithionite-based approach to reductively dissolve Fe in a wide

range of sediment samples (iron oxides), and concluded that a certain amount of organic matter in sediments was directly associated with and can be protected by iron oxides. Our current result expanded that study by showing that the release of intercalated organic matter was related to Fe reduction extent in clay minerals. In the present study, chemical reduction by DCB resulted in nearly complete release of ALA from N_{Au}-2 because it reduced 80-90% of the structural Fe(III), whereas smaller biological reduction (13.5-28.1%) largely preserved ALA within N_{Au}-2 interlayers because no significant changes occurred to the N_{Au}-2 structure.

The positive correlation between the amount of ALA release and the extent of Fe(III) reduction (Fig. 9) could be explained by reduction-related structural alterations (Lee et al., 2006) and reductive dissolution of ALA-N_{Au}-2. In chemically reduced ALA-N_{Au}-2, high extents of reductive dissolution were observed, as evidenced by extensive dissolution pits (Fig. 6H), the lack of Fe and C in these dissolution pits (Fig. 6K), and high aqueous concentrations of Si and Fe (Fig. 3). In contrast, the extent of dissolution was much less in bioreduced samples, as evidenced by lack of dissolution pits (instead rose-like textures observed, Fig. 6C), and lower aqueous Si and Fe concentrations (Fig. 3). All these observations were consistent with previous results suggesting that reductive dissolution could be extensive when the extent of Fe(III) reduction was > 30%, but insignificant when the extent of reduction was <30% (Jaisi et al., 2007; Dong et al., 2009). Although ALA was largely preserved within the bioreduced nontronite interlayers, its Fe and C contents were depleted relative to Si (Fig. 6F), suggesting partial dissolution.

Therefore, intercalated organic matter release may be associated with reductive dissolution of structural Fe(III) in ALA-NAu-2, but future work is needed to reveal the causal relationship between organic matter release and Fe(III) reduction.

4.3 Clay mineral transformation by methanogens in the presence of ALA

Many microbes can obtain energy from reduction of Fe(III) in solid minerals to support their growth (Lovley et al., 1996; Lovley 2000, 2004; Stucki, 2006). As a result, solid minerals undergo structural and chemical changes (Dong, 2012). In our earlier research, an extensive amount of vivianite was formed as a result of bioreduction of structural Fe(III) in NAu-2 by *M. mazei*, along with observations of many dissolution pits, high-charge smectite, and silica aggregates (Zhang et al., 2012). Such formation of new minerals during bioreduction implied that structural changes of nontronite by *M. mazei* may be irreversible. However, in the presence of ALA, dissolution pits, high-charge smectite, and vivianite were not observed after bioreduction, suggesting that bioreduction-induced mineralogical alterations may be small for ALA-intercalated nontronite.

Previous research has shown that the type of organic matter may determine the degree of mineralogical changes (Zhang et al., 2007a). When the intercalated organic matter was an essential nutrient for microbes (such as amino acid), its presence would be attractive to microbes and could result in a higher extent of bioreduction to promote mineralogical change. However, if the organic matter intercalated into the nontronite structure was toxic to cells (such as toluene), Fe(III) bioreduction would be inhibited and no mineralogical changes were observed. In our study, ALA is neither toxic nor

beneficial to the methanogens, so mineralogical alterations of NAu-2 were observed to be small, as expected.

4.4. Environmental Implications

Our experiments showed that the amount of organic matter released from nontronite depended on the extent of reduction of structural Fe(III) in nontronite. Although the bioreduction extents (13.5-28.1%) were much lower than those by chemical reduction, biological reduction is more relevant to natural environments because chemical reductant such as sodium dithionite is not commonly present in nature. Therefore, organic matter should be largely protected from microbial degradation if it is within smectite clay minerals. Such organic matter-smectite association could explain the positive correlation between the abundance of TOC and smectite in various sedimentary rocks (Mayer et al., 1985; Mayer, 1994; Keil et al., 1994; Ransom et al., 1998; Arnarson and Keil, 2001; Kennedy et al., 2002; Kennedy and Wagner, 2011). However, we note that our experiments may not be entirely representative of natural sediments because iron-rich smectite, i.e., nontronite, is not a common smectitic clay mineral. Nonetheless, this study does offer a mechanistic understanding of organic matter release from smectite clay minerals.

Certain expandable clay minerals are widely utilized for remediation of organic contaminants by intercalating/sequestering them into the interlayers of montmorillonite in order to isolate them from the environment (Mulligan et al., 2001; Walcarius and Mercier, 2010). The results of this study offer important insights into the role of microbial activities on clay mineral transformation and subsequent organic matter release. If the

clay mineral contains a certain amount of structural Fe(III) and natural metal-reducing microbes can reduce Fe(III), the organic contaminants should be largely protected within smectite structure. An improved understanding of iron biogeochemistry of clay minerals will be valuable for designing remediation technologies for sequestering organic contaminants.

5. CONCLUSION

During the reduction of structural Fe(III) in nontronite (NAu-2) either biologically by methanogens *M. maize* and *M. thermautotrophicus* or chemically by sodium dithionate, the intercalated ALA was partially released, and the extent of release was correlated with the extent of Fe(III) reduction. Bioreduction exhibited lower Fe(III) reduction with release of only ~2% ALA, whereas chemical reduction showed much higher Fe(III) reduction extent and release of nearly all the intercalated ALA. SEM observations and aqueous chemistry data support the argument that reductive dissolution was a main mechanism for ALA release from nontronite structure. Since biological reduction should be more prevalent than chemical reduction in natural environment, the results of this study suggest that organic matter should be largely preserved within the nontronite structure. In addition, ALA intercalation into the nontronite interlayer exhibited a stronger inhibition effect of methanogenesis than NAu-2 alone by *M. thermautotrophicus*, likely due to a combination of multiple factors including reduction potential and physical blocking of the electron transfer chain by ALA.

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FIGURE CAPTION

Fig. 1. A) XRD patterns showing ALA intercalation into the NAu-2 interlayer. The 001 peak of NAu-2 moved from 12.18 to 16.65 Å after intercalation. B) FTIR spectra for NAu-2, ALA, and ALA-NAu-2.

Fig. 2. Production of total Fe(II) in ALA-NAu-2 and NAu-2 by *M. mazei* and *M. thermautotrophicus* with time as measured by the 1,10-phenanthroline method. Initial cell concentration was 10^8 cells/mL. Averages of two measurements from duplicate experimental tubes were reported. The error bars represent the higher and lower values. Control A did not have any cells. (A). Reduction of structural Fe(III) in ALA-NAu-2 by *M. mazei*; (B). Reduction of structural Fe(III) in NAu-2 by *M. mazei*; (C). Reduction of

structural Fe(III) in ALA-NAu-2 by *M. thermautotrophicus*; (D). Reduction of structural Fe(III) in NAu-2 by *M. thermautotrophicus*.

Fig. 3 Aqueous concentrations of Si and Fe as a function of reduction extent for NAu-2 (A) and ALA-NAu-2 (B) suspensions in the presence of AQDS. In each plot, the first five data points are for bio-reduced samples and the last data point is for chemically reduced NAu-2 or ALA-NAu-2.

Fig. 4. Time-course decrease of TOC content (wt %) in ALA-NAu-2 and NAu-2 samples over the course of Fe(III) bio-reduction. Control A did not have any cells. The error bars represent the higher and lower values from duplicated measurements. (A). with *M. mazei*; (B). with *M. thermautotrophicus*.

Fig. 5. XRD patterns showing change of the d(001) spacing for ALA-NAu-2 before and after bio-reduction by *M. mazei* (A) and *M. thermautotrophicus* (B), respectively. The d(001) peak intensity in all treatments decreased after bio-reduction. C) XRD patterns of the DCB-reduced ALA-NAu-2 showing the same pattern as that for the DCB-reduced NAu-2.

Fig. 6. Secondary electron images showing NAu-2 and ALA-NAu-2 after 100 days of

bio-reduction. A) abiotic control of N Au-2; B) abiotic control of ALA-N Au-2; C) rose-like texture in bio-reduced ALA-N Au-2; D) EDS spectrum of unreduced N Au-2 in the abiotic control (from point A1 in Fig. 5A); E) EDS spectrum of unreduced ALA-N Au-2 in the abiotic control (from point A2 in Fig. 5B); F) EDS spectrum of rose-like textured particles in bio-reduced ALA-N Au-2 (from point A3 in Fig. 5C); G) plate-shaped clay particles and silica aggregates in bio-reduced ALA-N Au-2; H) net-shaped ALA-N Au-2 after DCB reduction; I) EDS spectrum of plate-shaped clay particles in bio-reduced ALA-N Au-2 (from point A4 in Fig. 5G); J) EDS spectrum of silica aggregates after bio-reduction of N Au-2 (from point A5 in Fig. 5G); K) EDS spectrum of net-shaped ALA-N Au-2 after DCB reduction (from point A6 in Fig. 5H). In all EDS spectra, the Au peak was from sample coating.

Fig. 7. Time-course methane production by *M. mazei* (A) and *M. thermotrophicus* (B) in all experimental treatments. The error bars represent the higher and lower values from duplicated measurements. Control B did not have any clay minerals. Control C was for the N Au-2 treatments (with ALA intercalation).

Fig. 8. FTIR spectra for ALA-N Au-2 and reduced ALA-N Au-2 (A-E) and for N Au-2 and reduced N Au-2 (F-H). A) The νCH_2 stretching bands in ALA-N Au-2, bio-reduced ALA-N Au-2, and DCB reduced ALA-N Au-2. B) δNH_3^+ deformation and $\nu_{\text{as}}\text{COO}^-$ asymmetrical stretching bands in ALA-N Au-2, bio-reduced ALA-N Au-2, and DCB

reduced ALA-NAu-2. C) OH-stretching bands in ALA-NAu-2, bio-reduced ALA-NAu-2, and DCB reduced ALA-NAu-2. D) Si-O stretching bands in ALA-NAu-2, bio-reduced ALA-NAu-2, and DCB reduced ALA-NAu-2. E) M₂-OH bending bands in ALA-NAu-2, bio-reduced ALA-NAu-2, and DCB reduced ALA-NAu-2. F) OH-stretching bands in NAu-2, bio-reduced NAu-2, and DCB reduced NAu-2. G) Si-O stretching bands in NAu-2, bio-reduced NAu-2, and DCB reduced NAu-2. H) M₂-OH bending bands in NAu-2, bio-reduced NAu-2, and DCB reduced NAu-2.

Fig. 9. A positive correlation between TOC release and the extent of Fe(III) reduction.

Those points with the reduction extent < 30% are for bio-reduced ALA-NAu-2 and the last few points (>80% reduction extent) are for chemically reduced ALA-NAu-2.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

ALA-NAu-2 Treatment

NAu-2 Treatment

Fig. 8

Fig. 9