Characterization of incubation experiments and development of an enrichment culture capable of ammonium oxidation under iron reducing conditions

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ABSTRACT

Incubation experiments were conducted using soil samples from a forested riparian wetland where we have previously observed anaerobic ammonium oxidation coupled to iron reduction. Production of both nitrite and ferrous iron were measured repeatedly during incubations when the soil slurry was supplied with either ferrihydrite or goethite and ammonium chloride. Significant changes in the microbial community were observed after 180 days of incubation as well as in a continuous flow membrane reactor, using 16S rRNA gene PCR-denaturing gradient gel electrophoresis, 454-pyrosequencing, and real-time quantitative PCR analysis. We believe that one of the dominant microbial species in our system (an uncultured *Acidimicrobiaceae* bacterium A6), belonging to the *Acidimicrobiaceae* family, whose closest cultivated relative is *Ferrimicrobium acidiphilum* (with 92% identity) and *Acidimicrobium ferrooxidans* (with 90% identity), might play a key role in this anaerobic biological process that uses ferric iron as an

electron acceptor while oxidizing ammonium to nitrite. After ammonium was oxidized to nitrite, nitrogen loss proceeded via denitrification and/or anammox.

Keywords: ammonium oxidation, iron reduction, *Actinobacteria*, nitrite, autotrophic, Feammox, anaerobic.

1. INTRODUCTION

The most common removal of nitrogen from soil environments is mineralization (for organic nitrogen), followed by nitrification and then denitrification (Canfield *et al*., 2010). In water-saturated sediments, such as wetland sediments and benthic sediments, there is little oxygen for significant nitrification by aerobic ammonium (NH_4^+) oxidation bacteria/ archaea (AOB/AOA). Nitrate (NO_3^-) is mainly delivered by groundwater discharging into such systems or surface water infiltration, although some nitrification does occur, such as in the vicinity of roots, where there is O_2 leakage. A novel anaerobic NH_4^+ oxidation process coupled to iron reduction was first noted in a forested riparian wetland in New Jersey (Clement *et al*., 2005). In this reaction, NH_4^+ is the electron donor, which is oxidized to nitrite (NO_2^-), and ferric iron [Fe(III)] is the electron acceptor, which is reduced to ferrous iron [Fe(II)]. The stoichiometry and change in free energy when ferrihydrite is the Fe(III) source is:

$$3Fe_2O_3 \cdot 0.5H_2O + 10H^+ + NH_4^+ \rightarrow 6Fe^{2+} + 8.5H_2O + NO_2^-$$

42 ($\Delta G_r \leq -145.08 kJ\ mol^{-1}$) (Equation 1, Supplementary Information 1. 1)

43

44 No proven pathway for the oxidation of NH_4^+ to NO_2^- in anaerobic environments has
45 been described in the literature before this process was reported. Using labeled $^{15}NH_4^+$
46 in a microcosm experiment, resulted in the production of $^{15}N_2$, which conclusively
47 showed that ammonium-N was converted to nitrogen gas (N_2) in these sediments under
48 iron reducing conditions (Shrestha *et al*., 2009). Either this same pathway for NH_4^+
49 oxidation, or a very similar one, was also observed in a biological reactor (Sawayama,
50 2006) and a tropical rainforest soil (Yang *et al*., 2012), and coined Feammox (Sawayama,
51 2006). These pathways have been reported to oxidize NH_4^+ to NO_2^- (Clement *et al*.,
52 2005; Shrestha *et al*., 2009), to NO_3^- (Sawayama, 2006), or directly to N_2 (Yang *et al*.,
53 2012), using Fe(III) as electron acceptor.

54 Our understanding of the Feammox process is still incomplete, particularly
55 information about the microorganism(s) responsible for it is lacking. This makes further
56 study into the mechanism of the Feammox process difficult. Here we focus on a series
57 of incubations and establishing a Feammox enrichment culture to identify the microbial
58 community responsible for the process described previously (Clement *et al*., 2005;
59 Shrestha *et al*., 2009). Soil samples were collected from the same location and used for
60 laboratory incubation experiments as well as to set up an enrichment system for
61 Feammox in a continuous flow membrane reactor. Various incubation conditions

62 [Fe(III) sources, inorganic carbon content, NH_4^+ concentration, $^{15}NH_4^+$, and acetylene gas
63 (C_2H_2) as a selected inhibitor] were used to study the Feammox mechanism. Molecular
64 biology methods, such as denaturing gradient gel electrophoresis (DGGE), 454
65 pyrosequencing, and real-time quantitative PCR (qPCR) analysis were used to investigate
66 the bacterial community change during incubations.
67

68 **2. METHODS**

69 **2.1 Sample collection and processing**

70 Soils for all the experiments described in this study were taken from a temperate forested
71 riparian wetland at the Assunpink Wildlife Management Area, New Jersey. Ten soil
72 cores were collected from 10 cm below the surface with polyethylene column containers
73 (8 cm diameter and 30 cm long) and transported to the laboratory within 2 hours. The
74 soil pH was between 3.5 and 4.5, and no Manganese oxide was detected. The detailed
75 physicochemical characteristic of these wetland soils have been described elsewhere
76 (Clement *et al*., 2005). Prior to all incubation experiments, soil slurry from the field site
77 was aerated for a month to degrade much of the labile organic carbon. After a 30 days
78 of aeration, the dissolved organic carbon (DOC) content was stable at 2.06 ± 0.20 mg g^{-1}.
79 Following the aeration treatment, the soil was divided into 400 × 10 g (air-dry equivalent)
80 subsamples, and added into 50mL serum vials, with 30 mL deionized water. The soil
81 slurries were purged thoroughly with a CO_2:N_2 (80:20) mixture, resulting in a final pH of
82 ~ 4 to 4.5. The vials were sealed tightly with rubber stoppers and were stored in an

anaerobic glove box for 30 days at ambient temperature to allow for stabilization before starting the incubations.

2.2 Batch incubation experiments

All incubations, addition of reagents, and sampling were conducted in an anaerobic glove box with a solution of resazurin as the redox indicator. Soil samples were first incubated with different Fe(III) sources to determine which source would yield a more active Feammox process: 6-line ferrihydrite (Fe_2O_3•$0.5H_2O$) or goethite [FeO(OH)] [prepared according to Cornell and Schwertmann, 2003] + NH_4^+ addition; ferric chloride + NH_4^+ addition; ferric citrate + NH_4^+ addition; either only ferrihydrite or NH_4^+ addition; and autoclaved soil with ferrihydrite + NH_4^+ addition (n = 30 per treatment). pH was adjusted to 4.5 in the ferrihydrite/goethite augmented samples, and to between 3.5 ~ 4.0 in the ferric chloride/citrate augmented samples. Soil-slurry samples, which were prepared to have an initial concentration of 12.0 mmol L^{-1} Fe(III) and/or 2.00 mmol L^{-1} NH_4^+, were incubated in a series of 50 ml vials with an oxygen-free headspace, created by purging with a CO_2:N_2 (80:20) mixture. Triplicate samples were collected destructively every two days to analyze iron and nitrogen species.

The second incubation was conducted to extend the anoxic incubation with ferrihydrite to 180 days, with repeated NH_4Cl additions after the NH_4^+ in solution was exhausted. The initial concentration of Fe(III) was 25.0 mmol L^{-1} and 1.00 mmol L^{-1} NH_4^+ was added on days 4, 24, and 60, furthermore, 0.20 mmol L^{-1} $NaHCO_3$ was added

104 on day 50 and day 90 of the incubation. On day 125, incubation vials were divided into
105 two sets to study the effect of different inorganic carbon contents on Feammox. Either
106 1.20 mmol L^{-1} or 0.20 mmol L^{-1} of $NaHCO_3$ plus 2 mmol L^{-1} of NH_4Cl were added to
107 each set. $NaHCO_3$ was then added every 10 days, which increased the soil pH to ~5 in
108 the samples amended with 1.20 mmol L^{-1} of $NaHCO_3$. For this incubation, samples
109 were collected every four days. Finally, soil samples collected on day 180 of the
110 incubations were used to enrich the Feammox bacteria in a membrane reactor. To study
111 how the organic carbon content affects the Feammox bacteria, 1.00 mmol L^{-1} sodium
112 citrate was also supplied on day 125 to four of the 1.20 mmol L^{-1} $NaHCO_3$ amended
113 samples.

114 In the third experiment, inorganic nitrogen species were quantified through
115 incubations in the presence of C_2H_2. Soil slurries were first incubated for 90 days in
116 eighty 50 mL vials, with an initial Fe(III) concentration of 25 mmol L^{-1}. One mmol L^{-1}
117 NH_4Cl and 0.20 mmol L^{-1} $NaHCO_3$ was added on days 24, 60, and 90. After this
118 incubation, 5 mL of pure C_2H_2 gas were added to 40 vials, which resulted in a finial C_2H_2
119 concentration of 100 μmol L^{-1}. Samples with and without C_2H_2 were then incubated
120 anaerobically for 20 days. The headspace gas was sampled every 24 hours for N_2O
121 analysis, and soil samples were analyzed every two days for Fe and N species.

122

123 **2.3 Continuous flow membrane Feammox reactor**

124 Soil samples collected on day 180 from the incubation with ferrihydrite, NH_4Cl, and

125 1.20 mmol L^{-1} $NaHCO_3$ additions were inoculated into a continuous flow membrane
126 reactor (Abbassi *et al*., 2014), which was operated under anaerobic conditions by
127 constantly purging N_2 trough the reactor's headspace at a room temperature (25℃), and
128 with a 48 hour hydraulic retention time.

129 The enrichment medium contained the following components per liter: 177 mg
130 NH_4Cl, 77.9 mg $(NH_4)_2SO_4$, 19.8 mg $NaHCO_3$, 71.0 mg $KHCO_3$, 9.00 mg KH_2PO4, 100
131 mg $MgSO_4 \cdot 7H_2O$,and 60.0 mg $CaCl_2 \cdot 2H_2O$. After autoclaving, 1 mL trace element
132 solution (Van de Graaf *et al*., 1996) was added to the medium. 50.0 mmol L^{-1}
133 ferrihydrite were added once every two weeks directly into the reactor. To aid in
134 maintaining anaerobic conditions, 0.10 - 0.20 mmolL^{-1} sodium citrate was feed to the
135 reactor about twice per month. pH was controlled at around 4~5, and dissolve oxygen
136 was < 0.10 mg/L. Samples form the outflow were collected every two days, and sludge
137 samples from reactor were collected and kept at -20 °C for molecular biology analysis.

138 Finally, ^{15}N isotope tracer incubations were conducted using slurries collected form
139 the stable Feammox membrane reactor. Five treatments (n = 3 per treatment) were
140 conducted: (1) control with only anoxic DI water; (2) $^{15}NH_4Cl$ addition; (3) $^{15}NH_4Cl$ +
141 Fe(III) addition; (4) $^{15}NH_4Cl$ and C_2H_2 addition; (5) $^{15}NH_4Cl$, C_2H_2, and Fe(III) addition.
142 The headspace gas of each 50mL incubation vial was sampled every 24 hours for $^{15}N_2O$
143 analysis (Supplementary Information 1.4).

144

145 **2.4 Chemical analyses**

146 For each sample collection during the incubations, a set of vials was destructively
147 sampled in a glove box under oxygen-free conditions and the pH was measured
148 immediately using a pH electrode. An extraction with 0.5N HCl was conducted for 24
149 hours at room temperature to determine acid-extractable Fe(II) and NH_4^+ concentrations
150 in the soils. Fe(II) was analyzed using the ferrozine assay method (Stookey, 1970;
151 Komlos *et al*., 2007). Extraction efficiency of Fe(II) was affected by the HCl
152 concentration and the extraction time. About 5-10 % more Fe(II) could be extracted with
153 either 1N HCl extraction over 24 hours or with 0.5 N HCl over 36 hours as opposed to
154 0.5 N HCl over 24 hours. Furthermore, after more Fe(II) was produced in the system
155 with increasing incubation time, the Fe(II) extraction efficiency improved. Only a 1-2%
156 difference was observed in the Fe(II) extracted over 24 hours using 0.5N vs. 1N HCl
157 towards the end of the incubation period. Clays, present in the soil incubations,
158 typically sorb Fe(II) more efficiently when the total Fe(II) is low, furthermore ferrihydrite
159 is slowly converted to magnetite, resulting in relatively different associations to different
160 phases of the Fe(II) over the duration of the incubation. All of which leads to
161 incomplete Fe(II) extractions, especially when the Fe(II) is low. Here we report Fe(II)
162 data obtained via 0.5N HCl extractions over 24 hours to ensure that the methods and
163 hence data are comparable to those reported by other researchers focusing on iron
164 reduction and iron bioavailability.

165 NH_4^+ was analyzed using a Dionex™ Ion Chromatograph (LC3000) with a CS-16
166 Colum and a CS-16 guard column (flow rate = 1.00 mL min^{-1}, detection limit = 0.012

ppm). NO_3^- and NO_2^- were extracted with DI water for 1 hour anaerobically, and measured via Ion Chromatography, using an AS-22 Column along with an AG-22 guard column (flow rate = 1.20 mL $min^{-1,}$ detection limit = 0.016 ppm). For the total organic carbon (TOC) and total nitrogen (TN) analyses a Shimadzu TOC-5000(A) was used. N_2O concentrations were determined on a gas chromatograph Shimadzu 2014 equipped with an electron capture detector.

2.5 DNA and RNA isolation

DNA and RNA samples were extracted from soils collected at the wetland prior to any laboratory incubation, from the samples taken at different time points during the incubation experiments and from the reactor. DNA was extracted from 500 mg soil or sludge samples using the FastDNA® spin kit for soil (MP Biomedicals, USA) as described by the manufacturer, and RNA using the FastRNA® pro Soil Direct Kit. The concentrations were measured using a Nano-drop 2000 spectrophotometer (Thermo Scientific, USA).

2.6 PCR-DGGE and 454 pyrosequencing Analysis

Bacterial universal 16S rRNA gene primer sets V3-2/V3-3 (Jensen *et al*., 1998) were used for PCR amplification (Table1). DGGE was performed with an 8% polyacrylamide gel containing a gradient from 40% to 80% denaturant using the gradient gel electrophoresis system (C.B.S. SCIENTIFIC, USA). The electrophoresis was

188 carried out at 60 V for 15 hours. After that the gel was stained with 0.1 μL mL^{-1} SYBR
189 Green I and visualized with an UV transilluminator. All visible bands were excised
190 from the gel and used as templates for re-amplification, using the primer set V3-1/ V3-2
191 (Jensen *et al*., 1998) and followed by cloning (Table1). PCR products were purified via
192 agarose gel extraction and cloned into a pGEM-T vector (Promega). A total of 10 to 30
193 positive recombinant clones for each band were identified by colony PCR, and were sent
194 for sequencing to avoid erroneous interpretations. DNA sequencing was then conducted
195 by Genewiz, Inc. Bacteria were classified and the phylogenetic tree of
196 *Acidimicrobiaceae*-related sequences was constructed using the Bayesian inference
197 (Huelsenbeck *et al*., 2001) (Supplementary Information 1.2). Sequences obtained in this
198 study are available in the GenBank database under accession numbers KC581755 -
199 KC581779. To further confirm the changes in the bacterial community, 454
200 pyrosequencing was performed with samples collected from the incubation on days 0, 30,
201 90, 160 and from the membrane reactor after 150 days of reactor operation.
202 Domain-specific primers Bact-338F1/909R, targeting the V3-V5 region of the 16S rDNA
203 of bacteria were amplified and sequenced following methods suggested by Pinto *et al*.
204 (2012) (Supplementary Information 1.2)
205
206 **2.7 Quantitative PCR (qPCR) assay**
207 qPCR experiments were carried using a StepOnePlus™ Real-Time PCR System
208 (Life Technologies, USA), represented by 16S rRNA genes, using primer sets

209 1055f/1392r for total bacteria (Harms *et al*., 2003), Amx368f/Amx820r for anammox
210 bacteria (Schmid *et al*., 2000; Schmid *et al*.,2003), acd320f - 432r which we developed
211 for *Acidobacteriaceae* bacteria, and acm342f - 439r which we developed for
212 *Acidimicrobiaceae* bacteria (Supplementary Information 1.3) (Table1). For the
213 detection of denitrifiers, AOB and AOA, denitrifying functional genes (*nirS and nirk*) ,
214 ammonia monooxygenase structural gene (*amoA*) were quantified with primer sets
215 NirS3F/NirS5R, NirK1F/NirK5R (Braker *et al*., 1998, amoA-1F/ amoA-2R(Rotthauwe *et*
216 *al*., 1997) for AOB-*amoA* and Arch *amoA*-F/ *amoA*-R for AOA-*amoA*(Francis *et al*.,
217 2005) , respectively (Rotthauwe *et al*., 1997). Primer pairs CrenamoA23f /616r, was
218 also used to quantify the thaumarchaeal *amoA* genes which represented acidophilic
219 ammonia oxidizers (Tourna *et al*., 2008), For DNA quantification, each qPCR mixture
220 (20 μL) was composed of 10 μL of SYBR Premix Ex Taq® II (Takara, Japan), 0.8 μL 10
221 μM of each primer, and ~ 10 ng DNA template. RNA quantification was conducted
222 through a real-time quantitative reverse transcription-PCR (RT-qPCR) analysis, by using
223 the One Step SYBR® PrimeScript® RT-PCR Kit (Takara, Japan) according to the
224 manufacturer's recommendations. Thermal cycling conditions for total 16S rDNA, *nirS*
225 and AOB-*amoA* gene numbers was initiated for 30 s at 94 °C, followed by 40 cycles of 5
226 s at 94 °C, 30 s at 57 °C, and 30 s at 70 °C. 16S rDNA numbers of anammox,
227 *Acidobacteriaceae* and *Acidimicrobiaceae* bacteria were performed at 56°C, 55 °C, and
228 58 °C as annealing temperature respectively, with the same program. For AOB-*amoA*
229 gene and thaumarchaeal *amoA* genes, annealing temperature was 53 °C and 55°C, and the

annealing time was adjusted to 45s. For RNA quantification, the cycling conditions were identical to those described for measuring gene numbers, with the exception that an initial incubation was conducted for 5 min at 42°C to facilitate reverse transcriptase activity. Each assay contained a standard using a serial dilution of plasmids containing specific target genes, independent triplicate templates for each soil sample, and triplicate no template controls (NTC).

3. RESULTS

3.1 Change in Fe and N species under different operational conditions

After incubating the pre-treated soil slurry with the four different Fe(III) sources for 30 days, only samples to which either ferrihydrite or goethite had been added showed measurable NH_4^+ oxidation (Fig. 1 and Fig. S1). In samples incubated with ferric citrate and NH_4Cl, Fe(III) reduction was much faster than in those supplied with Fe(III) oxides, but the NH_4^+ concentration remained fairly constant (Fig. 1). No detectable Fe(II) reduction or NH_4^+ oxidation was found the sterilized soils amended with ferrihydrite and NH_4Cl, (Fig. S2). Faster iron reduction and NH_4^+ removal was observed in ferrihydrite than in goethite-amended sediments (Fig. S1).

Since samples incubated with ferrihydrite and NH_4Cl resulted in the fastest NH_4^+ oxidation, the anaerobic incubation with ferrihydrite was extended to 180 days. Ferrihydrite as the Fe(III) source results in a larger negative $\triangle G$ value than goethite (Clement *et al*., 2005). The NH_4^+ oxidation rate increased as NH_4Cl was supplied repeatedly, especially after 125 days of incubation when the $NaHCO_3$ additions were increased from 0.20 to 1.20 mmol L^{-1} in addition to the 2.00 mmol L^{-1} NH_4^+ added. The increased $NaHCO_3$ dosing also increased the generation of Fe(II) (Fig. 2a,b). During

254 the 180-day incubation, the ratio of Fe (II) produced to NH_4^+ removed gradually
255 increased until it reached 5.3:1 by day 160 after which it remained stable (Fig. S6).
256
257 NO_2^- appeared within a few days after the addition of NH_4^+, with a maximum
258 concentration 0.44 ± 0.17 mmol L^{-1} in the second NH_4^+ oxidation cycle. NO_2^- did not
259 accumulate in the system and was immediately consumed after generation (Fig. S3a).
260 NO_3^- production showed a similar pattern to that of NO_2^- (Fig. S3a), and TN loss similar
261 to the decrease in NH_4^+ (Fig. S3b). During 180 days of incubation, the system
262 experienced a loss of TN of 57.2 ± 3.13 mg L^{-1}. The DOC content fluctuated slightly in
263 the early stage of incubation, but overall, the DOC concentration was relatively stable at
264 around 45~50 mg L^{-1} (Fig. S3b).
265 A 64.5% NH_4^+ removal, between inflow and outflow was achieved in the
266 membrane reactor after 150-days of operation.
267

268 **3.2 Phylogenetic analysis of the microbial community based on 16S rRNA gene**

269 All visible bands observed in the DGGE analysis (significant bands were marked, see
270 Fig. 3) were excised from the gel and sequenced after cloning. Clone libraries from 12
271 samples resulted in 721 sequences of partial 16S rRNA gene fragments, and six groups of
272 bacteria were classified via a phylogenetic analysis (Table 1 and Table S1). During this
273 180-day anaerobic incubation with ferrihydrite and NH_4Cl, the microbial communities
274 shifted dramatically and the microbial diversity decreased with time (Fig. 3, lane 1-4).

Some DGGE bands disappeared gradually with time, such as band A5 and band A7. Band A5, represents a dissimilatory iron-reducing bacteria, *Geobacter* sp., which existed in this Fe(III)-rich wetland soil and reappeared for a short time during the initial anaerobic incubation. Band A7, represents an ammonia-oxidizing bacterium, *Nitrosomonas sp.*, which showed a strong presence in the samples at 30 days of incubation and was attenuated after longer incubation times. In contrast, DGGE bands A6, A8 and A9 became more significant as the incubation time increased, showing that there were three groups of bacteria dominating in the system after 160 days of incubation. Band A6, represents a group of bacteria belonging to the *Acidimicrobiaceae* family. Bacteria from the *Acidobacteriaceae* family are represented by band A8. Some species in this family have been described as iron reducers and obligate heterotrophs (Kishimoto *et al*, 1991; Rowe *et al*., 2007; Coupland and Johnson, 2008). DGGE band A9 represents bacteria of the *Rhodocyclaceae* family. This family contains mainly denitrifying bacteria, which exhibit very versatile metabolic capabilities (Smith *et al*, 2005; Huang *et al*., 2011).

Microbial communities also differed between samples incubated with various Fe(III) sources, and between samples with or without the addition of inorganic carbon. Samples supplied with either ferric chloride or ferric citrate as the Fe(III) source plus NH_4^+, and samples supplied with just ferrihydrite and no NH_4^+, had a decreased diversity in their bacterial communities (Fig. 3, lane 5-7). Samples supplied with both organic carbon (1.00 mmol L^{-1} sodium citrate) and inorganic carbon (1.20 mmol L^{-1} $NaHCO_3$)

296 had a higher bacterial diversity (DGGE Fig.3, lane 8).

297 Changes in the microbial community after 180 days of incubation were also
298 confirmed via 454-pyrosequencing, and the obvious growth of *Actinobacteria,*
299 *Acidobacteria* and *β-Proteobacteria* groups (which band A6, A8 and A9 belong to) was
300 consistent with the DGGE results, where the *Actinobacteria* cell number increased the
301 most (Fig. 4). *Planctomycetes* phylum, with which anammox bacteria are affiliated, was
302 detected in the first 90 days of incubation, but disappeared or was below detection on day
303 160. *Actinobacteria* were also the dominant species in the Feammox enrichment reactor
304 based on the results of the 16S rDNA library obtained via pyrosequencing (Fig. 4).

305 The *Acidimicrobiaceae* bacterium, represented by band A6, which belongs to the
306 *Actinobacteria* phylum, was the dominant species in the incubation experiments after 180
307 days of incubation (14.8% in total 16S rRNA gene sequences) as well as in the membrane
308 reactor after 150 days of operation (40.2% in total 16S rRNA gene sequences). Its
309 similarity to other *Acidimicrobiaceae*-related sequences is shown using a phylogenetic
310 tree (Fig. 5). Unlike the bacteria represented by band A8 and A9, which were also
311 found in samples that did not show Feammox transformations, this *Acidimicrobiaceae*
312 bacterium was only detected in incubations (or reactor) that were augmented
313 simultaneously with ferrihydrite, NH_4Cl, and $NaHCO_3$ and have shown Feammox activity
314 (Fig. 3).

315

316 **3.5 Changes of bacterial abundance and activity during incubations and in the**

317 **reactor**

318 The total bacterial abundance determined via the 16S rRNA gene copy number,
319 decreased during the 180-day incubation (Fig. 6). Both, 16S rRNA gene and rRNA
320 fragment copies of *Acidimicrobiaceae* bacteria (DGGE band A6), increased during the
321 incubation, particularly after 90 days. The rRNA numbers increased slowly during the
322 first 3 months and doubled between day 130 and day 140 of the incubation period (Fig. 6).
323 rRNA as a biomarker for changes of protein level, even though not as specific as mRNA,
324 is a good indicator for bacterial activity (Poulsen *et al*., 1993; Park *et al*., 2010). The
325 abundance of *Acidobacteriaceae* bacteria (DGGE band A8), and that of denitrifiers
326 (represented by the number of *nirS* gene and *nirK* gene), increased over the 180-day
327 incubation (Fig. S4a, b, c), although less than the *Acidimicrobiaceae* bacteria (DGGE
328 band A6). Growth of *nirS* and *nirK* gene showed similar trends, although the number of
329 *nirK* gene was two orders lower than *nirS* (Fig. S4b, c). Increase in the denitrifier
330 activity was most likely stimulated by the NO_2^- generated via Feammox. The number of
331 the *amoA* gene, representing the abundance of ammonia-oxidizing bacteria, decreased
332 sharply with time and was hardly detected after 90 days of incubation (Fig. S4e).
333 Through quantification of thaumarchaeal *amoA* genes, none of the acidophilic ammonia
334 oxidizers was detected in our system.

335 In the Feammox reactor, the copy number of *Acidimicrobiaceae* bacterium A6,
336 Anammox bacteria and *nirS* gene were 0.37×10^7, 0.13×10^6, and 0.92×10^6 copies g^{-1} dw,
337 respectively, while the amoA gene was not detected.

338

339 **3.6 Changes of bacterial abundance and activities with $NaHCO_3$ amendment**

340 Abundance and activity of *Acidimicrobiaceae* bacteria, represented by band A6, were
341 compared between samples incubated under the same conditions except the amounts of
342 $NaHCO_3$ added (0.20 mmol L^{-1} vs. 1.20 mmol L^{-1}). From day 125 to day 180 of the
343 incubation, both 16S rRNA gene and rRNA fragment numbers of *Acidimicrobiaceae*
344 bacteria were higher in the soils with the higher inorganic carbon content. The 16S
345 rRNA gene copies of samples augmented with 1.20 mmol L^{-1} $NaHCO_3$ were four times
346 higher than those in samples that had been augmented with only 0.20 mmol L^{-1} $NaHCO_3$
347 (Fig. 6). The rRNA copies of the *Acidimicrobiaceae* bacteria, showed even larger
348 differences in response to the amounts of $NaHCO_3$ added. In the samples augmented
349 with 1.20 mmol L^{-1} $NaHCO_3$, the rRNA copy number increased from $0.04 \pm 0.06\times10^6$ to
350 $0.19 \pm 0.09\times10^6$ copies g^{-1} dw over 50 days of incubation (day 130 to day 180).
351 However, in the samples to which only 0.2 mmol L^{-1} $NaHCO_3$ were added on day 125, the
352 rRNA number gradually deceased from $0.29 \pm 0.10\times10^5$ to $0.19 \pm 0.05\times10^5$ copies g^{-1} dw
353 during the same 50 days incubation (Fig. 6).

354

355 **3.7 Nitrogen species changes in samples incubated in the presence of C_2H_2**

356 C_2H_2 can inhibit the oxidation of NH_4^+ to NO_2^- under aerobic conditions, and the
357 reduction of N_2O to N_2 as well as the anammox pathway under anaerobic conditions
358 (Yoshinari *et al.*, 1977; Jensen *et al.*, 2007; Kartal *et al.*, 2011). To gain further

359 insights into the nitrogen removal process observed, incubations with C_2H_2 were
360 conducted. After 20 days of incubation, less NH_4^+ was oxidized in the samples
361 amended with C_2H_2, compared to those incubated without C_2H_2 (Fig. S5a). NO_2^-, which
362 is postulated to be the direct product of the NH_4^+ oxidation, accumulated slowly in the
363 samples incubated with C_2H_2 (Fig. S5b). NO_3^- reached a higher concentration in
364 samples without C_2H_2 than in samples incubated with C_2H_2 (Fig. S5c). N_2O, a product
365 of NO_2^- reduction, accumulated in the samples incubated with C_2H_2, which inhibits the
366 reduction of N_2O to N_2 (Fig. S5d). Fe(II) production was not much affected by the
367 presence of C_2H_2, and after 20 days incubation was 4.36 ± 0.72 and 5.71 ± 0.67 mmol L^{-1}
368 in sample incubated with and without C_2H_2 , respectively.
369 In the ^{15}N isotope tracer incubations, detectable ^{15}N-N_2O was only found in samples
370 amended with both, $^{15}NH_4Cl$ and Fe(III), with ^{15}N-N_2O production rates 2.14±0.059 or
371 0.072±0.023 μg g^{-1} d^{-1} in samples incubated with or without C_2H_2 treatment (Table S2).
372
373 **DISCUSSION**
374 DGGE band A5 represents dissimilatory iron-reducing bacteria, which appeared for a
375 short time at the beginning of the anaerobic incubation. For longer incubation times
376 these heterotrophic bacteria decreased rapidly (Fig. 3). Over a 25 day incubation period,
377 more than three times the mass of Fe(II) was produced in samples amended with
378 ferrihydrite and NH_4^+ as compared to the samples amended only with ferrihydrite,

379 indicating that most of the Fe(III) reduction came from the Feammox reaction, and not
380 from dissimilatory Fe(III) reduction (Fig. 1a vs. Fig S2a).

381 AOB represented by DGGE band A7 (Fig. 3), as well as the *amoA* gene, decreased
382 after 30 days of incubation (Fig. S4e). Also, no AOA or acidophilic ammonia oxidizers
383 were detected although the pH condition seems suitable for them in these incubations.
384 Oxygen deficiency was the most likely reason for the decline in AOB in this system over
385 time (Laanbroek *et al*., 1994). Even though a small amount of AOB would be enough
386 for NH_4^+ oxidation, in our control samples to which no Fe(III) were added which AOB
387 do not required, no NH_4^+ consumption was detected (Fig. S2). Moreover, the decrease
388 in *amoA* gene at a time of increasing NH_4^+ oxidation also indicates that neither AOB nor
389 acidophilic ammonia oxidizers were the drivers of the NH_4^+ oxidation in the later
390 incubation times.

391 An uncultured *Acidimicrobiaceae* bacterium became the dominant species during
392 the 180-day anaerobic incubation period, increasing from 0.92% on day 0 in terms of cell
393 numbers to 14.8% on day 160. In the incubation experiments conducted (which
394 included controls with only NH_4^+, only iron, autoclaved, and various Fe(III) sources), this
395 *Acidimicrobiaceae* bacterium was only detected and growing in samples to which NH_4^+
396 was supplied as an electron donor, ferrihydrite was supplied as electron acceptor, and
397 $NaHCO_3$ was supplied as a carbon source (Fig. 1, Fig.3, Fig.S1, Fig S2). The
398 abundance and activity of this *Acidimicrobiaceae* bacterium increased along with the
399 Feammox activity during the incubations. During the incubation period its rRNA
400 changed from $(0.22 \pm 0.01)\times10^5$ copies g^{-1} dw to $(0.28 \pm 0.07)\times10^6$ copies g^{-1} dw,
401 indicating a substantial increase in its activity (Fig. 6). In the continuous flow

membrane reactor, which had a high NH_4^+ removal and Fe(III) reduction rate, this *Acidimicrobiaceae* bacterium was enriched from an initial 14.8% to 40.2% after 150 days operation, and no other known NH_4^+ oxidizers (AOB or anammox) were detected (Fig. 4). These results indicated that this *Acidimicrobiaceae* bacterium might play an important role in the Feammox reactions described in this study. According to a phylogenetic analysis, this bacterium has a 92% identity with *Ferrimicrobium acidiphilum* sp. (Table 1). *F. acidiphilum*, which belongs to the *Acidimicrobiaceae* family, was first isolated from mine environments (Johnson *et al*., 2009), and *F. acidiphilum* strain T23 is the only pure strain with a comprehensive characterization. Uncultured *Ferrimicrobium* sp. has been detected in mine water, but so far not in wetland soils (Gonzalez-Toril *et al*., 2003; Johnson *et al*., 2009; Bruneel *et al*., 2011). *Ferrimicrobium* sp. is an acidophilic heterotrophic ferrous iron oxidizing bacterium, which can also reduce Fe(III) under anoxic conditions (Johnson *et al*., 2009). The uncultured *Acidimicrobiaceae* bacterium, also has a 90% identity with *Acidimicrobium ferrooxidans* (Table 1), a facultative autotroph in the same family, which can reduce Fe(III) in anaerobic environments while oxidizing sulfide to sulfur and exists widely in soil environments (Clark and Norris, 1996; Bond *et al*., 2000; Hartmann *et al*., 2009).

According to a phylogenetic comparison with similar clones from studies reported in the GenBank (Fig. 5), and taking into account its special growth characteristics (stimulated by inorganic carbon, oxidizing NH_4^+ coupled to Fe(III) reduction), also its gradual activity increase with increased Feammox activity, as well as a strong link between it and a Feammox enrichment reactor, this uncultured *Acidimicrobiaceae* bacterium A6 is probably a previously unreported species in the *Acidimicrobiaceae* family that might be either responsible or play a key role in the Feammox process described here. *Acidimicrobiaceae* bacterium A6 was more active and the Feammox pathway was faster in samples with higher $NaHCO_3$ amendments (Fig. 2 and 6), which, in addition to the fact that ΔG in Equation 1 is negative, indicates that if this

429 *Acidimicrobiaceae* bacterium is actually responsible for conducting the Feammox
430 reaction as depicted in equation 1, it may be an autotroph. Growth of *nirS* gene
431 suggested that denitrification pathways were also active in the incubations described here.
432 NO_2^- that was being produced during the anaerobic NH_4^+ oxidation was reduced to N_2 by
433 denitrifiers, and NO_2^- did not accumulate in the system.

434 The Feammox reaction studied here proceeded only when iron oxides (ferrihydrite or
435 goethite) were supplied as electron acceptor, whereas samples incubated with ferric
436 chloride or ferric citrate as the Fe(III) source showed no measurable NH_4^+ oxidation (Fig.
437 1 and Fig. S1). In the incubations to which ferric citrate was added as the Fe(III) source,
438 Fe(III) was reduced rapidly by dissimilatory iron reducers, using organic carbon as
439 electron donor. The DGGE results for incubations with ferric citrate (Fig. 3, lane 7) show
440 that the most dominant species was an *Actinobacterium* (Table S1), known to reduce iron
441 under anaerobic conditions (Lin *et al*., 2007; Lentini *et al*., 2012). *Acidimicrobiaceae*
442 *bacterium* A6 was not detected in these incubations. Since acidic conditions as well as
443 minimal dissolved Fe(II) and NO_2^- concentrations are required to make the Feammox
444 reaction energetically favorable as shown in Equation 1, the presence of iron oxides as
445 the main Fe(III) source might may have helped to maintain the concentrations of Fe(II) in
446 solution below the detection limit through the incubation since iron oxides can sorb Fe(II)
447 and/or incorporate it into their structure.

448 Various NH_4^+ oxidation products, i.e. NO_3^-, NO_2^- and N_2, generated through the
449 Feammox process are thermodynamically feasible, and were reported in different
450 Feammox studies (Sawayama, 2006; Shrestha *et al*., 2009; Yang *et al*., 2012). Because
451 there was no initial nitrate or nitrite in the system, because all experiments were
452 conducted under strict oxygen free conditions, and because of the rapid decrease of *amoA*
453 genes, neither NO_3^- reduction nor aerobic NH_4^+ oxidation could be the reason for the
454 formation of NO_2^- during the incubations. In all incubations where NH_4^+ was removed,

455 the production of NO_2^- was observed. NO_2^- did not build up and, given the presence of
456 denitrifiers, it is likely that the NO_2^- produced was rapidly reduced.

457 When C_2H_2 was used to stop the reduction of N_2O to N_2, the total N_2O (0.72 ± 0.23
458 mmol L^{-1}) plus NO_2^- produced (0.13 ± 0.07 mmol L^{-1}) was equal to the NH_4^+ consumed,
459 showing that NH_4^+ was not oxidized directly to N_2 in our samples. ^{15}N-NH_4^+
460 incubations, as an extension of C_2H_2 treatment, showed that ^{15}N-N_2O built up when
461 $^{15}NH_4Cl$ was added as the NH_4^+ source (Table S2), demonstrating that NH_4^+ was oxidized
462 during the Feammox process rather than be adsorbed or taken uptake by microorganisms
463 in the system.

464 Although nitrification might happen in suboxic environments (oxygen ＜5μM,
465 Lam *et al*., 2007), NH_4^+ oxidation in the presence of C_2H_2 has never been reported.
466 C_2H_2 is an inhibitor of ammonia monooxygenase (AMO), and can restrain aerobic NH_4^+
467 oxidizers from using oxygen by binding covalently to AMO (Hynes and Knowles, 1982;
468 Hyman and Wood, 1985; Gilch *et al*., 2009). C_2H_2 can also inhibit the NH_4^+-activation
469 step of anammox cells, which use NO_2^- as the oxidant (Kartal *et al*., 2011). Therefore
470 these Feammox bacteria might differ from common NH_4^+ oxidizers, by using an
471 alternative NH_4^+ oxidation pathway that is not inhibited by C_2H_2, and AMO might not
472 play a role in Feammox. The fact that NH_4^+ oxidation was not affected by the presence
473 of acetylene is a further indication that AOB are not responsible for this process since
474 they would be affected by acetylene. Furthermore, in the isotope tracer incubations,
475 ^{15}N-N_2O was below the detection limit in samples to which Fe(III) was not supplied,
476 showing again that NH_4^+ oxidation proceeded only when iron was being reduced.

477 The role of anammox during the incubations was also evaluated. During the incubations
478 the ratio of NH_4^+ oxidized to Fe(III) reduced increased gradually from 1:1.9 to 1:5.3 (Fig.

479 S6), which is close to the stoichiometry of 1:6, shown in Equation 1. Although the
480 discrepancies in the Feammox stoichiometry between iron and NH_4^+ are attributed in part
481 to incompletely Fe(II) extraction, the influence of anammox activity in the earlier
482 incubations would have contributed to a lower Fe(II) produced to NH_4^+ removed ratio
483 than the theoretical value of 1:6.

484 The Feammox reaction became more dominant in terms of NH_4^+ oxidation after 125 days
485 of incubation due to a relative increase in the activity of the Feammox bacteria. A
486 parallel pathway to Feammox, such as anammox, could as mentioned above, explain the
487 lower stoichiometric ratio, especially at earlier incubation times. In the samples taken
488 before the incubation, $0.17 \pm 0.05 \times 10^6$ copies g^{-1} dw of anammox rRNA gene were found,
489 which decreased to $0.09 \pm 0.06 \times 10^5$ on day 130 (Fig. S4d). We postulate that anammox
490 was responsible for some initial NH_4^+ and NO_2^- removal, and denitrification became
491 more dominant for NO_2^- removal later during the incubation period (Fig. 5 and Fig.
492 S4b,d). NH_4^+ removal via Anammox in the early incubations may also explain why the
493 observed NH_4^+ oxidation rates and the abundance of *Acidimicrobiaceae A6* did not change
494 proportionally over the full incubation period.

495 The results and analyses described here have shown that a Feammox enrichment
496 reactor has the capacity of oxidize NH_4^+ coupled to iron reduction under anaerobic
497 conditions, and that an uncultured *Acidimicrobiaceae* bacterium A6, which became the
498 dominant species over time might be responsible for this Feammox reaction. Without
499 access to samples from other reported biological NH_4^+ oxidation by Fe(III) reducers
500 (Sawayama, 2006;Yang *et al*., 2012), it is not possible to know if the organisms for the
501 processes reported by these investigators are the same as those identified here.
502 Conclusive linkage between *Acidimicrobiaceae A6* and Feammox process requires the

503 isolation of the strain and then conduct incubations with the pure strain. At this point,
504 the observations (i) that NH_4^+ removal only occurred in samples when the presence of
505 *Acidimicrobiaceae* bacterium A6 was detected and when iron was being reduced, (ii) that
506 *Acidimicrobiaceae* A6 numbers increased gradually after sequential NH_4^+ and Fe(III)
507 additions, and (iii) the results from the enrichment culture which was operated for an
508 extended time period while only adding NH_4^+ and iron oxide sources and during which
509 *Acidimicrobiaceae A6* became the dominant bacterial species while no other known NH_4^+
510 oxidizer (AOB or anammox) was detected after 150 days of operation, indicate that
511 *Acidimicrobiaceae A6* is likely to play an important role of the oxidation of NH_4^+ under
512 iron reduction conditions.
513
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517
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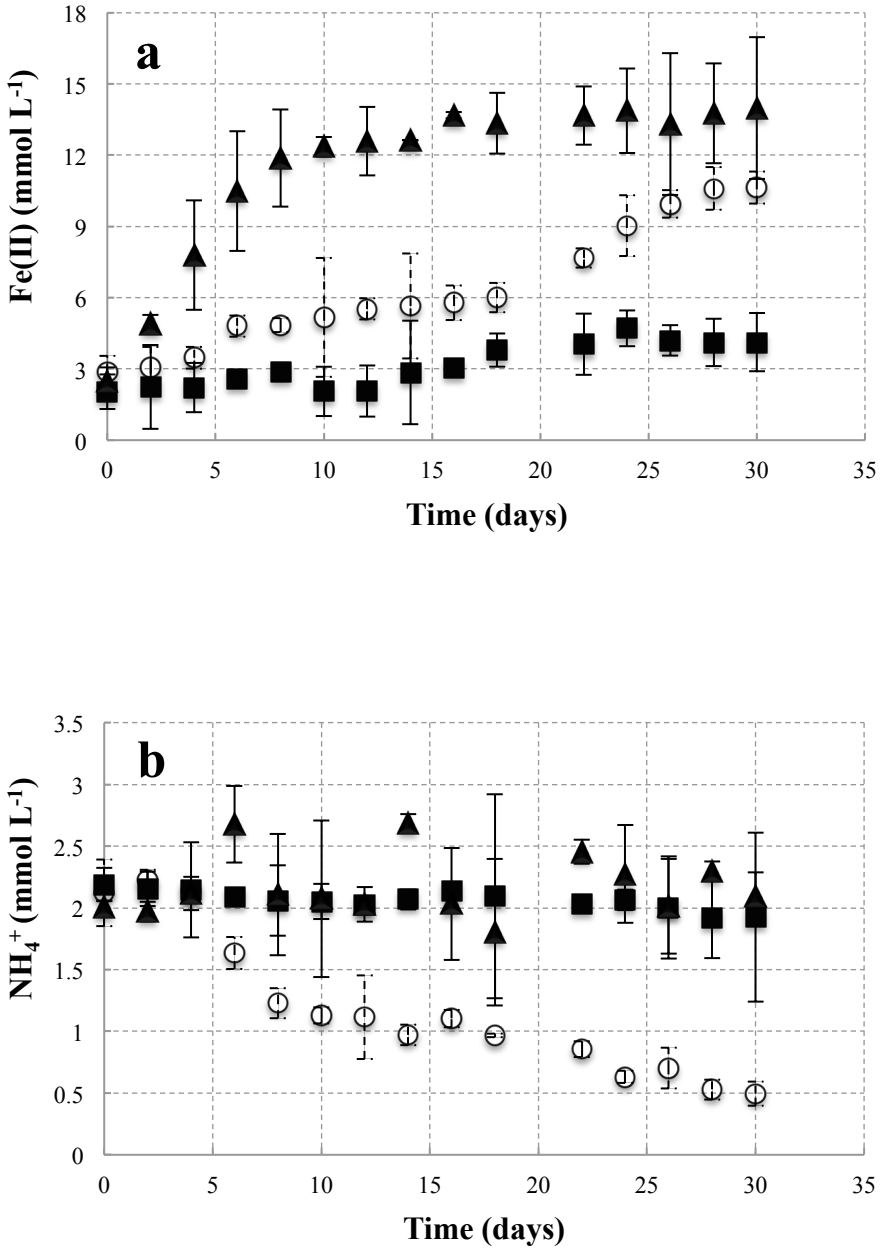

Figure 1. Concentration of Fe(II) and NH_4^+ in incubation with three different Fe(III) sources: ferrihydrite (○), ferric chloride (■), and ferric citrate (▲). The values represent the mean and standard error (n=3).

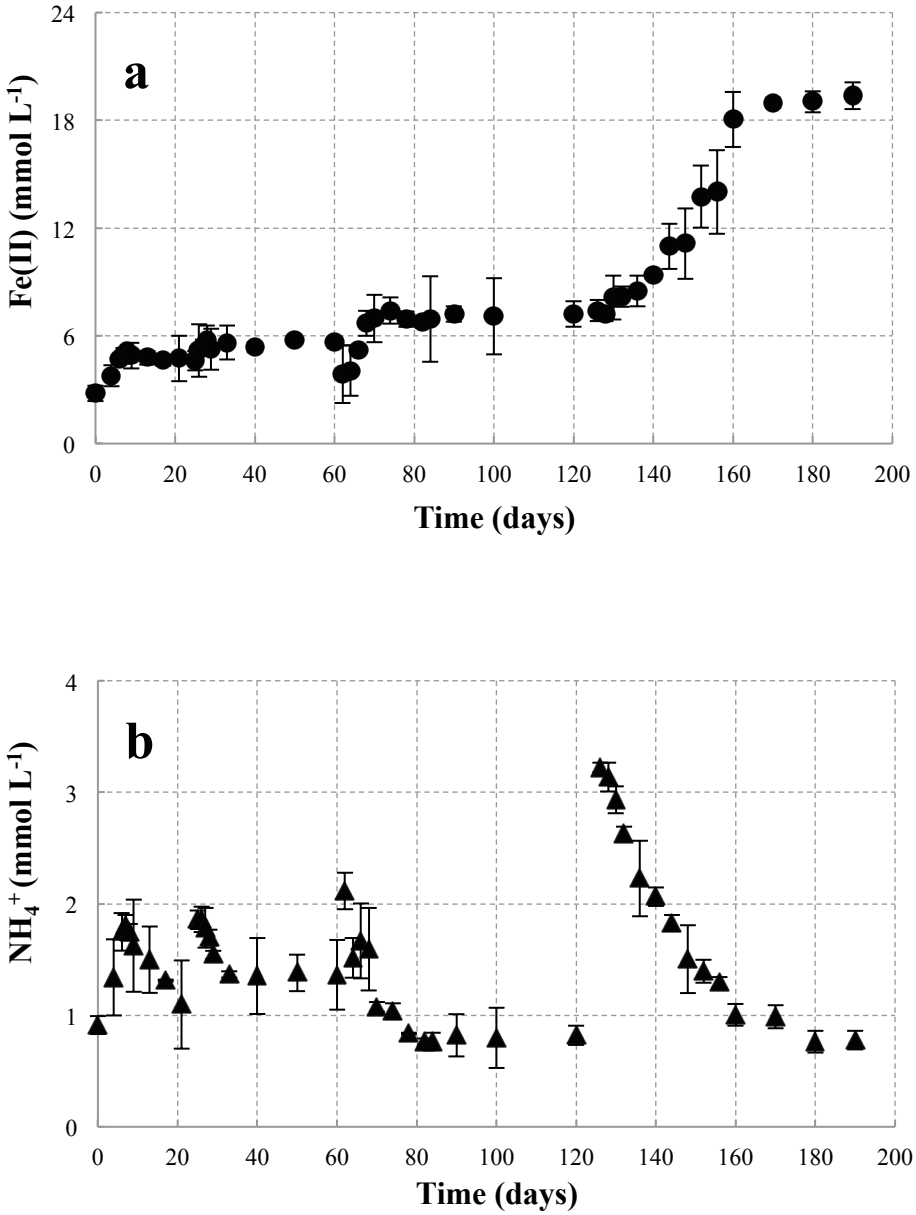

Figure 2. Concentration of (a) Fe(II) and (b) NH_4^+ during the 180 day incubation. 25 mmol L^{-1} Fe(III) was added on day 0. 1.0 mmol L^{-1} NH_4^+ was added on days 4, 24, and 60. 0.2 mmol L^{-1} $NaHCO_3$ was added on day 50 and day 90. 1.2 mmol L^{-1} + 2 mmol L^{-1} of NH_4Cl were added on day 125. The values represent the mean and standard error (n=3).

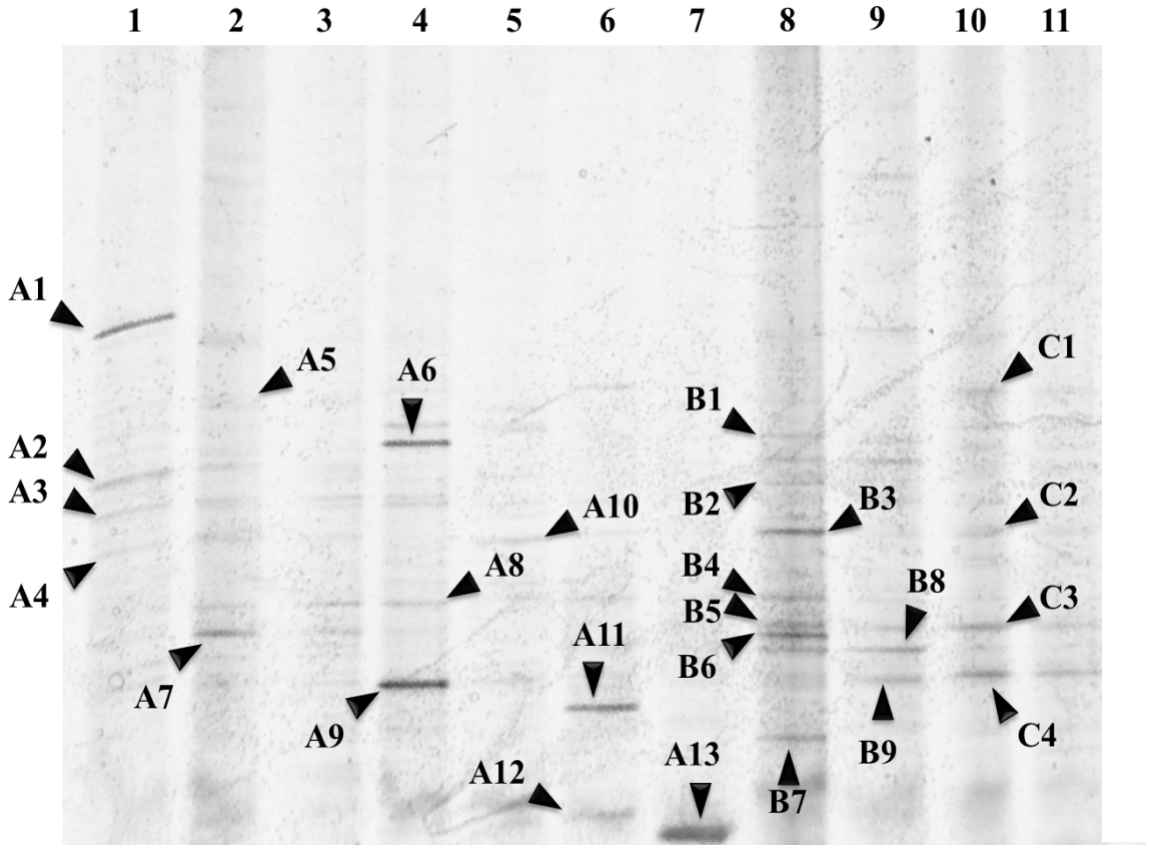

Figure 3. Comparison of DGGE analysis profiles of soil communities during anaerobic incubations. Samples from 0, 30, 90 and 160 days of incubation with ferrihydrite + NH_4Cl + $NaHCO_3$ (lane 1-4); 160 days of incubation with only ferrihydrite (lane 5); ferric chloride + NH_4Cl (lane 6); ferric citrate + NH_4Cl (lane 7); 120 days incubation with ferrihydrite + NH_4Cl + $NaHCO_3$ + organic carbon (band 8); ferrihydrite + NH_4Cl + organic carbon (lane 9). Samples from 6 and 120 days of incubation without any addition (lane 10 and 11) were use as controls.

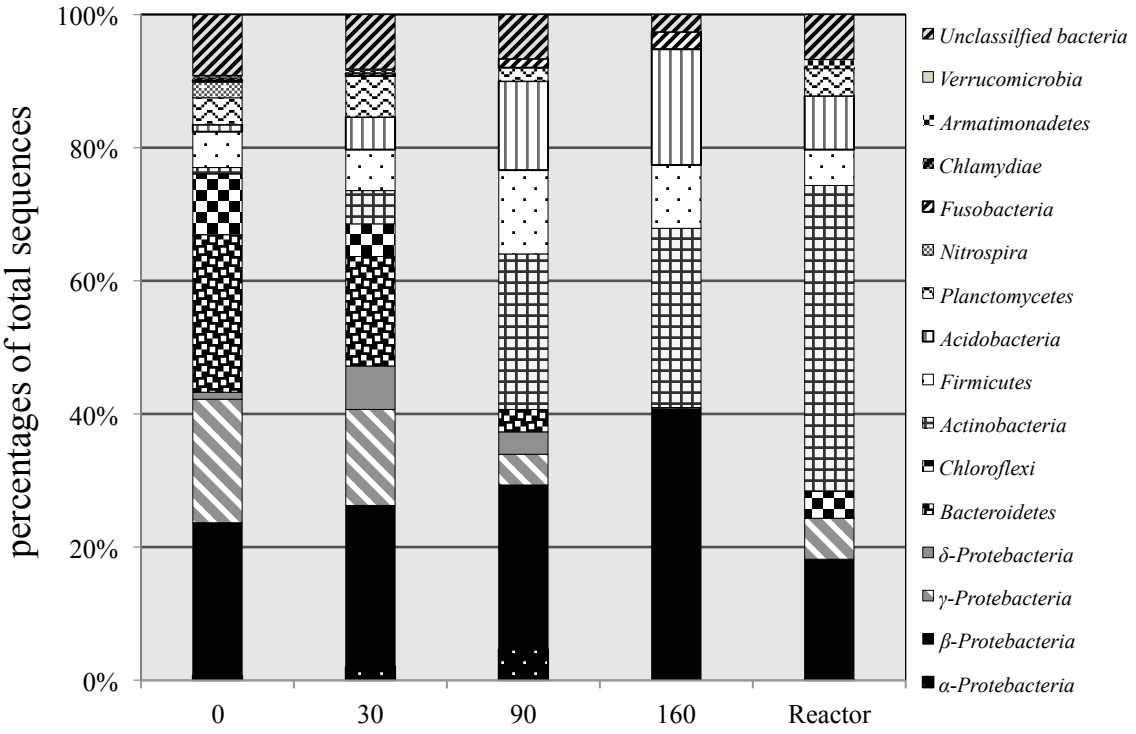

Figure 4. Relative abundance of bacterial phyla for each soil samples during anaerobic incubations (days 0, 30, 90, 160) and enrichment culture from the reactor.

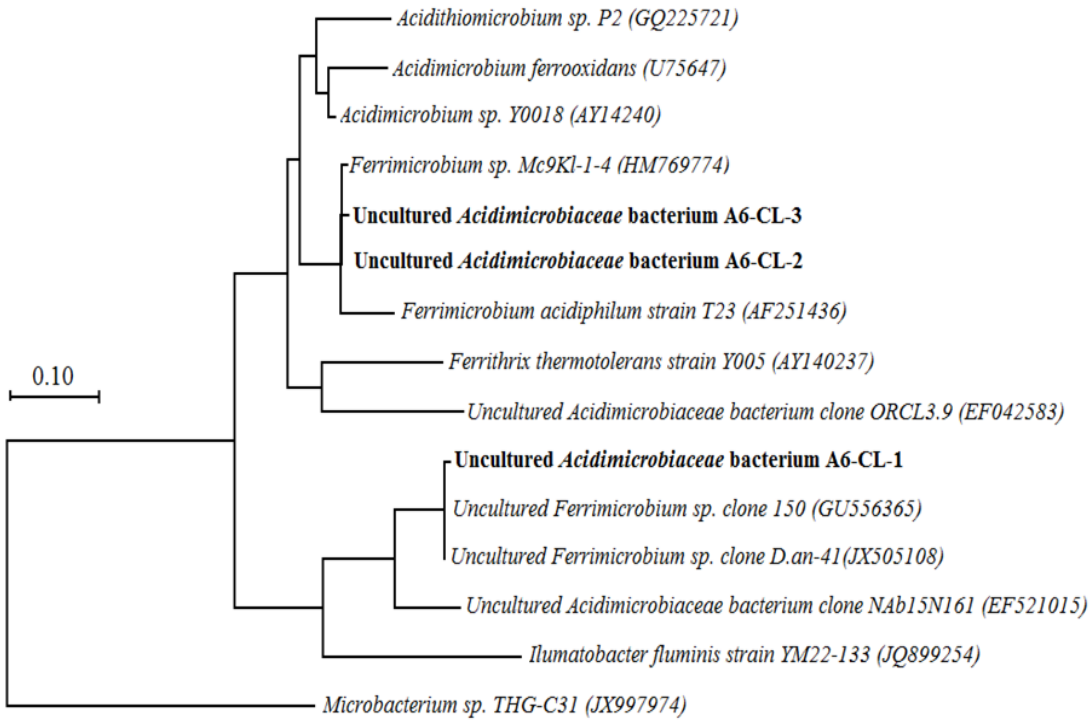

Figure 5. Phylogenetic tree of *Acidimicrobiaceae*-related sequences. The tree was constructed using the Bayesian inference (BI) method with 16S rRNA gene sequences from DGGE band A6 and bacteria from the *Acidimicrobiaceae* family from other studies. Sequences determined in this study are in bold. Bootstrap values were based on 1000 replicates each and are shown at the nodes with >50 % bootstrap support. The scale bar represents 10% sequence divergence.

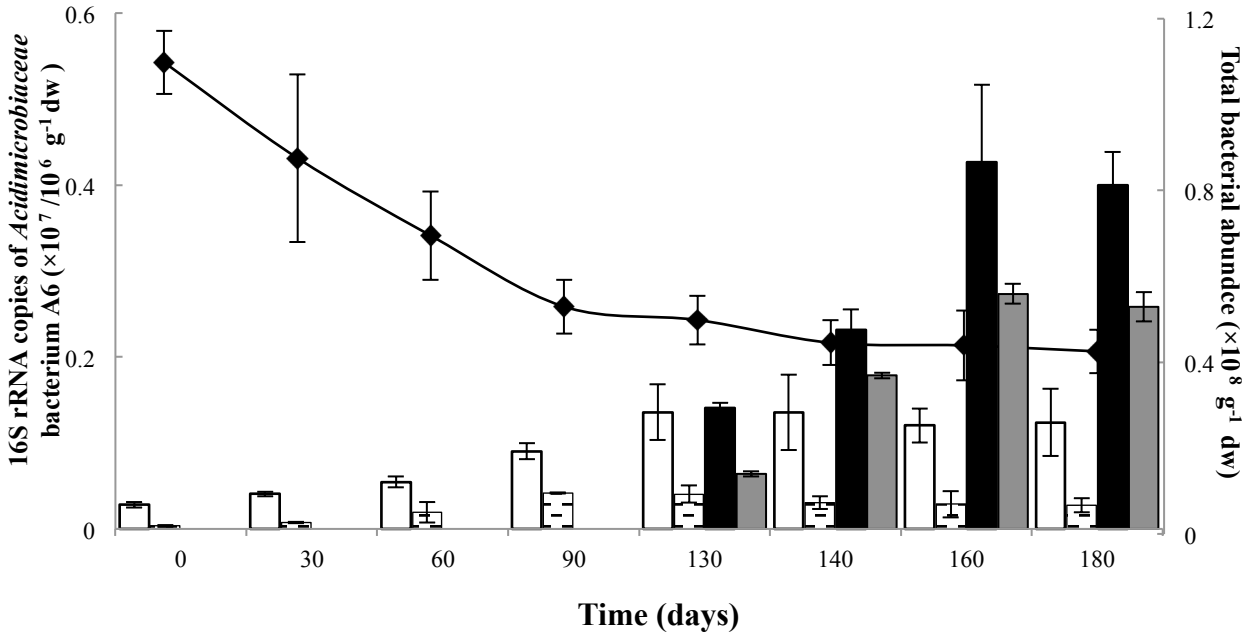

Figure 6. Abundance of total bacteria (◆) during 180 days of anaerobic incubation. 16S rRNA gene (■) and RNA (▒) copy numbers of *Acidimicrobiaceae* bacterium A6 in soil samples with 1.20 mmol L^{-1} $NaHCO_3$ addition. 16S rRNA gene (□) and RNA (▤) copy numbers of bacterium A6 with 0.20 mmol L^{-1} $NaHCO_3$ addition.

Table 1. DGGE and real time PCR primers used in this study.

Primer	Target gene	Sequence(5'-3')	Annealing temp.
V3-1	*16S rRNA*	CCT ACG GGA GGC AGC AG	56
V3-2	*16S rRNA*	ATT ACC GCG GCT GCT GG	56
V3-3	*16S rRNA*	ACG GGG GGC CTA CGG GAG GCA GCA G	56
1055f	*16S rRNA*	ATG GCT GTC GTC AGC T	57
1392r	*16S rRNA*	ACG GGG CGG TGT GTA C	57
Amx368f	*16S rRNA*	TTC GCA ATG CCC GAA AGG	56
Amx820r	*16S rRNA*	AAA ACC CCT CTA CTT AGT GCC C	56
acd320f	*16S rRNA*	CGG TCC AGA CTC CTA CGG GA	55
acd432r	*16S rRNA*	GAC AGG GTT TTA CAG TCC GAA GA	55
acm342f	*16S rRNA*	GCA ATG GGG GAA ACC CTG AC	58
acm439r	*16S rRNA*	ACC GTC AAT TTC GTC CCT GC	58
Nirs3F	*nirS*	CCT A(C/T)T GGC CGC C(A/G)C A(A/G)T	57
NirS5R	*nirS*	GCC GCC GTC (A/G)TG (A/C/G)AG GA A	57
NirK1F	*nirK*	GG(A/C) ATG GT(G/T) CC(C/G) TGG CA	56
NirK5R	*nirK*	GCC TCG ATC AG(A/G) TT(A/G) TGG	56
amoA-1F	*amoA*	GGG GTT TCT ACT GGT GGT	57
amoA-2R	*amoA*	CCC CTC KGS AAA GCC TTC TTC	57
Arch *amoA* F	*amoA*	STA ATG GTC TGG CTT AGA CG	53
Arch *amoA* R	*amoA*	GCG GCC ATC CAT CTG TAT GT	53
CrenamoA23f	*amoA*	ATG GTC TGG CTW AGA CG	55
CrenamoA616r	*amoA*	GCC ATA CAB CKR TAN GTC CA	55

Table 2. Sequence analysis of bands excised from DGGE gels of soil samples with Feammox Activity.

Phylogenetic group	Band	Related sequence	Identity (%)
Actinobacteria	A6, B1, D6	Ferrimicrobium acidiphilum strain T23 16S ribosomal RNA gene (AF251436)	92
		Acidimicrobium ferrooxidans strain TH3 16S ribosomal RNA gene (EF621760)	90
	A6	Uncultured Ferrimicrobium sp. clone D.an-41 16S ribosomal RNA gene (JX505108)	95
Acidobacteria	A8, D11	Uncultured Acidobacteria bacterium clone 3OL11 16S ribosomal RNA gene(GQ342349)	97
		Geothrix sp. culture clone AP-FeEnrich1 16S ribosomal RNA gene (JX828409)	94
BetaProteobacteria	A9,B9, C4, D14	Uncultured Rhodocyclus sp. clone W4S68 16S ribosomal RNA gene (AY691423)	97
	A9	Comamonas sp. 'ARUP UnID 223' 16S ribosomal RNA gene (JQ259419)	97