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

S. Huang and P. R. Jaffé

Department of Civil and Environmental Engineering, Princeton University, Princeton, NJ 08544, USA

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Correspondence to: P. R. Jaffé (jaffe@princeton.edu)

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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.

1 Introduction

The conventional removal of nitrogen from soil environments is mineralization (for organic nitrogen), followed by nitrification and then denitrification (Canfield et al., 2010). Saturated anoxic soils are not considered suitable for either aerobic or anaerobic ammonium (NH_4^+) oxidation because they lack dissolved oxygen or nitrite (NO_2^-) to serve as electron acceptors by aerobic or anaerobic (anammox) NH_4^+ oxidizing bacteria, respectively. 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 NO_2^-, and ferric iron [Fe(III)] is the electron acceptor, which is reduced to ferrous iron [Fe(II)]. The stoichiometry and

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change in free energy when ferrihydrite is the Fe(III) source is:

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

($\Delta G_r \leq -145.08\,kJ\,mol^{-1}$) (Eq. (1), Supplementary Information 1.1)

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

Our understanding of the Feammox process is still incomplete, particularly information about the microorganism(s) responsible for it is lacking. This makes further study into the mechanism of the Feammox process difficult. Here we focus on a series of incubations and establishing a Feammox enrichment culture to identify the microbial community responsible for the process described previously (Clement et al., 2005; Shrestha et al., 2009). Soil samples were collected from the same location and used for laboratory incubation experiments as well as to set up an enrichment system for Feammox in a continuous flow membrane reactor. Various incubation conditions [Fe(III) sources, inorganic carbon content, NH_4^+ concentration, and acetylene gas (C_2H_2) as a selected inhibitor] were used to study the Feammox mechanism. Molecular biology methods, such as denaturing gradient gel electrophoresis (DGGE), 454 pyrosequencing, and real-time quantitative PCR (qPCR) analysis were used to investigate the bacterial community change during incubations.

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2 Methods

2.1 Sample collection and processing

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

Soil samples were first incubated with different Fe(III) sources to determine which source could be used by the Feammox process: 6-line ferrihydrite ($Fe_2O_3 \cdot 0.5H_2O$) 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)

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

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

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2.3 Continuous flow membrane Feammox reactor

Soil samples collected on day 180 from the incubation with ferrihydrite, NH_4Cl, and 1.2 mmol L^{-1} $NaHCO_3$ additions were inoculated into a continuous flow membrane reactor (Abbassi et al., 2014), which was operated under anaerobic conditions by constantly purging N_2 trough the reactor's headspace at a room temperature (25 °C), and with a 48 h hydraulic retention time.

The enrichment medium contained the following components per liter: 177 mg 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 mg $MgSO_4 \cdot 7H_2O$, and 60.0 mg $CaCl_2 \cdot 2H_2O$. After autoclaving, 1 mL trace element solution (Van de Graaf et al., 1996) was added to the medium. 50.0 mmol L^{-1} ferrihydrite were added once every two weeks directly into the reactor. To aid in maintaining anaerobic conditions, 0.10–0.20 mmol L^{-1} sodium citrate was feed to the reactor about twice per month. pH was controlled at around 4 ~ 5, and dissolve oxygen was < 0.1 mg L^{-1}. Samples form the outflow were collected every two days, and sludge samples from reactor were collected and kept at −20 °C for molecular biology analysis.

2.4 Chemical analyses

For each sample collection during the incubations, a set of vials was destructively sampled in a glove box under oxygen-free conditions and the pH was measured immediately using a pH electrode. An extraction with 0.5N HCl was conducted for 24 h at room temperature to determine acid-extractable Fe(II) and NH_4^+ concentrations in the soils. Fe(II) was analyzed using the ferrozine assay method (Stookey, 1970; Komlos et al., 2007), and NH_4^+ was analyzed using a Dionex™ Ion Chromatograph (LC3000) with a CS-16 Colum and a CS-16 guard column (flow rate = 1.0 mL min^{-1}, detection limit = 0.012 ppm). NO_3^- and NO_2^- were extracted with DI water for 1 h anaerobically, and measured via Ion Chromatography, using an AS-22 Colum along with an AG-22 guard column (flow rate = 1.2 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

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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. 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 carried out at 60 V for 15 h. After that the gel was stained with 0.1 $\mu L\,mL^{-1}$ SYBR Green I and visualized with an UV transilluminator. All visible bands were excised from the gel and used as templates for re-amplification, using the primer set V3-1/V3-2 (Jensen et al., 1998) and followed by cloning. PCR products were purified via agarose gel extraction and cloned into a pGEM-T vector (Promega). A total of 10 to 30 positive recombinant clones for each band were identified by colony PCR, and were sent for sequencing to avoid erroneous interpretations. DNA sequencing was then conducted by Genewiz, Inc. Bacteria were classified and the phylogenetic tree of *Acidimicrobiaceae*-related sequences was constructed using the Bayesian inference (Huelsenbeck et al., 2001) (Supplementary Information 1.2). Sequences obtained in this study are available in the GenBank database under accession numbers KC581755–KC581779. To further con-

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firm the changes in the bacterial community, 454 pyrosequencing was performed with samples collected from the incubation on days 0, 30, 90, 160 and from the membrane reactor after 150 days of reactor operation. Domain-specific primers, targeting the V3-V5 region of the 16S rDNA of bacteria were amplified following methods suggested by Pinto et al. (2012)

2.7 Quantitative PCR (qPCR) assay

qPCR experiments were carried using a StepOnePlus™ Real-Time PCR System (Life Technologies, USA), represented by 16S rRNA genes, using primer sets 1055f/1392r for total bacteria (Harms et al., 2003), Amx368f/Amx820r for anammox bacteria (Schmid et al., 2000, 2003), acd320f (5'-CGG TCC AGA CTC CTA CGG GA -3') – 432r (5'-GAC AGG GTT TTA CAG TCC GAA GA -3') which we developed for *Acidobacteriaceae* bacteria, and acm342f (5'-GCA ATG GGG GAA ACC CTG AC -3') – 439r (5'-ACC GTC AAT TTC GTC CCT GC -3') which we developed for *Acidimicrobiaceae* bacteria (Supplementary Information 1.3). For the detection of denitrifiers and ammonia-oxidizing bacteria, denitrifying functional genes (*nirS*) and ammonia monooxygenase structural gene (*amoA*) were quantified with primer sets NirS3/NirS5 (Braker et al., 1998) and amoA-1F/amoA-2R, respectively (Rotthauwe et al., 1997). For DNA quantification, each qPCR mixture (20 µL) was composed of 10 µL of SYBR Premix Ex Taq® II (Takara, Japan), 0.8 µL 10 µM of each primer, and ~10 ng DNA template. RNA quantification was conducted through a real-time quantitative reverse transcription-PCR (RT-qPCR) analysis, by using the One Step SYBR® PrimeScript® RT-PCR Kit (Takara, Japan) according to the manufacturer's recommendations. Thermal cycling conditions for total 16S rDNA, *nirS* and *amoA* gene numbers was initiated for 30 s at 94 °C, followed by 40 cycles of 5 s at 94 °C, 30 s at 57 °C, and 30 s at 70 °C. 16S rDNA numbers of anammox, *Acidobacteriaceae* and *Acidimicrobiaceae* bacteria were performed at 56 °C, 55 °C, and 58 °C as annealing temperature respectively, with the same program. For RNA quantification, the cycling conditions were identical to those described for measuring gene numbers, with the exception that an initial incuba-

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tion 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 (Figs. 1 and 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 ΔG 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). NO_2^- appeared within a few days after the addition of NH_4^+, with a maximum concentration 0.44 ± 0.17 mmol L^{-1} in the second NH_4^+ oxidation cycle. NO_2^- did not accumulate in the system and was immediately consumed after generation (Fig. S3a). NO_3^- production showed a similar pattern to that of NO_2^- (Fig. S3a), and TN loss similar to the decrease in NH_4^+ (Fig. S3b). During

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180 days of incubation, the system experienced a loss of TN of $57.2 \pm 3.13\,mg\,L^{-1}$. The DOC content fluctuated slightly in the early stage of incubation, but overall, the DOC concentration was relatively stable at around $45 \sim 50\,mg\,L^{-1}$ (Fig. S3b).

A 64.5 % NH_4^+ removal, between inflow and outflow was achieved in the membrane reactor after 150 days of operation.

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

All visible bands observed in the DGGE analysis (significant bands were marked, see Fig. 3) were excised from the gel and sequenced after cloning. Clone libraries from 12 samples resulted in 721 sequences of partial 16S rRNA gene fragments, and six groups of bacteria were classified via a phylogenetic analysis (Tables 1 and S1). During this 180 day anaerobic incubation with ferrihydrite and NH_4Cl, the microbial communities 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).

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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} $NaHCO_3$) had a higher bacterial diversity (DGGE Fig. 3, lane 8).

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

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

3.3 Changes of bacterial abundance and activity during incubations and in the reactor

The total bacterial abundance determined via the 16S rRNA gene copy number, decreased during the 180 day incubation (Fig. 6). Both, 16S rRNA gene and rRNA frag-

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ment copies of *Acidimicrobiaceae* bacteria (DGGE band A6), increased during the incubation, particularly after 90 days. The rRNA numbers increased slowly during the first 3 months and doubled between day 130 and day 140 of the incubation period (Fig. 6). rRNA as a biomarker for changes of protein level, even though not as specific as mRNA, is a good indicator for bacterial activity (Poulsen et al., 1993; Park et al., 2010). The abundance of *Acidobacteriaceae* bacteria (DGGE band A8), and that of denitrifiers (represented by the number of *nirS* gene), increased over the 180 day incubation (Fig. S4a, b), although less than the *Acidimicrobiaceae* bacteria (DGGE band A6). Increase in the denitrifier activity was most likely stimulated by the NO_2^- generated via Feammox. The number of the *amoA* gene, representing the abundance of ammonia-oxidizing bacteria, decreased sharply with time and was hardly detected after 90 days of incubation (Fig. S4d).

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

3.4 Changes of bacterial abundance and activities with $NaHCO_3$ amendment

Abundance and activity of *Acidimicrobiaceae* bacteria, represented by band A6, were compared between samples incubated under the same conditions except the amounts of $NaHCO_3$ added (0.20 mmol L^{-1} vs. 1.20 mmol L^{-1}). From day 125 to day 180 of the incubation, both 16S rRNA gene and rRNA fragment numbers of *Acidimicrobiaceae* bacteria were higher in the soils with the higher inorganic carbon content. The 16S rRNA gene copies of samples augmented with 1.20 mmol L^{-1} $NaHCO_3$ were four times higher than those in samples that had been augmented with only 0.20 mmol L^{-1} $NaHCO_3$ (Fig. 6). The rRNA copies of the*Acidimicrobiaceae* bacteria, showed even larger differences in response to the amounts of $NaHCO_3$ added. In the samples augmented with 1.20 mmol L^{-1} $NaHCO_3$, the rRNA copy number increased from $0.04 \pm 0.06 \times 10^6$ to $0.19 \pm 0.09 \times 10^6$ copies g^{-1} dw over 50 days of incubation

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(day 130 to day 180). However, in the samples to which only 0.2 mmol L^{-1} $NaHCO_3$ were added on day 125, the rRNA number gradually deceased from $0.29 \pm 0.10 \times 10^5$ to $0.19 \pm 0.05 \times 10^5$ copies g^{-1} dw during the same 50 days incubation (Fig. 6).

3.5 Nitrogen species changes in samples incubated in the presence of C_2H_2

C_2H_2 can inhibit the oxidation of NH_4^+ to NO_2^- under aerobic conditions, and the reduction of N_2O to N_2 as well as the anammox pathway under anaerobic conditions (Yoshinari et al., 1977; Jensen et al., 2007; Kartal et al., 2011). To gain further insights into the nitrogen removal process observed, incubations with C_2H_2 were conducted. After 20 days of incubation, less NH_4^+ was oxidized in the samples amended with C_2H_2, compared to those incubated without C_2H_2 (Fig. S5a). NO_2^-, which is postulated to be the direct product of the NH_4^+ oxidation, accumulated slowly in the samples incubated with C_2H_2 (Fig. S5b). NO_3^- reached a higher concentration in samples without C_2H_2 than in samples incubated with C_2H_2 (Fig. S5c). N_2O, a product of NO_2^- reduction, accumulated in the samples incubated with C_2H_2, which inhibits the reduction of N_2O to N_2 (Fig. S5d). Fe(II) production was not much affected by the presence of C_2H_2, and after 20 days incubation was 4.36 ± 0.72 and 5.71 ± 0.67 mmol L^{-1} in sample incubated with and without C_2H_2, respectively.

4 Discussion

DGGE band A5 represents dissimilatory iron-reducing bacteria, which appeared for a short time at the beginning of the anaerobic incubation. For longer incubation times these heterotrophic bacteria decreased rapidly (Fig. 3). Over a 25 day incubation period, more than three times the mass of Fe(II) was produced in samples amended with ferrihydrite and NH_4^+ as compared to the samples amended only with ferrihydrite, indicating that most of the Fe(III) reduction came from the Feammox reaction, and not from dissimilatory Fe(III) reduction (Fig. 1a vs. Fig. S2a).

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Ammonia-oxidizing bacteria represented by DGGE band A7 (Fig. 3), as well as the *amoA* gene, decreased after 30 days of incubation (Fig. S4d). Oxygen deficiency was the most likely reason for the decline in ammonia-oxidizing bacteria in this system over time (Laanbroek et al., 1994). This decrease in *amoA* gene at a time of increasing NH_4^+ oxidation indicates that aerobic ammonia-oxidizing bacteria were not the drivers of the NH_4^+ oxidation in the later incubation times.

An uncultured *Acidimicrobiaceae* bacterium became the dominant species during the 180 day anaerobic incubation period, increasing from 0.92 % on day 0 in terms of cell numbers to 14.8 % on day 160. This *Acidimicrobiaceae* bacterium was only detected when $NaHCO_3$ was supplied as a carbon source, NH_4^+ as an electron donor, and ferrihydrite as electron acceptor (Fig. 3). The abundance and activity of this *Acidimicrobiaceae* bacterium increased along with the Feammox activity during the incubations. During the incubation period its rRNA changed from $(0.22 \pm 0.01) \times 10^5$ copies g^{-1} dw to $(0.28 \pm 0.07) \times 10^6$ copies g^{-1} dw, 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 (Fig. 4). These results indicate that this*Acidimicrobiaceae* bacterium may 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

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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), as well as its activity increase with increased Feammox activity, 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 (Figs. 2 and 6), which indicates that this *Acidimicrobiaceae* bacterium is an autotroph. Growth of *nirS* gene indicated that denitrification pathways were also active in the incubations described here. NO_2^- that was being produced during the anaerobic NH_4^+ oxidation was reduced to N_2 by denitrifiers, and NO_2^- did not accumulate in the system.

The Feammox reaction studied here proceeded only when iron oxides (ferrihydrite or goethite) were supplied as electron acceptor, whereas samples incubated with ferric chloride or ferric citrate as the Fe(III) source showed no measurable NH_4^+ oxidation (Figs. 1 and S1). Since iron oxides adsorb dissolved Fe(II) that is generated via the Feammox reaction, the concentrations of Fe(II) in solution were below the detection limit through the incubation. The acidic conditions of the incubations, lack of dissolved Fe(II) buildup due to sorption, and rapid removal of NO_2^- via denitrification makes the Feammox reaction, as shown in Eq. (1), energetically favorable. Various NH_4^+ oxidation products, i.e. NO_3^-, NO_2^- and N_2, generated through the Feammox process are thermodynamically feasible, and were reported in different Feammox studies (Sawayama, 2006; Shrestha et al., 2009; Yang et al., 2012). In our incubations amended with C_2H_2, the product of NH_4^+ oxidation through Feammox was NO_2^-. When C_2H_2 was used to stop the reduction of N_2O to N_2, the total N_2O ($0.72 \pm 0.23\,mmol\,L^{-1}$) plus NO_2^- produced ($0.13 \pm 0.07\,mmol\,L^{-1}$) was equal to the NH_4^+ consumed, showing that NH_4^+ was not oxidized directly to N_2 in our samples, and that NO_2^- is a major product of the NH_4^+ oxidation via Feammox. Although nitrification might happen in suboxic environments

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(oxygen < 5 µM, Lam et al., 2007), NH_4^+ oxidation in the presence of C_2H_2 has never been reported. C_2H_2 is an inhibitor of ammonia monooxygenase (AMO), and can restrain aerobic NH_4^+ oxidizers from using oxygen by binding covalently to AMO (Hynes and Knowles, 1982; Hyman and Wood, 1985; Gilch et al., 2009). C_2H_2 can also inhibit the NH_4^+-activation step of anammox cells, which use NO_2^- as the oxidant (Kartal et al., 2011). Therefore these Feammox bacteria might different from common NH_4^+ oxidizers, by using an alternative NH_4^+ oxidation pathway that is not inhibited by C_2H_2, and AMO might not play a role in Feammox.

The role of anammox during the incubations was also evaluated. During the incubations the ratio of NH_4^+ oxidized to Fe(III) reduced increased gradually from 1 : 1.9 to 1 : 5.3, which is close to the stoichiometry of 1 : 6, shown in Eq. (1). This indicates that the Feammox reaction became more dominant in terms of NH_4^+ oxidation after 125 days of incubation due to a relative increase in the activity of the Feammox bacteria. A parallel pathway to Feammox, such as anammox, could explain the lower stoichiometric ratio, especially at earlier incubation times. In the samples taken before the incubation, $0.17 \pm 0.05 \times 10^6$ copies g^{-1} dw of anammox rRNA gene were found, which decreased to $0.09 \pm 0.06 \times 10^5$ on day 130 (Fig. S4c). We postulate that anammox was responsible for some initial NH_4^+ and NO_2^- removal, and denitrification became more dominant for NO_2^- removal later during the incubation period (Figs. 5 and S4b, c).

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

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Table 1. 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

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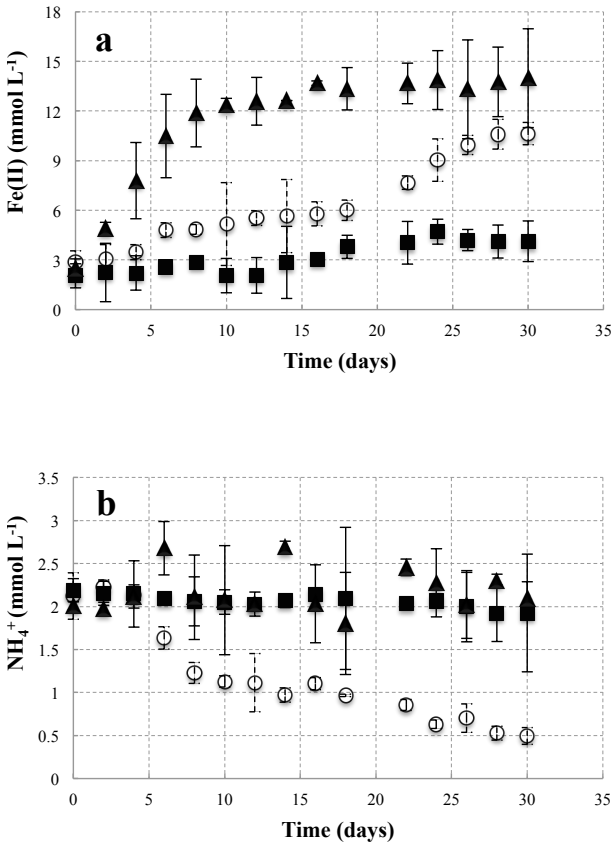

Figure 1. Concentration of Fe(II) and NH_4^+ in incubation with three different Fe(III) sources: ferrihydrite (◯), ferric chloride (■), and ferric citrate (▲). The values represent the mean and standard error ($n = 3$).

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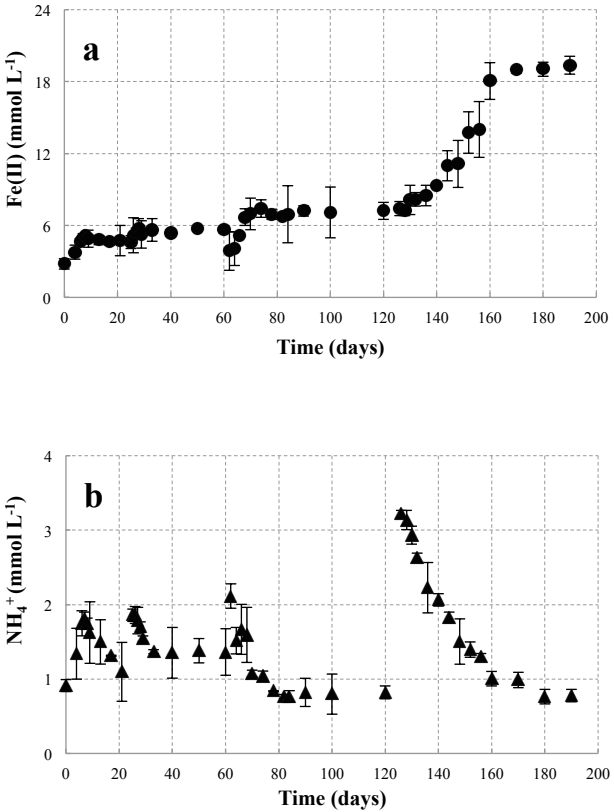

Figure 2. Concentration of **(a)** Fe(II) and **(b)** NH_4^+ during the 180 day incubation. 25 mmol L^{-1} Fe(III) was added on day 0. 1.0 mmol L^{-1} NH_4^+ was added on days 4, 24, and 60. 0.2 mmol L^{-1} $NaHCO_3$ was added on day 50 and day 90. 1.2 mmol L^{-1} + 2 mmol L^{-1} of NH_4Cl were added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

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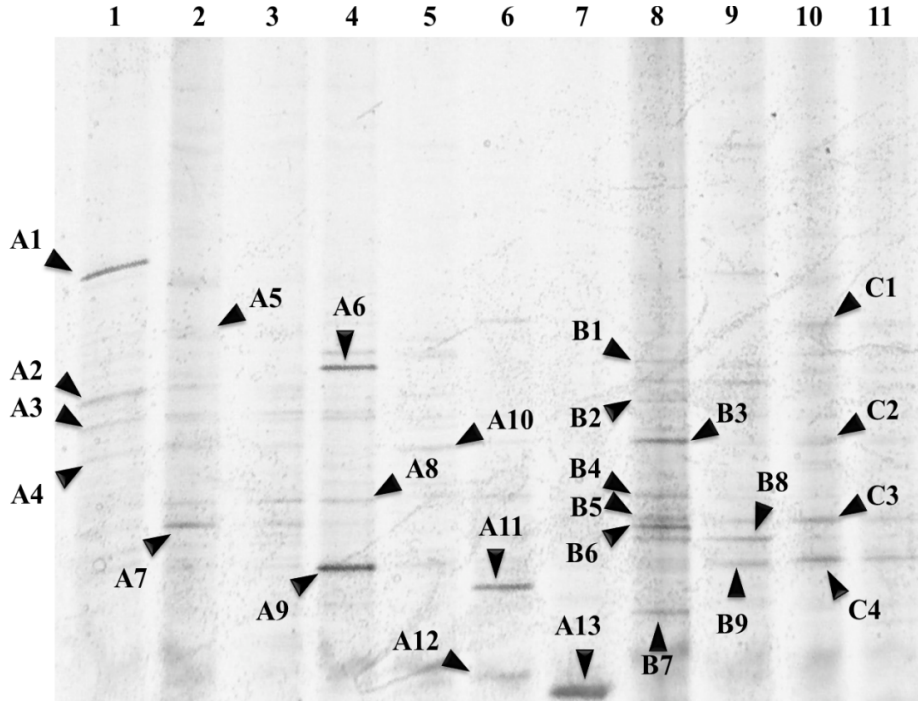

Figure 3. Comparison of DGGE analysis profiles of soil communities during anaerobic incubations. Samples from 0, 30, 90 and 160 days of incubation with ferrihydrite + NH_4Cl + $NaHCO_3$ (lane 1–4); 160 days of incubation with only ferrihydrite (lane 5); ferric chloride + NH_4Cl (lane 6); ferric citrate + 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.

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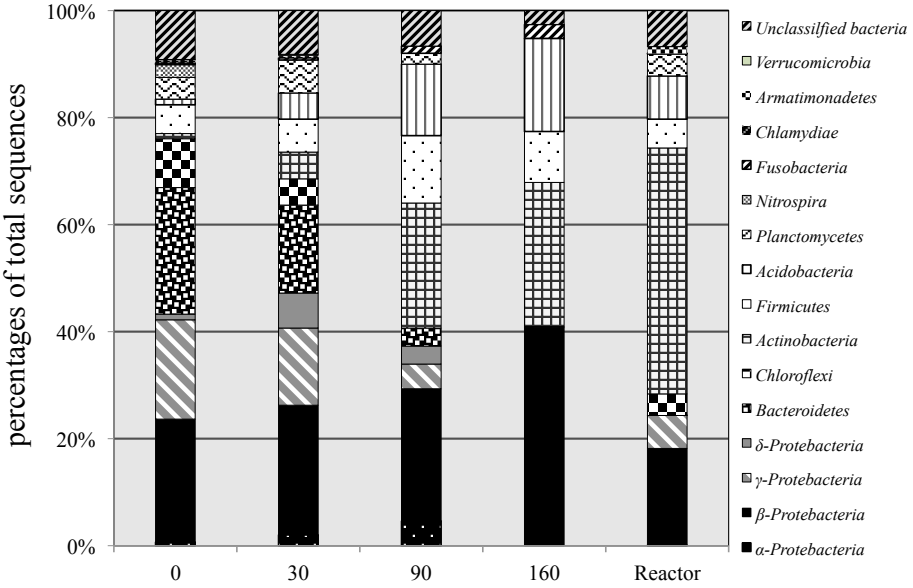

Figure 4. Relative abundance of bacterial phyla for each soil samples during anaerobic incubations (days 0, 30, 90, 160) and enrichment culture from the reactor.

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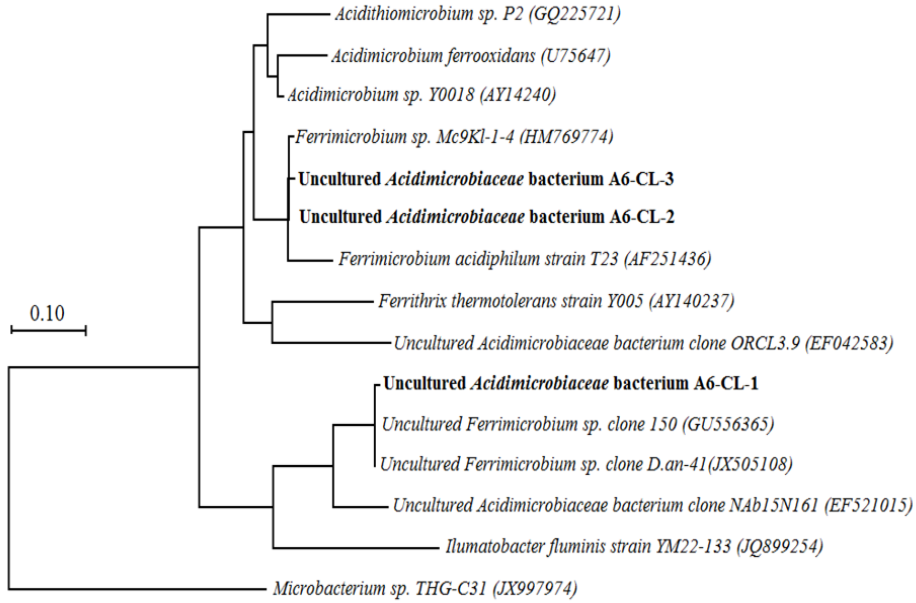

Figure 5. Phylogenetic tree of *Acidimicrobiaceae*-related sequences. The tree was constructed using the Bayesian inference (BI) method with 16S rRNA gene sequences from DGGE band A6 and bacteria from the *Acidimicrobiaceae* family from other studies. Sequences determined in this study are in bold. Bootstrap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

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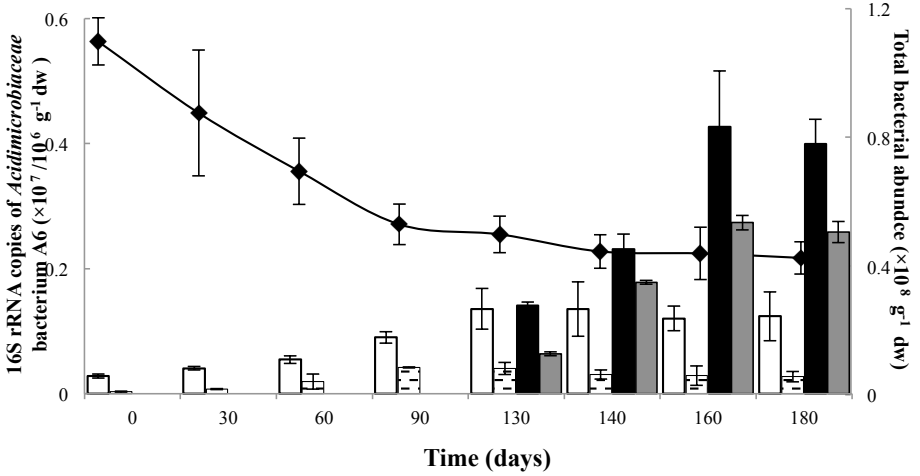

Figure 6. Abundance of total bacteria (◆) during 180 days of anaerobic incubation. 16S rRNA gene (■) and RNA (■) copy numbers of *Acidimicrobiaceae* bacterium A6 in soil samples with 1.20 mmol L^{-1} $NaHCO_3$ addition. 16S rRNA gene (□) and RNA (□) copy numbers of bacterium A6 with 0.20 mmol L^{-1}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

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