Biogeosciences

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 was 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 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 and organic carbon rich sediments or soils, such as wetland sediments, there is little oxygen for significant nitrification by aerobic ammonium (NH_4^+) oxidation bacteria/archaea (AOB/AOA). 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:

$$3Fe_2O_3 \bullet 0.5H_2O + 10H^+ + NH_4^+ \rightarrow 6Fe^{2+} \tag{1}$$

$$+8.5H_2O + NO_2^- \left(\Delta G_r \leq -145.08 \text{ kJ mol}^{-1}, \text{ Supplement 1.1} \right)$$

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 NO_3^- (Sawayama, 2006), or directly to N_2 (Yang et al., 2012), using Fe(III) as an 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, $^{15}NH_4^+$, 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.

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 oxides were detected. The detailed physicochemical characteristics 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 much of the labile organic carbon. After 30 days of aeration, 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 to 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

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 ($Fe_2O_3C \cdot 0.5H_2O$) 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 and ~ 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 2 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 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 or 0.20 mmol L^{-1} of NaHCO$_3$ plus 2 mmol L^{-1} of NH$_4$Cl 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} or NaHCO$_3$. For this incubation, samples were collected every 4 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 the 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. The Fe(III) concentration at the beginning of the incubations was 25 mmol L^{-1}. One mmol L^{-1} NH$_4$Cl and 0.20 mmol L^{-1} NaHCO$_3$ were 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 final 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 2 days for Fe and N species.

2.3 Continuous flow membrane Feammox reactor

Soil samples collected on day 180 from the incubation with ferrihydrite, NH$_4$Cl, and 1.20 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 through the reactor's headspace at room temperature (25°), and with a 48 h hydraulic retention time.

The enrichment medium contained the following components per liter: 177 mg NH$_4$Cl, 77.9 mg (NH$_4$)$_2$SO$_4$, 19.8 mg NaHCO$_3$, 71.0 mg KHCO$_3$, 9.00 mg KH$_2$PO$_4$, 100 mg MgSO$_4 \times$ 7H$_2$O, and 60.0 mg CaCl$_2 \times$ 2H$_2$O. After autoclaving, 1 mL trace element solution (Van de Graaf

et al., 1996) was added to the medium. $50.0\,\mathrm{mmol\,L^{-1}}$ ferrihydrite were added once every 2 weeks directly into the reactor. To aid in maintaining anaerobic conditions, 0.10–$0.20\,\mathrm{mmol\,L^{-1}}$ sodium citrate were fed to the reactor about twice per month. pH was controlled at around $4\sim5$, and dissolved oxygen was less than $0.10\,\mathrm{mg\,L^{-1}}$. Samples from the outflow were collected every 2 days, and sludge samples from the reactor were collected and kept at $-20\,^\circ\mathrm{C}$ for molecular biology analysis.

Finally, ^{15}N isotope tracer incubations were conducted using slurries collected form the stable Feammox membrane reactor. Five treatments ($n = 3$ per treatment) were conducted: (1) control with only anoxic deionized (DI) water; (2) ^{15}NH$_4$Cl addition; (3) ^{15}NH$_4$Cl + Fe(III) addition; (4) ^{15}NH$_4$Cl and C$_2$H$_2$ addition; and (5) ^{15}NH$_4$Cl, C$_2$H$_2$, and Fe(III) addition. The headspace gas of each 50 mL incubation vial was sampled every 24 h for ^{15}N$_2$O analysis (Supplement 1.4).

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). Extraction efficiency of Fe(II) was affected by the HCl concentration and the extraction time. About 5–10 % more Fe(II) could be extracted with either 1N HCl extraction over 24 h or with 0.5 N HCl over 36 h as opposed to 0.5 N HCl over 24 h. Furthermore, after more Fe(II) was produced in the system with increasing incubation time, the Fe(II) extraction efficiency improved. Only a 1–2 % difference was observed in the Fe(II) extracted over 24 h using 0.5 N vs. 1 N HCl towards the end of the incubation period. Clays, present in the soil incubations, typically sorb Fe(II) more efficiently when the total Fe(II) is low; furthermore, ferrihydrite is slowly converted to magnetite, resulting in relatively different associations with different phases of the Fe(II) over the duration of the incubation. All of which leads to incomplete Fe(II) extractions, especially when the Fe(II) is low. Here we report Fe(II) data obtained via 0.5 N HCl extractions over 24 h to ensure that the methods and hence data are comparable to those reported by other researchers focusing on iron reduction and iron bioavailability.

NH$_4^+$ was analyzed using a Dionex™ ion chromatograph (LC3000) with a CS-16 column and a CS-16 guard column (flow rate $= 1.00\,\mathrm{mL\,min^{-1}}$, detection limit $= 0.012\,\mathrm{ppm}$). NO$_3^-$ and NO$_2^-$ were extracted with DI water for 1 h anaerobically, and measured via ion chromatography, using an AS-22 column along with an AG-22 guard column (flow rate $= 1.20\,\mathrm{mL\,min^{-1}}$, detection limit $= 0.016\,\mathrm{ppm}$). For the total organic carbon (TOC) and total nitrogen (TN) analy-

ses, a Shimadzu TOC-5000(A) was used. N$_2$O concentrations were determined on a Shimadzu 2014 gas chromatograph 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 (Table 1). 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 $0.1\,\mathrm{\mu L\,mL^{-1}}$ 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 primer set V3-1/V3-2 (Jensen et al., 1998) and followed by cloning (Table 1). 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) (Supplement 1.2). Sequences obtained in this study are available in the GenBank database under accession numbers KC581755–KC581779. To confirm the changes in the bacterial community further, 454 pyrosequencing was performed with samples collected from the incubation on days 0, 30, 90, and 160, and from the membrane reactor after 150 days of reactor operation. Domain-specific primers Bact-338F1/909R, targeting the V3–V5 region of the 16S rDNA of bacteria, were amplified and sequenced following methods suggested by Pinto et al. (2012) (Supplement 1.2).

2.7   Quantitative PCR (qPCR) assay

qPCR experiments were carried out 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; Schmid et al., 2003), acd320f–432r, which we developed for

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

Acidobacteriaceae bacteria, and acm342f–439r, which we developed for *Acidimicrobiaceae* bacteria (Supplement 1.3) (Table 1). For the detection of denitrifiers, AOB and AOA, denitrifying functional genes (*nirS and nirk*), ammonia monooxygenase structural genes (*amoA*) were quantified with primer sets NirS3F/NirS5R, NirK1F/NirK5R (Braker et al., 1998) and amoA-1F/amoA-2R (Rotthauwe et al., 1997) for AOB-*amoA* and Arch *amoA*-F/*amoA*-R for AOA-*amoA* (Francis et al., 2005), respectively. Primer pairs Cren-amoA23f/616r were also used to quantify the thaumarchaeal *amoA* genes that represented acidophilic ammonia oxidizers (Tourna et al., 2008). 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 AOB-*amoA* gene numbers were 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, 55 and 58 °C as annealing temperatures, respectively, with the same program. For the AOB-*amoA* gene and the thaumarchaeal *amoA* genes, the annealing temperatures were 53 and 55 °C, and the annealing time was adjusted to 45 s. 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 Supplement 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 in 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 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 the 180-day incubation, the ratio of Fe(II) produced to NH_4^+ removed gradually increased until it reached 5.3 : 1 by day 160, after which it remained stable (Fig. S6).
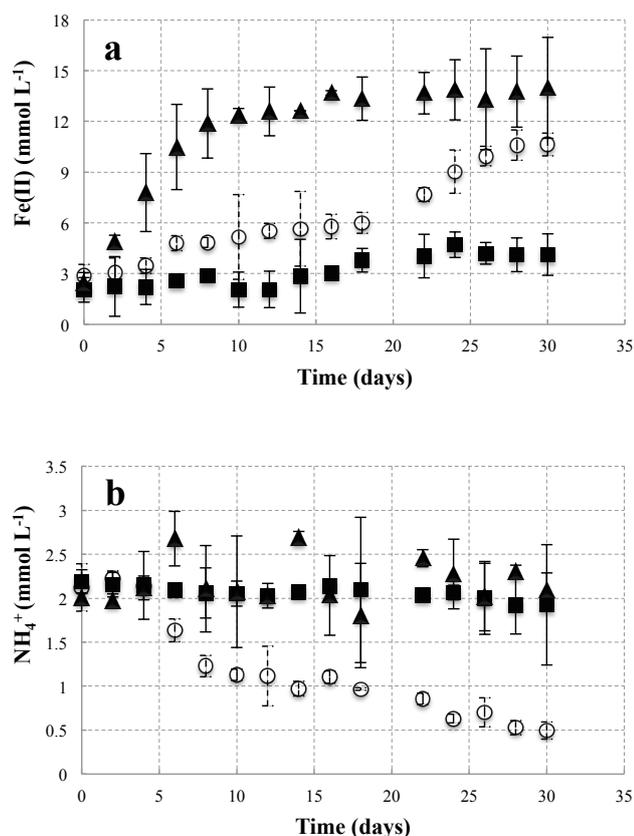
Figure 1. Concentration of Fe(II) and NH_4^+ in incubation with three different Fe(III) sources: ferrihydrite (∘), ferric chloride (■), and ferric citrate (▲). The values represent the mean and standard error ($n = 3$).

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 ($n = 3$).

NO_2^- appeared within a few days after the addition of NH_4^+, with a maximum concentration of $0.44 \pm 0.17\,mmol\,L^{-1}$ 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 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 the 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 sam-

ples resulted in 721 sequences of partial 16S rRNA gene fragments, and six groups of bacteria were classified via a phylogenetic analysis (Table 1 and Supplement Table 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, lanes 1–4). Some DGGE bands disappeared gradually with time, such as band A5 and band A7. Band A5 represents a dissimilatory iron-reducing bacterium, *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
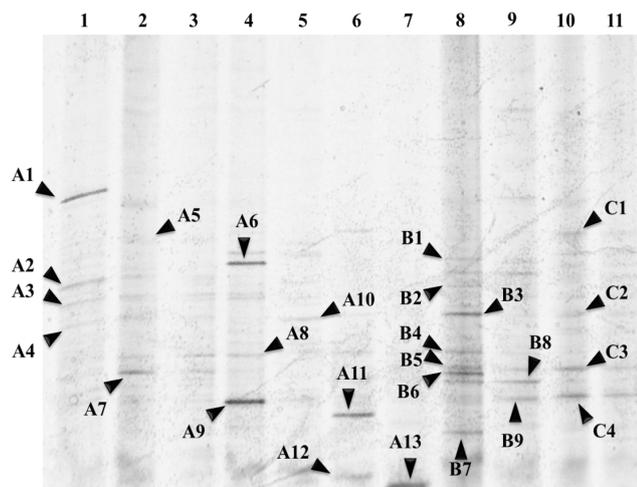
Figure 3. Comparison of DGGE analysis profiles of soil communities during anaerobic incubations. Samples from 0, 30, 90 and 160 days of incubation with ferrihydrite + NH_4Cl + $NaHCO_3$ (lanes 1–4); 160 days of incubation with only ferrihydrite (lane 5); ferric chloride + NH_4Cl (lane 6); ferric citrate + 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 (lanes 10 and 11) were used as controls.

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

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, lanes 5–7). Samples supplied with both organic carbon ($1.00\,\text{mmol}\,\text{L}^{-1}$ sodium citrate) and inorganic carbon ($1.20\,\text{mmol}\,\text{L}^{-1}$ $NaHCO_3$) 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 bands A6, A8 and A9 belong to) was consistent with the DGGE results, where the *Actinobacteria* cell number increased the most (Fig. 4). The *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 enrich-

ment 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 % of total 16S rRNA gene sequences) as well as in the membrane reactor after 150 days of operation (40.2 % of total 16S rRNA gene sequences). Its similarity to other *Acidimicrobiaceae*-related sequences is shown using a phylogenetic tree (Fig. 5). Unlike the bacteria represented by bands A8 and A9, which were also found in samples that did not show Feammox transformations, this *Acidimicrobiaceae* bacterium was only detected in incubations (or reactors) that were augmented simultaneously with ferrihydrite, NH_4Cl, and $NaHCO_3$, and have shown Feammox activity (Fig. 3).

3.3 Changes in 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 fragment 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 in protein level, even though not as specific as mRNA, is a good indicator of 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 copies of the *nirS* gene and the *nirK* gene), increased over the 180-day incubation (Fig. S4a, b, c), although their growth over that period was less than that of the *Acidimicrobiaceae* bacteria (DGGE band A6). An increase in copies of the *nirS* and *nirK* genes showed similar trends, although the number
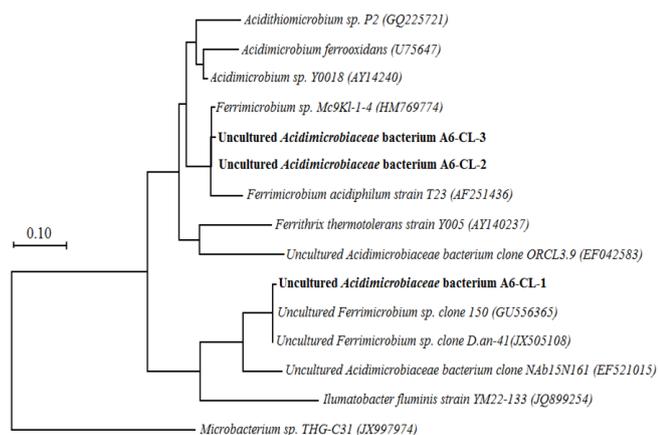
Figure 5. Phylogenetic tree of *Acidimicrobiaceae*-related sequences. The tree was constructed using the Bayesian inference (BI) method with 16S rRNA gene sequences from DGGE band A6 and bacteria from the *Acidimicrobiaceae* family from other studies. Sequences determined in this study are in bold. Bootstrap values were based on 1000 replicates each and are shown at the nodes with less than 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 $1.20\,\mathrm{mmol\,L^{-1}}$ NaHCO$_3$ addition. 16S rRNA gene (□) and RNA (⊠) copy numbers of bacterium A6 with $0.20\,\mathrm{mmol\,L^{-1}}$ NaHCO$_3$ addition.

of *nirK* gene copies was 2 orders of magnitude lower than that of *nirS* (Fig. S4b, c). An 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. S4e). Through quantification of thaumarchaeal *amoA* genes, none of the acidophilic ammonia oxidizers was detected in our system.

In the Feammox reactor, the copy numbers of *Acidimicrobiaceae* bacterium A6, Anammox bacteria and the *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 in 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 for the amounts of NaHCO$_3$ added ($0.20\,\mathrm{mmol\,L^{-1}}$ vs. $1.20\,\mathrm{mmol\,L^{-1}}$). 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 $1.20\,\mathrm{mmol\,L^{-1}}$ NaHCO$_3$ were four times higher than those in samples that had been augmented with only $0.20\,\mathrm{mmol\,L^{-1}}$ 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 $1.20\,\mathrm{mmol\,L^{-1}}$ 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 (day 130 to day 180). However, in the samples to which only $0.2\,\mathrm{mmol\,L^{-1}}$ 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-day incubation (Fig. 6).

3.5 Nitrogen species changes in samples incubated in the presence of C$_2$H$_2$

C$_2$H$_2$ 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$_2$H$_2$ were conducted. After 20 days of incubation, less NH_4^+ was oxidized in the samples amended with C$_2$H$_2$, compared to those incubated without C$_2$H$_2$ (Fig. S5a). NO_2^-, which is postulated to be the direct product of the NH_4^+ oxidation, accumulated slowly in samples incubated with C$_2$H$_2$ (Fig. S5b). NO_3^- reached a higher concentration in samples without C$_2$H$_2$ than in samples incubated with C$_2$H$_2$ (Fig. S5c). N_2O, a product of NO_2^- reduction, accumulated in samples incubated with C$_2$H$_2$, which inhibits the reduction of N_2O to N_2 (Fig. S5d). Fe(II) production was not much affected by the presence of C$_2$H$_2$, and after 20 days incubation was 4.36 ± 0.72 and $5.71 \pm 0.67\,\mathrm{mmol\,L^{-1}}$ in sample incubated with and without C$_2$H$_2$, respectively.

In the ^{15}N isotope tracer incubations, detectable ^{15}N–N$_2$O was only found in samples amended with both, ^{15}NH$_4$Cl and Fe(III), with ^{15}N–N$_2$O production rates 2.14 ± 0.059 or $0.072 \pm 0.023\,\mathrm{\mu g\,g^{-1}\,d^{-1}}$ in samples incubated with or without C$_2$H$_2$ treatment (Table S2).

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).

AOB represented by DGGE band A7 (Fig. 3), as well as the *amoA* gene, decreased after 30 days of incubation (Fig. S4e). Also, no AOA or acidophilic ammonia oxidizers were detected, although the pH condition seems suitable for them in these incubations. Oxygen deficiency was the most likely reason for the decline in AOB in this system over time (Laanbroek et al., 1994). Even though a small amount of AOB would be enough for NH_4^+ oxidation, in our control samples to which no Fe(III) was added, which AOB do not require, no NH_4^+ consumption was detected (Fig. S2). Moreover, the decrease in the *amoA* gene at a time of increasing NH_4^+ oxidation also indicates that neither AOB nor acidophilic ammonia oxidizers were 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. In the incubation experiments conducted (which included controls with only NH_4^+, only iron, autoclaved, and various Fe(III) sources), this *Acidimicrobiaceae* bacterium was only detected and growing in samples to which NH_4^+ was supplied as an electron donor, ferrihydrite was supplied as electron acceptor, and $NaHCO_3$ was supplied as a carbon source (Figs. 1, 3, S1, S2). 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, 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 with 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 (Figs. 2 and 6), which, in addition to the fact that ΔG in Eq. (1) is negative, indicates that if this *Acidimicrobiaceae* bacterium is actually responsible for conducting the Feammox reaction as depicted in Eq. (1), it may be an autotroph. Growth of the *nirS* gene suggested 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). In the incubations to which ferric citrate was added as the Fe(III) source, Fe(III) was reduced rapidly by dissimilatory iron reducers, using organic carbon as electron donor. The DGGE results for incubations with ferric citrate (Fig. 3, lane 7) show that the most dominant species was an *Actinobacterium* (Table S1), known to reduce iron under anaerobic conditions (Lin et al., 2007; Lentini et al., 2012). *Acidimicrobiaceae* bacterium A6 was not detected in these incubations. Since acidic conditions as well as minimal dissolved Fe(II) and NO_2^- concentrations are required to make the Feammox reaction energetically favorable as shown in Eq. (1), the presence of iron oxides as the main Fe(III) source may have helped to maintain the concentrations of Fe(II) in solution below the detection limit through the incubation since iron oxides can sorb Fe(II) and/or incorporate it into their structure.

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). Because there was no initial nitrate or nitrite in the system, because all experiments were conducted under strict oxygen-free conditions, and because of the rapid decrease in *amoA* genes, neither NO_3^- reduction nor aerobic NH_4^+ oxidation could be the reason for the formation of NO_2^- during the incubations. In all incubations where NH_4^+ was removed, the production of NO_2^- was observed, although NO_2^- did not accumulate. Most of the NO_2^- produced from the NH_4^+ oxidation was reduced rapidly by denitrifiers, which were present in the incubations.

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

Although nitrification might happen in suboxic environments (oxygen $< 5\,\mu M$, 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 differ 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 fact that NH_4^+ oxidation was not affected by the presence of acetylene is a further indication that AOB are not responsible for this process since they would be affected by acetylene. Furthermore, in the isotope tracer incubations, ^{15}N–N_2O was below the detection limit in samples to which Fe(III) was not supplied, showing again that NH_4^+ oxidation proceeded only when iron was being reduced.

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 $1:5.3$ (Fig. S6), which is close to the stoichiometry of $1:6$, shown in Eq. (1). Although the discrepancies in the Feammox stoichiometry between iron and NH_4^+ are attributed in part to incompletely Fe(II) extraction, the influence of anammox activity in the earlier incubations would have contributed to a lower Fe(II) produced to NH_4^+ removed ratio than the theoretical value of $1:6$.

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,

as mentioned above, 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 the anammox rRNA gene were found, which decreased to $0.09 \pm 0.06 \times 10^5$ on day 130 (Fig. S4d). 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 (Fig. 5 and Fig. S4b, d). NH_4^+ removal via anammox in the early incubations may also explain why the observed NH_4^+ oxidation rates and the abundance of *Acidimicrobiaceae* A6 did not change proportionally over the full incubation period.

The results and analyses described here have shown that a Feammox enrichment reactor has the capacity to 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. Conclusive linkage between *Acidimicrobiaceae* A6 and the Feammox process requires the isolation of the strain and then the conducting of incubations with the pure strain. At this point, the observations (i) that NH_4^+ removal only occurred in samples when the presence of *Acidimicrobiaceae* bacterium A6 was detected and when iron was being reduced, (ii) that *Acidimicrobiaceae* A6 numbers increased gradually after sequential NH_4^+ and Fe(III) additions, and (iii) the results from the enrichment culture that was operated for an extended time period while only adding NH_4^+ and iron oxide sources and during which *Acidimicrobiaceae* A6 became the dominant bacterial species, while no other known NH_4^+ oxidizer (AOB or anammox) was detected after 150 days of operation, indicate that *Acidimicrobiaceae* A6 is likely to play an important role in the oxidation of NH_4^+ under iron reduction conditions.

The Supplement related to this article is available online at doi:10.5194/bg-12-769-2015-supplement.

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