1 **Environmental Factors affecting the presence of *Acidimicrobiaceae* and Ammonium**
2 **Removal under Iron-reducing Conditions in Soil Environments**

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13 ***Abstract***

14 The oxidation of ammonium (NH₄⁺) under iron reducing conditions, also referred to as
15 Feammox, has been described in recent years by several investigators. The environmental
16 characteristics in which the Feammox process occurs need to be understood in order to
17 determine its contribution to the nitrogen cycle. In this study, through extensive field
18 sampling at various locations in the US (mostly New Jersey) and South China, soil
19 chemical analyses, serial incubation experiments, analysis of microbial communities, and
20 using canonical correspondence analyses, it was determined that the soil pH, as well as its
21 Fe(III) and NH₄⁺ concentration were the most important factors controlling the
22 distribution of *Acidimicrobiaceae*, which have been linked to the Feammox reaction.
23 Under the conditions that favored the presence of Feammox bacteria and their oxidation
24 of NH₄⁺ under iron reducing conditions, denitrification bacteria were also abundant.
25 However, the presence of known nitrous oxide (N₂O) reducers was limited under these
26 conditions, implying that at locations where the Feammox process is active, conditions
27 might favor a higher N₂O:N₂ ratio for the nitrogen (N) end products. To determine the
28 effect of dissolved oxygen (DO) on NH₄⁺ oxidation under iron reducing conditions,
29 incubations were conducted at two different DO levels. In incubations with DO < 0.02
30 mg/L, and when NH₄⁺ oxidation was noticeable, the proportion of *Acidimicrobiaceae*
31 with respect to total bacteria increased, while the proportion of other known NH₄⁺
32 oxidizing bacteria (aerobic ammonia-oxidizing bacteria and anammox bacteria)

decreased. In incubations of the same soil but with DO = 0.8~1.0 mg/L the opposite trend was observed, indicating that Feammox favored very low or no DO in these incubations.

1. Introduction

Ammonium (NH_4^+) oxidation is an important step in the nitrogen cycle in soil and aqueous environments. Numerous studies have focused on the aerobic oxidation of NH_4^+ to nitrite (NO_2^-), the first and rate limiting biological step during nitrification. In addition to nitrification, NH_4^+ can be oxidized via anammox, which is the biological conversion of NH_4^+ as the electron donor and NO_2^- as the electron acceptor to nitrogen gas in the absence of oxygen (Jetten, 2008). In addition to these two NH_4^+ oxidation pathways, there is a newly discovered NH_4^+ oxidation process coupled to iron (Fe) reduction that has been referred to as Feammox (Sawayama, 2006; Yang *et al.*, 2012; Huang and Jaffé, 2015; Gilson *et al.*, 2015). Feammox is a process that can be described as the oxidation of NH_4^+ under iron reducing conditions, with iron oxides [Ferric iron, Fe(III)] as the electron acceptor. In this reaction Fe(III) is reduced to ferrous iron Fe(II), while NH_4^+ is transformed to NO_2^- , nitrogen gas (N_2), or other nitrogen forms (Clément *et al.*, 2005; Shrestha *et al.*, 2009; Yang *et al.*, 2012; Huang and Jaffé, 2015). So far the Feammox process has been reported in riparian wetland soils in New Jersey (Clément *et al.*, 2005; Shrestha *et al.*, 2009; Huang and Jaffé, 2015), in tropical rainforest soils in Puerto Rico (Yang *et al.*, 2012), and in paddy soils in China (Ding *et al.*, 2014). In our previous study, conducted with soil samples obtained from a forest riparian wetland (Assunpink Wildlife Management Area, NJ, USA), active Feammox reactions were always accompanied with the growth of *Actinobacteria*. *Acidimicrobiaceae* bacterium A6, a previously unreported species in the *Acidimicrobiaceae* family, has been identified as playing a key role in the Feammox process (Huang and Jaffé, 2015).

So far little information is available on the environmental factors that affect the Feammox process. The stoichiometry and change in free energy for Feammox when ferrihydrite is the Fe(III) source was given as (Huang and Jaffé, 2015):

($\Delta\text{Gr} \leq -145.08\text{kJ mol}^{-1}$, the species concentrations and pH for that calculation were described in Huang and Jaffé, 2015).

Equation 1 indicates that soils with bioavailable Fe(III) phases and low pH as well as conditions that prevent the buildup of dissolved Fe(II) and NO_2^- should favor the Feammox process.

Environmental factors that affect aerobic ammonia-oxidizing bacteria (AOB) and anammox bacteria are well known and have been studied extensively. In soil environments, the most studied AOB belong to the genera *Nitrosomonas* and *Nitrosococcus* (Juretschko *et al.*, 1998; Purkhold *et al.*, 2000; Kowalchuk and Stephen 2001; Arp *et al.*, 2002). Anammox bacteria, which use NO_2^- as the electron acceptor, belong to five genera: *Kuenenia* (Speth *et al.*, 2012), *Brocadia* (Kartal *et al.*, 2008), *Anammoxoglobus* (Kartal *et al.*, 2007), *Jettenia* (Quan *et al.*, 2008; Nikolaev *et al.*, 2015), and *Scalindua* (Schmid *et al.*, 2003). The first four were enriched from sludges from wastewater treatment plants (Schmid *et al.*, 2003; Kartal *et al.*, 2007; Kartal *et al.*, 2008; Quan *et al.*, 2008; Speth *et al.*, 2012). *Scalindua* dominates the marine environment, but is also found in some freshwater ecosystems and wastewater treatment plants (Schmid *et al.*, 2003; Zhu *et al.*, 2010). These two groups of bacteria (AOB and anammox) are adapted to environments with close to neutral pH (Nicol *et al.*, 2008; Jaroszynski *et al.*, 2011). AOB are usually present in environments with a pH of 6.5-7.5 and in the presence of dissolved oxygen (DO) levels of at least 3 mg/L (Guo *et al.*, 2009). Various field and laboratory studies have shown that anammox activity is optimal at a pH of 7.8 - 8.0. Both AOB and anammox bacteria are autotrophic, therefore the organic carbon concentration in soils does not directly affect their NH_4^+ oxidation rate (Fukushima *et al.*, 2013). The organic carbon concentration does of course affect the DO levels in soils, which as said above affect both AOB and anammox activity.

Ammonia-oxidizing Archaea (AOA) are known to be able to oxidize NH_4^+ under acidic conditions (Treusch *et al.*, 1985; Zhang *et al.*, 2011; He *et al.*, 2012). To date two AOA, *Nitrosopumilus maritimus* and *Nitrososphaera viennensis*, have been isolated and described (Walker *et al.*, 2010; Pester *et al.*, 2011). In soil environments, the activity of AOA depends on the pH, sulfide levels, and phosphate levels (Xia *et al.*, 2011; Zhang *et al.*, 2011; He *et al.*, 2012). AOA are better adapted than AOB to ammonia-limited oligotrophic conditions, including low-pH environments where ammonia is mostly in the form of NH_4^+ (Martens-Habbenha *et al.*, 2009). These acidophilic NH_4^+ oxidizers and their specific thaumarchaeal *amoA* genes have been found in several acidic soils (pH < 4.50) (Tourna *et al.*, 2008; Pester *et al.*, 2011). AOB, AOA, and anammox bacteria often coexist in hypoxic environments, where AOB and/or AOA provide the NO_2^- for the anammox reaction (Xiao *et al.*, 2009; Xia *et al.*, 2011; Wang *et al.*, 2012). Therefore, Feammox bacteria, which also produce NO_2^- while oxidizing NH_4^+ , may enhance anammox activity in environments where AOB/AOA are less active. Batch incubations of soil samples at sites where Feammox reaction was thought to be occurring have shown that after NH_4^+ was oxidized to NO_2^- by the Feammox process, NO_2^- transformation proceeded via denitrification and/or anammox (Shrestha *et al.*, 2009; Yang *et al.*, 2012; Huang and Jaffé, 2015). As shown by equation 1, removal of NO_2^- should make the Feammox reaction more favorable, but the coexistence of Feammox with denitrifiers and/or anammox bacteria and how they interact in acidic soil environments needs further study.

The aim of this study was to (1) investigate how different environment factors affect the presence of *Acidimicrobiaceae* bacteria and the Feammox reaction in acidic soil environments, and (2) determine the link between the *Acidimicrobiaceae* bacteria and/or the Feammox reaction and other microorganisms responsible for nitrogen transformations in various soil/ sediment environments. This was achieved through extensive field sampling at various locations, which included soil chemical analyses, serial incubation experiments, analysis of microbial communities, and using a canonical correspondence analyses to link the Feammox reaction as well as the presence of *Acidimicrobiaceae* to key soil chemical and microbiological properties. These experiments were then

augmented with incubation experiments to further explore the effect and expand the range of key variables like pH, NO_2^- , and dissolved oxygen.

2. Methods and materials

2.1 Sample collection

Although no rigorous studies on the environmental factors affecting the Feammox process have been conducted to-date, the Feammox reaction has only been reported for acidic soils (pH 3.5 - 5.5) with abundant iron oxides and for vegetated conditions (Shrestha *et al.*, 2009; Yang *et al.*, 2012; Ding *et al.*, 2014; Huang and Jaffé, 2015). Furthermore, calculations of the change in free energy for the Feammox process, as described by equation 1, have shown that the reaction is only thermodynamically favorable under acidic conditions (Huang and Jaffé, 2015). Therefore, all samples collected for this study were from locations with a pH < 7.5. The majority of samples were collected from vegetated soils/sediments with high water/moisture content. Release of oxygen by wetland-plant roots can, as described below, result in the formation of Fe(III) plaques on root surfaces, and soil with high water content results in lower DO and/or anoxic micro locations. Soils for the experiments described in this study were taken from several locations in central New Jersey, at Tims Branch at Savannah River in South Carolina, both in the United States, and at several locations in the Guangdong province in China (Table 1). A total of 66 locations were selected covering 4 different types of soils/sediments: wetland soils (W), river sediments (R), forest soils (F), and paddy soils (P) (Table 1). For each location where samples were collected with a corer, three 20 cm length soil cores were collected within a 2m × 2m sampling plot. Soil samples were separated into three layers, 0-5, 5-10 and 10-20 cm. The three soil samples from the same soil layer were then combined and homogenized into a single soil sample representing that layer. Soils collected at depths from 0-5 cm and 5-10 cm exhibited no obvious difference in both their chemical and microbial properties. Therefore, for the purpose of the analyses presented here, soil samples were classified into surface samples (S, 0 to 5 cm deep) and subsurface samples (D, 10 to 20 cm deep) (Table 1). The majority of soil samples were obtained following the outlined protocol. Paddy soil and river samples were not obtained with a corer, in which case no subsurface samples were

collected since these grab samples were obtained from a depth up to 10 cm, and three replicate soils were homogenized into a single sample. In a few occasions a complete core was not obtained and S and D samples are from adjacent locations but not from the same core and labeled as a different sample number. All samples were transported to the laboratory within 2 hours from collection and then stored at 4 °C. All soil/sediment analyses were conducted and incubation experiments initiated within 2 weeks from the sampling date.

2.2 Analysis of chemical properties

Physicochemical characteristic of these soils/sediments, including pH, Eh, gravimetric water content (u), Fe concentration [Fe (III) plus Fe(II)], nitrogen species (NO_3^- , NO_2^- and NH_4^+), sulfate (SO_4^{2-}), phosphate (PO_4^{3-}), and dissolved organic carbon (DOC) were determined. pH and Eh were measured immediately using a pH and ORP electrode probe. Gravimetric water content was determined from the difference of the initial sample weight and when the sample weight was constant after oven drying at 80°C. A 0.5N HCl extraction was conducted for 24 hour at room temperature to determine the bioavailable Fe(III), extractable Fe(II), and NH_4^+ concentrations in the soils. Total Fe was measured by adding 20 μL of 6.25 M hydroxylamine hydrochloride, which reduces Fe(III) to Fe(II), to 1 mL of HCl-extracted sample. Dissolved Fe(II) was then measured using the ferrozine assay method (Stookey, 1970; Komlos *et al.*, 2007). Bioavailable Fe(III) was taken as the difference between the total Fe and the total Fe(II) measured via the ferrozine assay method without the addition of hydroxylamine. 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). Because of an overlap of the potassium (K^+) and NH_4^+ peaks in the IC chromatogram, extraction was done with 0.5 N HCl instead of KCl, as has been described in previous studies (Huang and Jaffé, 2015). NO_3^- , NO_2^- , SO_4^{2-} and PO_4^{3-} concentrations were measured via 1-hour anaerobic DI water extractions followed by Ion Chromatography analyses, 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 DOC analyses a Shimadzu TOC-5000(A) was used.

2.3 *Feammox reaction activity incubation*

Soil samples from each location were divided into 12×10 g (air-dried equivalent) subsamples, and added into 50 mL serum vials, with 30 mL deionized (DI) water. The soil slurries were purged thoroughly with a $\text{CO}_2:\text{N}_2$ (80:20) mixture (gas flow was 20 mL/min, for three minutes), and then incubated under anaerobic conditions in sealed vials in an anaerobic glove box (Coy Laboratory Products Inc.). The glove box uses a palladium catalyst and was supplied with an anaerobic gas mix containing 5% H_2 and 95% N_2 to scrub any oxygen that may enter the glove box.

Anaerobic incubations were performed for 110 days and NH_4^+ was analyzed regularly. Incubations were started, and once NH_4^+ was relatively stable (after ~ 15 days), samples were amended in the glove box with varying amounts of 1 N NH_4Cl to achieve in each vial a total concentration of $2 \text{ mmol}\cdot\text{L}^{-1}$ of NH_4^+ . This was the start of incubation “Stage I”, which had a duration of 30 days. At the end of Stage I, the NH_4^+ concentration in each vial was again adjusted to $2 \text{ mmol}\cdot\text{L}^{-1}$, and incubations continued. This second phase is labeled “Stage II” and was selected as the next 50 days (day 30 to 80) of incubation, over which the NH_4^+ concentration remained relatively stable. “Stage III” is the incubation period starting on day 80, when samples were amended again in the glove box with NH_4Cl and 0.5 N ferrihydrite to achieve in each vial a total concentration of $2 \text{ mmol}\cdot\text{L}^{-1}$ NH_4^+ and $10 \text{ mmol}\cdot\text{L}^{-1}$ Fe(III). Controls consisted of autoclaved sterilized soils that were prepared identically and incubated with the same treatments. Three replicate subsamples were collected from each of two vials ($n=2$) by destructively sampling on each sampling day. Subsamples collected on each sampling day were kept at -20°C for further molecular biology analysis.

2.4 *Feammox incubations under different dissolved oxygen levels*

Soil samples W11D, R5, and F7D (Table 1), which have shown NH_4^+ removal under iron reducing conditions and include wetland soils, river sediment, and forest soils were selected to study the relationship between the NH_4^+ oxidation processes and the microbial communities during incubations under two different low dissolved oxygen (DO)

concentrations. Soil samples were divided into 12×10 g (air-dry equivalent) subsamples, and added into 50 mL serum vials, with 30 mL deionized (DI) water. Incubation vials were divided into two sets, labeled L-DO for low DO: L-DO-0.02 and L-DO-0.8. For the L-DO-0.02 incubations, vials were first purged thoroughly with a $\text{CO}_2:\text{N}_2$ (80:20) gas mixture, and then incubated in a glove box. The DO concentrations in these vials ranged from an initial concentration of $0.02 \text{ mg} \cdot \text{L}^{-1}$ to below the detection limit. The L-DO-0.8 incubations were conducted with DO concentrations between $0.8 \sim 1.0 \text{ mg} \cdot \text{L}^{-1}$. This DO was achieved by swirling the vial gently while exposed to the atmosphere for one hour per day. On day 30, 60, and 90, NH_4Cl and ferrihydrite were added to both incubation sets to adjust the concentrations to $2 \text{ mmol} \cdot \text{L}^{-1} \text{ NH}_4\text{Cl}$ and $6 \text{ mmol} \cdot \text{L}^{-1}$ ferrihydrite. The initial pH was adjusted to between 5.5-6.0 (pH adjusted with HCl and/or NaOH) for all samples of this incubation. During these 120-day incubations, pH increased from 6.0 to 6.5, which is consistent with our previous studies (Shrestha *et al.*, 2009; Huang and Jaffé, 2015). Triplicate samples were collected from two vials for each set on days 0, 30, 60, 90, 120 for iron and nitrogen species analysis after which samples collected on these days were kept at -20°C for further molecular biology analysis. DO levels were confirmed when a vial was destructively sampled.

2.5 Incubation as a function of pH and NO_2^- concentrations

After 120 days of the anoxic incubations described above, slurries from incubation W11D were used to conduct further incubations to determine how varying the pH and NO_2^- concentration over a wider range than observed in our field samples may affect the putative Feammox reaction in a sample where NH_4^+ removal under iron reducing conditions was detected. This sample was selected because it had among the highest proportion of *Acidimicrobium* (14.9%) with respect to the total bacterial population. Slurry samples were divided into 30×10 mL subsamples, added into 50 mL serum vials, with 30 mL deionized (DI) water, purged thoroughly with a $\text{CO}_2:\text{N}_2$ (80:20) gas mixture, and then sealed with a rubber stopper and separated into two sets. To determine the effect of pH, these enrichment cultures were incubated with the pH adjusted to 2, 4.5, 6.5, and 8. To determine the effect of NO_2^- levels, enrichment cultures were amended with different amounts of NO_2^- , resulting in final NO_2^- concentrations of 0.02, 0.2, 1.0, and 2.0

mmol·L⁻¹. Hence, each set of incubations had five treatments including one autoclaved control, and each treatment had three replicates. The initial concentration of Ferrihydrite in all of these incubations was 25 mmol·L⁻¹ and that of NH₄⁺ was 2 mmol·L⁻¹. Triplicate subsamples were collected with syringes every two days for a month to analyze for dissolved iron and nitrogen species, as well as to monitor the numbers of *Acidimicrobiaceae* bacteria.

2.6 DNA isolation and *Feammox* primers modification

DNA samples were extracted from soils collected prior to any laboratory incubation, and from the samples taken at different time points during the incubation experiments. DNA was extracted from 500 mg triplicate soil samples using the FastDNA[®] spin kit for soil (MP Biomedicals, USA) as described by the manufacturer. The concentrations of DNA were measured using a Nano-drop 2000 spectrophotometer (Thermo Scientific, USA). Primer sets acm362f/539r and acm342f/439r (Table S1) were designed to target *Acidimicrobiaceae* family and *Acidimicrobiaceae* bacterium A6, respectively (Huang and Jaffé, 2015). The efficiency of these two primers was improved by a touchdown temperature gradient test and their accuracy was checked using their sequenced relevant PCR products. The accuracy of these primers was further confirmed by comparing the quantitative PCR results to the pyrosequencing analysis.

2.7 Quantitative polymerase chain reaction assay (qPCR)

qPCR experiments were carried out using a StepOnePlus Real time PCR system (Applied Biosystems, USA) for total bacteria and anammox bacteria represented by 16S rRNA genes, using primer sets 1055f/1392r (Harms *et al.*, 2003) and Amx368f/Amx820r (Schmid *et al.*, 2000; Schmid *et al.*, 2003) respectively (Table S1). *Acidimicrobiaceae* bacteria were enumerated with primer acm362f/539r and acm342f/439r (Table S1). For the detection of denitrifiers, denitrifying functional genes (*narG*, *nirS*, *nosZ*,) were quantified with primer sets NarG1/NarG3, NirS3/NirS5, NosZ-1f/NosZ-2, nrfA-2f/2r (Braker *et al.*, 1998, Scala and Kerkhof 1998) (Table S1). For the detection of *nrfA* gene, primer nrfA-2f/2r (Smith *et al.*, 2007) was used. For the detection of the bacterial *amoA* gene, primer amoA1f/ amoA2r (Rotthauwe *et al.*, 1997) was used. Primer pairs

CrenamoA23f /616r, were used to quantify the thaumarchaeal amoA genes which represented acidophilic ammonia oxidizers (Tourna *et al.*, 2008). qPCR analysis from each location on serially diluted (10^{-10} to 10^{-4} times) DNA samples were done to prevent inhibitor effects on qPCR efficiency. Each qPCR mixture (20 μ L) was composed of 10 μ L of SYBR Premix Ex Taq[®] II (Takara, Japan), 0.8 μ L 10 μ M of each primer, and $\sim$ 10 ng DNA template. Thermal cycling conditions for total 16S rDNA, *narG*, *nirS*, *nosZ* and *amoA* gene numbers was initiated for 30 s at 94 $^{\circ}$ C, followed by 40 cycles of 5 s at 94 $^{\circ}$ C, 30 s at 57 $^{\circ}$ C, and 30 s at 70 $^{\circ}$ C. 16S rDNA numbers of anammox and *Acidimicrobiaceae* bacteria A6 were performed at 56 $^{\circ}$ C and 58 $^{\circ}$ C as annealing temperature respectively, with the same program. 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).

2.8 454-pyrosequencing analysis

To further confirm the changes in the bacterial community, 454 pyrosequencing was performed with samples collected from the incubation of samples W11D, R5 and F7D on days 0 and 120 under L-DO incubations I and II. Domain-specific primers, targeting the V3-V5 region of the 16S rDNA of bacteria were amplified following methods suggested by Pinto *et al* (2012). To amplify a 16S rRNA gene fragment of the appropriate size and sequence variability for 454 pyrosequencing, specific primers Bact-341F (CCTACGGGNGGCWGCAG) /909R(CCGTCAATYHTTTRAGT), targeting the V3-V5 region of the 16S rRNA gene of bacteria were chosen (Pinto *et al.*, 2012). The PCR conditions used were 94 $^{\circ}$ C for 2 min, 20 cycles of 94 $^{\circ}$ C, 45 s denaturation; 55 $^{\circ}$ C, 45 s annealing and 72 $^{\circ}$ C, 1 min extension; followed by 72 $^{\circ}$ C, 6 min. After 20 rounds of amplification, another 3 rounds of amplification were done to add the *a* and *b* adapters required for 454 pyrosequencing to specific ends of the amplified 16S rRNA fragment for library construction. Approximately 4~6 ng/ μ l of 16S rRNA gene fragment from each soil sample was required to construct the nine libraries for 454 sequencing. Polymerase chain reaction products were cleaned using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions, quantified using a Qubit Fluorometer

(Invitrogen), and then sent for 454-pyrosequencing using a Roche/454 GS FLX sequencer.

A total of 227268 partial 16S rRNA sequences were obtained from the nine soil samples. The sequences were then evaluated with the DOTUR program to further reduce errors as outlined previously (Schloss and Handelsman, 2005). Briefly, after trimming, pre-clustering, removal of chloroplast sequences and alignments, a total of 20512-27141 sequences remained for each library. These sequences were clustered with the average neighbor algorithm with a 3% dissimilarity cutoff, which resulted in 2862-6316 Operational Taxonomic Units (OTUs).

2.9 Statistical analysis

Correlations between NH_4^+ oxidation coupled with iron reducing conditions in the presence of *Acidimicrobiaceae* bacteria, as well as bacterial distribution and environmental factors were analyzed using the canonical correspondence analysis (CCA) (Dang *et al.*, 2008). Analyses of Spearman rank, multivariate and stepwise linear regressions of environmental and bacterial abundance variables were carried out using SPSS v12 on both raw and log-transformed data. All data were analyzed using one-way analysis of variance (ANOVA) with a significance level $p < 0.05$.

3. Results

3.1 Chemical properties of soil/sediment samples collected from different locations

Soil and sediment samples collected for this study cover a wide range of chemical properties as shown in Table S2 and S4: pH ranged from 3.03 to 7.62; Fe(III) from 76.81 mg kg^{-1} to 1772.29 mg kg^{-1} ; Fe(II) from 8.03 mg kg^{-1} to 477.14 mg kg^{-1} ; NH_4^+ from 5.12 mg kg^{-1} to 2079.08 mg kg^{-1} ; NO_3^- from 4.12 mg kg^{-1} to 852.8 mg kg^{-1} ; NO_2^- from 0.34 mg kg^{-1} to 152.8 mg kg^{-1} ; SO_4^{2-} from 2.32 mg kg^{-1} to 114.6 mg kg^{-1} ; PO_4^{3-} from 1.90 mg kg^{-1} to 57.1 mg kg^{-1} ; DOC from 8.08 g kg^{-1} to 55.20 g kg^{-1} .

Most of the wetland and forest soil samples were acidic, with more than half of them having a pH lower than 6. Soils/sediments collected for this study from wetlands and

forests had higher DOC and iron concentrations compared to that of paddy soils and river sediments (Table S2). pH values of surface soil/sediment samples (0-5 cm) collected from wetlands and forests were slightly higher than that of the subsurface samples (10-20 cm). River sediment samples were sulfate rich but had lower concentrations of Fe(III) and Fe(II) (Table S2b). pH values and NH_4^+ concentrations were higher in sediments collected from the Pearl River in China than that from Tims Branch at Savannah River in US. The average NH_4^+ concentration in the Pearl River sediments was 872 mg/kg which was ~ 100 times higher than that in sediments from Tims Branch at Savannah River (Table S2b). Due to fertilizer applications, paddy soil samples contained high concentrations of NH_4^+ and PO_4^{3-} . Samples W10D to W14D that were collected from a wetland near agricultural fields also had high concentrations of NH_4^+ (Table S2a).

3.2 Distribution of nitrogen transformation microorganisms in soil samples

Total bacteria abundance ranged from 1×10^9 to 1×10^{10} cells/g soil/sediment for all 217 samples. Selected denitrifying functional genes (*narG*, *nirS*, *nirK* and *nosZ*) were found at all sampling locations (Table 2, S3, and S5). Sediments have a higher amount of denitrifying genes and a larger proportion of denitrifying bacteria than forest soils, which is consistent with results obtained by other studies (Liu *et al.*, 2010; Huang *et al.*, 2011). However, the distribution of each type of denitrifying functional gene was different. *narG* genes were more abundant in wetland sediments, and were higher in samples W15S to W18D (Table 2). For nitrite reductase genes, river sediments had the highest number of *nirS* genes while *nirK* genes were more prevalent in soils collected from forest soils (Table S3a, b). Dissimilatory nitrate reduction gene, *nrfA*, which can reduce NO_2^- to ammonia, was also higher in river sediments. Compared to other types of soils, paddy soil samples had larger proportions of *nosZ* genes, which are responsible for coding the N_2O reductase (Table S3a, c).

The abundance of NH_4^+ oxidizers also differed between the various types of soils and depths. AOB (represented by the *amoA* gene) had a higher presence in forest soil samples , especially in surface soil samples. AOA (represented by the *amoA* gene of AOA) were detected in half of the wetland and forest soil samples, all of which were subsurface

samples (Table 2 and Table S3). In river sediment samples, AOA were more abundant than AOB, especially in samples R7-10, where the number of *amoA* genes of AOA was 5 times higher than those of AOB (Table S3a). Anammox bacteria were prevalent in paddy soil samples, but not widespread in other soil/sediment samples. The presence of *Acidimicrobiaceae* and *Acidimicrobiaceae* bacterium A6, were detected in 21 and 20 of the 66 samples. Most of the samples where *Acidimicrobiaceae* bacteria were detected were subsurface samples from wetland and forest soils, while only four samples from paddy soil and river sediments (R5, R6, P2, P3) showed the existence of *Acidimicrobiaceae* bacteria and then with a relatively lower number than in wetland and forest soil samples (Table 2 and Table S3).

3.3 NH_4^+ consumption and Fe(II) production during anaerobic incubations

To monitor signs for possible Feammox activity in different samples, Fe(II) production and NH_4^+ consumption of three sequential incubation stages were analyzed. None of the autoclaved control samples showed any NH_4^+ removal or Fe (II) production during the incubations, and none had DNA amplified during the qPCR analysis. Therefore the changes in NH_4^+ and Fe(II) concentrations in the non-autoclaved incubations were attributed to biotic reactions. Increase of Fe(II) with time in each incubation stage was considered evidence for Fe(III) reduction, while decrease of NH_4^+ was taken as NH_4^+ removal. After 80 days of anaerobic incubation, NH_4^+ removal that was stimulated by the addition of Fe(III) in Stage III was considered to be associated with the Feammox reaction.

During incubation Stage I (day 0 to 30), most samples showed NH_4^+ removal with removal rates ranging from 3.33 to 50 $\mu\text{mol day}^{-1}$. For samples collected from the same location in forest areas, shallow soils samples (0 cm to 5 cm) showed more anoxic NH_4^+ removal ($p < 0.05$) than subsurface samples (10 cm to 20 cm) (Fig. S1c). Among the four types of soil samples studied, paddy soils had higher NH_4^+ removal rates during the initial stage of these incubations, with an average NH_4^+ removal rate of 20 $\mu\text{mol day}^{-1}$ (Fig. S1a). During incubation Stage II, detectable ($> 0.01 \text{ mmol L}^{-1}$) NH_4^+ removal was found in only 23 samples, none of which was a river sediment sample (Fig. S1a). The average

NH₄⁺ removal rates during Stage II 0.70, 2.72 and 2.04 μmol day⁻¹ in wetland, forest, and paddy soil samples, respectively (Figs. 1a, S1a, c). At the end of Stage II, the remaining NH₄⁺ concentrations were around 1.87-2.03 mmol L⁻¹, hence the much lower NH₄⁺ removal rate during Stage II can not be attributed to low NH₄⁺ levels. During incubation Stage III (day 80 to 110), noticeable NH₄⁺ removal (> 3.33 μmol day⁻¹) was detected in only 19 samples. Among these samples, 11 samples did not exhibit any NH₄⁺ removal during Stage II, and these samples showed significantly higher (*p*<0.01) NH₄⁺ removal during Stage III than during the previous stages (Figs. 1a, S1a, c). Except for samples R5, R6, P2, P3, NH₄⁺ removal under iron reduction was most prevalent in wetland and forest soils, which is consistent with the distribution of *Acidimicrobiaceae* bacteria. Only samples in which *Acidimicrobiaceae* bacteria were detected showed noticeable NH₄⁺ removal during Stage III, after Fe (III) was added on day 80 (Table 2 and Table S3).

Fe(II) production could have been due to heterotrophic iron reduction as well as due to Feammox activity. *Geobacter sp.* decreased from 2.12×10⁷ to 7.11×10⁶ cells/L during the full incubation period, indicating that conditions were not favorable for sustained heterotrophic iron reduction. During Stage I, all samples showed substantial iron reduction, producing between 1.42 and 5.93 μmol day⁻¹ of Fe(II) (Figs. 1b, S1b, d). During Stage II, iron reduction decreased markedly to around 1.2-18.4% compared to those in Stage I, although less so in a small set of the forest soils that were rich in iron and carbon. These forest soil samples showed an Fe(II) production around 0.54 μmol day⁻¹ during Stage II (Fig. S1d). During Stage III, after augmenting the samples with NH₄⁺ and ferrihydrite, Fe(II) production increased noticeable (*p*<0.01) as compared to Stage II, producing between 3.44 and 36.21 μmol day⁻¹ of Fe(II).

For further statistical analyses, soil/sediment samples were divided into those in which *Acidimicrobiaceae* bacteria were detected and samples where *Acidimicrobiaceae* bacteria were not detected, without distinguishing for the sample origin or depth. Results, shown in Table 3 demonstrate that there was significantly more NH₄⁺ removal in Stage III in the presence of *Acidimicrobiaceae* bacteria (*p*<0.01), while significantly more Fe(II) was produced during Stages II and III in the presence of *Acidimicrobiaceae* bacteria (*p*<0.01).

The same statistical results were reached when analyzing samples in this manner but also dividing them by their source (not shown). These results also show the need for long-term incubations to clearer isolate the possible contributions of Feammox bacteria to NH_4^+ removal and Fe(II) production.

3.4 Canonical correspondence analysis of Feammox distribution in soil samples

Correlations between the concentrations of chemical soil/sediment properties and the abundance of different nitrogen oxidizing/reducing microorganisms are shown in Table S6. The numbers of *Acidimicrobiaceae* family and *Acidimicrobiaceae* bacterium A6 were correlated ($R^2=0.967$, $p=0.05$). Therefore, using any of them to represent putative Feammox bacteria for the CCA analysis yielded similar results (Fig 2 and S2).

The CCA analysis of the distribution of *Acidimicrobiaceae* bacterium A6 in response to environmental variables is shown in Figure 2a. The first two CCA axes (CCA1 and CCA2) explain 69.5 % of the total variance in the bacterial abundance and 78.6% of the cumulative variance of the bacteria-environment relationship. Fe(III) concentration show positive correlations with the distribution of *Acidimicrobiaceae* bacterium A6 ($p = 0.021$), while NO_2^- showed a negative correlation with the distribution of *Acidimicrobiaceae* bacterium A6 ($p = 0.01$). pH [H^+] also shows a strong correlation with the distribution of the putative Feammox bacterium (higher *Acidimicrobiaceae* bacterium A6 abundance at lower pH).

The CCA of the relationship between the distributions of nitrogen oxidizing/reducing microorganisms in response to environmental variables is shown in Figure 2b. The first two CCA axes (CCA1 and CCA2) explain 79.4 % of the total variance in the microbial composition and 83.2% of the cumulative variance of the bacteria-sampling location relationship. CCA1 represents the pH level of the soils, showing that Feammox bacteria A6 and AOA were more abundant in low pH soils/sediments (Fig. 2b). CCA2 represents the vertical profile of soil/sediment samples, and soils/sediment samples collected from different depths were separated. Microorganisms detected in soils/sediments collected from similar depths clustered together. Aerobic/anammox NH_4^+ oxidation bacteria were more prevalent in surface soil samples, while the presence of *Acidimicrobiaceae* bacteria

and denitrifiers was higher in soil samples collected from depths below 10 cm.

3.5 Incubations and ammonium oxidation for different dissolved oxygen levels

Three different soil samples were selected to conduct incubations under different low dissolved oxygen levels. Sample a1 is a subsample of W11D before the low-DO incubation was initiated. Sample a2 is a subsample of W11D taken on day 120 of incubation L-DO-0.02 ($\text{DO} < 0.02 \text{ mg} \cdot \text{L}^{-1}$), and sample a3 is a subsample of W11D taken on day 120 of L-DO-0.8 ($\text{DO} = 0.8 \sim 1.0 \text{ mg} \cdot \text{L}^{-1}$). Similarly, sample b1, b2 and b3 were subsamples of sample R5, sample c1, c2 and c3 were subsamples of sample F7D collected before and after the same low dissolved oxygen incubations. After 120 days of the L-DO-0.02 incubations, 3.75 mmol L^{-1} , 2.98 mmol L^{-1} , and 4.95 mmol L^{-1} of NH_4^+ were consumed in samples W11D, R5, and F7D, respectively. While during 120 days of the L-DO-0.8 incubations, a total of 3.92 mmol L^{-1} , 2.90 mmol L^{-1} , and 4.76 mmol L^{-1} of NH_4^+ were consumed in samples W11D, R5, and F7D, respectively. In all of these samples, α -, β -, γ -, and δ -Proteobacteria were major fractions of the total bacterial community (Fig 3 and S3). Compared to sample a1 and c1, sample b1 had a higher fraction of β - and δ -Proteobacteria, 32% and 16%, respectively. These four groups of microorganisms remained fairly constant as a percentage of the total population during incubation L-DO-0.8, except a noticeable increase of α -Proteobacteria in sample b1 (Fig. S3c). *Planctomycete*, the phylum to which anammox bacteria belong, increased during incubation L-DO-0.8 in all samples and either decreased or stayed constant during all L-DO-0.02 incubations (Fig 3 and S3). *Scalindua* was the dominant anammox bacteria found in these all samples. *Actinobacteria* exhibited a marked increase, from 4% to 22% of the overall population, during the L-DO-0.02 (sample a2) and much lower increase from 4% to 8% during the L-DO-0.8 (sample a2) of sample W11D (Fig 3). The trends for *Actinobacteria* during the incubations of samples R5 and F7D were similar (Fig S3).

Substantial changes in the composition of NH_4^+ oxidation bacteria were observed within the β -Proteobacteria and *Actinobacteria*. *Nitrosomonas* and *Nitrosospira* were the AOB detected within the β -Proteobacteria of the wetland samples W11D, and they became more dominant during incubation L-DO-0.8 while they became a much lower fraction of the overall population during incubation L-DO-0.02 (Fig 3). *Nitrosospira* is the only

AOB that was still detected after incubation L-DO-0.02, decreasing from 15% to 1% (Fig 3c). The other two samples showed the same trends during the incubations, with different ratios of *Nitrosomonas* and *Nitrosospira* (Fig S3). In contrast to AOBs, *Acidimicrobiaceae* bacteria were not the major proportion of *Actinobacteria* before these incubations, but significantly increased after 120 days in both low DO incubations. In sample W11D, within the *Actinobacteria* groups, *Acidimicrobiaceae* bacteria increased from 5% to 68% and to 47% during incubation L-DO-0.02 and L-DO-0.8, respectively. Increases consistent with these changes were also found in the other sample incubations (Fig 3 and S3).

3.5 Effect of incubations with different pH and NO_2^- concentrations

NH_4^+ oxidation coupling with iron reducing conditions proceeded at the highest rate in sample W11D with pH adjusted to 4.5. When the pH increased to 6.5, the NH_4^+ removal rate and Fe reduction rate decreased, and no detectable NH_4^+ removal was observed until day 8 of the incubations. The Feammox reaction did not appear to proceed at all when the pH was either 2.0 or 8.0 (Figs. S4 a, b). Abundance of *Acidimicrobiaceae* bacteria increased over 14 days of anaerobic incubation in samples with the adjusted pH ranging from 4.5 to 6.5 and slightly decreased in samples with pH adjusted to 8. For pH adjusted to 2, although little or no NH_4^+ removal was noticed, the *Acidimicrobiaceae* bacteria number increased. There was no significant difference in the number of *Acidimicrobiaceae* bacteria in samples incubated at a pH of 4.5 and 6.5, which after two weeks of incubation had 2.37×10^4 and 2.41×10^4 copies mL^{-1} , respectively (Fig. S4c).

The apparent Feammox activity was sensitive to the NO_2^- concentration in the samples. The reaction proceeded when NO_2^- concentrations were in the range of $0.02 \text{ mmol} \cdot \text{L}^{-1}$ to $0.2 \text{ mmol} \cdot \text{L}^{-1}$, and was completely inhibited when the NO_2^- concentration reached $1.0 \text{ mmol} \cdot \text{L}^{-1}$ (Figs. S5 a, b). The abundance of *Acidimicrobiaceae* in soil samples was higher in incubations with low NO_2^- , which also had higher NH_4^+ removal and Fe reduction (Fig. S5 c).

4. Discussion

Soil/sediment samples that in Stage III of the incubations showed NH_4^+ oxidation after the addition of ferrihydrite were considered as possibly being Feammox active. It should be emphasized that without using ^{15}N tracers in combination with acetylene block, such as used by Huang and Jaffé, 2015, we cannot estimate what proportion of NH_4^+ removal was due to Feammox, nor can we estimate Feammox rates. Thus, we refer here generally to NH_4^+ removal under Fe-reducing conditions.

The pH values of samples that were considered as possibly being Feammox active were all in a range of 3.92 to 6.21 (Tables S2 and Figs. 1, S1), and *Acidimicrobiaceae bacterium* A6, the putative Feammox bacteria was present in all them (Tables 2). The CCA analysis of Feammox bacteria vs. environmental variables, shows that the pH value, Fe(III) concentration, and NH_4^+ concentration were the most important factors controlling the distribution of *Acidimicrobiaceae bacterium* A6 (Fig. 2a). The CCA analysis of the *Acidimicrobiaceae* family vs. environmental variables showed similar results (Fig. S2).

Roots, specially from wetland plants, are known to transport oxygen from the atmosphere into saturated solid/sediments, which will result in the oxidation of Fe(II) and production of iron oxide phases. Therefore more Fe(III) was found in saturated sediments that were vegetated than in non-vegetated sediments/soils, thereby providing Fe(III) rich environments that facilitate or enhance the Feammox reaction.

Although DO affects known NH_4^+ oxidation pathways, it is not straight forward to conduct accurate DO measurements in soils under field conditions, especially because DO varies between pores and micro pore structures where specific bacteria might be active. Therefore, incubations of selected soil/sediment samples for which the Feammox reaction was thought to be occurring, were conducted under two different low DO levels ($\text{DO} < 0.02 \text{ mg/L}$ and $\text{DO} = 0.8\sim 1.0 \text{ mg/L}$), to evaluate NH_4^+ oxidation and microbial communities shifts for these different DO conditions. During these incubations, the community structure became more uniform in all incubations, but more so for the incubations at lower DO levels (Fig. 3 and Fig. S3). During the L-DO-0.02 incubations,

the proportion of *Acidimicrobiaceae* bacteria increased and that of other NH_4^+ oxidation bacteria (aerobic ammonia-oxidizing bacteria and anammox bacteria) decreased, while for the L-DO-0.8 the opposite trend was observed. These results indicate that *Acidimicrobiaceae* and hence Feammox can conduct NH_4^+ oxidation under DO levels that are too low for AOB to thrive.

Surface soils collected in this study show a higher abundance of AOB and NO_3^- reducers, (Table 2, S2 and Fig. 1b). With increasing depth, AOB were gradually replaced by AOA that have lower oxygen requirements, as well as anammox that use NO_2^- and Feammox that use Fe (III) as electron acceptors for NH_4^+ oxidation (Table 2 and Fig. 1b). pH played an important role in the distribution and aggregation of nitrogen-transforming microorganisms (Fig. 2b). Only a few autotrophic nitrifying bacteria can carry out nitrification in low (5-7) pH environments. AOA are considered to be the main NH_4^+ oxidation microorganisms in acidic soils (Boer *et al.*, 1991; Boer and Kowalchuk 2001; Nicol *et al.*, 2008). Hence it is likely that the main processes for NH_4^+ oxidation in acidic soils is driven by AOA in the presence of low DO and possibly by Feammox when DO is too low for AOA activity.

The CCA analyses show a negative relationship between *Acidimicrobiaceae* and NO_2^- levels (Fig 2a and S2), which is consistent with the incubation experiments with different NO_2^- concentrations (Fig S5). Furthermore, the CAA analyses show an association of *Acidimicrobiaceae* with *nirK* (Fig 2b). Therefore, these results indicate that conditions that result in the removal of NO_2^- may favor Feammox activity. With the exception of N_2O reducers, denitrifiers are active over a wide pH range, (Yamulki *et al.*, 1997; Liu *et al.*, 2010; Pan *et al.*, 2012). The translation/assembly of N_2O reductase is sensitive to low pH, and measurable N_2O reduction only occurs at pH 6.1-7.0 (Pan *et al.*, 2012; Butterbach-Bahl *et al.*, 2013). Hence, at the low pH values observed here where Feammox may be active, denitrification of Feammox-produced NO_2^- could result in higher $\text{N}_2\text{O}:\text{N}_2$ as the nitrogen end-product in such soils/sediments.

In summary, bacteria that have been linked to the Feammox reaction require acidic

soils/sediments, the presence of Fe(III) phases, removal of NO_2^- , are favored by higher NH_4^+ levels, and require very low DO to complete anoxia. Under these conditions, results presented here show that of the known ammonium oxidizing microorganisms, the Feammox bacteria were dominant. Since iron rich acidic soils are common, Feammox might play an important role in the global nitrogen cycling.

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Table 1. Locations of Soil/Sediment Samples

Samples collected in New Jersey, USA (2011-2014)														
Sample ID	W1S	W2S	W6S	W7S	W8S	W9S	W11S	W12S	W13S	F1S	F2S	R1	R2	R3
Depth (cm)	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-10	0-10	0-10
Sample ID	W1D	W3D	W4D	W5D	W7D	W8D	W9D	W10D	W11D	W12D	W13D	W14D	F1D	F2D
Depth (cm)	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20

Samples collected in South Carolina, USA (2013-2014)										
Sample ID	W15S	W16S	W17S	W15D	W16D	W17D	W18D	R4	R5	R6
Depth (cm)	0-5	0-5	0-5	10-20	10-20	10-20	10-20	0-10	0-10	0-10

Samples collected in Guang Dong, China (2014-2015)														
Sample ID	F3S	F4S	F5S	F6S	F7S	F8S	F9S	F10S	P1	P2	P3	P4	R7	R8
Depth (cm)	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-10	0-10
Sample ID	R9	R10	F3D	F4D	F5D	F6D	F7D	F8D	F9D	F10D	P5	P6	P7	P8
Depth (cm)	0-10	0-10	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20	10-20

W = wetland sediments
R = river sediments
F = forest soils
P = paddy soils
S = surface samples, 0 - 5 cm
D = subsurface samples, 10- 20 cm

* Sampling dates are given in Table S7.

Table 2 The Mean values of abundance nitrogen oxidizing/reducing microorganism in wetland sediment samples.

Sample	W1S	W2S	W6S	W7S	W8S	W9S	W11S	W12S	W13S	W15S	W16S	W17S	W1D	W3D
<i>Total bacteria</i> (1×10^9 /g dw)	1.73	1.62	1.92	2.19	1.02	2.03	2.19	2.01	2.18	1.88	1.84	0.77	2.34	2.12
<i>narG</i> (1×10^7 /g dw)	1.65	1.43	1.51	2.10	1.18	0.22	2.00	1.19	2.38	4.72	4.57	4.99	2.10	1.91
<i>nirS</i> (1×10^7 /g dw)	0.10	0.07	2.19	2.23	1.01	1.10	0.52	0.58	1.09	0.83	0.33	0.60	0.07	0.12
<i>nirK</i> (1×10^7 /g dw)	0.34	0.05	0.07	0.12	0.43	0.23	0.03	0.34	0.04	0.19	0.45	0.34	0.03	0.83
<i>nosZ</i> (1×10^7 /g dw)	0.03	0.02	0.03	0.01	0.08	0.08	0.20	0.08	0.07	0.19	0.21	0.09	0.02	0.02
<i>nrfA</i> (1×10^8 /g dw)	1.23	1.84	0.73	0.74	0.84	0.66	1.92	1.83	0.34	1.30	1.56	2.93	1.03	0.45
<i>amoA(AOB)</i> (1×10^8 /g dw)	0.23	0.36	0.66	0.58	0.40	0.49	0.73	0.61	0.62	0.68	0.87	0.60	0.12	0.13
<i>amoA(AOA)</i> (1×10^8 /g dw)	-	-	1.06	-	0.18	-	1.94	0.87	0.08	-	-	-	-	-
<i>Anammox</i> (1×10^8 /g dw)	0.02	0.20	0.07	0.29	0.05	0.17	0.02	0.13	0.20	1.31	1.21	1.67	0.06	0.10
<i>Acidimicrobiaceae</i> (1×10^8 /g dw)	-	-	-	-	-	-	0.09	0.18	-	-	-	-	-	-
<i>A6</i> (1×10^8 /g dw)	-	-	-	-	-	-	0.08	-	-	-	-	-	-	-
Sample	W4D	W5D	W7D	W8D	W9D	W10D	W11D	W12D	W13D	W14D	W15D	W16D	W17D	W18D
<i>Total bacteria</i> (1×10^9 /g dw)	1.02	0.89	2.98	2.01	1.78	2.13	2.56	2.19	1.69	1.98	1.02	0.78	0.68	0.71
<i>narG</i> (1×10^7 /g dw)	2.35	2.56	1.89	1.58	0.41	1.67	2.33	1.02	1.00	1.58	3.94	3.77	3.91	3.49
<i>nirS</i> (1×10^7 /g dw)	0.61	0.67	2.79	0.99	1.00	0.72	0.79	0.68	0.58	0.67	0.31	0.40	0.59	0.53
<i>nirK</i> (1×10^7 /g dw)	1.04	0.32	0.10	0.33	0.15	0.56	3.52	3.07	3.13	4.15	0.23	0.01	0.67	0.28
<i>nosZ</i> (1×10^7 /g dw)	0.10	0.14	0.01	0.23	0.02	0.22	0.22	0.37	0.42	0.31	0.22	0.02	0.16	0.21
<i>nrfA</i> (1×10^8 /g dw)	1.45	1.03	0.12	0.18	0.23	0.56	0.23	0.93	0.13	0.95	1.76	1.03	0.45	1.23
<i>amoA(AOB)</i> (1×10^8 /g dw)	0.10	0.12	0.26	0.02	0.20	0.11	0.17	0.11	0.10	0.21	0.29	0.32	0.12	0.10
<i>amoA(AOA)</i> (1×10^8 /g dw)	0.07	0.09	-	0.14	-	1.27	1.10	0.17	0.08	1.01	0.18	0.14	-	0.06
<i>Anammox</i> (1×10^8 /g dw)	0.02	0.02	0.58	0.16	0.18	0.10	0.03	0.10	0.01	-	1.07	1.17	0.77	0.72
<i>Acidimicrobiaceae</i> (1×10^8 /g dw)	0.58	0.29	-	0.10	-	0.18	0.65	0.25	0.43	0.41	-	-	-	0.12
<i>A6</i> (1×10^8 /g dw)	0.42	0.22	-	0.10	-	0.14	0.58	0.19	0.41	0.27	-	-	-	0.16

Table 3. Mean and *p* values (two-tailed Student's *t*-test) of NH₄⁺ consumption and Fe (II) production between samples with and without *Acidimicrobiaceae* bacterium A6, during anaerobic incubation Stage I, II and III.

	NH ₄ ⁺ consumption (mmol·L ⁻¹)			Fe (II) production (mmol·L ⁻¹)		
	<i>p</i>	Mean (samples with A6)	Mean (samples without A6)	<i>p</i>	Mean (samples with A6)	Mean (samples without A6)
Stage I	0.518	0.724	0.953	0.060	3.465	2.532
Stage II	0.512	0.087	0.131	0.18E-3	0.349	0.078
Stage III	1.72E-15	0.483	0.018	1.26E-11	3.429	1.210

Figure 1. Anaerobic incubations of wetland samples. Stage I incubation day 15 to day 30, stage II day 45 to day 60, and stage III day 80 to day 95. Decrease of NH_4^+ between two sampling days during the incubation was taken as NH_4^+ consumption (a), and increase of Fe(II) was considered as Fe(III) reduction (b). Samples in which *Acidimicrobiaceae* were detected are marked with *.

Figure 2. Ordination plots of the canonical correspondence analysis (CCA) for the first two dimensions of CCA of the relationship between the distribution of (a) *Acidimicrobiaceae* bacterium A6 and (b) nitrogen oxidizing/reducing microorganisms vs. environment factors of all soil/sediment samples. W = wetland sediments; R = river sediments; F = forest soils; P = paddy soils

Figure. 3. Relative abundance of bacterial phyla for sample W11D during incubations with different DO levels. Detailed composition of *β-Proteobacteria* and *Actinobacteria* are shown in separated figures. (a) Sample a1, W11D subsample on day 0. (b) Sample a2, W11D subsample on day 120 of Incubation L-DO-0.02 (DO <0.02 mg · L⁻¹). (c) Sample a3, W11D subsample on day 120 of L-DO-0.8 (DO= 0.8 ~ 1.0 mg · L⁻¹).

b

β -Proteobacteria

Actinobacteria

c

β -Proteobacteria

Actinobacteria