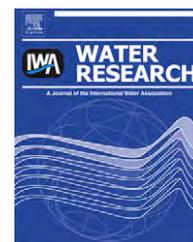


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Temporal and spatial distributions of ammonia-oxidizing archaea and bacteria and their ratio as an indicator of oligotrophic conditions in natural wetlands

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ABSTRACT

Ammonia-oxidizing organisms play an important role in wetland water purification and nitrogen cycling. We determined soil nitrification rates and investigated the seasonal and spatial distributions of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in three freshwater wetlands by using specific primers targeting the *amoA* genes of AOA and AOB and real-time quantitative polymerase chain reaction (qPCR). The nitrifying potentials of wetland soils ranged from 1.4 to 4.0 $\mu\text{g g}^{-1} \text{day}^{-1}$. The specific rates of ammonia oxidation activity by AOA and AOB at the Bee Hollow wetlands were 1.9 $\text{fmol NH}_3 \text{ cell}^{-1} \text{day}^{-1}$ and 36.8 $\text{fmol NH}_3 \text{ cell}^{-1} \text{day}^{-1}$, respectively. Soil nitrification potential was positively correlated with both archaeal and bacterial *amoA* abundance. However, the gene copies of AOA *amoA* were higher than those of AOB *amoA* by at least an order of magnitude in wetland soils and water in both summer and winter over a three year study period. AOB were more sensitive to low temperature than AOA. The *amoA* gene copy ratios of AOA to AOB in top soils (0–10 cm) ranged from 19 ± 4 to 100 ± 11 among the wetland sites. In contrast, the ratio of the wetland boundary soil was 10 ± 2 , which was significantly lower than that of the wetland soils ($P < 0.001$). The $\text{NH}_4^+ - \text{N}$ concentrations in wetland water were lower than 2 mg/L throughout the study. The results suggest that ammonium concentration is a major factor influencing AOA and AOB population in wetlands, although other factors such as temperature, dissolved oxygen, and soil organic matter are involved. AOA are more persistent and more abundant than AOB in the nutrient-depleted oligotrophic wetlands. Therefore, ratio of AOA *amoA* gene copies to AOB *amoA* gene copies may serve as a new biological indicator for wetland condition assessment and wetland restoration applications.

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1. Introduction

Wetlands are collectively comprised of swamps, marshes, bogs and fens that are inundated by surface water or groundwater. The protracted occurrence of flooding supports the growth of specially adapted plants (hydrophytes) and development of characteristic wetland soils (Mausbach and Parker, 2001). Wetlands featuring characteristic hydrology, hydric soil and vegetation play an important role in ecosystems and environmental sustainability. They provide important functions such as storm and flood control, sediment retention, nutrient removal, carbon storage, and wildlife and fisheries habitation (Cabezas and Comín, 2010; Fink and Mitsch, 2007; Huang and Pant, 2009; Richardson et al., 2011; Sooknah and Wilkie, 2004).

Maintaining and restoring the chemical, physical, and biological integrity of wetlands is one of the goals of the Clean Water Act in the United States. To maintain biological integrity, wetland biological assessment (bio-assessment) becomes essential and an index of biological integrity (IBI) is used to evaluate the health of wetlands (USEPA, 2002a). The IBI combines multiple indicators of biological condition into an index value, which can be compared to that of reference wetlands to determine the relative health of individual wetlands (USEPA, 2002a). A variety of biological assemblages such as algae, amphibians, birds, fish, macro-invertebrates, and vascular plants have been proposed to develop the IBI in wetland bio-assessment (USEPA, 2002a; b). Microorganisms such as bacteria and archaea have been rarely used in wetland bio-assessment because of their complexity and a lack of understanding of their occurrence in wetland soils and water. The rapid development of molecular techniques during the past decade, however, has made it possible to develop a new assessment tool using bacteria and archaea. Such a tool is expected to be more important and relevant to wetland biological assessment because wetland microorganisms play a key role in regulating biogeochemical fluxes across different types of wetlands (USEPA, 2002a).

Ammonia-oxidizing organisms including ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) are essential in nitrification processes that oxidize ammonia to nitrite and nitrate, the key and often rate-limiting step in nitrogen (N) removal and N cycling. AOB are often considered to be major contributors to soil ammonia oxidation (Prosser, 1990; Schleper and Nicol, 2010). *Nitrosomonas* and *Nitrospira* lineages are of prime importance in the brackish and freshwater wetland sediments and bogs (Coci et al., 2005; Morales et al., 2006). However, recent studies show that AOA are abundant in hot springs (de la Torre et al., 2008; Zhang et al., 2008), seawater (Francis et al., 2005; Lam et al., 2007; Mincer et al., 2007), marine sediments (Beman and Francis, 2006; Santoro et al., 2008), soils (Adair and Schwartz, 2008; Beman and Francis, 2006), and rhizosphere (Chen et al., 2008; Herrmann et al., 2008). Furthermore, AOA override AOB in several terrestrial and aquatic systems (Francis et al., 2005; Leininger et al., 2006; Martens-Habbena et al., 2009; Tourna et al., 2008).

Both AOA and AOB have the same functional *amoA* encoding the α -subunit of ammonia monooxygenase (AMO),

which catalyzes the first step in ammonia oxidation. The enzyme is encoded by the genes *amoA*, B, and C in AOB and AOA (Nicol and Schleper, 2006). They are considered to be fundamental players in ammonia oxidation in soils (Jia and Conrad, 2009; Offre et al., 2009). Notwithstanding, there has been continuing debate about the relevant importance of these groups in ammonia oxidation. In recent studies, although AOA were more abundant than AOB, bacteria, not archaea, were found to be the active ammonia oxidizers in agricultural soils, suggesting that population abundance alone cannot determine the role of AOA in ammonia oxidation (Di et al., 2009; Jia and Conrad, 2009). Other studies, however, suggest that AOA are well adapted to growth in the nutrient limiting (oligotrophic) environment (Martens-Habbena et al., 2009) with unique ecological or niche separation (Erguder et al., 2009; Gubry-Rangin et al., 2010; Santoro et al., 2008).

It is thus apparent that AOA and AOB are widespread in the environment. So far, the composition of the ammonia oxidizers and their activities have been investigated mainly in wastewater treatment systems (Cao et al., 2011; Park et al., 2006), lakes, rivers (Xia et al., 2004) and estuaries (García-Lledó et al., 2011; Herrmann et al., 2011), but rarely in natural wetland ecosystems. Little is known about the competition and interactions between the ammonia oxidizers under low nitrogen loading, temperature, and dissolved oxygen conditions. The objectives of this study were to determine the spatial and seasonal distributions of AOA and AOB in natural wetlands and to evaluate whether the ratio of AOA to AOB could be used for wetland condition assessment.

2. Materials and methods

2.1. Wetland site description

Three Missouri freshwater wetlands were selected for chemical and biological monitoring over a three year study period. Bee Hollow Conservation area, (Macon County, MO) is an oxbow complex containing 36 acres of land and holds water year-round, unless severe drought occurs. Essentially it witnesses no flooding because of an upstream lake (Long Branch Lake) and receives water mainly from upland surface drainages and rainfall. The wetland was infested with cattails and duckweeds. The wetland soil is a silty clay loam texture with organic C content of 2.9%. Van Meter State park (Saline County, MO) is a Missouri River floodplain marsh encompassing 151 acres, but it is not affected by Missouri River flooding because of levee protection. It receives water from upland drainages and precipitation with water volume controlled by a flood gate. The prominent vegetation around the monitoring station includes cattails and *Carex hyalinolepis* (shoreline sedge). The soil texture class is clay and the organic C content was measured to be 2.4%. Pershing State park (Linn County, MO) is a Locust Creek floodplain wet prairie comprising of 643 acres, shrub swamp and bottomland hardwood forest. It receives water from rainfall and Locust Creek flooding. Excessive flooding has been exacerbated by logjams, and as a result, increased sedimentation has occurred. The exotic invasive reed canary grass, buttonbush,

pin oaks and ash trees are some of the featuring vegetation at this location. The soil is silt loam with the organic C content of 1.6%. The soil samples extracted from the boundary of Van Meter was of alluvial nature and showed characteristics entirely different from the wetlands, suggesting accumulation of nutrients from the runoff and organic material being cyclic accumulated over time.

2.2. Soil and water field sampling and chemical analysis

Field sampling was conducted in the experimental installations located at each of the afore-mentioned wetland sites. Soil samples were collected using a push core sampler marked at 25 inches from the wetland sites in summer and winter seasons over a three year period. At each wetland site, soil was collected from the top layer (0–10 cm) and the lower layer (10–25 cm) from at least four different plots (i.e., >4 cores per site) using a sterile spatula, stored in heavy-duty sterile Ziploc bags, and placed on ice immediately after sampling. For comparison, soils from the wetland boundary impacted by agricultural practices were collected for chemical and microbial analyses as well. Water samples were collected from the same locations as the soil samples and stored in sterile plastic bottles. All water and soil samples were transferred immediately to the lab and stored at 4 °C before analysis. Water quality parameters such as pH, dissolved oxygen (DO), temperature, and phosphorous concentration were monitored onsite. In the lab, the samples were tested for chemical oxygen demand (COD), ammonia-N (NH_4^+ -N), nitrate-N (NO_3^- -N), nitrite-N (NO_2^- -N), and Total Kjeldal Nitrogen (TKN) following the Standard Methods (APHA, 2002). All tests were conducted in triplicate.

2.3. Microbial analysis

DNA was extracted from wetland soil samples (0.5 g) and 500 mL of water samples using Ultraclean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's protocol. For soil microbial analysis, the unpooled soils from each core were air dried and directly used for DNA extraction to assess the microbial richness at each wetland (Manter et al., 2010). In brief, around nine 0.5-g soil samples per wetland site were used (total volume, 4.5 g) for DNA extraction. The DNA concentrations were determined by measuring absorbance at 260 nm (A_{260}), assuming that 1 A_{260} unit corresponds to 50 μg of DNA per mL. We further confirmed the intact DNA by electrophoresis on a 1% agarose gel. Aliquots (10 μL) from each of the nine DNA extracts generated with the DNA isolation kit were then combined to produce a single pooled DNA sample for quantitative real-time PCR (qPCR) assay.

2.4. Quantitative polymerase chain reaction analysis

Quantitative real-time PCR (qPCR) assays were applied to determine the amount of genes encoding *amoA* in AOA and AOB in the wetland and boundary samples during the summer and winter seasons over a period of three years: August and December of 2009, February and June of 2010 and February and June of 2011. To determine the amount of AOB

amoA genes, primers *amoA*-1F (GGGGTTTCTACTGGTGGT) and *amoA*-2R (CCCCTCKGSAAAGCCTTCTTC) (Rotthauwe et al., 1997) were used and quantified with SYBR[®] Green PCR Master Mix (Applied Biosystems, New Jersey, USA) as a fluorescent dye. Each reaction was performed in a 25 μL volume containing 10 ng sample DNA, 20 μmol of each primer and 12.5 μL of SYBR[®] Green PCR Master Mix. PCR cycling conditions were as follows: 15 min at 95 °C, 45 cycles of 1 min at 95 °C, 1 min at 54 °C, 1 min at 72 °C, followed by final extension reaction for 10 min at 72 °C and a dissociation step at 95 °C for 15 s. PCR product specificity was confirmed on 2% agarose gels showing specific product bands at the expected size of 491 bp (Rotthauwe et al., 1997).

For AOA, the crenarchaeal *amoA* gene copy numbers were also quantified with SYBR[®] Green PCR Master Mix as a fluorescent dye. Clones positive for the crenarchaeal *amoA* gene were amplified using the PCR with the primers CrenAmoAQ-F (5' GCA RGT MGG WAA RTT CTA YAA) and CrenAmoAModR (5' AAG CGG CCA TCC ATC TGT A) (Mincer et al., 2007) that generated a PCR product of ca. 200 bp long. Each reaction was performed in a 25 μL volume containing 10 ng sample DNA, 10 μmol of each primer and 12.5 μL of SYBR[®] Green PCR Master Mix. PCR cycling conditions were as follows: initial denaturation for 15 min at 94 °C, amplification 45 cycles, denature for 15 s at 94 °C, primer annealing for 30 s at 52 °C, extension for 30 s at 72 °C and 1 s at 78 °C to assure stringent product detection, followed by final extension reaction for 15 min at 72 °C and a dissociation step at 95 °C for 15 s (Mincer et al., 2007).

For comparison purposes, qPCR was used to quantify bacterial 16S rRNA gene copy number using primers 1055f (ATGGCTGTCGTCAGCT) and 1392r (ACGGCGGTGTGTAC) (Ferris et al., 1996). To reduce potential false positive signals, TaqMan-based detection was applied and the TaqMan probe 16S Taq1115 (6-FAM)-CAACGAGCGCAACCC-(TAMRA) was modified from the 1114f primer. The PCR Mix had a total volume of 25 μL consisting of TaqMan Universal PCR Master Mix, 20 μmol primers, 10 μmol TaqMan probe and 5 μL of the DNA sample. The PCR program for 16S rRNA bacterial quantification was 10 min at 95 °C, 45 cycles of 30 s at 95 °C, 60 s at 50 °C, and 45 s at 72 °C.

The copy numbers of *amoA* gene of AOA and AOB and 16S rRNA in all the samples were determined in triplicate and all PCR runs included negative controls (containing no DNA) to avoid potential contamination. The abundance of target genes in each sample was calculated by the parallel quantitative PCR of dilutions of the standards and by comparing threshold cycles (Ct) obtained in each PCR run with those of DNA standards. Real-time PCR assays were performed on a 7500 Real-Time PCR System (Applied Biosystems, CA) equipped with a fluorescence detector and 7500 SDS system software version 1.4 (Applied Biosystems, CA). The threshold cycle is defined as the cycle at which the fluorescence intensity crosses over a level where the amplification enters a logarithmic growth phase. Ct of each PCR reaction was determined automatically by detecting the cycle at which the fluorescence exceeded the calculated threshold. The data were linear for 10^1 – 10^8 *amoA* gene copies for per gram of soil or per mL of water for soil and water samples, respectively (lowest detection limit being 10^1 copies/g or copies/mL). To make standard curves plotted as Ct

versus log concentration of template, plasmids containing copies of 16S rRNA of total bacteria, crenarchaeal *amoA* gene of *Nitrosopumilus maritimus* and the bacterial *amoA* gene of *Nitrosomonas europaea* were constructed and used as standards. Amplified fragments from extracted DNA were cloned using a pCR4-TOPO TA Cloning kit (Invitrogen, CA). Cloned inserts were amplified by using primers that targeted vector sequences as described in details in Di et al. (2010), and in the manual for the kit. In all the cases, the R^2 of the standard curves were >0.99 .

2.5. Nitrification potential in wetland soils

Soil nitrification potential was determined according to the shaken-slurry method (Hart et al., 1994) with slight modification. Twenty grams of air dried soil from each wetland sample was preincubated at room temperature and then mixed with phosphate buffer (0.25M K_2HPO_4 ; 0.03M KH_2PO_4) containing ammonium sulfate (0.03M) in a total volume of 100 mL in a 125 mL Erlenmeyer flask. An aliquot (2 mL) of potassium chlorate solution (final concentration = 9 mM) was added to stop the second step of nitrification (oxidation of nitrite to nitrate). The flask was shaken on a rotary mixer for 5 min and then allowed to stand for another 5 min. The upper part of the slurry was filtered and the filtrate was stored in a refrigerator. The remnant soil in the flask was incubated at room temperature for 48 h. Nitrite concentrations at the beginning and end of the incubation were determined using the colorimetric nitrite assay at 543 nm (APHA, 2002), and the nitrification potential was calculated in $\mu\text{g NO}_2^- \text{N/g}$ of dry soil/day.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was applied to compare the differences in chemical and microbial properties between groups at a significance level (α) of 0.05. Correlation analysis of microbial abundances within soil and water samples was performed using linear regression analysis.

3. Results and discussion

3.1. Wetland water quality and nitrogen concentration

The concentrations of TKN, ammonia nitrogen ($\text{NH}_4^+ \text{N}$) and nitrate-nitrogen ($\text{NO}_3^- \text{N}$) in water at the wetland sites, namely Bee Hollow, Pershing, Van Meter and the wetland boundary for the winter and summer seasons over the three year time period were averaged and are shown in Fig. 1. The chemical properties in these selected wetland sites were generally consistent with the results reported by others (Johnston et al., 1984). During the winter sampling seasons, the water temperature ranged from 4 °C to 6 °C and the samples collected from Pershing, Van Meter and the boundary of Van Meter displayed a neutral (pH = 7.2) to basic (pH = 8.4) pH values. The TKN concentrations at Pershing and Van Meter wetlands were both 4.7 ± 0.2 mg/L while the boundary wetland TKN was measured at 2.3 ± 0.5 mg/L. Consistently, the concentrations of $\text{NH}_4^+ \text{N}$ in Pershing and Van Meter

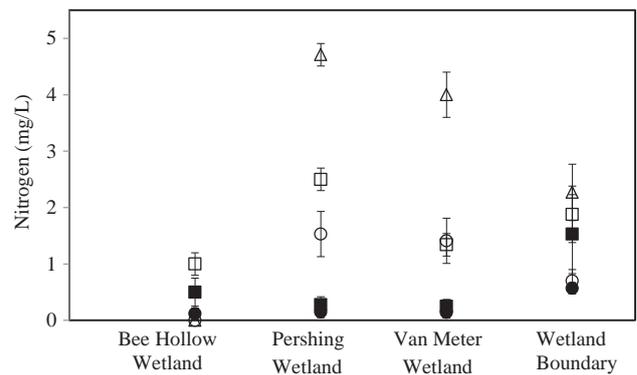


Fig. 1 – Average concentrations of nitrogen species in wetland water in the winter (open symbols) and summer seasons (filled symbols). TKN (triangles), ammonia (circles), nitrate (squares). Error bars indicate one standard deviation of the average over the three year period.

wetlands were 1.5 ± 0.4 mg/L and 1.4 ± 0.4 mg/L, respectively (Fig. 1).

There was a significant difference in the $\text{NH}_4^+ \text{N}$ and $\text{NO}_3^- \text{N}$ concentrations between summer and winter in all sites ($P = 0.002$ and $P = 0.003$, respectively). In summer, the low concentrations of $\text{NH}_4^+ \text{N}$ (0.1 ± 0.1 mg/L to 0.5 ± 0.4 mg/L) and $\text{NO}_3^- \text{N}$ at 0.3 ± 0.1 mg/L to 1.5 ± 0.7 mg/L in wetland water indicated good nitrification and denitrification. Furthermore, low dissolved oxygen concentrations (0.4–3.6 mg/L) in water were recorded at the wetlands but not at the boundary water (where the DO was 5 mg/L). The predominant plant species (cattails and duckweeds) in the Bee Hollow wetland with high soil organic carbon (2.9%) blocked light to algae and other aquatic plants that could be oxygen providers while microbial respiration at high temperatures in summer continued to consume oxygen in water. As a result, the Bee Hollow wetland had the lowest dissolved oxygen level (0.4 mg/L). Hence, all wetland water bodies in this study exhibited low concentrations of nitrogen species and low DO, especially in the summer, thereby creating a nutrient limiting ecological niche that could preferably support the growth of AOA (Martens-Habben et al., 2009), as described in detail below.

3.2. Microbial abundance in wetland soils

The AOB and AOA gene copies in the wetland soils over three years were averaged and their seasonal and spatial changes in winter and summer are shown in Figs. 2 and 3, respectively. In the winter seasons, the bacterial 16S rRNA gene copy numbers ranged from $1.02 \pm 0.5 \times 10^8$ to $1.4 \pm 0.5 \times 10^8$ copies/g dry soil in the top layer soils (top soils). For comparison, the AOB gene copy numbers ranged from $1.1 \pm 0.5 \times 10^4$ to $1.01 \pm 0.5 \times 10^7$ copies/g dry soil, while the AOA numbers ranged from $1.5 \pm 0.2 \times 10^6$ to $1.7 \pm 0.2 \times 10^7$ copies/g dry soil (Fig. 2a). At the same sampling site, the gene copies of AOA were higher than AOB by more than an order of magnitude, indicating the higher resistance of AOA to low temperature than AOB. At the Pershing wetlands, AOB were completely washed out of the top soils in winter. The lower layer soils (subsoils) had

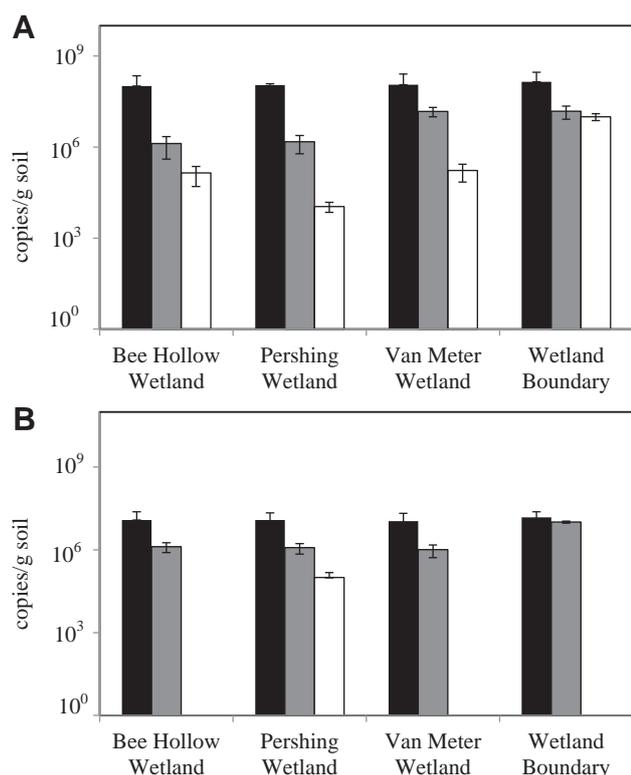


Fig. 2 – The gene copy numbers for *amoA* of AOB and AOA and bacterial 16S rRNA in wetland top soils (A) and bottom soils (B) in winter. Error bars indicate one standard deviation of the average over the three year period ($n = 9$). 16S rRNA bacteria, black; AOA, gray; AOB, white. Except for Pershing wetland, no bacterial *amoA* gene copies were detected in bottom soils in winter.

relatively lower gene copy numbers of AOB and AOA than the top soils. AOA seemed to thrive better than their bacterial counterpart (AOB) as the DO concentration decreased quickly with soil depth (Cook, 1995).

Similar trends of the spatial distribution of microbial population in the wetland soils were observed in the summer (Fig. 3). The gene copies of bacterial 16S rRNA ranged from $1.02 \pm 0.5 \times 10^{12}$ to $1.1 \pm 0.5 \times 10^{12}$ copies/g dry soil throughout the wetland top soils, which was statistically higher than those in winter ($P = 0.003$). Similarly, the AOB gene copies increased, ranging from $1.2 \pm 0.2 \times 10^6$ to $1.9 \pm 0.1 \times 10^7$ copies/g dry soil in top soils while the AOA copies ranged from $1.25 \pm 0.5 \times 10^7$ to $1.5 \pm 0.2 \times 10^8$ copies/g dry soil in top soils (Fig. 3a). These numbers are in accordance with those reported in marsh lands (Moin et al., 2009). Again, in each wetland site, the hydric soil harbored more AOA than AOB. The boundary soil samples posed a similar trend of increase in AOA and AOB gene copies with AOA *amoA* gene copy numbers higher than those of AOB ($P = 0.002$). In spite of a 12-month interval among the three years of sampling, all samples collected displayed similar microbial results with low error bars (Figs. 2, 3), suggesting that AOA and AOB compositions were very stable at these wetland sites.

The spatial change of microbial gene copy numbers at different soil depths was significant. In general, there were

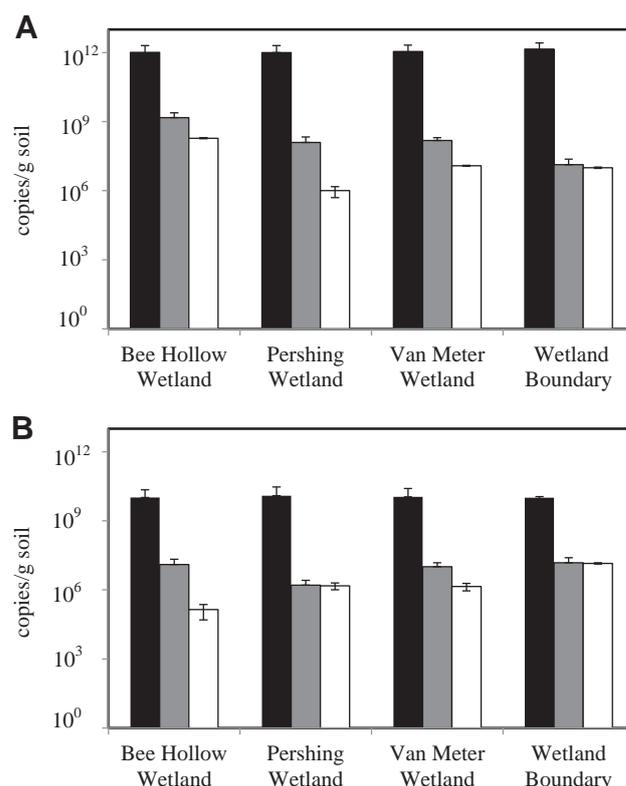


Fig. 3 – The gene copy numbers for *amoA* of AOB and AOA and bacterial 16S rRNA in wetland top soils (A) and bottom soils (B) in summer. Error bars indicate one standard deviation of the average over the three year period ($n = 9$). 16S rRNA bacteria, black; AOA, gray; AOB, white.

more bacteria available in the top soil than in the subsoil samples owing to the available nutrients and oxygen. More anaerobic conditions in the subsoils could lead to the accumulation of organic matter which stimulated AOA *amoA* transcript levels (Chen et al., 2008). Temperature, however, appears to be a more significant factor affecting spatial bacterial distribution. Similar results were observed for soil archaeal community with significant reduction of AOA from the top soils in winter (Fig. 2a) compared to those in summer (Fig. 3a). Since the subsoil was less prone to temperature changes, we were able to quantify the AOA in winter (Fig. 2b).

3.3. Microbial abundance in wetland water

The microbial gene copy numbers during summer and winter in the wetland water samples, averaged over a three year study period are shown in Fig. 4. The bacterial 16S rRNA gene copy numbers ranged from $1.0 \pm 0.5 \times 10^7$ to $1.8 \pm 0.5 \times 10^7$ copies/mL throughout the wetland water in summer, which was statistically higher than those in winter ($P = 0.001$). The results are consistent with a recent study in a simulated creek ecosystem where 10^5 – 10^7 gene copy numbers were reported (Herrmann et al., 2011). The AOB and AOA gene copies ranged from $1.0 \pm 0.1 \times 10^5$ to $0.4 \pm 0.5 \times 10^7$ copies/mL and $1.9 \pm 0.5 \times 10^6$ to $1.0 \pm 0.2 \times 10^7$ copies/mL in summer, respectively (Fig. 4a). By using Pearson's correlation, we

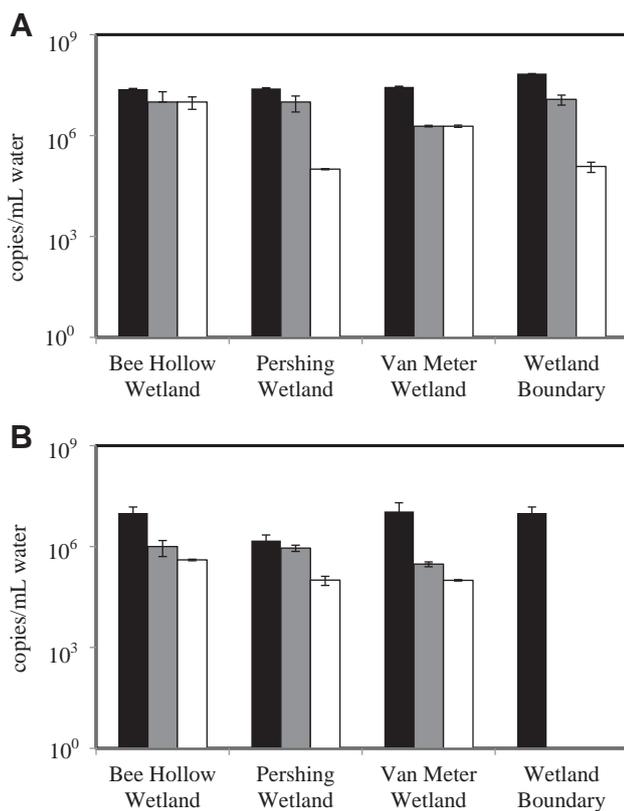


Fig. 4 – The gene copy numbers for *amoA* of AOB and AOA and bacterial 16S rRNA in wetland water in summer (A) and winter (B). Error bars indicate one standard deviation of the average over the three year period ($n = 9$). 16S rRNA bacteria, black; AOA, gray; AOB, white. Both AOA and AOB were missing in wetland boundary water samples.

established a linear relationship of archaeal or bacterial *amoA* gene copy numbers between the wetland water samples and top soil samples in summer with R^2 ranging from 0.83 to 0.99 (Table 1), suggesting that there is a linear relationship between the abundance of ammonia-oxidizing organisms in soils and in wetland water.

3.4. Links between potential nitrification activity and the abundance of ammonia oxidizers in wetland soils

The soil nitrification rates in Bee Hollow, Pershing and Van Meter wetlands were $4.0 \pm 0.3 \mu\text{g g}^{-1} \text{day}^{-1}$, $1.4 \pm 0.13 \mu\text{g g}^{-1} \text{day}^{-1}$ and $3.2 \pm 0.2 \mu\text{g g}^{-1} \text{day}^{-1}$ respectively. While the variation may be

associated with soil textures in the wetlands, the results were consistent with the rates in other studies, with the range of $0.1\text{--}5 \mu\text{g g}^{-1} \text{day}^{-1}$ in forest and cropland soils (Huang et al., 2012; Isobe et al., 2012). With the assumption of 2.5 copies AOB *amoA* gene per cell and 1 copy archaeal *amoA* gene per cell (Harms et al., 2003; Santoro et al., 2010), the specific rates of ammonia oxidation activity by AOA and AOB at the Bee Hollow wetlands were $1.9 \text{ fmol NH}_3 \text{ cell}^{-1} \text{day}^{-1}$ and $36.8 \text{ fmol NH}_3 \text{ cell}^{-1} \text{day}^{-1}$, respectively. These results were consistent with other studies, where the cell-specific rates for AOA and AOB were estimated to be between 0.2 and $15 \text{ fmol cell}^{-1} \text{day}^{-1}$ and between 1 and $6 \text{ fmol cell}^{-1} \text{day}^{-1}$, respectively (Santoro et al., 2010; Wuchter et al., 2006). The soil nitrification potential was correlated with AOB *amoA* gene copies ($R^2 = 0.98$) better than with AOA *amoA* gene copies ($R^2 = 0.91$), suggesting that AOA may not use NH_3 oxidation as their sole source of energy and may grow heterotrophically (Di et al., 2010; Long et al., 2012). Others, however, did not find significant correlation between nitrification rates and the abundance of either AOA or AOB *amoA* genes (Wankel et al., 2011). Although little is known about the relative contribution of AOB and AOA to nitrification activity, recent studies indicate higher nitrification rates at the lower-nutrient sediments than at the nutrient-rich sites (Wankel et al., 2011). Furthermore, sites with high nitrification primarily contained genes of *Nitrosospira*-like bacteria and phylogenetically diverse AOA whereas sites with low nitrification rates were dominated by *Nitrosomonas*-like *amoA* sequences and archaeal *amoA* sequences similar to those from the soil (Wankel et al., 2011). Further research is needed to determine the relative contribution of AOB and AOA to the process of nitrification in different natural wetland soils.

3.5. Implications of AOA/AOB ratio and its potential application in wetland biological assessment

Archaeal *amoA* was more abundant than bacterial *amoA* in all the wetlands in this study. Low nitrogen loading and high organic carbon appeared to facilitate nitrification in an oligotrophic environment with the abundance of the archaeal ammonia oxidizers, which have high affinity for $\text{NH}_4^+\text{-N}$, as shown by an extremely low half saturation constant ($K_m = 133 \text{ nM total ammonium}$) (Martens-Habbena et al., 2009). The ratios of AOA *amoA* gene copies to AOB *amoA* gene copies (hereafter referred to as AOA/AOB) in top soils (0–10 cm) for the Pershing and Van Meter wetlands were 19 ± 4 and 21 ± 6 , respectively (Fig. 5). This ratio was the highest at 100 ± 11 at the Bee Hollow where the soil organic carbon content was the highest among the wetlands studied. On the contrary, the AOA/AOB in the boundary top soil

Table 1 – Linear correlation between the gene copy numbers of 16S rRNA bacteria, AOA, and AOB in wetland water and top soil samples.

Wetland sites	16S rRNA bacteria		AOA		AOB	
	Water (copies/mL)	Soil (copies/g)	Water (copies/mL)	Soil (copies/g)	Water (copies/mL)	Soil (copies/g)
Bee Hollow	$2.4 \pm 0.5 \times 10^7$	$1.02 \pm 0.5 \times 10^{12}$	$2.0 \pm 0.1 \times 10^7$	$0.5 \pm 0.6 \times 10^8$	$1.0 \pm 0.5 \times 10^7$	$1.9 \pm 0.1 \times 10^7$
Pershing	$2.8 \pm 0.5 \times 10^7$	$1.0 \pm 0.5 \times 10^{12}$	$1.0 \pm 0.5 \times 10^7$	$1.2 \pm 0.5 \times 10^8$	$1.0 \pm 0.1 \times 10^5$	$1.0 \pm 0.2 \times 10^6$
Van Meter	$1.8 \pm 0.5 \times 10^6$	$1.1 \pm 0.5 \times 10^{12}$	$1.2 \pm 0.5 \times 10^6$	$1.5 \pm 0.5 \times 10^7$	$1.0 \pm 0.1 \times 10^6$	$1.2 \pm 0.1 \times 10^7$
R^2	0.83		0.99		0.98	

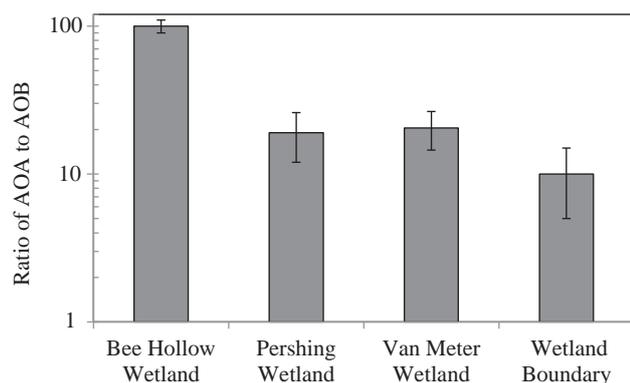


Fig. 5 – Ratio of the *amoA* gene copy number from AOA and AOB in wetland top soils in summer. Error bars indicate one standard deviation of the average over the three year period.

impacted by agricultural practices was 10 ± 2 , which was significantly lower than the wetland soils ($P < 0.001$). The $\text{NH}_4^+ - \text{N}$ concentrations in wetland water were lower than 2 mg/L throughout the study (Fig. 1). The increased relative abundance of AOA in the wetland soils under low-nutrient conditions suggested that such niche differentiation may form the grounds for the coexistence of AOA and AOB with the dominance of AOA (Lam et al., 2007). The changing patterns in AOA and AOB abundances in response to nutrient inputs were in line with recent findings that AOA prefers low-nutrient environments whereas AOB dominates in high nutrient soils (Schleper, 2010; Verhamme et al., 2011). Ammonia-oxidizing archaea are adapted to low-nutrient environments and can be inhibited by ammonia at high concentrations (Pratscher et al., 2011). Indeed, there were more AOB than AOA in aerobic sludge samples with high nitrogen loading. The ratio of AOB to AOA increased significantly up to ~ 2000 to ~ 4000 with increasing ammonium loading, touting AOB as the main players in the nitrification reactors (Ye and Zhang, 2011). In a constructed wetland system treating wastewater containing high ammonium concentrations, more AOB were detected than the archaeal counterpart (Sims et al., 2012). Under eutrophic conditions, the AOB *amoA* genes outnumbered the archaeal *amoA* genes with higher diversity of AOB (Wei et al., 2011). Hence, ammonium concentration clearly is a major factor influencing AOA and AOB population (Gregory et al., 2010; Höfferle et al., 2010).

The highest AOA/AOB at the Bee Hollow wetlands coincided with the highest soil organic carbon in the wetland, suggesting that the high soil organic carbon content and low DO assisted AOA to survive better than AOB (Moin et al., 2009). AOA were recently shown to be actively involved in autotrophic ammonia oxidation and exhibited a diverse and dynamic activity in carbon assimilation in an agricultural soil, presumably via the hydroxypropionate–hydroxybutyrate cycle (Pratscher et al., 2011). However, studies also demonstrated that AOA in soil and sludge were heterotrophic or mixotrophic (Jia and Conrad, 2009; You et al., 2009). It is known that *Crenarchaeota* are capable of using dissolved organic carbon, such as amino acids, as a carbon source (Herndl et al., 2005; Wuchter et al., 2006). Other factors may also influence

the AOA/AOB ratio in wetland soils (Mußmann et al., 2011). Low DO appeared to be one of the most important factors that favor the growth of AOA (Erguder et al., 2009). At the low-oxygen fresh and brackish stations, the crenarchaeal *amoA* is nearly 10 times more abundant than bacterial *amoA* (Santoro et al., 2008). Factors such as temperature, soil pH, salinity and C/N ratios (Mosier and Francis, 2008) may also affect the AOA/AOB ratio in the different wetland soils. For instance, archaeal *amoA* abundance and diversity increased with soil pH (Gubry-Rangin et al., 2011). Further study is necessary to verify the suspected factors influencing AOA/AOB in a variety of wetlands, including riverine floodplains, marshes, meadows, shrub swamps, bogs and fens, and wetlands in arid regions.

The biological indicators of ecosystem integrity are increasingly being sought out for its use in wetland biological assessment and wetland restoration. The most common and effective way to assess the health or biological condition of wetlands is to measure the biological community conditions first and in concert with physical and chemical assessment. Our findings suggest that AOA are more pervasive under low DO conditions and more abundant than AOB in the wetlands under the nutrient-depleted oligotrophic conditions. Although it is difficult to use at low temperatures because of higher sensitivity of AOB than AOA, the ratio of soil AOA to AOB is a promising biological indicator of “wetland health” to infer eutrophic or oligotrophic wetland conditions. Furthermore, by determining the diversity and abundance of AOA or AOB in the wetland systems coupled to nitrification rate measurements, valuable insights into the physiological and biological tolerances and ecological niches of the different nitrifying organisms can be drawn (Wankel et al., 2011).

4. Conclusions

The nitrifying potentials of wetland soils ranged from 1.4 to $4.0 \mu\text{g g}^{-1} \text{day}^{-1}$. The specific rates of ammonia oxidation activity by AOA and AOB at the Bee Hollow wetlands were $1.9 \text{ fmol NH}_3 \text{ cell}^{-1} \text{day}^{-1}$ and $36.8 \text{ fmol NH}_3 \text{ cell}^{-1} \text{day}^{-1}$, respectively. There was a strong correlation between the abundance of AOA and AOB and nitrification activity in wetland soils. The summer season depicted the tendency of more complete nitrification owing to higher AOA and AOB numbers than in winter. Although factors such as temperature, dissolved oxygen conditions and soil organic matter could affect the abundance of ammonia-oxidizing organisms, the results suggest that AOA are more persistent and more abundant than AOB in the nutrient-depleted (oligotrophic) wetlands. The ratio of AOA to AOB may therefore serve as a new biological indicator for wetland biological assessment and wetland restoration applications.

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