Ammonia Oxidation Potential and Microbial Diversity in Sediments from Experimental Bench-Scale Oxygen-Activated Nitrification Wetlands

By

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ABSTRACT

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Ammonia pollution, commonly from sewage treatment plants and agricultural activities, can degrade surface waters by causing eutrophication and exhibiting toxicity to aquatic biota. Constructed treatment wetlands can be used to treat a wide array of waste waters, but low oxygen concentrations characteristic of these systems will limit ammonia removal because ammonia must be first oxidized to nitrate before being denitrified to nitrogen gas. My study evaluated the effects of dissolved oxygen enhancement on ammonia oxidation and microbial community diversity in bench-scale wetland mesocosms. Sediments were extracted from oxygenated and non-oxygenated (control) wetland microcosms. Oxygen-activated wetland microcosms underwent side-stream oxygenation with pure oxygen gas and had DO levels > 10 mg/L; DO in controls was typically < 1 mg/L. Potential rates of ammonia oxidation measured in sediments were significantly higher in oxygenated mesocosms (2.6 ± 0.80 mg-N/g-dw•d) versus control mesocosm (0.48 ± 0.20 mg-N/g-dw•d). These rates were higher than those typically measured in agricultural soils (0.0012-1.9 mg-N/g-dw•d) and aquatic sediments (0.0010-1.2 mg-N/g-dw•d). Bacterial 16S rDNA libraries were generated using universal PCR primers. *Nitrosomonas*, a genus of nitrosofying bacteria typically found in nitrifying wastewater treatment plants that are commonly responsible ammonia oxidation, were only observed in the oxygenated systems. Other notable differences in microbial diversity included more species of filamentous cyanobacteria, sulfur-reducing bacteria, and denitrifiers in the low DO control mesocosms. The

combination of high ammonia oxidation potential and the presence of ammonia oxidizing bacteria in sediments from the oxygenated mesocosm support the hypothesis that oxygenating wetland sediments supports higher rates of biological ammonia oxidation in the experimental wetlands.

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INTRODUCTION

Nitrification, the biological oxidation of ammonia to nitrate, is an increasingly important removal mechanism used in a number of treatment processes to control ammonia pollution. Nitrification is a two-step, oxidative process where ammonia (NH_4^+) is converted to nitrite (NO_2^-) that is subsequently oxidized to nitrate (NO_3^-). This process occurs in terrestrial, aquatic and sedimentary soils across the globe. The overall process is carried out by two phylogenically different groups of obligate aerobes: nitrosofying bacteria and nitrifying bacteria (Schmidt, 1982). Nitrosofying bacteria or ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrite in the first step of the process. Participating microbial genera commonly found in natural ecosystems and engineered treatment systems include *Nitrosomonas, Nitrosospira* and *Nitrosococcus*. Nitrifying bacteria or nitrite oxidizing bacteria (NOB) are responsible for oxidizing nitrite to nitrate. Common genera responsible for this in natural and constructed systems include *Nitrobacter* and *Nitrospira*.

Substrate concentrations at each step can limit total nitrification. Because nitrite is converted to nitrate faster then ammonia is converted to nitrite, overall nitrification rates are largely limited by ammonia oxidation (Schmidt, 1982; Caffrey *et al*., 2007; Kadlec and Knight, 1995), indicating that AOB are comparatively more sensitive to environmental factors, such as pH and temperature, and substrate concentrations (Schmidt, 1982). A number of studies have indicated that ammonia and oxygen utilization rates differ among species will affect species dominance and biomass yield (Limiyakorn *et al.*, 2007; Metcalf and Eddy, 2003; Geets *et al*., 2006; Tchobanoglou*s et al.*, 2003; Gorra *et al*., 2007). While bacterial species do oxidize substrates at different rates, temperature likely plays a larger role in oxidation rates compared

with species dominance, substrate concentration and pH. The optimum temperature range for nitrification is 20-40 ˚C (Schmidt, 1982) with lower temperature significantly decreasing AOB activity (Groeneweg *et al.*, 1994). High temperatures can also inhibit ammonia oxidation. Oxygen solubility decreases as temperature increases, and although nitrification can proceed at dissolved oxygen (DO) concentrations as low as 0.05 mg-O_2/L (Abeliovich, 1987), low oxygen concentrations are not favorable. High temperatures also increase heterotrophic respiration requirements, further reducing oxygen concentrations.

The successful use of constructed treatment wetlands (CTWs) to treat N pollution in point and non-point discharges has increased substantially over the past 20 years (Kadlec and Knight, 1996; Mitsch *et al*., 2000; Horne and Fleming-Singer, 2005). Anthropogenic activities have elevated nitrogen (N) discharges to aquatic systems thereby presenting us with a wide range of challenging management issues. N pollution, mostly resulting from the extensive use of N fertilizer, poses a number of serious threats to surface and ground water quality. One of the primary environmental impacts of N pollution from eutrophication of surface waters including excessive phytoplankton growth, depressed DO levels, and fish kills. Estuaries and other coastal waters, such as the Hood Canal, Washington (Newton *et al*., 2005) and waters as the mouth of the Mississippi River (Weir, 2005), are particularly sensitive to N pollution because primary productivity is N limited. N in the form of ammonia can also be extremely toxic to aquatic biota, especially during algal blooms when high pH favors the formation of toxic un-ionized ammonia (Thurston *et al*., 1981). From a human health perspective, nitrate pollution in groundwater poses a risk for methemoglobinemia or 'blue baby syndrome' in infants. Roughly 7% of US drinking water wells have been shut down because of nitrate contamination (Horne, 2001). In Washington State, 1.5% of public water systems exceed the nitrate standard of 10 mg-N/L; the rate is as high

as 20-25% for single family wells in heavily polluted agricultural areas in the Tri-Cities, Spokane County, and Whatcom County (GWPC, 1996). Because wetlands are predominately reducing environments, conventional CTWs are not very effective at removing ammonia because an oxidative step (nitrification) is required to initiate the process before a reductive step (denitrification) (Kadlec and Knight, 1996; Vymazal, 2005). Ammonia removal in CTWs requires around five times the surface area needed to remove nitrate alone (Palmer and Beutel, 2009). This critical performance limitation has led to a number of investigators to examine the feasibility of increasing rates of nitrification in CTWs by enhancing DO, but levels in wetlands with limited success (Cottingham *et al*., 1999; Thullen *et al*., 2002; Wu *et al*., 2001; Jamieson *et al*., 2003).

Palmer and Beutel (2009) evaluated a novel approach to enhance ammonia removal in CTWs through the use of side-stream oxygenation of wetland water using pure oxygen gas, a process they termed ‘oxygen-activated nitrification wetlands. Oxygenation has been used to enhance DO levels in lakes, reservoirs and rivers (Beutel and Horne, 1999; Speece, 1996), but it has not been used to date in CTWs. Using replicate bench-scale wetland mesocosm loaded with synthetic secondary effluent (10 mg-N/L ammonia), Palmer and Beutel (2009) found that side-stream oxygenation resulted in near complete conversion of influent ammonia to nitrate; areal removal rates of ammonia were an order of magnitude greater in oxygen-activated wetland mesocosms compared to non-oxygenated control mesocosms.

The objective of the current applied research project was to evaluate how oxygenation affects ammonia oxidation potential and microbial diversity is mesocosm sediments. Sediments were the focus of the study because nitrification rates tend to be much higher in sediments versus water because sediments provide bacteria with a substrate on which to grow at relative high

densities (Schmidt, 1982). Sediment bacteria dominated the nitrification process in aerobic wetlands becuase the water column is so shallow, although the water column could dominate if the sediment-water interface was fully anaerobic. The working hypothesis of this study is that, relative to low oxygen conditions, oxygenation in wetlands loaded with secondary effluent (i.e., high ammonia and low biochemical oxygen demand) will select for a unique microbial cohort in sediments with high capacity of nitrification. To examine this hypothesis, I collected sediment from oxygen-activated and non-oxygenated wetland mesocosms and: (1) performed a set of short-term nitrification assays in which ample ammonia was added to sediment slurries, chlorate was used to block the bioconversion of nitrite to nitrate, and the rate of nitrite accumulation was used to estimate oxidation potential, and (2) identified bacterial 16s rDNA using traditional PCR libraries and analysis relative to Gen Bank resources. With the use of N fertilizer predicted to increase three-fold over the next forty years (Tilman *et al*., 2001), it is imperative to develop and evaluate novel strategies to control N pollution. A better understanding of the microbial ecology of oxygen-activated nitrification wetlands will help inform such efforts.

METHODS

Mesocosm Setup

Four experimental wetland mesocosms were constructed in the laboratory during the summer of 2007 (Fig. 1). Mesocosms consisted of glass aquariums (50.8 cm (l) x 25.4 cm (w) x 45.7 cm (h)) filled with plants (*Typha* spp.), associated mineral sediment (organic content < 3 %) and water. All mesocosm contents were collected from a mature CTW in Moscow, Idaho where ammonia oxidation was active. Thickness of the sediment-rhizome zone was approximately 20 cm and overlying water depth was 23 cm. Water volume in each mesocosm was 29.5 L and

surface area was 0.129 m^2. Approximately 17 plants were placed in each mesocosm yielding a plant density 134 plants/m^2. The mesocosms were fed synthetic secondary effluent composed of de-ionized water, dried cheese whey, ammonia chloride, and sodium bicarbonate. The influent had a chemical oxygen demand of 20 mg/L, a biological oxygen demand of 10 mg/L, and a total N concentration of 10 mg-N/L, which consisted almost entirely of ammonia. The average flow rate was 5.6 L/d resulting in a hydraulic retention time of 5 d and a hydraulic loading rate of 4.3 cm/d, values typical of high rate CTW systems (Mitch and Jørgensen, 2004). Mesocosms were exposed to natural light and supplementary indoor plant lighting for 12 hr/d. Room temperature was maintained near 20 ^oC for the duration of the experiment, which lasted about 8 weeks. Additional details of mesocosm construction and operation can be found in Palmer and Beutel (2009).

Duplicate mesocosms underwent two different treatments, oxygenation (Oxygen A, Oxygen B) and no oxygenation (Control A and Control B). In oxygen-activated mesocosms, a side-stream of water was pumped out of the influent end of the mesocosm, bubbled with pure oxygen gas, and returned to the mesocosm (Fig. 1). DO levels in the oxygen-activated and control mesocosms ranged from 5-20 mg/L and < 0.5 mg/L, respectively.

Ammonia Oxidation Potential

Ammonia oxidation potentials were measured using the short-term nitrification assay described by Hart *et al.* (1994). Approximately 15 g of wet surficial sediment was collected in the last weeks of the experimental incubations from the entrance, middle and exit of the mesocosms (Figure 1). Sediment was placed in a 250 ml Erlenmeyer flask with 90 ml of 0.5 mM phosphate buffer, 0.2 ml of 0.25 M ammonium sulfate, and 1 ml of 1.0 M potassium chlorate. Chlorate blocks the biological conversion of nitrite to nitrate (Belser and Mays, 1980;

Torstensson, 1993; Hoffman *et al*., 2007; Smorczewski and Schmidt, 1991). With the conversion of ammonia to nitrite being the rate-limiting step in overall nitrification (Chu *et al*., 2008, Limpiyakorn *et al*., 2007), nitrite accumulation is an analog for nitrification or ammonia oxidation potential. Sediment slurries were placed on a shaker table at 200 rpm for 12 hours. Aliquots of 5 mL were removed from each flask approximately every 3 hours. Samples were filtered through 0.45 μm filters and analyzed immediately for nitrite concentration using standard colorimetric techniques (APHA, 1998). Sediment samples were also dried at 105 ^{o}C to determine dry weight, and ammonia oxidation rates were normalize to sediment dry weight (dw). Ammonia oxidation potential in mg-N/g-dw/d was calculated as the slope of the linear regression of accumulated nitrite mass versus time divided by sediment dry weight.

Microbial Diversity

Small samples (~1-2 g) of surficial sediment were carefully collected near the entrance of the wetland mesocosm (Fig. 1). The sediments were frozen at -80 ˚C until DNA extraction. DNA was extracted from centrifuged sediment samples using the MoBIO Laboratories UltraClean Soil DNA Kit (MO BIO Laboratories, Carlsbad, CA) and protocol. The manufacturer's protocol was modified slightly: soil samples were incubated at 70 ˚C for 10 minutes after the addition of solution S1, the prescription for samples that are difficult to lyse, and the samples were rinsed several times with solution S4 to ensure high quality DNA. Extracts were checked by agarose gel electrophoresis.

16s rRNA sequences were amplified from the purified genomic DNA using the universal bacteria primers 16s 27F (5'-AGAGTTTGA TCCTGGCTCAG-3') and 16s 805R (5'-CCGTCAATTCCTTTRAGTTT-3') (Besser *et al*., 2008) by PCR (50 uL, including 5 uL DNA

template (~ 5 ng of total DNA), 1 X Taq buffer (Thermo Fisher Scientific Inc., Foster City, CA), 2.0 mM $MgCl_2$, 1.0 mM dNTPs, 20uM forward primer, 20 uM reverse primer, 2 U Taq DNA polymerase). PCR amplification included 2 min at 94 °C followed by 35 cycles of denaturing (94 °C, 1 min), primer annealing (55 °C, 45 s), and chain extension (72 °C, 90 s); cycling was followed by a final extension phase at 72 °C for 10 minutes.

PCR products were cloned into a PCR 2.1 vector using TOPO TA Cloning kit (Invitrogen Corp., Carlsbad, CA) and 96 clones were picked for each library. Using T7 (5'-CCCTATAGTGAGTCGTATTAC-3') and M13 (5'-CAGGAAACAGCTATGA-3') primers, PCR amplification included 2 min at 94 °C followed by 35 cycles of denaturing (95 °C, 30 s), primer annealing (62 °C, 1 min), and chain extension (72 °C, 60 s); cycling was followed by an incubation at 72 °C for 10 minutes. Presence of insert was confirmed using gel electrophoresis on a 1% agarose gel.

The crude PCR product (T7/M13 primers) was sent to Functional Biosciences, Inc. at the University Research Park in Madison, WI (http://www.functionalbio.com/) for automated sequencing. The forward and reverse compliment sequences were aligned in Squencher 4.0 (Gene Codes, Ann Arbor, MI). Michigan State University's (MSU) Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) was used to compare the genera in both treatments. Further identification was completed using BLASTn through the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Ammonia Oxidation Potential

The time series of nitrite accumulation in ammonia oxidation assays varied considerably between sediments from oxygen-activated versus control wetland mesocosms (Fig. 2). All sediment samples from the control mesocosms showed a slow nitrite accumulation up to 5 μg-N over the course of the assay. In contrast, all sediment samples from oxygen-activated mesocosms accumulated high levels of nitrite, up to 15-25 μg-N by the completion of the assay. Though sediments from Oxygen B showed a delayed response, with nitrite only accumulating at the end of the assay, nitrite levels were similar in magnitude at the end of the assay as those observed in sediments from Oxygen A. In both sets of oxygen-activated sediment samples, nitrite accumulation was somewhat lower in sediments sampled from the exit. Ammonia oxidation potentials ranged from 1.2- 3.5 mg-N/g-dw/d in sediments from oxygen-activated mesocosms and 0.4-0.8 mg-N/g dw/d in sediments from control mesocosms (Fig. 3). Rates from the duplicates showed similar spatial patterns: decreasing with distance in oxygen-activated mesocosms and peaking slightly in the middle in control mesocosms. Pooling the samples by treatment, ammonia oxidation potentials were 2.6 ± 0.80 mg-N/g-dw/d (average ± standard deviation, n = 6) in oxygen-activated sediments and 0.48 ± 0.20 mg-N/g-dw/d in control sediments, and rates were significantly higher in sediments from oxygen-activated mesocosms (one-tailed t-test assuming unequal variances; $P < 0.001$). The number of nitrifying bacteria in sediments was estimated based on the maximum activities per cell for *Nitrosomonas* sp. and *Nitrosolobus multiformis* of 0.023 picomoles per cell per hour (Hart *et al*., 1994). Values in wetland mesocosm ranged from a minimum of 6×10^7 cells/g-dw in the entrance and exit of control mesocosms to a peak of 5×10^8 cells/g-dw in the entrance of the oxygen-activated

mesocosms. This indicated that oxygenation also increased ammonia oxidizing bacterial cell counts.

Microbial Diversity

The identifiable species resulting from the BLAST search are complied in Table 1. The species listed in the oxygen-activated and control columns were unique to the indicated treatment. The central column lists species found in both treatments. Phylogenic analysis of the results indicated that ammonia oxidizing bacteria (*Nitrosomonas oligotropha*) were affiliated with the oxygen-activated mesocosms but not the controls. Nitrifying bacteria belonging to the genus *Nitrospira*, responsible for oxidizing nitrite to nitrate, were found in both treatments. Although *Nitrospira* directly participate in nitrification, they are not responsible for ammonia oxidation. Species diversity among the oxygen-activated and control mesocosms was comparable with approximately 20% of the identified common to both treatments. The remaining 80% of identifiable species (39 and 40 species, respectively) were specific to the oxygen-activated and control treatments (Table 2).

Species richness is also reflected in the common metabolic processes of each treatment. I further categorized species by the preferred metabolic pathways (Fig. 5). The oxygen-activated mesocosms displayed a higher diversity of anoxygenic phototrophic bacteria with eight different species were identified, five of which were unique to the oxygen-activated treatment (Table 3). Diversity among aerobic phototrophs, primarily consisting of cyanobacteria, was much greater in the control mesocosms. Comparing the different treatments, eight different aerobic phototrophs were identified in the control mesocosms while only one was identified in the oxygen-activated mesocosm. Fermenting bacteria were common to both treatments. Approximately the same numbers of species were identified, but none were common to both treatments, indicating high

species diversity among fermenting bacteria in both treatments. However, three species from each treatment belong to the same genera. Five different sulfur reducing species were identified in the control mesocosm, but two of these were also present in the oxygen-activated treatment. Methanotrophs and iron reducing bacteria were also identified. These bacteria were a smaller fraction of the total identifiable species so it is difficult to assert whether diversity varied between the oxygen-activated and control mesocosm.

DISCUSSION

Ammonia Oxidation Potential

Rates of ammonia oxidation potential measured in sediments from oxygen-activated wetland sediments (1.2-3.5 mg-N/g-dw/d) were significantly higher then those measured in other soils and sediments (0.0010-1.9 mg-N/g-dw•d) (Table 1). The increased rates are attributed to enhanced DO. Potentials sequentially dropped as oxygen was consumed along the length of the activated mesocosm (Fig. 3). This pattern was noticeably absent from the control mesocosms and illustrates the general effectiveness of the oxygenation. A comparison of ammonia oxidizing potential in surficial, rhizosphere, and bulk submerged soils further highlights the relationship between ammonia oxidation potential and oxygen. Holding ammonium concentrations constant, ammonia oxidation potentials are greatest in the surficial and rhizosphere sediments of rice fields (Bodelier and Frenzel, 1999; Nicolaisen *et al*., 2004). DO diffusing from shallow water above the surficial sediments provides enough oxygen to fuel limited ammonia oxidation in surficial sediments, and oxygen released from root stimulates nitrification in the rhizosphere. Studies investigating root oxygen release rates in common wetland and rice plants have documented release rates large enough to fuel oxidative reactions, such as nitrification, in the rhizosphere

(Wiebner *et al*., 2002; Jespersen *et al*., 1998; Inubushi *et al*., 2002; Yoshida, 1981, Wu *et al.*, 2001). Rhizosphere oxidation could also explain the pattern of ammonia oxidation potential in my studies control mesocosms. Sediments from the central sampling sites had the highest ammonia oxidation potential and *Typha spp.* densities in the mesocosm.

Because nitrification can occur when in oxygen concentrations are as low as 0.05 mg/L, ammonia availability often limits nitrification rates (Bothe *et al*., 2000; Albeliovich, 1987). The comparatively higher ammonia oxidation potentials documented in this study are partially attributed to enhanced ammonia availability. Ammonium oxidation is the rate-limiting step in nitrification and slow growing nitrifying bacteria are sensitive to ammonia concentrations (Chu *et al*., 2008, Limpiyakorn *et al*., 2007); high substrate availability enhances substrate utilization and subsequently increases ammonia oxidation potentials (Tchobanoglou*s et al.*, 2003). Similar conclusions were established when Gorra *et al*. (2007) documented specific effects of ammonium concentration on ammonia oxidation potentials in sediments from a CTW. Sediments from an established CTW were treated with 2.5 mM and 25 mM ammonium. Ammonia oxidization potential associated with the 25 mM treatment was consistently and significantly higher. The results indicated that ammonia oxidation potentials were limited by nitrifying species' sensitivity to low ammonium concentrations. Arable soils treated with ammonia reflected similar results. Comparing ammonia oxidation potentials of arable soils pre-incubated in ambient air, ammonia, carbon monoxide and methane, ammonia oxidation potentials were highest in the ammonia incubation (Bender and Conrad, 1994). Increasing ammonia concentrations also increases ammonia oxidizing bacterial cell counts. Okano *et al*. (2004) compared ammonia oxidizing bacterial growth yields of soils treated with 1.5 mM and 7.5 mM ammonia. At the completion of the seven-day study, these soils had growth yields of 5.6

x 10^6 cells/µmol and 1.8 x 10^7 cells/µmol. Prior to the treatment, AOB were approximately 0.4 % of the total bacterial populations. Post-treatment AOB populations increased to 3.1 and 5.7% of total bacterial populations in the 1.5 and 7.5 mM treatments.

The estimated AOB cell counts in our study (6 x 10^7 cells/g-dw in the entrance and exit of control mesocosms to a peak of 5 x 10^8 cells/g-dw in the entrance of the oxygen-activated mesocosms) were higher than cell counts in wastewater treatment plant sludge and arable soils, as determined by Okano *et al.* (2004) (0.5-1.5 x 10^7 cells/g-dw) and Mendum, *et al.* (1999)(1.4 x 10^4-6.5 x 10^6 cells/g-dw), but comparable to those documented by Urakawa *et al*. (2006)(5.7-8. x 10^8 cells/g-dw) in a canal receiving wastewater. Other AOB counts in fertilized arable soils (~6.2x 10^7 cells/g -dw) were very similar to the estimated values of this study (Hermansson and Lindgren, 2001). Unfertilized soils evaluated in this latter study had AOB populations approximately one-third of the fertilized cell counts, corroborating the researchers' hypothesis that N fertilization enhances AOB biomass yield and nitrification. Total bacterial cell counts could not be estimated in our samples via the nitrification assay or conventional PCR, so it is impossible to determine the fraction of AOB to total bacteria in sediments from the wetland sediments. But the ongoing application of real time PCR will allow for such an evaluation (see Future Research subsection).

Microbial Diversity

A key finding in this study was the presence of *Nitrosomonas oligotropha,* a common AOB, in sediments from the oxygen-activated wetland mesocosms; no AOB were isolated in sediments from control mesocosm. *Nitrospira spp*., a nitrite oxidizer, was found in sediments

from both mesocosms. Three key observations in the sediments from the oxygen-activated mesocosms, the presence of AOB, and high rates of ammonia oxidation potential, supports the contention by Palmer and Beutel (2009) that oxygenation will 'activate' wetland sediments and led to higher rates of biological ammonia oxidation in the experimental wetlands. Results from the present study regarding the dominant species of nitrifying bacteria in oxygen-activated sediments are similar to a number of studies of AOB species diversity in wastewater treatment reactors. Ammonia utilization varies among AOB (Metcalf and Eddy, 2003). As a result, species diversity will also vary with ammonia concentration, which has been the focus of a number of AOB studies of nitrifying activated sludge. Limpiyakporn *et al*. (2007) treated reactors with four different ammonium concentrations to determine effects on ammonia oxidizing community. Results indicated *Nitrosomonas oligotropha* dominance at 2 mM, 5 mM and 10 mM ammonium treatments. *Nitrosomonas europaea* and *Nitrosococus mobilis* were dominant in the 30 mM ammonium treatment. Although present at the beginning of the 30 mM treatment, *N. oligotropha* was undetected in the reactor by the second week, indicating that *N. europaea* and *Nitrosococus mobilis* out-competed *N. oligotropha* at high ammonium concentrations. Suwa *et al.* (1994; 1997) and Bollmann and Laanbroek (2006) documented similar *N. oligotropha* dominance at low ammonium concentrations in both activated sludge and estuarine sediments.

DO utilization varies among species, indicating that oxygen concentration will also affect community diversity and activity. Guo *et al.* (2009) documented nitrification and total AOB population changes during high and low oxygen treatments. Nitrification rates were greater in the high DO reactor. The accompanying flouresence in-situ hibridization (FISH) analysis confirmed that AOB populations in the high and low DO reactors ranged from 9-12% and 6-8%,

respectively, and signified that high DO enhances both nitrification and AOB populations. In a study documenting the effects of DO on specific ammonia-oxidizing bacterial communities, Park and Noguera (2004) documented a clear species differential between the high and low DO chemostat reactors (~8.5 and < 0.24 mg-DO/L) within the first 56 days after start up. *N. oligotropha* dominated in the high DO chemostat reactor during the first four months of study, at which time dominance shifted to *N. europaea*. *N. europaea* remained dominant in the low DO reactor (<0.24 mg/L) throughout the duration of the study. Similar to findings by Beutel and Palmer (2008), the high DO reactor nitrified over 90% of the ammonium within days of initial start up. Complete nitrification was eventually reached in the low DO reactor but it took approximately one month. Community changes to a full scale WWTP were also examined in the Park and Noguera (2004) study. At the completion of the three month study, *N. oligotropha* and *N. europaea* were dominant in the high and low DO reactors (<7.4 and < 0.8 mg/L).

Some studies have examined AOB in environmental systems, but only a fraction of these have looked at nitrifier diversity in sediments from aquatic settings such as lakes, rivers and wetlands. A study examining ammonia-oxidizing communities in wetlands found that the dominant species were member of the "phylogenically young" *Nitrosospira* lineage (Gorra *et al.*, 2007). *Nitrosospira spp.* are beta-proteobacterial AOB but they do not belong to the same genera as *Nitrosomonas* (Dworkin *et al.*, 2006). *Nitrosospira* is often the dominant AOB genera in submerged soil systems like wetlands (Haleem *et al.*, 2003; Ikenaga *et al.*, 2003, Hails *et al.* 2004, *Ibekwe, et al.*, 2003). This is a noted difference from wastewater treatment plants, which are generally dominated by *Nitrosomonas spp.* (Suwa *et al.*, 1994; Suwa *et al.*, 1997; Bollmann and Laanbroek, 2006; Park and Noguera, 2004; Limpiyakorn *et al.*, 2005; Limpiyakorn *et al.*, 2007). The fact that *Nitrosomonas oligotropha*, rather than *Nitrosospira spp.*, was the dominant

AOB in oxygenated sediments from the experimental mesocosms suggests that the oxygen-activated treatment wetlands were more of a 'treatment systems' than a 'natural' wetland. Bernhard *et al.* (2005) determined that salinity affected AOB community diversity. The results from low, mid, and high salinity locations showed that seasonal and community diversity decreased as salinity increased. The dominant species were *N. oligotropha* and *N. ureae*, which correlated to findings in other estuaries (Bollman and Laanbroek, 2002).

Species richness was comparable in the separate treatments (Fig. 4); approximately forty different species were identified in each treatment (Table 2). Although some species were present in both treatments, the majority of the identifiable species were unique to each treatment. The control and oxygen-activated lineage diversities were comparable to those found in a shallow eutrophic lake (Tamaki *et al.*, 2005). Other studies outlining wetland species diversity showed that plants had negligible effects on diversity (Baptista *et al*., 2008; Gorra *et al*., 2007). Because the wastewater fed to each of the treatments was identical, the oxygenation likely had the largest effect on species diversity.

The low DO in the control wetland mesocosms did not reduce microbial species diversity. Instead, bacteria with low substrate utilization rates proliferated by out-competing other organisms. Filamentous cyanobacteria diversity burgeoned in the low DO control mesocosms (Table 1 and 2), which coincided with findings by Metcalf and Eddy (2003) in low oxygen wastewater reactors. Other species lack the low oxygen substrate utilization rates characteristic of filamentous bacteria, which allows these bacteria to out compete other species. Large portions of the identified species in the control mesocosms were these filamentous aerobic phototrophs (Fig. 5). Nitrite oxidizing species were among the bacteria found in the control mesocosm. Ammonia oxidization is the rate-limiting step during nitrification (Chu *et al*., 2008,

Limpiyakorn *et al*., 2007) but oxygen concentrations in the control mesocosms were high enough for some nitrification to proceed (Bothe *et al*., 2000; Albeliovich, 1987). Thus, it is unlikely that the low oxygen concentration in the control is solely responsible for the absence of AOB and the low oxidation rates. Instead, fast growing heterotrophic bacteria likely out competed the slow growing lithotrophic AOB for the limited oxygen supply (Metcalf and Eddy, 2001; Madigan and Martinko, 2004). In contrast, the high oxygen concentrations in oxygen-activated mesocosms may have been toxic to some heterotrophic bacteria, which provided comparatively slower growing lithotrophic AOB with opportunities to prosper. Mikell *et al.* (1986) documented the biomass of four heterotrophic bacteria in benthic sediments underlying perpetually high DO waters of an Antarctic lake. Maximum cell density fell as a result of the high DO concentrations, suggesting that elevated DO concentrations inhibited the heterotrophic species diversity. Thus, oxygen-activation could have inhibited heterotrophic diversity while simultaneously enhancing lithotrophic diversity.

Sulfur- and iron-reducing bacteria were identified in both treatments, but more sulfur-reducing species were identified in the control mesocosms. In addition, nitrate-reducing (denitrifying) bacteria were identified only in the control sediments. Thus, while high oxygen concentrations in the oxygen-activated mesocosms enhanced ammonia oxidation, it appears to have inhibited anaerobic metabolic processes such as sulfate and nitrate reduction. Similarly, fermenting bacteria were identified in both treatments but none of the species were held in common. Again, this difference is attributed to different oxygen tolerance levels of fermenting bacteria, as well as the difference in overall environmental conditions in the oxygen-activated and control mesocosms.

CONCLUSIONS

The primary goal of this research was to determine the effects of oxygenation on microbial activity and diversity in CTW sediments. Using the chlorate inhibition technique, the ammonia oxidation potential was calculated as nitrite mass from the soil accumulated over time. Traditional PCR techniques were also used to identify the microbial communities in each treatment. During the nitrification assay, all sediment samples from oxygen-activated mesocosms accumulated high levels of nitrite while the control mesocosms showed a slow nitrite accumulation over the course of the assay. Pooling and comparing the samples by treatment, ammonia oxidation potentials were significantly higher in the oxygen activated mesocosms (2.6 ± 0.80 mg-N/g-dw/d) then the control mesocosms (0.48 ± 0.20 mg-N/g-dw/d) and values documented in other studies. The increased rates under oxygenated conditions are attributed to enhanced DO availability at the sediment-water interface in the wetlands, and resulting increased rates of ammonia oxidation by AOB. This proposition is supported by the observation that nitrification potential in sediments dropped from the inlet to the exit as oxygen and ammonia were consumed along the length of the activated mesocosms, a pattern absent from the control mesocosm. *Nitrosomonas oligotropha* and *Nitrospira* sp. were identified in the oxygen activated sediments; no AOB were isolated in sediments from control mesocosm. The presence of AOB combined with the high rates of ammonia oxidation potential in oxygen-activated sediments supports the contention by Palmer and Beutel (2009) that oxygenation 'activates' wetland sediments and leads to higher rates of biological ammonia oxidation in the experimental wetlands. Species richness was comparable in each treatment. The oxygenation did not enhance

diversity but appears to have selected for a different cohort of species able to thrive in a more oxygenated sediment-water interface.

FUTURE RESEARCH

Real Time PCR for AOB and AOA

Traditional PCR efforts indicated that a disparity exists between the nitrifying communities in the sediments of the oxygen-activated and control communities. Real-time PCR is a method of quantifying the number total bacteria and target bacteria. A number of studies have used real-time PCR to quantify AOB populations in sludges and sediments (Ward *et al.*, 2000; Limpiyakorn *et al.*, 2005). Using real-time PCR, quantification of total and ammonia oxidizing bacteria from sediments in this study is currently under way. This will help to determine if oxygenation enhanced only the nitrification process or if it successfully augmented the fraction of AOB to total bacterial cells, which then subsequently increased nitrification rates. Expanding research on the importance of ammonia oxidizing archaea (AOA) in aquatic and soil systems suggests that AOA may be partially responsible for the high ammonia oxidation potentials (Chen *et al*., 2008; Leininger, *et al*., 2006). Quantifying the AOA populations, in addition to AOB, through real-time PCR will help to identify the species responsible for the majority of ammonia oxidation in the mesocosms. The primer sets quantifying total bacteria and archaea and ammonia oxidizing bacteria and archaea were selected from published studies (Quan *et al.*, 2008; and He *et al*., 2007). Results of the analysis are pending. These efforts are currently underway in collaboration with ongoing PhD student Stephen Dent and Dr. Doug Call's research staff.

Trace Gas Emissions

A growing issue related to CTWs is their tendency to emit significant amounts of two key trace gasses responsible for global warming: methane (CH_4) and nitrous oxide (N_2O) (Søvik and Kløve, 2007). While nitrous oxide emissions from CTWs tend to be an order of magnitude lower than methane emission (Søvik *et al*., 2006), nitrous oxide is over ten times as potent as methane from a warming perspective (IPCC, 2001); thus both trace gasses are of concern. To implement CTWs on a more sustainable basis, we must fully understand what environmental factors control greenhouse gas emissions from CTWs. In collaboration with research staff from the WSU Laboratory for Atmospheric Sciences, I performed preliminary trace gas emission measurements on the experimental wetland mesocosms described in this study. Our main question was whether the oxygen-activated nitrification wetlands exhibited higher rates of nitrous oxide emissions associated with elevated levels of nitrification and denitrification, thereby offsetting the overall environmental benefit of ammonia removal. Nitrous oxide is a common intermediate nitrogen-oxide species emitted during the reduction of nitrate to dinitrogen gas (Firestone, 1982). Nitrous oxide has also been measured during nitrification in soils and marine environments, presumably forming as an intermediate during the oxidation of hydroxylamine to nitrite (Schmidt, 1982). Using standard protocols for the environmental measurement of trace gas fluxes (TGPDC, 2003), we measured nitrous oxide fluxes from duplicate oxygen-activated and control mesocosms (Fig. 6). Preliminary results showed that nitrous oxide fluxes from oxygen-activated wetlands were about twice those in the controls (3-5.5 versus 1.5-3 $\mu l/m^2/d$). Further research is needed to document trace gas emissions from oxygen-activated wetlands and, in a broader context, to determine how and if wetland oxygenation might be used to minimize emissions of trace gas from CTWs.

Treatment of Halogenated Aliphatic Compounds

Nitrifying bacteria employ very unique enzymes including ammonia monooxygenase to convert ammonia to hydroxylamine and hydroxylamine oxdioreductase to convert hydroxylamine to nitrite. A number of studies have documented the degradation of a wide range of halogenated compounds by ammonia oxidizing bacteria including dichloromethane, dibromomethane, chloroform, trichloroethane, trichloroethylene (TCE) and vinyl chloride (Yang *et al*., 1999; Vannelli *et al*., 1990; Arciero *et al*., 1989). These studies are typically done in highly controlled environments using well defined isolates and pure cultures. Further study is needed in the context of natural treatment systems with mixed and highly diverse microbial populations, such as oxygen-activated nitrification wetlands, to evaluate their potential to degrade halogenated aliphatic compounds and resulting changes in microbial communities associated with such treatments. In collaboration with Dr. Richard Watts, an expert in *in situ* chemical oxidation of organic pollutants, I performed an initial experiment to evaluate the capacity of the experimental wetland mesocosms in this study to degrade TCE. Approximately 10 mg/L of TCE and 20 mg-N/L of ammonia were added to each mesocosm, which were operated in batch mode (i.e., influent flow was discontinued). Ammonia and TCE (via gas chromatography) were tracked over time. Unfortunately, at the beginning of the batch experiment an excess of organic matter was also added to the mesocosms, resulting in an extreme drop in dissolved oxygen in both the oxygen-activated and control mesocosms. As a result, nitrifying microorganisms, which are particularly sensitive to environmental conditions and not at all resilient (Schmidt, 1982), were inhibit and/or kill. This was confirmed by a total lack of ammonia removal in oxygenated wetlands during the batch experiment. Experimental

results of the effort were inconclusive. It is quite possible that oxygen-activated nitrification wetlands, with their extreme rates of ammonia removal as documented by Palmer and Beutel (2009), could also yield high rates of degradation of a range of halogenated aliphatic compounds.

Immobilized Cell Biotechnology

A relatively new approach to enhance ammonia removal in a range of wastewaters is immobilized-cell biotechnology (Jung *et al*., 2004; Rostron *et al*., 2001). Cell immobilization involves the purposeful entrapment and encapsulation of select microbial cells in small beads or pellets of support media made up of natural (e.g., calcium alginate) or synthetic polymers (e.g., polyethylene glycol). Because these pellets can easily be retained in treatment reactors using screens, immobilized nitrifying microorganisms do not get washed out of the reactors resulting in more effective treatment (i.e., shorter hydraulic retention times and smaller reactors). In one study that looked at ammonia removal in livestock wastewater, the use of immobilized nitrifiers resulted in a drop in the required hydraulic retention time from 10 days to 12 hours (Vanotti and Hunt, 2000). Further research is needed to evaluate how immobilized-cell biotechnology could interface with CTWs to enhance ammonia removal. Perhaps immobilized nitrifiers in biodegradable media, in concert with oxygenation or without oxygenation, could be intermittently added to a CTW with the result of enhancing rates of nitrification and shrinking the required size and cost of the treatment wetland.

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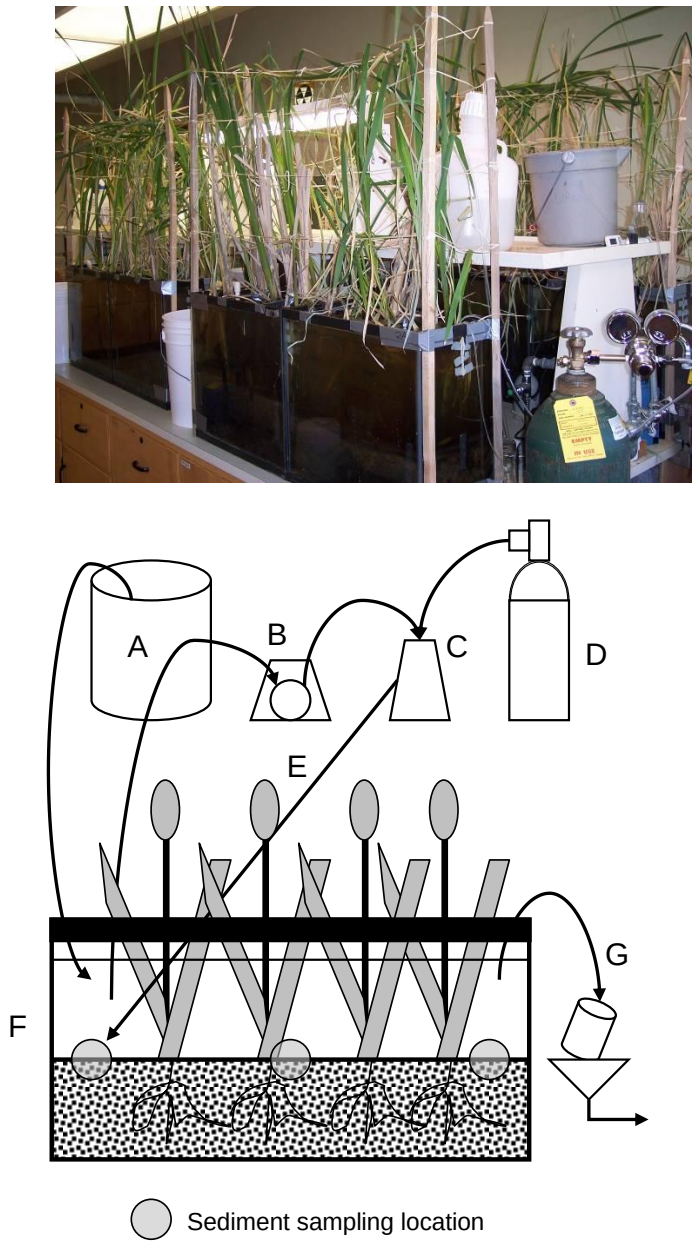

Figure 1. Top: Photo of experimental setup. Bottom: Schematic of oxygen-activated wetland mesocosm and sediment sampling locations. Note that control wetland mesocosms had no side-stream oxygenation components. (A) synthetic wastewater inflow via gravity-feed line; (B) side-stream pump feeding oxygenation chamber; (C) oxygenation chamber; (D) pure oxygen gas cylinder feeding oxygenation chamber; (E) oxygenated side-stream return line; (F) aquarium with water, wetland sediment, and cattail plants; and (G) siphon outlet structure and effluent sampling point. Modified from Palmer and Beutel (2009).

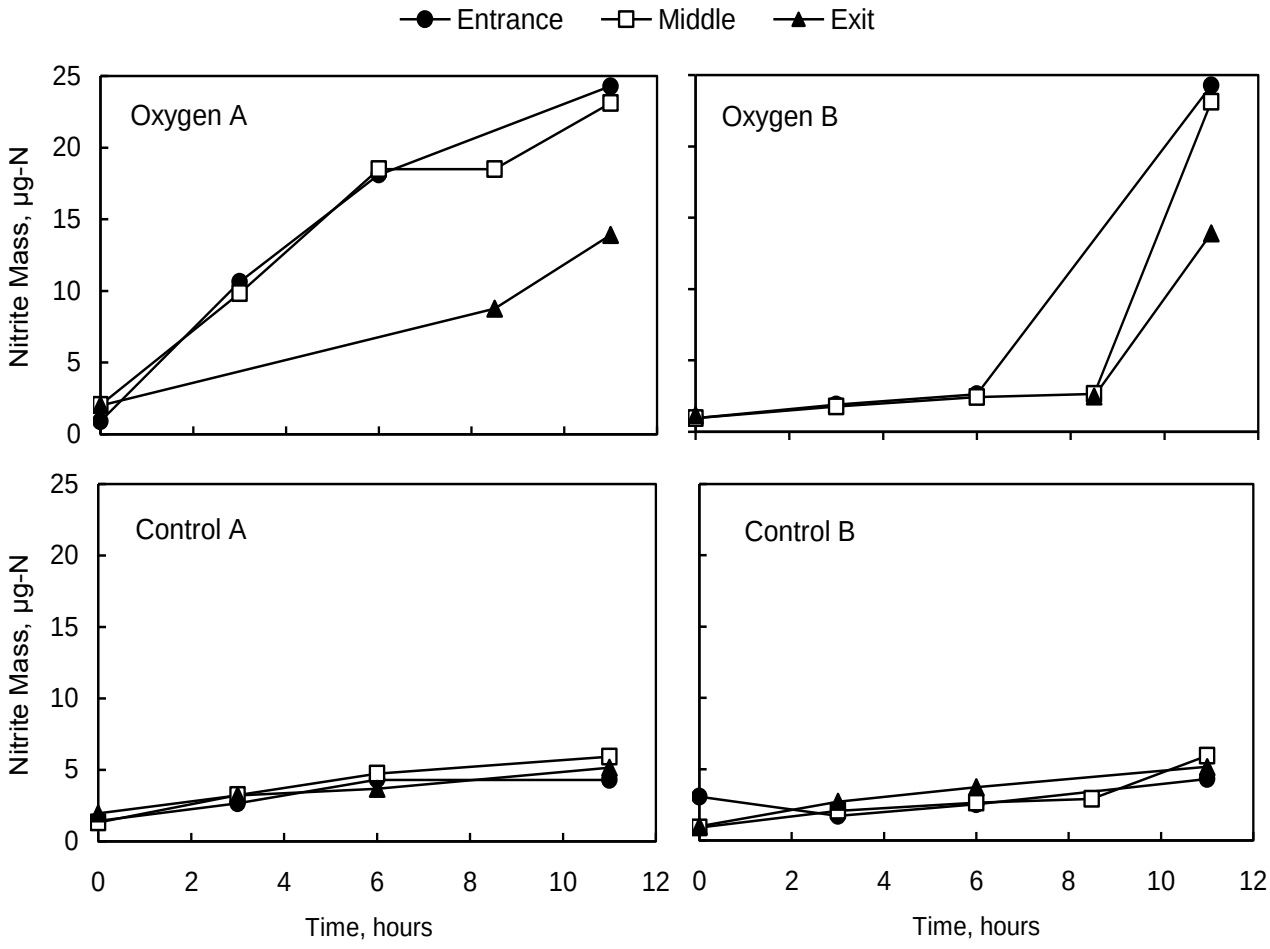

Figure 2. Accumulation of nitrite mass during ammonia oxidation potential assays with surficial sediments sampled at the entrance, middle and exit of duplicate oxygen-activated wetland mesocosms (Oxygen A, Oxygen B) and duplicate control wetland mesocosms (Control A, Control B).

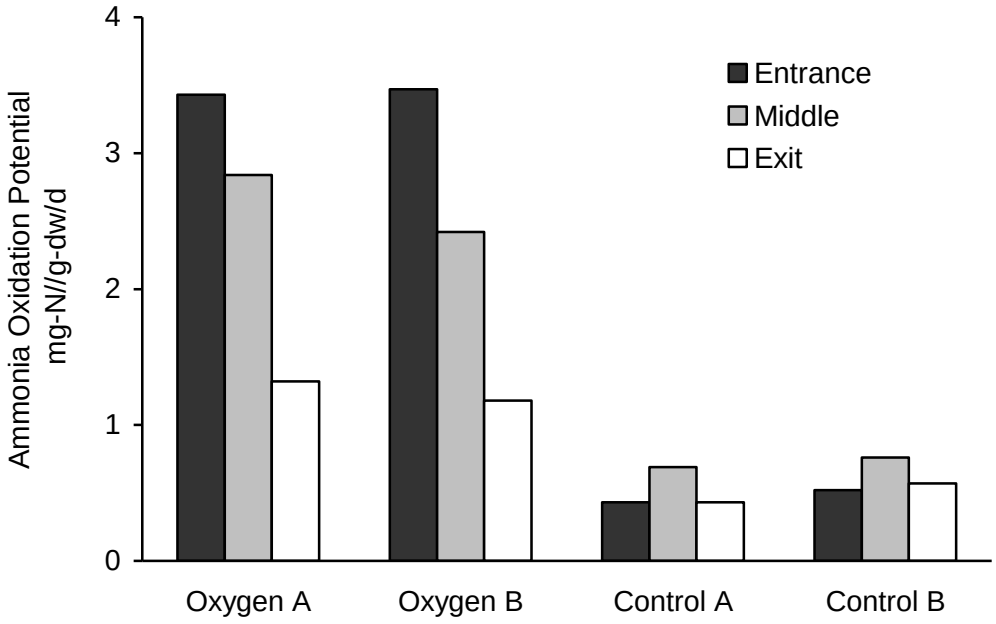

Figure 3. Ammonia oxidation potential measured in surficial sediments sampled at the entrance, middle and exit of duplicate oxygen-activated wetland mesocosms (Oxygen A, Oxygen B) and duplicate control wetland mesocosms (Control A, Control B).

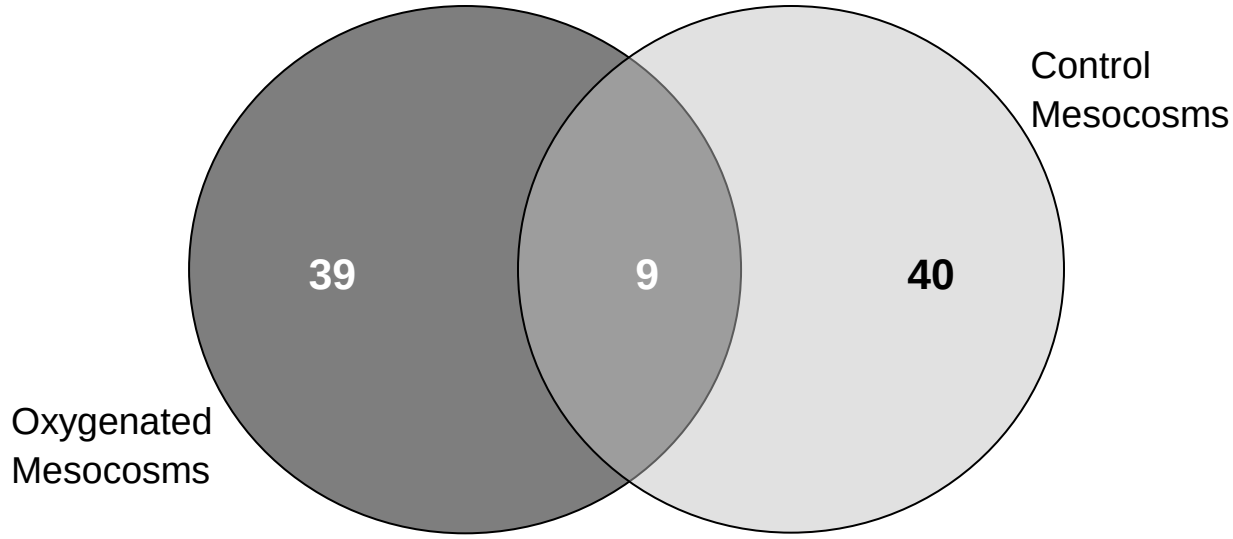

Figure 4. Venn diagram of identifiable microbial species measured in surficial sediments from oxygen-activated and control wetland mesocosms.

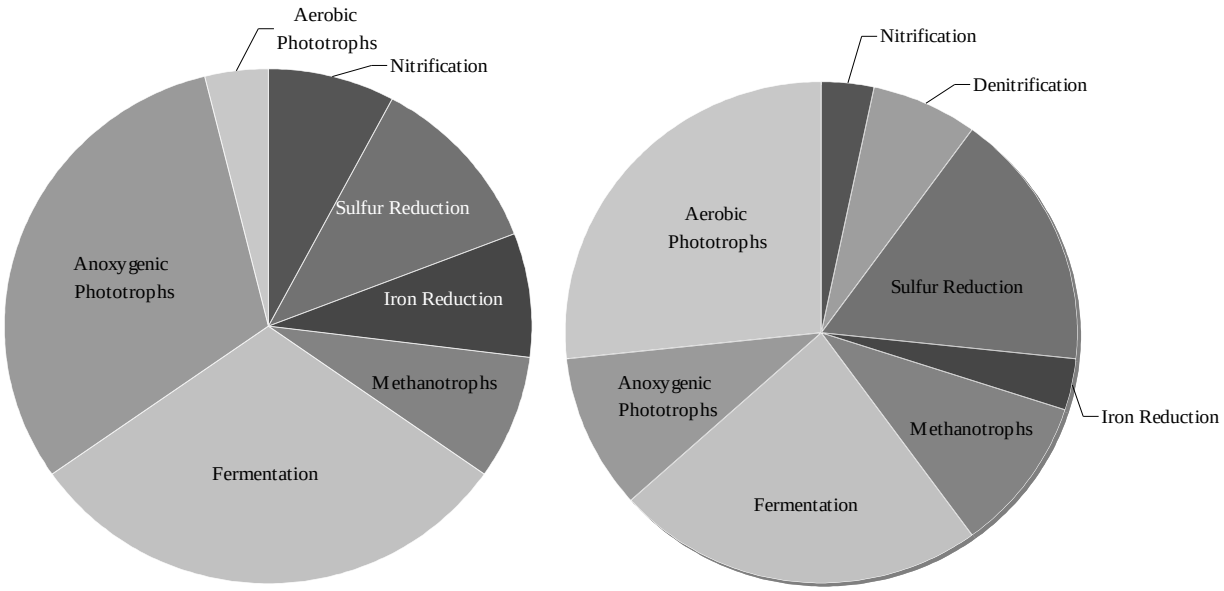

Figure 5. Microbial species richness measured in surficial sediments from oxygen-activated (left) and control (right) wetland mesocosms according to preferred metabolic capability. Note that areas represent number of species and not the microbial numerical populations.

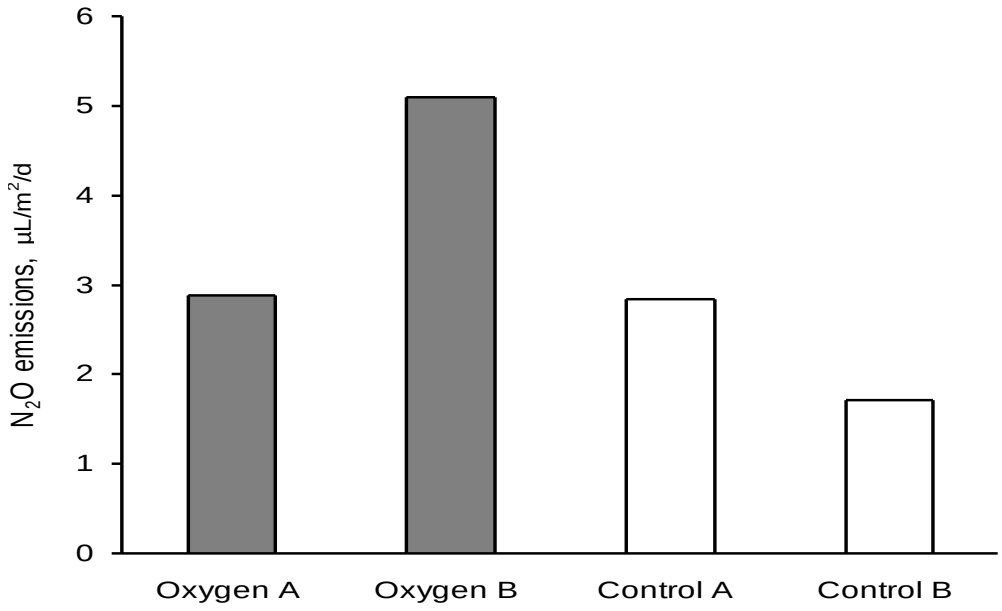

Figure 6. Preliminary results from trace gas measurements made in duplicate oxygen-activated wetland mesocosms (Oxygen A, Oxygen B) and duplicate control wetland mesocosms (Control A, Control B). Measurements were made in collaboration with staff from the WSU Laboratory for Atmospheric Research following standard protocol detailed in TGPDC (2003).

Table 1. Ammonia oxidation potentials of terrestrial soils and aquatic sediments under various treatment regimes.

Ammonia Oxidation Potentials

Media	Treatment/Soil Zone	Ammonia Oxidation Potential (mg-N/g dry weight•d)	Reference
Experimental constructed wetland sediments	Oxygen-activated	1.2-3.5	This study
	Control	0.43-0.76	
Constructed wetland sediments	25 mM ammonium treatment:		Gorra *et al.*, 2007
	root zone	0.049-1.2	
	bulk zone	0.031-0.38	
	2.5 mM ammonium treatment:		
	root zone	0.024-0.31	
	bulk zone	0.0010-0.31	
Rice field soils	Root Zone	0.0046-0.015	Bodelier and Frenzel, 1999
	Bulk Zone	0.0033-0.0047	
Rice field soils	Surface soil	0.018	Nicolaisen *et al.*, 2004
	Bulk soil	0.0094	
	Rhizosphere	0.013	
Grass land and Forest	Forest	0.6-1.8	Hoffman *et al*., 2007
	Agriculture	1.9	
	Natural Grassland	0.3-0.25	
Meadow, forest, rice paddy	Air	0.015	Bender and Conrad, 1994
	Methane	0.016	
	Ammonia	0.020	
	Carbon Monoxide	0.016	
Agricultural soils		0.0012-0.0067	Kurola, et. al. 2005
Agricultural soils		0.0024-0.0029	Berg and Rosswall, 1987
Agricultural soils (long-term fertilization)	Fallow	0.050	He *et al*., 2007
	No fertilizer	0.036	
	Fertilizer	0.0062-0.024	
	Fertilizer + Organic Matter	0.11	

Table 2. A comparison of bacterial species identified in surficial sediments from oxygen-activated and control wetland mesocosms.

Species Diversity (according to BLAST results)		
Oxygen-Activated Mesocosms	**Both**	**Control Mesocosms**
Acidobacterium sp.	*Chlorobium phaeobacteroides*	*Acidithiobacillus thiooxidans*
Actinopolymorpha rutilus	*Anaerolinea thermolimosa*	*Amphora coffeaeformis*
Agrobacterium tumefaciens	*Caldilinea aerophila*	*Anaerolinea thermolimosa*
Arthrobacter oxydans	*Chloroflexus sp.*	*Arenimonas sp.*
Arthrobacter rhombi	*Dechloromonas sp.*	*Attheya longicornis*
Azospira oryzae	*Desulfobacca acetoxidans*	*Azospira oryzae*
Bacillus benzoevorans	*Desulfobacterium indolicum*	*Bacillus longiquaesitum*
Blastopirellula sp.	*Flavobacterium sp.*	*Bacillus nealsonii*
Chlorobium limicola	*Nitrospira sp.*	*Bacteriovorax sp.*
Clostridium aldrichii		*Brevundimonas vesicularis*
Clostridium beijerinckii		*Clostridium acetobutylicum*
Clostridium carboxidivorans		*Clostridium cellulosi*
Cystobacter violaceus		*Clostridium hveragerdense*
Cytophaga sp.		*Codakia orbicularis*
Dechloromonas hortensis		*Cystobacter ferrugineus*
Desulfobulbus rhabdoformis		*Dechloromonas hortensis*
Geobacter lovleyi		*Desulfocapsa sp*
Geobacter pelophilus		*Desulfofaba fastidiosa*
Haliscomenobacter sp.		*Desulforhopalus sp.*
Magnetic coccus		*Dickieia ulvacea*
Nitrosomonas oligotropha		*Flavobacterium aquatile*
Nocardioides sediminis		*Flavobacterium hercynium*
Phenylobacterium lituiforme		*Flavobacterium succinicans*
Pirellula staleyi		*Geobacter sp.*
Pseudomonas aeruginosa		*Geothermobacter ehrlichii*
Pseudomonas nitroreducens		*Holophaga sp.*
Rhodobacter changlensis		*Methylobacter tundripaludum*
Rhodobacter ovatus		*Methylocystis parvus*
Rhodobacter sphaeroides		*Methyloversatilis sp.*
Rhodoplanes sp.		*Microlunatus phosphovorus*
Rhodopseudomonas palustris		*Nanofrustulum shiloi*
Smithella sp.		*Navicula phyllepta*
Spirobacillus cienkowskii		*Nitzschia closterium*
Sterolibacterium denitrificans		*Phaeodactylum tricornutum*
Syntrophorhabdus aromaticivorans		*Rhodocyclus sp.*
Syntrophus aciditrophicus		*Salinisphaera sp.*
Syntrophus gentianae		*Salinispora arenicola*
Syntrophus sp.		*Sphingobacterium sp.*
Thalassiosira gravida		*Sphingomonas sp.*
		Syntrophus sp.

1 Table 3. Common metabolic processes and associated species found in surficial sediments of the
2 oxygen-activated and control wetland mesocosms.

	Bacterial Species (according to BLAST analysis)	
Metabolism	Oxygen-Activated Mesocosms	Control Mesocosms
Nitrification	*Nitrosomonas oligotropha* *Nitrospira sp.*	*Nitrospira sp.*
Denitrification		*Pseudomonas aeruginosa* *Pseudomonas nitroreducens*
Sulfur Reduction	*Desulfobacca acetoxidans* *Desulfobacterium indolicum* *Desulfobulbus rhabdoformis*	*Desulfobacca acetoxidans* *Desulfobacterium indolicum* *Desulfocapsa sp. Cad626* *Desulfofaba fastidiosa* *Desulforhopalus sp.*
Iron Reduction	*Geobacter lovleyi* *Geobacter pelophilus*	*Geobacter sp.*
Methanotroph	*Bacillus longiquaesitum* *Bacillus nealsonii*	*Methylobacter tundripaludum* *Methylocystis parvus* *Methyloversatilis sp.*
Fermentation	*Syntrophorhabdus aromaticivorans* *Syntrophus aciditrophicus* *Syntrophus gentianae* *Syntrophus sp.* *Thalassiosira gravida* *Clostridium aldrichii* *Clostridium beijerinckii* *Clostridium carboxidivorans*	*Clostridium acetobutylicum* *Clostridium cellulosi* *Clostridium hveragerdense* *Anaerolinea thermolimosa* *Flavobacterium aquatile* *Flavobacterium hercynium* *Flavobacterium succinicans*
Anoxygenic Phototrophs	*Rhodobacter changlensis* *Rhodobacter ovatus* *Rhodobacter sphaeroides* *Rhodoplanes sp.* *Rhodopseudomonas palustris* *Anaerolinea thermolimosa* *Caldilinea aerophila* *Chloroflexus sp.*	*Anaerolinea thermolimosa* *Caldilinea aerophila* *Chloroflexus sp.*
Aerobic Phototrophs	*Thalassiosira gravida*	*Phaeodactylum tricornutum* *Attheya longicornis* *Navicula phyllepta* *Amphora coffeaeformis* *Dickieia ulvacea* *Nanofrustulum shiloi* *Nitzschia closterium* *Navicula phyllepta*

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