The Presence and Functionality of Ammonia Oxidizing Bacteria in the Constructed Treatment Wetland at the John Heinz National Wildlife Refuge

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Abstract

The vast majority of research done on constructed treatment wetlands (CTWs) has focused on outdoor installations, with little work done on indoor systems. Previous work on the indoor CTW at the John Heinz National Wildlife Refuge focused on whether the lack of floral diversity in the system would negatively impact its ability to remove various chemical parameters, but despite their great importance in bioremediation the microbial communities in these CTWs have never been studied. In this study, the presence and functionality of ammonia oxidizing bacteria was examined in various points of the treatment system using both culture-dependent and culture-independent DNA-based techniques. In addition, the overall ability of the treatment system to filter out nitrogenous compounds was measured, to demonstrate how its functionality may have changed over the multi-year period since the last study. Water chemical testing revealed that there was a functional shift observed in each section of the treatment system, with much higher levels of ammonium leaving the first aerobic section than before and with the second anaerobic section now eliminating seemingly all forms of inorganic nitrogenous compounds. Despite more levels of ammonium leaving the aerobic section than the anaerobic one, functional assays only indicated ammonia oxidizing activity from the microbial communities isolated from plant roots taken from the aerobic section than the anaerobic one. Metagenomics analysis of the microbial communities isolated from plant roots taken from both sections of the treatment system indicate a dominance of *Proteobacteria* throughout the entire system, with higher levels of *Acidobacteria*, *Verrucomicrobia*, and *Planctomycetes* in the aerobic section. The higher percentage of *Planctomycetes* in the aerobic marsh compared to the anaerobic one might be correlated with the brackish water in this component of the CTW, as this condition gives ammonia oxidizing bacteria a competitive advantage over other bacterial types. Furthermore, the absence of *Nitrospirae* may be correlated with high levels of nitrite in the aerobic section, which would also indicate ammonia oxidation is occurring. Both of these findings suggest that conditions do exist for these bacteria to function well *in situ*, but some other properties of the marsh may inhibit their activity. For example, the presence of *Actinobacteria* in the anaerobic marsh could indicate that this section of the CTW Is not fully anaerobic, which may explain why ammonia oxidation occurs there. Due to the great potential of indoor CTWs, and the scarcity of data available on them, more work needs to be done fully understand the microbial dynamics and long-term functionality of these systems.

Introduction

The importance of wetlands for groundwater filtration has long been known, and something that has provided immense value to both natural ecosystems and the human population. For example, the absorption of excess nutrients from influent groundwater into wetlands protects downstream and adjacent waterways from eutrophication and its subsequent anoxia, which not only helps maintain their water quality and wildlife populations but also makes them more economically useful, through fishing and recreational use, and safe, through the prevention of toxic algal blooms, for people (Hammer 1989). Because wetlands are highly efficient at water purification, people have taken advantage of this and built artificial ones to use for wastewater treatment. When compared to traditional mechanized systems of wastewater treatment these artificial wetlands require more space and have a delayed onset between their construction and functional use, however these constructed treatment wetlands (CTWs) require less energy input and physical upkeep to maintain, are more cost efficient, can provide aesthetic value, and serve as an educational tool to demonstrate the functional significance of these landscapes in nature (Kadlec and Wallace 2009).

Numerous examples of wastewater CTWs exist and their value for purifying wastewater has been thoroughly demonstrated, but the vast majority of wastewater CTWs in use are outdoor systems, and very few indoor ones exist. One principle reason for this is the large land requirement needed to have a CTW big enough to filter influent wastewater well enough for functional use. According to Sustainable Sanitation and Water Management, a company which constructs CTWs for wastewater treatment, a wetland surface area of as high as $3m^2$ per person equivalent (the amount of people that generate waste the CTW will have to treat) is needed to filter wastewater before either reuse or discharge into the environment (Tilley et al. 2014). In addition, to most effectively remove NH_4^+, which is of particular concern in wastewater treatment, an increased hydraulic retention time of the influent water in the wetland is needed, which may lead to an even larger size needed for the CTW than originally planned (Simeral). Effective treatment wetlands also should contain a diverse plant community (Engelhardt and Ritchie 2002, Ebrahimi et al. 2013), which may lead to increases in planned CTW size in order to include the necessary floral diversity. Furthermore, the potential large size of the plants, and machinery needed for wetland upkeep, also takes up space. However, one main advantage of an indoor CTW over an outdoor one is the ability to more tightly control the growing and living conditions of the plants and microorganisms in the treatment system. In nature both temperature and precipitation fluctuate seasonally affecting the biological activity of the organisms in any given landscape, such as the ability of plants to grow and absorb nutrients and microorganisms to metabolize various chemicals in the ground, meaning the efficiency of an outdoor CTW may change temporally stemming from these climactic variations (Jing and Lin 2004, Braeckevelt et al. 2011, Garfí et al. 2012). Having a CTW indoors would give the system operators more control of these environmental parameters, allowing them to adjust them to yield the most biological activity, and hence greatest efficacy, of the wastewater treatment system throughout the entire year, rather than certain portions of it. Thus, though challenges exist for the construction of indoor CTWs, their great potential for high-efficiency wastewater treatment regardless of ambient seasonality calls for an effort to build more of them and study the ones already in existence to better understand their functionality.

Though the vast majority of wastewater CTWs are outdoors, an indoor one is in use to clean the wastewater from the Cusano Environmental Center at the John Heinz National Wildlife Refuge in Tinicum, PA. Constructed in 2001, this system, located within a greenhouse, was designed to filter wastewater produced from the various daily activities of the environmental center and either recycle it as grey water to use for flushing toilets or release into the surrounding environment (Applied Water Management Inc.). Before being treated by the CTW wastewater produced by the environmental center is first channeled to a large pretreatment tank, where solid materials drop out of suspension and are degraded by anaerobic bacteria. Upon reaching the CTW, the water passes first through the aerobic section of the system and then through the anaerobic section. These two sections of the treatment system are physically separated from one another and located laterally adjacent to one another (Figure 1). Just like natural wetlands, the chemicals in the influent water are removed physically, through mechanisms such as sedimentation and sorption to soil particles and other material in the planting medium, and biologically, through microbial degradation and biologic uptake and assimilation by plants, bacteria, and any other organism living in the system (Novitzki et al. 1997). Also similarly to wetlands in nature, this system theoretically should be able filter out a wide array of the influent chemicals due to the presence of both aerobic and anaerobic soils. After passing through both sections, the water is exposed to UV radiation, which kills all microorganisms living in it, before being either recycled as toilet water or expelled into the environment (Applied Water Management, Inc.).

Originally in each section of the marsh there was a mixture of native and tropical plants, but one summer the cooling system within the greenhouse broke, causing many of the local plant species to perish and the tropical ones to dominate; currently the aerobic section of the marsh contains predominantly elephant ear (*Colocasia esculenta*) and canna (*Canna* L. hybrid) plants, while the anaerobic section has mainly umbrella papyrus (*Cyperus alternifolius* L.) (Gerhart 2012). The resulting lack of floral diversity and shift towards a system containing mainly non-native ones caused concern about changes in the system's functionality. A loss in biodiversity in the CTW I concerning as it has been shown that high plant diversity minimizes competition and results in more complete chemical elimination as different plant species preferentially uptake different nutrients through niche differentiation (Gilbert 2004). In addition, certain plants are innately better at taking up certain chemicals over others, and a treatment system with different uptake specializations among the plant species present should also provide better water remediation. Furthermore, certain plants physically and chemically alter the soil which makes other plants more efficient at chemical uptake, and hence bioremediation (Engelhardt and Ritchie 2002).Finally, different plants species also have tendencies to harbor different rhizobia-associated microbial communities, leading to a more wide array of *in situ* chemical metabolism (Faulwetter et al. 2011). With knowledge in mind, questions exist about the current floral species present in the ability of the current floral species present in the CTW at the Cusano Environmental Center to filter wastewater in the absence of high plant species diversity in the system. For example, while it has been demonstrated in CTWs that umbrella papyrus is effective at removing NH_4^+ and much of the chemical oxygen demand in wastewater, previous work has revealed that it does not remove phosphorus as well (Ebrahimi et al. 2013). Previous work has also indicated that while canna (in this case *Canna indica*) can survive in treatment systems with high levels of ammonia (Konnerup and Brix 2010), as would be the case in those that treat wastewater, compared on average to other plants used in these systems it is not as effective as removing both NO_3^- and NH_4^+ (Zhang et al. 2009). Furthermore, while a recent study deemed elephant ear effective at removing the different forms of nitrogen and phosphorus containing compounds and chemical oxygen demand, it cannot translocate heavy metals past its roots very well, making its harvest for heavy metal removal difficult (Madera-Parra et al. 2015).

Stemming from these concerns, a former student within the Department of Earth and Environmental Science at the University of Pennsylvania decided to test the functionality of the treatment system after this loss of diversity. He uncovered that although the system was still able to filter out a lot of the excess nutrients in the influent wastewater, levels of NO_3^- were higher than expected leaving the anaerobic marsh. Moving forward, his principle suggestion for improving the ability of the anaerobic marsh to eliminate NO_3^- was to restore the original composition of the vegetation in this section presumably through enhancing its ability to reduce the incoming NO_3^- to nitrogen gas from the aerobic one. However, the plants used in constructed wetlands represent only one component of their overall functionality, and to fully understand both how these systems work and how to improve them it is imperative to study some of the other factors which may impact their efficacy.

In addition to the floral composition in these systems, the microorganisms present, both attached to the plant roots and living elsewhere throughout the planting medium, are critical for the filtration of influent wastewater in CTWs. It has long been known that microorganisms are critical for bioremediation of toxic agents and are vital for nutrient cycling and chemical removal in natural wetlands, making them central for proper CTW function. However, while much data has been generated

about both CTW function and the impact different plant species can have on it, relatively few studies exist that focus on the microorganisms present in these systems. In systems studied previously, microbial biomass and diversity tend to be greatest at the soil surface and decrease significantly with depth (Tietz et al. 2007, Truu et al. 2005), indicating that microbes may have the greatest impact on bioremediation closer towards the surface. In addition, the plants used in wetlands greatly impact the types and diversity of bacteria present in the system, which also has great implications for the capacity of the CTW to filter influent water (Sleytr et al. 2009, Faulwetter et al. 2011). Certain bacteria are also critical for the removal of certain chemical species. For example, without bacteria from certain genera such as *Nitrosomonas* and *Nitrosospira*, adequate removal of ammonium cannot occur (Faulwetter et al. 2008).

Because microorganisms play such a critical role for CTW function more information about how their presence and function in these systems needs to be examined, and this knowledge would also assist with the proper maintenance of the John Heinz National Wildlife Refuge CTW.

In addition to studying the treatment system as it is currently, it is important to examine how its functionality has changed over a multi-year period. In a previous study (Sartoris et al. 1999) observing the nitrogen transformations in CTWs over a multi-year timescale, conditions became less suitable for microbial transformation of ammonium into nitrate because levels of oxygen within the wetland had dropped considerably over time. It was hypothesized that the intense nutrient load into the treatment system had initially fostered so much microbial growth and metabolism that all the oxygen within the soil had been consumed, leaving it anaerobic and unfavorable for nitrification to occur. Also, at times levels of available carbon for microbial metabolism may fluctuate in these systems, and because nitrification requires carbon this will affect the amount of ammonium the system will be able to convert into nitrate at different points in time (Sartoris et al. 1999). Conversely, there could be a build-up of extra carbon from dead roots and other organic matter, reducing the penetration of oxygen into the soil, and favoring the growth of anaerobic microorganisms over aerobic bacteria. This may then hinder the elimination of ammonium from the aerobic section of the treatment system as some aerobic bacteria oxidize ammonium. Furthermore, the build-up of organic matter from decaying plant and microbial parts may fill the soil pore spaces, reducing oxygen penetration and occluding surfaces where bioremediating bacteria live. Because bioremediation generally takes place on such surfaces, occlusion results in less contact with the water being treated. Filling up the pore spaces would also decrease the hydraulic retention time of the first marsh, meaning that more influent wastewater would pass through without remediation. This decreased hydraulic retention time (Reed 1993), coupled with the reduction in bioremediative surface area, may account for more chemicals passing through the system untreated than before. Additionally, even though the plants in wastewater CTWs foster nutrient removal through direct uptake and stimulation of microbial bioremediation, plants can also act as a source of nutrient addition into the wastewater. When plants are actively growing they intake more nutrients to support their increased biomass production. However, right after their peak growth rate the plants will begin to die back and exude more nutrients into the planting medium than they uptake, causing a net increase of nutrients into the system, working against the goal to clean the wastewater (Sartoris et al. 1999). For the wastewater CTW in the current study, plants are harvested when the vegetation gets too large but it

is unknown whether this occurs after the plant maximum growth rate, another factor which may influence the amount of certain chemicals of interest leaving the treatment system.

The aim of the current project was explore the microbial community within both sections of this CTW while still continuing to monitor levels of ammonium and nitrate at different points in the system, to assess the level at which the CTW it is operating and determine if there has been any shift in functionality. The goal of the project was to determine which bacteria were present and functional in both sections of the marsh, specifically focusing on those involved with ammonia oxidation. Ammonia oxidation is not only a critical step within the nitrogen cycle to remove excess nitrogenous waste compounds, but also useful in the study of treatment wetlands because it sheds light onto the other physical parameters, such as oxygen content, in the system, because it is only under specific conditions that this transformation occurs. All of the data and insight uncovered from this study will be relayed to the refuge management to assist with their management of the system moving forward.

Methods

Water chemical testing

To determine the treatment system's current efficacy levels of ammonium, nitrate, and phosphorus, each of which are common in wastewater and whose removal is critical before effluent discharge into the environment, each was measured at three points in the CTW: 1) before entry into the first aerobic marsh, 2) after exiting the first marsh and entering the second, and 3) after exiting the entire system (Figure 1). Water samples from each location were collected using 50mL bottles and brought back to the University of Pennsylvania Department of Earth and Environmental Science facilities for spectrophotometric analysis. In total, 33 water samples were collected from each sampling spot, and were predominantly analyzed the same day as collection (only a few were stored at 4 degrees Celsius overnight before analysis). Spectrophotometric determination of each chemical being measured was done using both HACH instrumentation and protocols. Levels of ammonium were measured via salicylate digestion using HACH methods 10031 (high range) and 10023 (low range), and levels of nitrate were measured via chromotropic acid digestion using method 10020.

Root collection

To determine the composition and functionality of bacteria in the treatment system root samples were taken from plants within both marshes. Roots were harvested from four locations within each marsh in the late fall (November 2014). The aerobic marsh was broken up into six equal sized sections based on vertical (top, middle, bottom) and horizontal (left, right) position (Figure 1). The anaerobic marsh was divided into quadrants (Figure 1). Despite being planted in gravel, the roots were surrounded by soil, and the roots were harvested at a depth when this soil was moist, the depth at which bioremediation of the wastewater begins. In addition, roots were also collected from greater depths by pulling them up from the ground beneath where the digging had stopped. An emphasis was placed on collecting the tips of the roots because they would provide more surface area for bacterial contact. Only soil directly attached to the roots was included in the sampling. Aseptic techniques were used, such as rinsing the small gardening tool used for digging and root collection with ethanol and sterile water before collection from the next location, to avoid cross-contamination. The roots were

placed in sterile Whirl-Pak® bags and then into a cooler with an ice pack and transported back to be stored at 4 degrees Celsius in a microbiology laboratory at the University of Pennsylvania for further testing.

Another root sample was taken during the winter (January 2015) from each marsh and handled similarly as those from the previous sampling period, except this time two root samples were collected from each location. From the aerobic marsh roots were harvested from the bottom-left section and those from the anaerobic component were again taken from the "middle" (Figure 1).

Detection of ammonia oxidation

Initial testing to determine the presence of ammonia oxidizing bacteria from each root sample was conducted the same day as root collection. Four grams of roots from each sample were mixed with 50mL of phosphate buffer (pH 7) and shaken at 100rpm at 30 degrees Celsius for two hours using the New Brunswick Scientific Excella E25 Incubator Shaker Series in order to dislodge the bacteria attached to the roots or any soil particles collected. Three mL of the resulting solution was mixed with 7mL liquid ammonia oxidizing medium, and incubated at 30 degrees Celsius for one week (protocol adapted from Weber and Legge 2010). All samples from the first collection period were incubated under aerobic conditions, while one of the root sample bacterial solutions from each location from the second collection period was incubated within an anaerobic chamber for two days and exposed to aerobic conditions for the remainder of incubation as described in more detail in the cultivation methods below.

After one week the amount of ammonia, nitrite, and nitrate in each root sample bacterial solution was determined using the API Freshwater Master Test Kit. For each sample, 1mL of the solution was mixed with the appropriate amount of testing solution for each test (8 drops for the ammonia testing solution, and 5 and 10 for those testing nitrite and nitrate respectively). After 5 minutes the resulting color change was recorded and measured using the appropriate color scoring cards provided in the test kit, with a more significant color change being correlated to the amount of the chemical present (protocol adapted from Leboffe and Pierce 2010) (Figure 2). This test served as an indirect measure of the presence of ammonia oxidizing bacteria and their relative amounts between the different marshes, with a higher nitrate level recorded from bacterial root wash solutions from a given marsh implying more of these bacteria present in this section. Because it is the ammonia oxidizing bacteria that would be converting the ammonium from the medium into nitrate, the level of nitrate indicated by this test is positive correlated with levels of ammonium oxidizing bacteria in the root wash solution. As controls, one tube of only phosphate buffer, and another of only the medium, were incubated and subjected to the same API Freshwater Master Test Kit chemical testing, to ensure that both the phosphate buffer itself did not influence the results of the testing and the medium itself did not on its own oxidize into nitrate, yielding a false positive for the presence of ammonia oxidizing bacteria in the sample.

Relative ammonia oxidizing bacterial enumeration between marshes

Additionally, a subset of root sample bacterial solution was serially diluted (from 10^0 to 10^{-3}using water) and 100ul of each dilution transferred to a solid medium designed for ammonia oxidizing bacterial cultivation, along with several dilutions of these bacterial solutions. Because no growth was seen in this experiment, the bacterial solutions from roots collected from the second sampling period were collected and serially diluted only after 18 hours (as opposed to 1 week in the first trial) The root sample bacterial solutions were serially diluted as described above, 100ul transferred to the same solid

medium as previously mentioned above, and incubated at 30 degrees Celsius for 3 days under aerobic conditions. Additionally, as indicated above, the bacterial solutions from each marsh that had been incubated within the anaerobic chamber were introduced to aerobic conditions to permit the serial dilution of the solution onto the solid media. These plates were then incubated anaerobically at 30 degrees Celsius for 24 hours within the anaerobic chamber. This was done to see whether there would be any difference in colony count or composition of the microorganisms when grown either with or without oxygen being present.

Root DNA extraction and analysis of associated microbial community

DNA was extracted from the roots taken from each marsh during the second collection period, using the PowerSoil DNA Isolation Kit from MO BIO Laboratories, Inc. and the associated protocol. Though mainly roots were targeted for bacterial DNA extraction, some soil attached to the roots was also included for each sample. The isolated DNA was stored at -20 degrees Celsius before being sent to the Microbial Community Systems Laboratory at the Argonne National Laboratory in Argonne, IL for metagenomics analysis. Using a primer designed for the 16S rRNA gene, the bacterial community structure and relative abundance at the phylum level was determined for each root sample using PCR, Illumina Next Generation Sequencing, and bioinformatics data analysis protocols established by the Argonne National Laboratory.

Results/Discussion

Water Chemical Testing

Interestingly, significant differences were observed between the levels of each chemical at the different water sampling points compared to those measured in the previous study of this treatment system. Whereas in the previous study consistently low levels of ammonium were discharged from the aerobic section, as would be expected, currently much more ammonium is passing through (Figure 3), notably more than the 3mg/L level set by those who designed the system (Applied Water Management, Inc.). Additionally, previously the level of nitrate leaving the anaerobic section was higher than both expected and what is permitted by the EPA, currently the levels of each chemical measured leaving the system are much lower, and at levels permitted legally for discharge (Figure 4). In the previous study the aerobic section (Marsh one) worked a lot more closely to what would be expected with regards to ammonia oxidation than how it is functioning currently, indicating that conditions in this section are no longer suitable for ammonia oxidation. Even though at various sampling points a decrease in ammonium is observed between what is entering and leaving the aerobic section, this decrease is not accompanied by an increase in nitrate, as would be expected if the ammonium was being oxidized. In general levels of nitrate did not increase from the influent water to the system from that leaving the aerobic section, further indicating that potentially the amount of ammonium being oxidized is lower than expected.

Ammonia Oxidation Potential for Root Samples from Each Section

Ammonia oxidation into nitrate was measured in all bacterial solutions washed from roots from the aerobic section of the marsh, though more nitrate was measured from samples taken during the first sampling period than the second. The wash solutions cultivated aerobically and anaerobically from the first marsh during the second root sampling period both had the same about ammonia oxidation

detected. Conversely, no measurable ammonia oxidation was observed from bacterial solutions washed from roots from the anaerobic marsh, regardless of sampling period or the presence of oxygen during cultivation. The samples from the roots from each location detected high levels of ammonium, indicating that ammonium was free and available for use by any ammonium oxidizing bacteria present. Nitrite was only detected in one of the wash solutions taken from the aerobic marsh during the first sampling period (Figure 5 and 6). Unfortunately, because there was no positive control it is difficult to determine the level of ammonium oxidation on a more absolute scale for each sample, but these results still serve well as a basis of comparison between the different sampling points.

At first these results seem intuitive, because more ammonia oxidation (stemming from a greater ammonium oxidizing bacterial presence) should be expected in the aerobic section of the marsh than the anaerobic one. Yet, because the above HACH chemical readings seemingly indicate that more ammonium is getting oxidized in Marsh two than Marsh one, these results may seem contradictory. Because the root samples from Marsh one contained functional ammonia oxidizing bacteria, the elevated levels of ammonium leaving this section must be attributed to another factor, such as these bacteria's ability to function *in situ* or any other physical characteristic of the marsh which hinder ammonia oxidation. Because no ammonium was converted into nitrate for the root wash solution from Marsh two, the elimination of ammonium from this section as indicated by the HACH testing must be due to some other factor.

Relative Colony Counts and Morphologies

A lawn of bacterial growth, defined as mat of colonies which are not physically separated or distinct from one another, was noted for undiluted and 10^{-1} bacterial root solutions from both marshes grown both under aerobic and anaerobic conditions, and from the 10^{-2} dilutions of all solutions grown under both conditions there were too many colonies to count. From the 10^{-3} dilution grown under aerobic conditions 25 colonies were isolated from the root wash solution from Marsh one while 24 were isolated from Marsh two, and from this dilution under both conditions of oxygen presence from root wash solutions from both marshes there were too few colonies to be considered meaningful. Replating on TSA yielded differences in colony morphologies between both marshes (Figure 7 and 8). While the results of the colony counts were similar, the observed growth only represents the culturable fraction of the entire rhizobia-associated microbial communities from each marsh grown on this specific solid medium at these specific incubation conditions, and these results do not take into account the large percent of bacteria not observed here. Because of this, these results cannot yield insight into the comparative total amount of bacteria with ammonia oxidizing capacity between both marshes. However, because root wash samples from both marshes did contain ammonia oxidizing bacteria, but ammonia oxidation was only noted from root wash samples taken from Marsh one from the functional assay, perhaps Marsh one contains much more potent ammonia oxidizers, such as members from the *Nitrosomonas* or *Nitrosospira* genera, than Marsh 2. The idea of there being a difference in the microbial community composition between the different sections of the treatment system is further supported by the difference in colony morphologies noted during the TSA plating.

Root Microbial Community Analysis

The microbial communities from both sections of the treatment system were dominated by *Proteobacteria*, a phylum which includes both ammonium and nitrite oxidizing bacteria. Several notable differences existed between the microbial communities isolated from roots collected in each marsh

(Figure 9). Marsh one had a relatively high amount of *Planctomycetes*, a phylum that usually thrives in brackish conditions, indicating that the salinity of this section may have been higher than one would expect. Interestingly, ammonia oxidizing bacteria are not as negatively impacted as other bacterial types upon increasing the salinity of their environment, meaning that in this section of the treatment system they could have a competitive advantage and thus more ammonium could be oxidized (Bassin et al. 2011). However, as indicated above, ammonium was detected in water samples taken from the outlet of the aerobic section suggesting that, although these ammonia oxidizing bacteria are likely functional *in situ,* excess nutrients may have been released by the plants and other organic matter in Marsh one. Interestingly, *Nitrospirae* were not detected in the metagenomics analysis of samples collected from marsh one. While *Nitrobacter* were not identified, the *Proteobacteria*, which dominated the community composition in both marshes, contains the genus *Nitrobacter*, so it is possible that they are present within Marsh one. In wastewater treatment plants previous work has demonstrated that the two dominant forms of nitrite oxidizing bacteria are *Nitrospira* and *Nitrobacter*, and the relative amounts of each sheds insight into the relative amount of nitrite, and hence ammonia oxidation, taking place in the system. *Nitrospira* tend to dominate when levels of nitrite are low with the reverse being true for *Nitrobacter* (Nogueira and Melo 2006). Taking these results into account, the absence of *Nitrospira* and possible presence of *Nitrobacter* may mean that levels of nitrite, and hence ammonia oxidation, are high within Marsh one. In this case, the release of ammonium by Marsh one may indicate that the system's ability to process ammonium is overwhelmed, possibly due to the to pore space clogging issues mentioned above. Lastly, in Marsh two the percentage of *Actinobacteria* relative to the total microbial community diversity was high than what was observed in Marsh one. Because *Actinobacteria* are obligate aerobes (McNeil and Brown 1994), their presence in Marsh two suggest that oxygen levels in this section of the treatment system are higher than previously thought.

Ideally, for the metagenomic analysis more than just one root sample per marsh would have been taken, but due to financial restrictions that was all that could be done. Having DNA extractions carried out from different points in each marsh would have given a more representative view of the microbial structure of each section, and perhaps could have even given insight into any localized anomalies. In spite of the limitations, the data obtained does provide some insight into marsh functioning, as well as directing future avenues to explore.

Future Directions

Future work will need to include more root sampling points from different points within each section of the treatment system. This will allow for meaningful statistical analysis between the different sampling points for the *ex situ* determination of ammonia oxidation potential for the microbial plant root communities from each marsh, the colony counts, and any metagenomics analyses. This will also provide more insight into any localized patterns of functionality within each marsh.

While the change in functionality of Marsh one might have been expected as per the literature presented in the introduction, it is more difficult to explain why Marsh two functions differently than before. Although Marsh two was intended to be anaerobic, much of the ammonium presumably coming from the first marsh is eliminated by the time the influent water exits this section of the treatment system. This, in conjunction with the dramatic reduction in nitrate by the anaerobic marsh, may lead one to conclude that this marsh is in fact carrying out the role of both marshes with regards to nitrogen elimination. Interestingly, in recent years another component of the nitrogen cycle, anommox

(anaerobic oxidation of ammonium), has garnered much attention for its role in nitrogen removal. During anommox ammonium is anaerobically oxidized with nitrite as the electron acceptor by way of the bacteria that can perform this metabolism, eventually producing dinitrogen (N_2) gas as the end product. Despite that bacteria capable of anammox function better at different oxygen conditions than ammonia oxidizing bacteria, increasing the concentration of ammonium oxidizing bacteria will positively influence the function of anammox bacteria, due to increased nitrite being leaked into the surroundings (Zhu et al. 2011). Thus, one possibility is that even though the presence and functionality ammonium oxidizing bacteria should be low in the anaerobic marsh, the large amount of ammonium making it to this section may be stimulating these bacteria just enough so they are making sufficient levels of nitrite for the anommox bacteria to function effectively. Not only would this explain the extent of ammonium elimination by the second marsh, but also it would explain why little nitrate is found in its effluent because the end product is N_2 gas, which is free to leave the planting medium. Anammox should not heavily contribute to the nitrogen cycle in the first marsh because oxygen levels are too high. While this metabolic pathway and the extent it contributes to both the nitrogen cycle and nitrogen removal in CTWs is still being investigated, it has been demonstrated to play a significant role in nitrogen removal in oxygen-deficient marine environments (Daalsgard, Thamdrup, and Canfield 2005), so potentially it may also be more significant here. Primers do exist for anammox bacteria, so a number of assays could be performed to determine the extent of their influence in Marsh two.

For both marshes, it is important to determine what exactly is happening to the nitrogen that enters the CTW. ^{15}N-labeled ammonium can be introduced to each marsh so that future researchers could track where it actually winds up (Ambrosano et al. 2011). For example, if ^{15}N-labeled ammonium is added to the inlet of Marsh one and high levels are detected entering Marsh two (within a relatively short time frame), then this would indicate that much of the ammonium entering the system passes through the aerobic marsh unaltered. Conversely, if low levels of ^{15}N-labeled ammonium are detected at the inlet of Marsh two, this would indicate that much of the ammonium leaving Marsh one is generated by the organic material within that section of the treatment system. ^{15}N-labeled ammonium can also be added to Marsh two, and if a large percentage of the labeled nitrogen is leaving as N_2 gas, this would indicate that the ammonia oxidation does occur in the anaerobic marsh. If not, then some other process would be leading to nitrogen removal in this section of the treatment system. Finding out where exactly the nitrogen that enters each system winds would be pivotal for understanding how this CTW functions.

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Figures

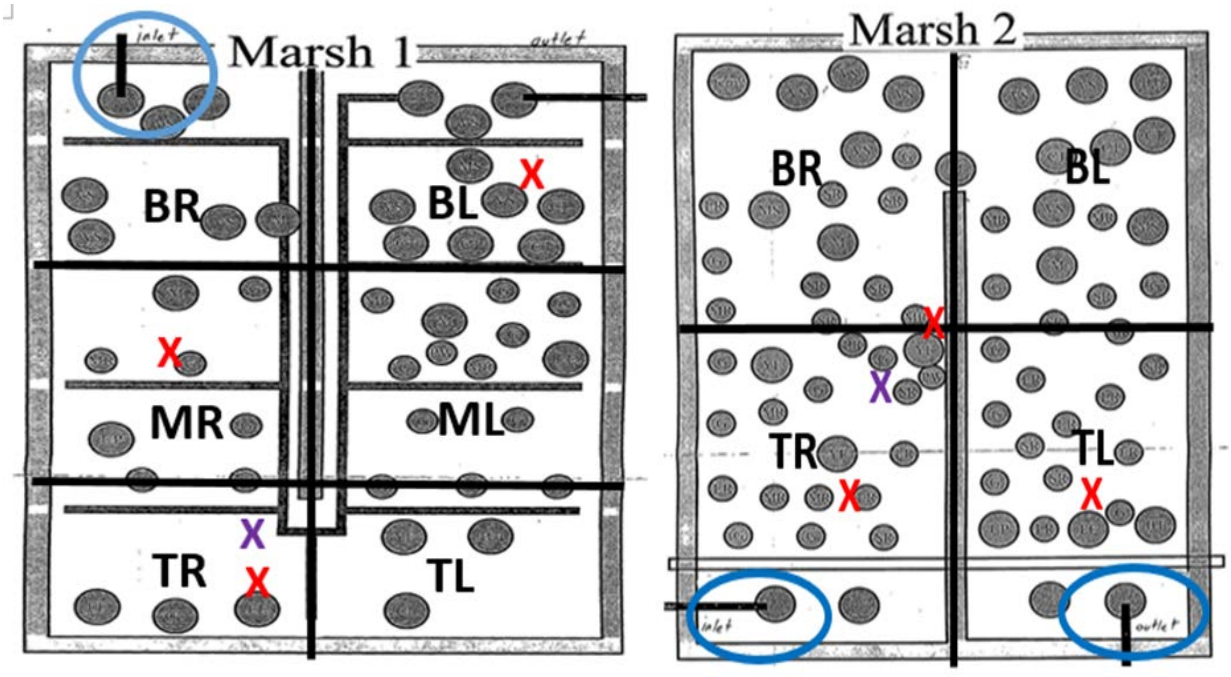

Figure 1. Schematic of both sections of the treatment system. Water sampling points are circled in blue, points of root collection for first sampling period are indicated by red X's, and points of root collection for second sampling period (also roots used for metagenomics analysis) indicated by purple X's. Abbreviations for marsh sections are: BR- back right, BL- back left, MR- middle right, ML- middle left, TR- top right, TL- top left.

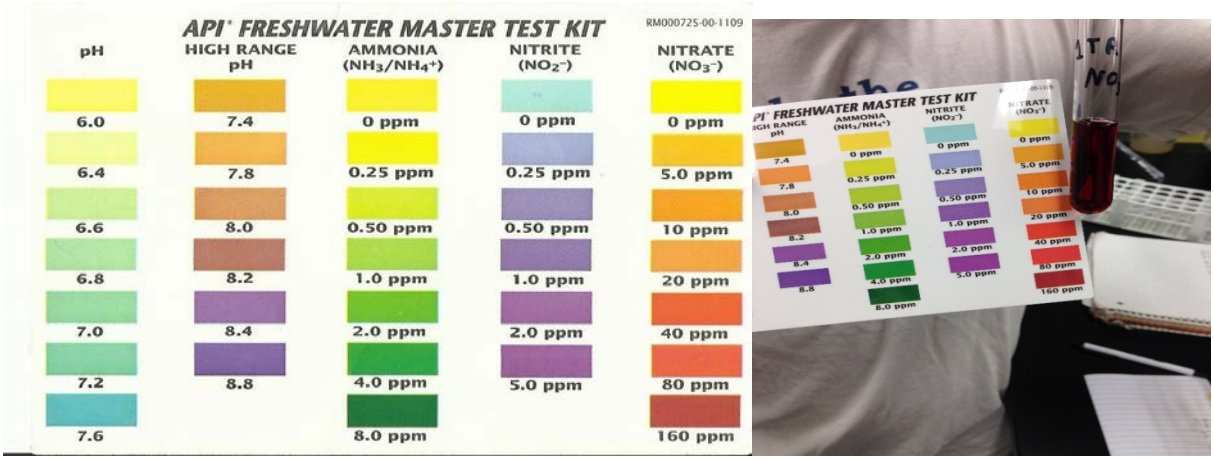

Figure 2. Color scoring card for API Freshwater Master Test Kit, and demonstration of how it is used to gauge chemical content of a solution.

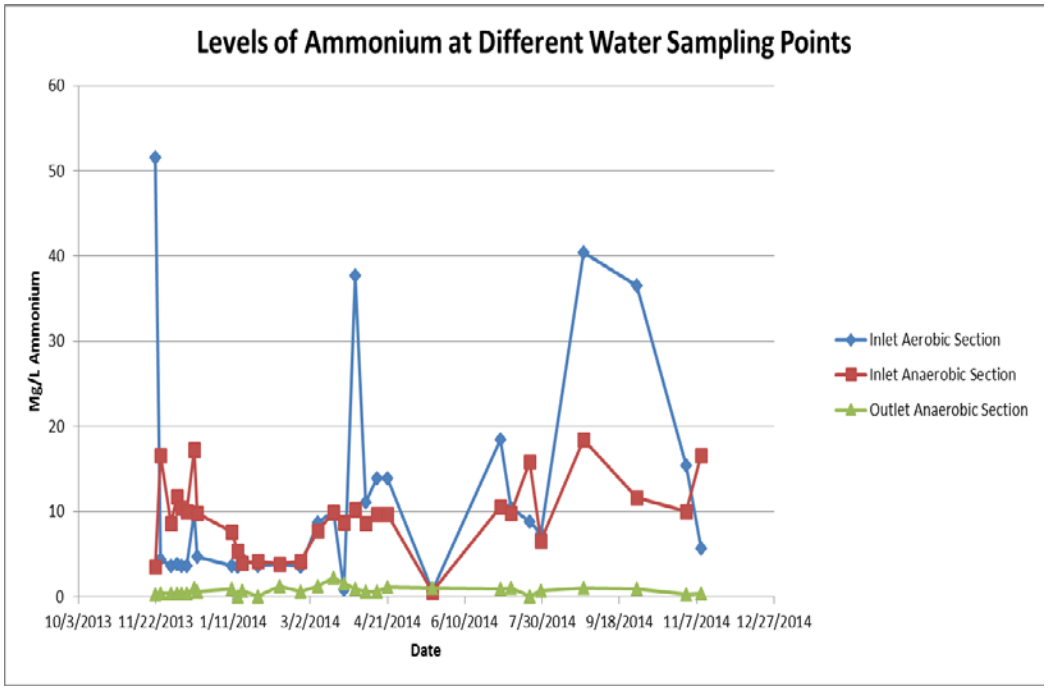

Figure 3. Levels of ammonium detected at three water sampling points.

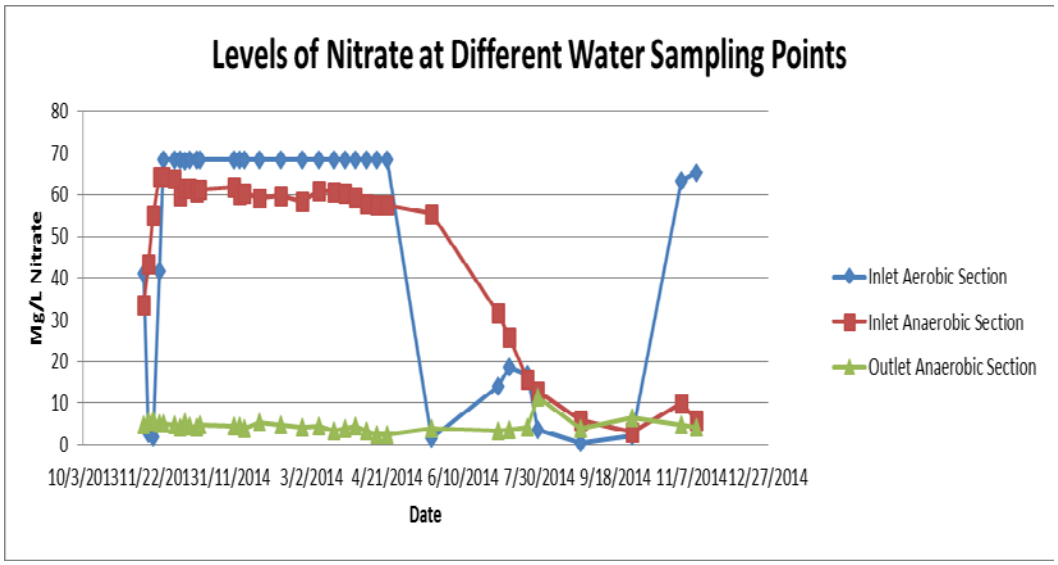

Figure 4. Levels of nitrate detected at three water sampling points.

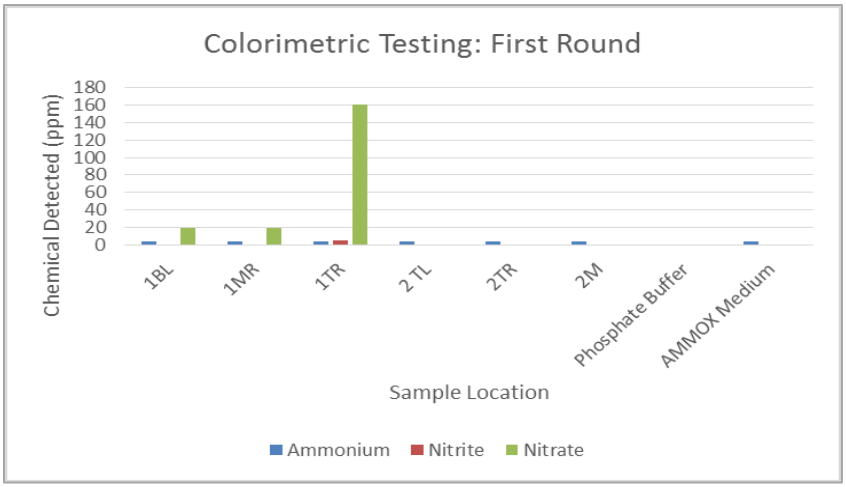

Figure 5. Results from API Freshwater Master Test Kit for first round of root sampling.

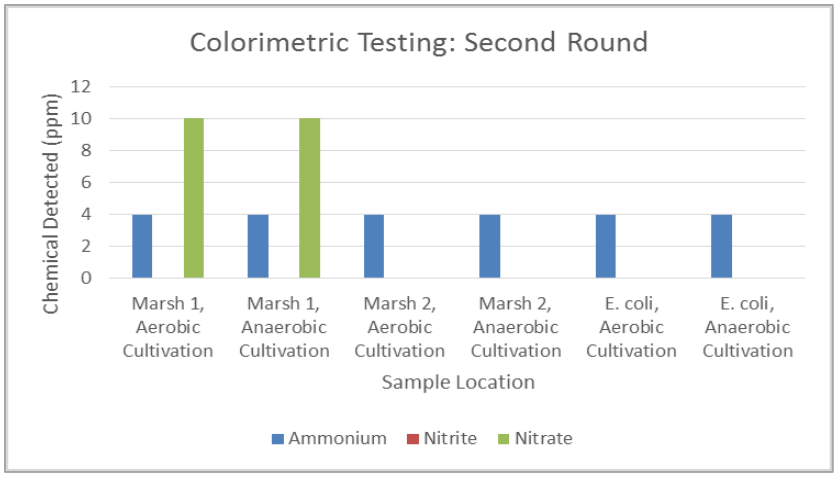

Figure 6. Results from API Freshwater Master Test Kit for second round of root sampling.

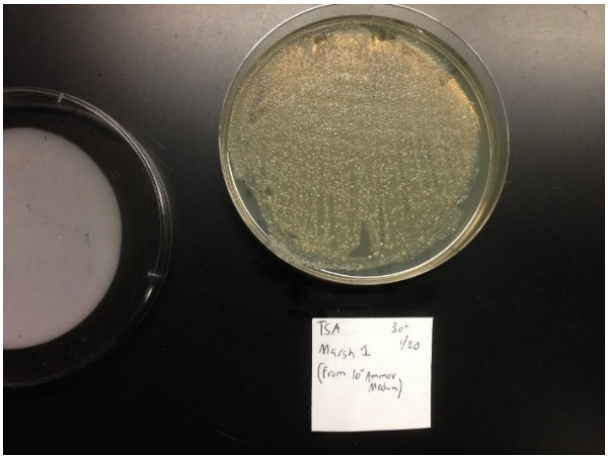

Figure 7. Bacterial growth from aerobic marsh on TSA plate.

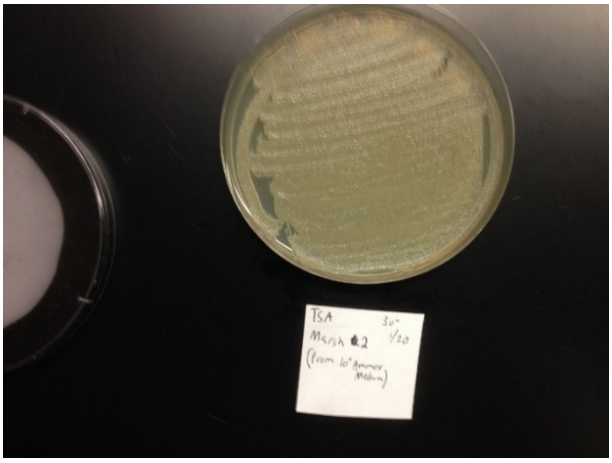

Figure 8. Bacterial growth from anaerobic marsh on TSA plate.

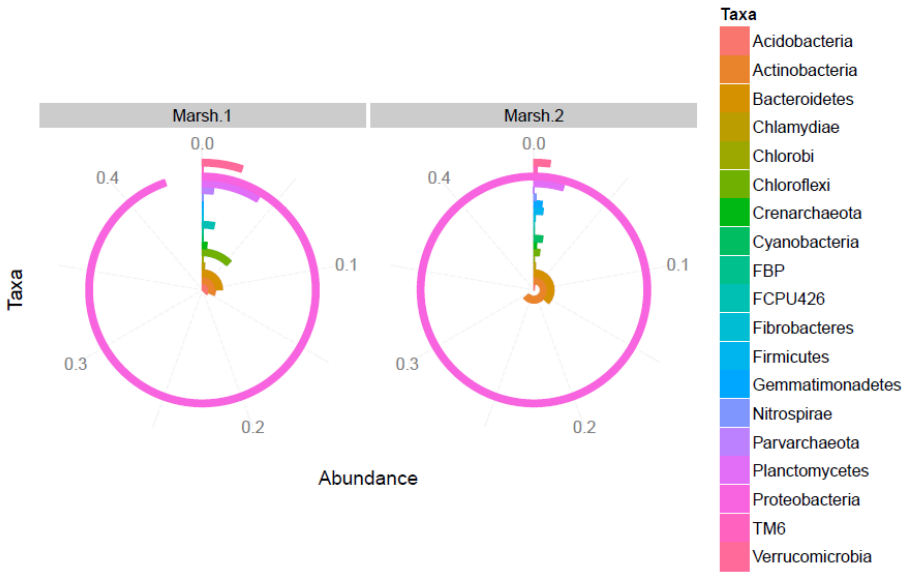

Figure 9. Metagenomic analysis of microbial communities taken from root samples from both sections of the treatment system.