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Microbial Diversity in Continuous Flow Constructed a Wetland for the Treatment of Swine Waste

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Abstract

Contaminant removal may largely be a function of many microbial processes in constructed wetlands. However, the role of microbial diversity for the removal of swine waste in constructed wetlands is limited. Here, we used 454/GS-FLX pyrosequencing to assess archaeal, bacterial, and fungal composition within a surface flow constructed wetland to determine their spatial dynamics and contaminant removal within the wetland. We analyzed our data using UniFrac and principal coordinate analysis (PCoA) to compare community structure and specific functional groups of bacteria, archaea, and fungi in different sections of the wetland. PCoA analysis showed that, bacterial, archaeal, and fungal composition were significantly different (p=0.001) for the influent compared to the final effluent. Our results showed that, the wetland system contained relatively higher proportions of bacteria and fungi than archaea. Most of the bacteria and archaea that were associated with nitrogen removal were affiliated with *Nitrosomonas* which are ammonia oxidizing bacteria (AOB), *Candidatus Solibacter*, an anaerobic ammonia oxidizing bacteria (Anammox), as well as *Nitrosopumilus*, ammonia oxidizing archaea (AOA). The detection of AOB, Anammox, and AOA in this wetland shows abundance and diversity of these microorganisms that are responsible for nitrification processes in constructed wetlands.

Keywords: Constructed wetland; Contaminants; Pyrosequencing; Microbial communities; Swine; Wastewater

Introduction

Constructed wetland (CW) is a natural process for the treatment of waste from a variety of sources [1-3]. They are cost-effective, ecologically-friendly, and a simple alternative to conventional technologies for wastewater treatment [3,4]. It has also been shown that, significant benefits to human populations in both [2] developed and developing countries can be achieved through constructed wetlands, and these benefits may include water-quality improvement, water reclamation, conservation of habitat for species, flood control, recreational and educational activities [1,4-7]. North Carolina is one of the largest swine producing states in the United States [2], and waste disposal was traditionally done by flushing into anaerobic lagoons and then later sprayed on agricultural fields. This resulted in swine waste from large swine farms polluting surface and well waters [8]. During hurricane Floyd in 1999 many North Carolina hog waste lagoons overflew [2] and polluted many surface water systems with fecal indicator bacteria from compromised septic and municipal sewage systems, and livestock waste lagoons [8]. After the storm, North Carolina invested resources [2] into new, cost-effective technologies for waste disposal, and one such technology was constructed wetlands for the treatment of swine waste. As a result, a pilot constructed wetland for the treatment of swine waste was evaluated at [2] North Carolina Agricultural and Technical State University (Greensboro NC, USA). The wetland system uses [2] natural plants for the removal of nitrogen (N), phosphorus (P), solids, and Chemical Oxygen Demand (COD) from treated swine wastewater, resulting in a cleaning final effluent [9].

The removal efficiency by constructed wetlands has been shown to be a function of diverse microbial communities [10]. However, there is a general lack of information on the diversity and changes of the microbial communities in long-term constructed wetlands treating swine waste [2]. Work on bacterial diversity in constructed wetlands is well documented, whereas the role played by archaea and fungi is not clear. The main contaminants from swine waste [2] may include nutrients, salts, microbes, including pharmaceutically active compounds, and their removal involves complex physical, chemical, and biological processes [2]. The aim of the current study was to compare the composition of microbial communities in a continuous surface flow constructed wetland used for the treatment of swine waste using pyrosequencing.

Materials and Methods

Experimental site and sampling

The experiment was conducted at a swine research facility at North Carolina Agricultural and Technical State University farm in Greensboro, NC, USA. This was a continuous flow constructed wetland originally built in March 1996 [11] (Figure 1), and planted with *Typha latifolia* L. (broadleaf cattail) and *Schoenoplectus americanus* (Pers-American bulrush). The wetland has six cells and each cell is 40 m long and 11 m wide. In 2003, a modification was made to cells 5 and 6 [11] to achieve a continuous marsh system with a slope of 0.33% (Figure 1). The new sections were planted with giant bulrushes (*Scirpus californicus*) as previously noted [11]. Sixty-five to 115 pigs were managed in the swine house between January 2007 and January 2012. Samples were collected from eight different points from different sections of the wetland in

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duplicate in November 2012. Grab samples were collected sequentially from effluent from the swine house, lagoon 1, lagoon 2, and the 8,000 L storage tank. More samples were collected from continuous wetland cell influent, final effluent samples, storage pond, and the final recycled effluent. The wetland cell received 10 kg/ha/day with a hydraulic load of 3.8 m^3/day. Samples were stored on ice and taken to the laboratory for further analysis. All samples were analyzed for ammonium (NH^+_4-N), nitrate (NO_3-N), total-phosphate (TP) and available-phosphate (PO_4^{3-}) using a flow injection analysis instrument (Lachat-QuikChem 8000, Loveland, CO, USA), as well as carbon (C) and nitrogen (N) concentrations using the Perkin-Elmer 2400, CHNS/O series II Analyzer (Shelton, CT, USA) [4].

DNA extraction and Pyrosequencing

Total bacterial DNA was extracted from effluent samples using Power Water DNA kits (MO BIO, Inc., Solana Beach, CA), according to the manufacturers' protocol. DNA extracts from duplicate samples (200 ng) were pooled. Extracted DNA (2μL) was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington DE), and run on a 1.0% agarose gel before it was used for pyrosequencing. DNA was stored at -20° C prior to pyrosequencing analysis. The DNA samples (15.0 μl) were then submitted to Research and Testing Laboratories (Lubbock, TX) for polymerase chain reaction (PCR) optimization and pyrosequencing analysis. Bacterial tag-encoded FLX amplicon pyrosequencing was conducted on the 16S rRNA gene for amplification of bacteria and archaea sequences, whereas the microbial tag-encoded FLX amplicon was used for amplification of 18S rRNA gene sequences in fungi. Pyrosequencing (TEFAP) procedures described earlier [12] based on 16S rRNA genes for archaea (V3-V5 regions) and bacteria (V1-V3 regions) with primer pairs, 340F90 (GYGCASCAGKCGMGAAW)/806R96 (GGACATCVSGGGTATCTAAT) and 28F

(GAGTTTGATCNTGGCTCAG)/519R (GTNTTACNGCGGCKGCTG), respectively, were employed. The fungal 18S rRNA gene was amplified using the primer pair, SSUF (TGGAGGGCAAGTCTGGTG)/ funSSUR(TCGGCATAGTTTATGGTTAAG). Pyrosequencing data were analyzed using the dist. seqs function in MOTHUR, version 1.9.1 [13]. Raw reads were treated as previous described [14] using the Pyrosequencing Pipeline Initial Process of the Ribosomal Database Project (RDP), to sort the data, trim off the adapters, barcodes and primers using the default parameters, and to remove ambiguous 'N' [15]. Sequence libraries were further resampled to obtain similar numbers of sequences for diversity and richness estimations [16]. All sequence reads with a quality score 20 and a read length 200 bp were removed. Shannon's diversity index values (H'), and Chao estimates were calculated from MOTHUR as well as operational taxonomic units (OTUs, 97% similarity). We used RDP Classifier with a bootstrap cutoff of 80% for taxonomic classification of the bacterial sequences. We used the keep first 200 bp commands in MOTHUR to eliminate sequencing noise resulting in a sequence read fragment covering the 18S region and aligned to the SILVA database for further analysis of data as stated above.

Statistics and analysis of pyrosequencing data

SAS version 9.1 [17] was used to conduct analysis of variance (ANOVA) to determine differences in wetland properties. PCoA and UniFrac analysis were carried out using MOTHUR to group microbial communities of different samples into different taxonomic groups using the RDP Classifier. RDP Complete Linkage Clustering was also used to generate OTUs and weighted UniFrac from all samples [18]. The relaxed neighbor-joining algorithm in Clearcut (version 1.0.9) [19] was used for the construction of phylogenetic trees and the parsimony test (P-values of 0.05) in Treeclimber [20] was used for between-site comparisons.

Results

Nutrient removal

There were significant decreases ($P<0.05$) in total N, ammonium (NH^+_4), and total suspended solids (TS) in wetlands from the lagoon to the final effluent (Figure 2). The removal rates of N and NH^+_4 in this study were above 70% from the influent to the effluent and this was similar to what had been previously reported [9,21]. The removal rate of total and organic phosphorus was low between the lagoon and the effluent samples. The removal rates of total and suspended solids were significantly higher ($P<0.01$) between lagoon and the final effluent [18].

Community composition, diversity, and estimated richness

A total of 6354, 81234, and 50719 sequence tags for archaea, bacteria, and fungi, respectively, were generated through 454 pyrosequencing (Tables 1-3). The total numbers of OTUs were 661, 8429, and 1946 for archaea, bacteria, and fungi, respectively (Table S1-S3). For the archaea, the highest number of sequences was found in storage pond while the lowest was in the final effluent (Table 1). For bacterial the highest sequence tag was from lagoon while the lowest was from mid marsh (Table 2). Furthermore, for fungi, the highest sequence tag was found in samples collected from storage pond and the lowest from mid marsh (Table 3). We normalized our data to the smallest sequence tag, for reanalysis to show normal distribution of variances (Table S1-S3). Shannon diversity index (*H'*) showed variations in diversity among the different wetland segments with the highest archaeal and fungal

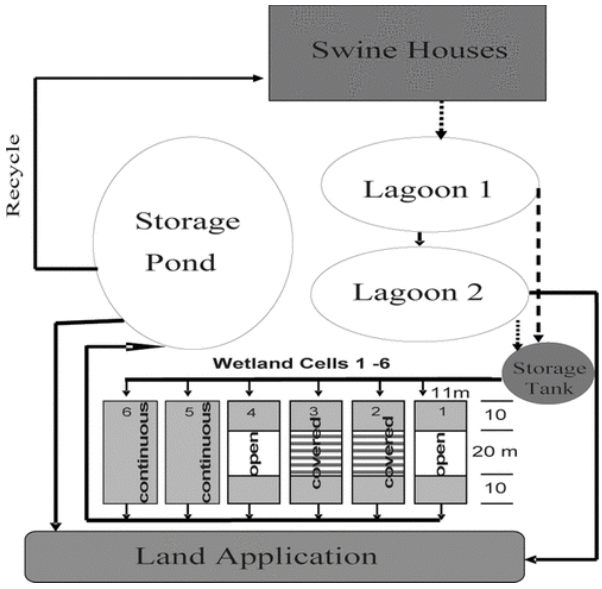

Figure 1: Wetland cells 40 m long 11 m wide constructed in 1995. Each cell consisted of 11 m by 10 m marsh at both influent and effluent and 11 m by 20 m pond section separating the marshes and planted with *Typha latifolia* L. (broadleaf cattail) and *Scirpus americanus* (bulrush) in March 1996. The marsh and pond sections of wetlands have previously been described [2,4].

Group	nseqs	OTUs	chao	invsimpson	npshannon	simpson	coverage
S1	630	51	132.2	5.333953	2.361805	0.187478	0.953968
S2	952	111	354.83333	7.096459	3.043943	0.140915	0.919118
S3	619	92	202.5	16.108388	3.530358	0.062079	0.915994
S4	3318	171	381	3.398484	2.161431	0.294249	0.968354
S5	257	76	130.66667	16.472709	3.830253	0.060706	0.840467
S6	280	89	149.05556	33.759723	4.180865	0.029621	0.832143
S7	163	41	167.5	11.094958	3.192706	0.090131	0.858896
S8	135	30	48.2	7.97619	2.842945	0.125373	0.896296

Table 1: Total sequence tags for archaea generated from pyrosequencing from the wetland land units.

Group	nseqs	OTUs	chao	invsimpson	npshannon	simpson	coverage
S1	9201	751	1440.81	14.230392	4.141051	0.070272	0.953918
S2	11967	1683	3677	22.425723	5.147448	0.044592	0.916688
S3	12385	1233	2765.64	7.080288	4.102311	0.141237	0.940654
S4	10181	1222	2585.45	19.936472	4.683588	0.050159	0.927414
S5	6934	1268	2907.43	20.243401	5.272591	0.049399	0.88852
S6	10800	1758	3380.38	64.356874	6.053123	0.015538	0.914907
S7	10795	822	1809.5	25.159356	4.438501	0.039747	0.955998
S8	8971	1719	3653.54	87.078426	6.120541	0.011484	0.890313

Table 2: Total sequence tags for bacteria generated from pyrosequencing from the wetland land units.

Group	nseqs	OTUs	chao	invsimpson	npshannon	simpson	coverage
S1	10340	135	345.789474	2.212453	1.255139	0.451987	0.991296
S2	8814	102	224.0625	1.167906	0.52713	0.856234	0.992852
S3	6987	556	1186.123457	27.501675	4.283528	0.036361	0.954201
S4	1761	180	390.416667	11.555549	3.552191	0.086539	0.942646
S5	4609	472	947.561644	12.476529	4.024419	0.08015	0.942721
S6	5554	201	439.214286	3.572661	2.243241	0.279903	0.979114
S7	9204	117	289.117647	1.770473	1.058795	0.564821	0.991634
S8	3450	183	414.875	5.192079	2.690632	0.192601	0.969275

Table 3: Total sequence tags for fungi generated from pyrosequencing from the wetland land units.

diversity in the continuous flow effluent, while the highest bacterial diversity was from manure influent to the lagoon 1.

From our pyrosequencing data, the majority of archaeal sequences belonged to the phyla, *Euryarchaeota* (88.7%) and *Crenarchaeota* (5.21%), and at the class level *Methanomicrobia* (42.1%), *Thermoplasmata* (26.25%), and *Methanobacteria* (17.35%) were dominant (Table 4). Bacterial sequences primarily comprised of the phyla, *Proteobacteria* (36.58%), *Bacteroidetes* (18.15%), *Firmicutes* (11.86%) (Table 5). The most abundant fungal phylum was the Basidiomycota (86.3%) (Table 6).

Using the greengenes database, we identified potential sequences at the genus level for specific functional groups of microorganisms that could perform specific functions in constructed wetlands (Table 7). These sequences were mainly from bacteria and archaea. We had a relatively low abundance of *Nitrosomonas,* which are ammonia oxidizing bacteria (AMO) in the midsection of the wetland. However, high sequences of a carbon degrader (CD) *Roseburia* were detected in this section of the wetland. *Flavobacterium, Rhodobacter, Thauera*, and *Methylophilus* are all denitrifying bacteria (DN), and as expected were found in relatively high abundance in this section of the wetland. *Hydrogenophaga* (hydrogen oxidizing bacteria) was also found in relatively high abundance. *Candidatus Solibacter*, an anaerobic ammonia oxidizing bacterium (Anammox), and *Nitrosopumilus*, an ammonia oxidizing archaea (AOA), were detected in this section of the wetland in relatively low numbers. The detection of both Anammox and AOA was not surprising since both are known to perform major functions in major water columns [22].

Spatial phylogenetic structure of microbial community from different segments of the wetland

We applied PCoA (Figure 3), and the UPGMA hierarchical clustering analysis (data not shown) to determine the distribution of microbial phylogenetic similarities and sorted them into different groups by applying UniFrac distance matrix using the UniFrac web interface in MOTHUR. The pattern for archaeal community dynamics was different from that of bacteria (Figure 3A). An archaeal sample from the swine house and lagoons clustered to the right, whereas, samples from storage tanks, wetland cells effluent, mid marsh effluent, and the final effluent for land application clustered to the left. These segments clustered closer to each other, which was the opposite for bacteria. As stated earlier, these four segments have already gone through the wetland lagoons and wetland cells, where most of the biological interactions with complex communities are occurring for the degradation of major compounds from the swine house waste (Figure 1). Bacterial community structures from wetland samples collected from the swine house and lagoons 1and 2 were significantly different ($p<0.0001$) based on parsimony tests (Figure

3B) as wetland compounds were strong structural factors influencing bacterial assemblages (R^2=0.63, p=0.0024). The PCoA (Figure 3B) showed samples from swine house and lagoons clustering to the left while samples from storage tanks, wetland cells effluent, mid marsh effluent, and the final effluent for land application clustered to the right. Furthermore, bacterial community structures from these four segments of the wetland were significantly different based on the parsimony test (p<0.001), and this was confirmed by hierarchical clustering analysis (data not shown) with Jackknife supporting values. In general, the pattern of separations found in bacteria was also observed with fungal community structures in the different wetland segments.

Discussion

We employed 454 pyrosequencing techniques to quantify archaea, bacteria, and fungi community structures in a continuous flow constructed wetland. One major advantage of pyrosequencing is high-throughput and a large dataset of sequences, which can identify many microorganisms in a single analysis. As shown in this study, and from our previous study with bacteria [3], 16S rDNA sequences from different segments of the wetland may be used to determine the efficiency of the constructed wetland. This approach can monitor changes of microbial communities as waste flows through the wetland and has high potential for improving water quality based on bacterial concentrations. However, this technique does not quantify the members in the community in terms of cell number in the water samples, nor does it imply viability of the organism, but may demonstrate the presence of potential DNA sequences as shown in our previous study [23]. Here, we showed the diversity of microbial communities in relation to nutrient content in different sections of the constructed wetland fed with anaerobic lagoon swine wastewater. It has also been reported that, the bulk of the water quality improvement in constructed wetlands is due to microbial activities [1,2,24]. Archaeal, bacterial, and fungal OTUs from the influent were higher than from the effluent of the wetland. These results were associated with nutrient content differences in the different segments of the wetland where the concentrations of TN, NH_4^-, TP and PO_4^{3-} decreased from lagoon to the final effluent (Figure 2). The results can be partly explained by the decrease in nitrogen and phosphorous, which are essential nutrients for microbial growth. This is different from the research results reported by Ipsilantis and Sylvia [25], where microbial counts did not consistently increase under elevated nutrient conditions due to carbon limitation. Nitrogen removal in wetlands has been documented to be the result of microbial activities, which play roles in the pathways of Anammox [26] and nitrification-denitrification [27]. In addition, P removal may partially depend on microbial activities via mineralization [26,28] and immobilization.

We used pyrosequencing in this study to investigate the microbial diversity in the wetlands system because changes in environmental variables will likely have some influence on changes in OTUs. Archaea from storage tank samples displayed the highest species richness, diversity and evenness than samples from other segments of the wetland while results from the final effluent showed the lowest number of sequences; OTUs, and other diversity indices (Table 1). However, for bacteria and fungi, the highest diversity came from samples from wetland effluent, which has gone through the continuous marsh section of the wetland. The abundance, richness, and diversity of bacterial and fungal communities were considerably higher than archaea in a majority of the wetland cells (Table 2 and 3). This was similar to wetlands planted with *Vetiveria zizanioides* or *Juncus effusus* L. that showed much higher bacterial abundance but lower archaeal abundance [29]. In our study bacteria and fungi outnumbered archaea in all the wetland cells.

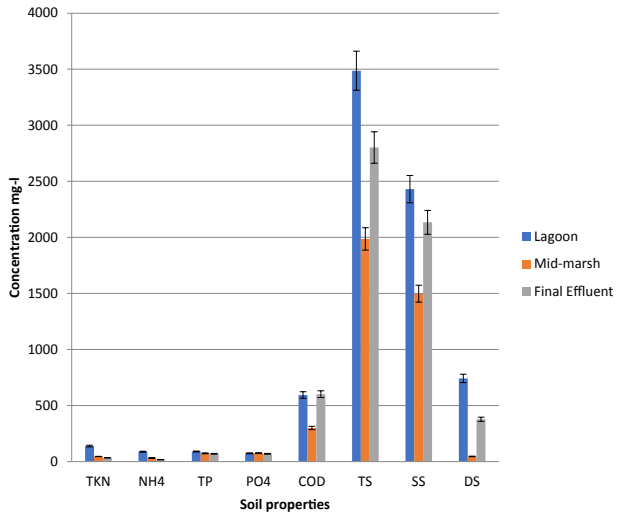

Figure 2: Nutrient removal in wetland samples collected in duplicated.

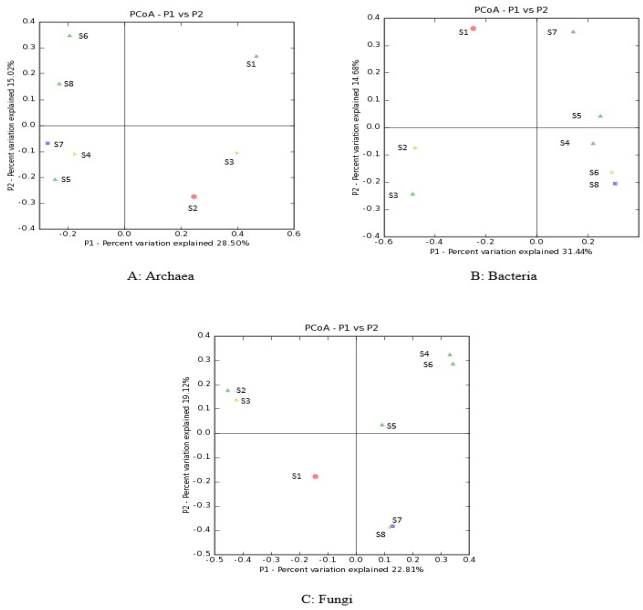

Figure 3: The distribution of microbial phylogenetic similarity sorted into different groups by applying principal coordinates analysis (PCoA) spatially. All data were subjected to a matrix of UniFrac distances using the UniFrac web interface in MOTHUR.

Results from PCoA analysis confirmed that, TN, NH_4^-, TP and PO_4^{3-}, were strongly correlated with the distribution of microbial species on which NH_4^-, TP and PO_4^{3-} concentrations had significant effects. These results suggested that, high levels of nutrient status promoted diversity and distribution of microbial species within the community. It has also been shown that, shifts in the structure of bacterial communities can be associated with changes in a number of soil properties including soil texture and soil nitrogen availability [30,31]. On the other hand, Calheiros et al. [32] reported that, bacterial diversity in constructed wetland may be a significant driver influencing the final effluent quality. Ogier et al. [33] and Dubernet et al. [34] reported that, the difference in the relative abundance of community members may affect the detection

of certain species due to competition during PCR. However, there may well be some species whose presence was obscured because they were not detected by pyrosequencing. Our study showed that, when wetland samples were analyzed spatially, community structures from wetland samples associated with the swine house and lagoons 1 and 2 were significantly different (P<0.0001) based on parsimony test of UniFrac data (Figure 3A) from storage tanks, wetland cells effluent, storage pond, and the final effluent. Microbial communities in wetland cells are highly responsive to perturbation, dissolved organic matter concentration, and chemical stress, among others [35-38].

Pyrosequencing data showed high levels of *Bacillus* which is one of the largest Eubacteria found in soil. Some bacilli are phosphate solubilizing and are capable of surviving in extreme conditions due to their ability to form spores. Han and Lee [39] reported that, the inoculation of certain species of *Bacillus* may increase soil P availability. The high concentration of P in swine wastewater will limit the assimilative capacity of wetland soil and the presence of such bacteria in our constructed wetlands for swine wastewater treatment could impair the capabilities of P nutrient removal. A high relative abundance of potential denitrifying bacteria, *Flavobacterium*, was detected in mid-section of the wetland. *Flavobacterium* associated with this wetland had been shown to be closely related to nitrifying bacteria [2]. However, these authors noted that even though these bacteria demonstrated phylogenic similarity, it does not mean that they have denitrifying potential. Therefore, the existence of denitrifying bacteria may be a contributing factor to the decreasing trend of nitrogen concentration from the influent to the effluent end in our wetland.

Another important pathway for the removal of nitrogen from this wetland is through the Anammox process. During this process, ammonium and nitrite are converted to dinitrogen gas [40]. Our research findings suggested the existence of Anammox bacterial sequences, *Candidatus Solibacter*, and this bacterium is a strict anaerobic autotroph. It is also known for its extremely slow growth resulting in limited applications [41]. Partial-nitrification with Anammox in constructed wetlands with higher removal efficiency of total nitrogen than conventional methods has also been reported [42], thus confirming the role of these group of bacteria in water quality improvements in constructed wetlands. It is possible that Anammox bacteria played a role in nitrogen removal in our wetland. It has been reported that Anammox uses carbon dioxide as its carbon source to produce biomass ($CH_2O_{0.5}N_{0.15}$) and nitrite as electron acceptor for ammonium oxidation, and electron donor for the reduction of carbon dioxide [40]. In our study, we identify six OTUs of *Candidatus Solibacter,* whose sequences were 100% similar to *Candidatus Solibacter* from different sections of our wetland. This wetland has a high concentration of ammonium and nitrite, and as mentioned above [40], the Anammox bacteria require both ammonium and nitrite, which can be found at or near the aerobic–anaerobic interface of sediments and water bodies to function efficiently. Our samples were collected in the continuous flow section of the wetland which provides the most ideal region for the enrichment of Anammox. In constructed wetlands, ammonium diffuses upwards and meets the oxygen that is diffusing downwards, and create aerobe-anaerobe zone for most of the microbial activities associated aerobic bacteria or crenarchaeal ammonium oxidizers. In our study, about 5.1% of archaea were *Crenarchaeota* (Table 4). We also detected by pyrosequencing *Nitrosopumilus* which is a major ammonia oxidizing archaea [22,29,43]. These authors have shown that both bacteria and archaea communities can play important roles in biogeochemical processes in constructed wetland system.

In conclusion, the different sections of the wetland had different nutrient status reflecting different bacterial communities and diversities, permitting different bacteria to play important roles in nutrient removal within the wetland. The removal rates of N and NH_4^+ in this study were above 70% from the influent to the effluent and this signifies the important roles of different microbial groups, played in contaminant removal. Most important, is the improvement of water quality from the final effluent since this water could be spread on the pasture. Therefore, the removal of the main pollutants from the swine wastewater would have a beneficial effect on the ground and surface water and in most of the swine producing regions of the world. This project is an innovative model for waste management for the swine industry and other confined-animal facilities.

Taxon	Total	S1	S2	S3	S4	S5	S6	S7	S8
Archaea	100	100	100	100	100	100	100	100	100
Crenarchaeota	1.2	0.16	0	0.16	1.45	1.97	2.5	1.84	8.15
Euryarchaeota	97.64	99.84	100	99.68	98.01	94.49	92.14	90.18	78.52
pMC2A384	0.08	0	0	0	0.09	0	0	0.61	0.74
unclassified archaea	1.09	0	0	0.16	0.45	3.54	5.36	7.36	12.59
Full sequence percentages of archaea classes									
Taxon	**Total**	**A.MU**	**A.SW**	**A.LA**	**A.MI**	**A.MM**	**A.ME**	**A.SP**	**A.FE**
C2	1.01	0	0	0.16	1.33	1.97	1.79	1.84	4.44
Methanobacteria	15.62	62.38	20.59	22.46	2.53	18.9	18.57	33.13	19.26
Methanomicrobia	59.34	0	3.36	25.04	93.64	65.35	54.29	52.76	52.59
pMC2A209	0.02	0	0	0	0.03	0	0	0	0
Thaumarchaeota	0.16	0.16	0	0	0.09	0	0.71	0	2.96
Thermoplasmata	22.3	37.46	76.05	52.02	1.84	4.72	17.86	4.29	2.96
unclassified archaea	1.09	0	0	0.16	0.45	3.54	5.36	7.36	12.59
unclassified Crenarchaeota	0.02	0	0	0	0	0	0	0	0.74
unclassified euryarchaeota	0.38	0	0	0.16	0	5.51	1.43	0	3.7
unclassified pMC2A384	0.08	0	0	0	0.09	0	0	0.61	0.74

Table 4: Full sequence percentages of archaea at the phylum and class levels.

Taxon	Total	S1	S2	S3	S4	S5	S6	S7	S8
Acidobacteria	0.07	0.08	0	0.06	0.04	0.5	0	0.02	0
Actinobacteria	1.31	2	0.39	3.21	1.05	0.74	0.08	2.43	0.12
Armatimonadetes	0.01	0.01	0	0	0.09	0.03	0	0	0
Bacteroidetes	18.15	10.02	14.08	10.38	11.14	18.23	28.73	17.01	39.15
Chlorobi	0.02	0	0	0.03	0.09	0	0	0	0
Chloroflexi	0.13	0.07	0.08	0.24	0.39	0.23	0	0.01	0
Deferribacteres	0	0	0	0	0	0	0	0	0.01
Fibrobacteres	0.05	0	0.03	0	0	0	0.3	0	0.09
Firmicutes	11.86	0.22	8.44	0.6	1.97	0.42	47.56	0.29	34.89
Fusobacteria	0.01	0	0.04	0	0	0	0	0	0.04
Gemmatimonadetes	0.02	0.02	0.01	0.01	0	0.12	0	0.01	0
Lentisphaerae	0.01	0	0.03	0.01	0.02	0	0	0	0.01
OD1	0	0	0	0.01	0	0	0	0.01	0
Planctomycetes	0.01	0.02	0	0.01	0.02	0.06	0	0.02	0
Proteobacteria	36.58	27.39	58.49	60.1	66.36	29.59	1.19	29.13	7.44
Spirochaetes	0.54	0	0.79	0.05	0.12	0.09	2.17	0	0.94
SR1	0	0	0.01	0	0	0	0	0	0
Tenericutes	0.12	0	0.43	0.15	0.06	0.03	0.02	0	0.18
TM7	0.06	0.11	0	0.06	0.04	0.06	0	0.19	0
unclassified bacteria	30.33	57.98	17.13	24.24	18.38	47.59	19.94	49.95	17.13
Verrucomicrobia	0.73	2.09	0.04	0.86	0.24	2.32	0.01	0.94	0
Total	100	100	100	100	100	100	100	100	100

Table 5: Full sequence percentages of bacteria at the phylum level.

Taxon	Total	S1	S2	S3	S4	S5	S6	S7	S8
Basidiomycota	87.26	97.26	92.58	76.48	81.03	85.98	89.09	95.56	45.36
unclassified fungi	12.74	2.74	7.42	23.52	18.97	14.02	10.91	4.44	54.64
Full taxonomy RDP by order									
	Total	**F.FE**	**F.LA**	**F.ME**	**F.MI**	**F.MM**	**F.MU**	**F.SP**	**F.SW**
Agaricomycetes	85.84	96.87	91.89	70.92	80.18	83.9	88.13	95.3	44.09
unclassified basidiomycota	1.43	0.4	0.69	5.57	0.85	2.08	0.95	0.26	1.28
unclassified fungi	12.74	2.74	7.42	23.52	18.97	14.02	10.91	4.44	54.64
Full taxonomy RDP by family									
	Total	**F.FE**	**F.LA**	**F.ME**	**F.MI**	**F.MM**	**F.MU**	**F.SP**	**F.SW**
Agaricales	83.74	96.66	90.89	60.46	78.48	81.58	87.95	94.99	42.7
unclassified Agaricomycetes	2.1	0.2	1	10.46	1.7	2.32	0.18	0.3	1.39
unclassified basidiomycota	1.43	0.4	0.69	5.57	0.85	2.08	0.95	0.26	1.28
unclassified fungi	12.74	2.74	7.42	23.52	18.97	14.02	10.91	4.44	54.64
Full taxonomy RDP by genus									
	Total	**F.FE**	**F.LA**	**F.ME**	**F.MI**	**F.MM**	**F.MU**	**F.SP**	**F.SW**
Pleurotaceae	81.84	95.66	90.67	51.94	75.41	80.17	87.94	93.67	42.55
unclassified Agaricales	1.9	1.01	0.22	8.52	3.07	1.41	0.02	1.33	0.14
unclassified Agaricomycetes	2.1	0.2	1	10.46	1.7	2.32	0.18	0.3	1.39
unclassified basidiomycota	1.43	0.4	0.69	5.57	0.85	2.08	0.95	0.26	1.28
unclassified fungi	12.74	2.74	7.42	23.52	18.97	14.02	10.91	4.44	54.64
Full taxonomy RDP by species									
	Total	**F.FE**	**F.LA**	**F.ME**	**F.MI**	**F.MM**	**F.MU**	**F.SP**	**F.SW**
Hohenbuehelia	81.84	95.66	90.67	51.94	75.41	80.17	87.94	93.67	42.55
unclassified Agaricales	1.9	1.01	0.22	8.52	3.07	1.41	0.02	1.33	0.14
unclassified Agaricomycetes	2.1	0.2	1	10.46	1.7	2.32	0.18	0.3	1.39
unclassified basidiomycota	1.43	0.4	0.69	5.57	0.85	2.08	0.95	0.26	1.28
unclassified fungi	12.74	2.74	7.42	23.52	18.97	14.02	10.91	4.44	54.64

Table 6: Full sequence percentages of some dominant Fungi from phylum to species.

AOB	Nitrosomonas	11
CD	Roseburia	124
DN	Flavobacterium	1626
DN	Rhodobacter	28
DN	Thauera	57
DN	Methylophilus	3
HOB	Hydrogenophaga	7116
anammox	CandidatusSolibacter	6
AOA	Nitrosopumilus	4

AOB- ammonia oxidizing bacteria, CD- carbon degrader, DN- denitrifying bacteria, HOB-hydrogen oxidizing bacteria, anammox- anaerobic ammonia oxidizing bacterium, AOA- ammonia oxidizing archaea.

Table 7: Bacteria and archaea with potential contribution for removal of contaminants from constructed wetlands.

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