Comparison of nutrient retention efficiency between vertical-flow and floating treatment wetland mesocosms with and without biodegradable plastic

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ABSTRACT

Treatment wetlands are ecological systems that are engineered to improve polluted water quality through macrophyte, soil, and microbial remediation and are used commonly for urban and agricultural runoff treatment. However, constructed wetlands used for marine aquaculture effluent treatments are understudied when compared to their freshwater counterpart. We compared the nutrient retention and the microbial communities of two types of constructed wetland mesocosms, a vertical-flow treatment wetland (VFTW) and floating treatment wetland (FTW) in subtropical south Florida. To enhance nutrient retention efficiency, we implemented biodegradable plastic (polycaprolactone), as an external carbon source and monitored the performance of VFTW and FTW for the treatment of marine aquaculture effluent. Polycaprolactone surface were covered by various cyanobacterial genera including *Oscillatoria*, *Leptolyngbya*, *Brasilonema*, and *Trichormus* and some plastic-degrading bacteria such as *Pseudomonas*. The presence of a biodegradable plastic in FTW improved the overall performance of nitrogen removal (nitrite plus nitrate) by 14% through denitrification. The pattern of nutrient removal between two treatment wetland mesocosms were significantly different ($p < 0.01$), with over 87-91% retention of total nitrogen in VFTW and no retention in FTW, the latter due to poor retention of nitrite plus nitrate and production of organic nitrogen from the system not present in inflow waters. Total phosphorus was retained in both mesocosm types, with higher retention (74-81%) in the VFTW than in the FTW (17-40%). The nutrient retention in VFTW was higher overall compared with FTW mesocosms regardless of biodegradable plastic presence.

Keywords: biodegradable plastic, vertical-flow treatment wetland, floating treatment wetland, aquaculture, microbial community

1. INTRODUCTION

Wetland construction or wetland restoration has been effective in water quality enhancement through nutrient reductions from agricultural and urban runoff (Fink and Mitsch, 2004; Nahlik and Mitsch, 2006; Mitsch et al., 2012, 2015; Griffiths and Mitsch, 2017). Treatment wetlands are ecological systems that are engineered to treat polluted water through macrophyte, soil, and microbial remediation and have some varieties (Vymazal, 2007). Vertical-flow treatment wetlands (VFTWs) are fed inflows intermittently or continuously with a relatively short hydraulic residence time (Stottmeister et al., 2003; De Lange et al., 2013) and effective for solids removal from the water column and nutrient cycling by means of phytoremediation and microbial processes (e.g. denitrification and nitrification) (Fuchs et al., 2011; De Lange et al., 2013).

A floating treatment wetland (FTW) is a relatively new phytoremediation technique to reduce the impact of excess nutrient loading within the waterbody itself. FTWs consist of aquatic or terrestrial plants grown hydroponically on a floating mat directly in the open water of the system allowing for direct treatment of eutrophic waters (Hubbard et al., 2004; Vymazal, 2007; Headley and Tanner, 2011; Zhao et al., 2012; Olguín et al., 2017; Pavlineri et al., 2017). The plant roots are exposed directly to the water column instead of buried in a sand or gravel substrate allowing for nutrients to be absorbed hydroponically, reducing the nutrient load internally (Zhou and Wang, 2010; Headley and Tanner, 2011; White and Cousins, 2013). The development of an extensive root system along with microbial biofilm formation provide for the main nutrient removal pathway in this type of wetland system (Headley and Tanner, 2011). FTWs have been shown to be effective at treatment of high nutrient wastewaters (e.g. stormwater, sewage, agriculture) (Headley and Tanner, 2011; Yeh et al., 2015; Chen et al.,

2016). Plant roots have a greater surface area exposure in the water column, which allow for greater bacterial colonization and unique rhizosphere microbial functions (Zhao et al., 2012; White and Cousins, 2013; Urakawa et al., 2017). These two wetland designs (VFTW and FTW) have been proven to be effective for agriculture and storm water treatments (Faulwetter et al., 2011; Zhang et al., 2013b; Liu et al., 2016; Fu et al., 2017; Urakawa et al., 2017).

Since treatment wetlands have been specifically designed for wastewater treatment removing high nutrients and suspended solids (Turcios and Papenbrock, 2014; Mitsch and Gosselink, 2015), it is possible to apply treatment wetlands to remediate aquaculture wastewater as a cost-effective approach (Brown et al., 1999; Lin et al., 2010; Liang et al., 2017). One of the most common treatment wetlands for aquaculture effluent is characterized as subsurface flow construction, which has a sand or gravel substrate, where water flows either vertically (vertical-flow) or horizontally (horizontal-flow), and treated water is either reused in a closed system or discharged in an open system (Konnerup et al., 2011; Mitsch and Gosselink, 2015). Enhancement of aquaculture wastewater treatment capacity could be possible through the addition of various external carbon sources such as methanol, glucose, starch, and cellulose (Wu et al., 2014). Several studies aimed to explore different denitrification activity with external carbon in constructed wetlands, use of periphyton as a producer of organic carbon (Sirivedhin and Gray, 2006), and addition of different sugars (e.g. glucose and fructose) to wetland influents (Lin et al., 2002; Lu et al., 2009).

Using biodegradable plastic as an external carbon source in treatment wetlands is a new approach and two benchtop scale wetland microcosms were previously designed with the use of a cornstarch/polycaprolactone blend (Shen et al., 2015) and poly-3-hydroxybutryate-co-3-hydroxyvalerate/polyacetic acid (PHBV/PLA) (Yang et al., 2018). However, no application has

been made in a medium scale outdoor treatment wetland and understanding the microbial community composition in a treatment wetland with biodegradable plastic is the next question to improve the performance of nutrient removal.

In this study, we evaluated nutrient retention efficiency between vertical-flow and floating treatment wetland mesocosms with and without biodegradable plastic (polycaprolactone) for treatment of marine aquaculture effluent to enhance nutrient cycling (e.g. denitrification). Likewise, determining how microbial community composition could change with the addition of a biodegradable plastic and how microbial community composition differs between vertical-flow and floating treatment wetland mesocosms was concurrently studied for a better understanding of microbial community composition of these two treatment wetland systems.

2. MATERIALS AND METHODS

2.1 Vertical-flow treatment wetland construction

In May 2016, the experimental units were constructed at the Everglades Wetland Research Park of Florida Gulf Coast University (26°06.452´N, 81°46.334´W) in two rows of four wetland mesocosms (1.33 m x 0.47 m x 0.61 m polyethylene tubs) with one row modeling vertical-flow treatment wetlands (VFTW) and one row modeling floating treatment wetlands (FTW) in a batch system (**Fig. 1**). Vertical-flow constructed mesocosms were filled with a 10 cm layer of gravel followed by an approximate 30 cm of sand fill according to methods outlined in Ahn et al. (2001) and Ahn and Mitsch (2002) (**Fig. 1**). Cordgrass (*Spartina patens*) was collected from a nearby 23-ha restored brackish marsh (5 ppt) at the Naples Botanical Garden

(26°06.181´N, 81°46.534´W) (Zhang et al., 2017a) and planted in August 2016. Salinity was gradually increased from 0 to 5 ppt to acclimate plants for 10 months prior to starting the experiment. Two mesocosms were incorporated with a 1 cm layer of polycaprolactone beads (3.5 mm in diameter, IC3D, TechTack Moldable plastic) buried at a depth of 9 cm (1.82 kg) within the upper substrate layer prior to effluent feeding to mesocosms to allow for settling of the plastics, which are buoyant in water, as upper substrate layer was no longer densely packed after burial. Two other mesocosms were used as a control without biodegradable plastic incorporation. A nutrient removal experiment was conducted during June 2017.

2.2 Floating treatment wetland construction

Four floating mat treatment mesocosms were filled with lake water pumped up from an adjacent lake. Each mat had 18 plantings (9 cm diameter) spaced 25 cm apart from the center of each hole (**Fig. 1**). Seven-cm cordgrass (*S. patens*) plants were placed in aerator pots seated within the floating mats. Artificial saltwater (Instant Ocean) was used to adjust salinity to be 5 ppt. A recirculating bioreactor system was equipped in all four floating treatment wetlands: two mesocosms had bioreactors with polycaprolactone (PCL) plastic beads as a reactor medium with two mesocosms having empty bioreactors used as control. The recirculating bioreactor setup consisted of 250 mL biodegradable beads (472 g) in AQUAMAXX bioreactors (1 L volume) connected with a filter pump (Cobalt MJ-1200) and a flow nozzle controlled to a flowrate at 1 L min^{-1}.

2.3 Upstream tank setup

A 560-liter upstream tank (dimensions 1.00 x 0.8 x 0.7 m^3) housed 10 Pinfish (*Lagodon rhomboides*) used to generate the brackish aquaculture wastewater (**Fig. 2**). A filtration system

consisted of a canister filter with ultraviolet sterilizer lamp (55.9 cm h x 35.6 cm d, Red Sea brand) and a 3.8 L bioreactor (NextReef, MR1 XL) with polypropylene plastic fill (2.54 cm Bio Barrels, Pentair) and filtration pump (Maxi-jet Pro Powerhead, Pentair). The brackish aquaculture wastewater was fed manually once a week for one month prior to starting the experiment and then every six days after start for two months to both systems. Average inflow parameters such as temperature, salinity, DO, and nutrient concentration are shown in **Table 1**.

2.4 Water sampling and chemical analysis

Water samples were collected in 250 mL autoclaved polypropylene sampling bottles (ThermoScientific Nalgene) from the outflow pipe of mesocosms (**Fig. 1**) and stored at -20°C until analysis. The hydraulic loading rate of the vertical-flow systems were set to be 3.03 L day^{-1} (48.4 cm day^{-1}), manually fed to the system from the upstream tank, which allowed for a complete flow-through of three days to the outflow pipe. The hydraulic loading rate (HLR) was determined according to Mitsch and Gosselink (2015) using the following equation, $q = 100Q / A$, where q = (HLR), (cm day^{-1}), Q = inflow rate, m^3 day^{-1}, and A = wetland surface area, (m^2). Water quality parameters such as water temperature, pH, salinity, and dissolved oxygen (DO) were measured in the FTW mesocosms using a YSI Pro Plus meter. Turbidity was determined using a Trilogy fluorometer with a turbidity module (Turner Design). Ammonia concentration was colorimetrically conducted using a Spectronic Genesys 20 spectrophotometer (Thermo Scientific) using a standard sodium salicylate method. Nutrients in water samples were colorimetrically determined using a SmartChem Autoanalyzer to measure nitrate-nitrite nitrogen and Total Kjeldahl nitrogen (TKN) according to EPA guidelines 353.1 and 351.2 respectively (USEPA, 1993b, a). Total nitrogen was determined from the combined TKN and nitrate-nitrite

concentrations. Total phosphorus (TP) concentration was determined according to the EPA guideline 365.1 (USEPA, 1993c).

2.5 Plant tissue samples

Aboveground plant tissue samples (9 cm^2) were randomly collected from all mesocosms at the start and end of the experiment. Changes in plant stem height were measured for an estimate of daily growth rate over each system period.

2.6 Microscopy

Water samples were collected from the outflow pipes of each mesocosm and fixed with formalin (2% final concentration [vol/vol]). Cells were stained with 4', 6-diamidino-2-phenylindole (DAPI), then part of the fixed water samples (0.8 mL) were filtered onto black 0.22-µm polycarbonate isopore membrane filters (GTBP, MilliporeSigma) with a standard hand vacuum pump operation. An anti-bleaching agent was used as the mounting medium (AF1; Citifluor). Cells were observed under 600x magnification using an Olympus BX51 epifluorescence microscope system. For each filter, more than 10 random fields were viewed to determine cell numbers.

2.7 Sample collection for microbial analysis

Biodegradable plastics were collected in clean 50 mL plastic centrifuge tubes from those embedded in the VFTW mesocosms and bioreactors on the FTW mesocosms. Root samples were collected using sterilized scissors and stored in 50 mL centrifuge tubes, consisting of a mixture of 0 - 15 cm depth segments from two distinct locations within each mesocosm. Soil samples were collected from two distinct locations in each vertical-flow mesocosm at a depth of 5 cm. The collected soil samples were vortexed for homogenization after initial collection. Water

194 samples (250 mL) collected from all FTW mesocosms were filtered using 0.2 µm cellulose
195 nitrate membrane filters (47 mm diameter, Fischer Scientific Nalgene Analytical Test Filter) for
196 further DNA extraction. All samples were stored at -20°C for DNA extraction.

197 *2.8 High throughput sequencing*

198 DNA samples were extracted from biofilm on PCL beads, root, soil, and water filter
199 using the MagAttract PowerSoil DNA KF kit (Qiagen) according to the manufacturer's
200 instructions. Extracted DNA was eluted into 100 µL EB solution. Archaeal and bacterial 16S
201 rRNA genes were amplified using the primer set, 515yF (5'GTGYCAGCMGCCGCGGTAA)
202 and 926pfR (5'CCGYCAATTYMTTTRAGTTT) (Parada et al., 2016) tagged with the Illumina
203 i5 forward (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and i7 reverse
204 (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) sequencing primer. Each PCR
205 reaction contained 25 µL reactions with Qiagen HotStar Taq master mix, equal amount of
206 forward and reverse primers (5 µM each), and 1 µL of DNA template (1 to 20 ng). Thermal
207 cycling consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for
208 30 sec, annealing at 54°C for 40 sec, and extension at 72°C for 1 min, with a final extension of
209 10 min at 72°C. PCR product from the first stage was then transferred to a second PCR based on
210 qualitatively determined concentrations with primers for the second PCR based on the Illumina
211 Nextera PCR primers forward (AATGATACGGCGACCACCGAGATCTACAC-[i5 index]-
212 TCGTCGGCAGCGTC) and reverse (CAAGCAGAAGACGGCATACGAGAT-[i7 index]-
213 GTCTCGTGGGCTCGG). The second stage amplification was run with the same as the first
214 except for 10 cycles instead of 35 cycles. Amplicons were visualized with eGels (Life
215 Technologies), products were pooled equimolar with each size selected quantified using the

Quibit 2.0 fluorometer (Life Technologies). Amplicons were then loaded on an Illumina MiSeq (Illumina) 2 x 300 flow cell at 10 pM (RTL Genomics).

For analysis, FASTQ formatted files were merged using the PEAR Illumina paired-end read merger (Zhang et al., 2013a). Prefix dereplication was completed using the algorithm of USEARCH (Edgar et al., 2011). Clustering at a 3% divergence level was conducted using the USEARCH (Edgar et al., 2011). Operational taxonomic unit (OTU) selection was performed using UPARSE-OTU algorithm (Edgar, 2013). Chimera checking was completed using UCHIME (Edgar, 2010) and detected chimera sequences were removed. Representative OTUs were used to determine taxonomic information through a basic local alignment search tool (BLAST) at National Center for Biotechnology Information (NCBI), and MG-RAST (Meyer et al., 2008). The high-throughput sequence datasets were deposited in GenBank under BioProject number PRJNA496041.

2.9 Data analysis

The significant differences were determined when $p < 0.05$. Tukey-Kramer method was employed in conjunction with a one-way analysis of variance (ANOVA) using JMP data analysis software (SAS Institute) according to Lehman (2005) for testing statistical differences among multiple mesocosm settings. Student's t-test was also implemented to determine if two sets of data were significantly different from each other. All statistics (one-way ANOVA, Tukey-Kramer, and Student's t-test) were completed using two-tailed and unpaired data analyses. Data were presented by mean ± standard deviation unless otherwise noted. General statistics of high-throughput sequence data were performed using MG-RAST (Meyer et al., 2008). Diversity index calculations (Shannon index, Menhinick's richness and Pielou's evenness indices) were implemented using Microsoft Excel.

3. RESULTS AND DISCUSSION

3.1 Physical parameters

Average rainfall over the experimental period was 7.6 ± 0.9 mm day^{-1} measured using the real-time hydrologic, water quality monitoring, and meteorological field station at the Everglades Wetland Research Park (Zhang et al., 2017a), which was less than the calculated HLR 48.4 cm day^{-1}, with an average rain gauge depth of 1.2 ± 1.3 cm. Physical parameters were measured for FTW mesocosms with an average water temperature of 29.9°C, salinity of 5.1 ppt, pH of 7.95, and DO of 3.2 mg L^{-1}. Turbidity (NTU, nephelometric turbidity unit) was high in the VFTW mesocosms (23.5 ± 3.0 NTU and 47.0 ± 7.3 NTU with and without biodegradable plastic, respectively) and low in the FTW mesocosms (1.71 ± 0.1 NTU and 2.26 ± 0.2 NTU) due to the impact of soil.

3.2 Effect of biodegradable plastics for nutrient removal

Total nitrogen retention was significantly different ($p < 0.001$, $n = 8$, one-way ANOVA) and higher in the vertical-flow system (**Table 2**). TN retention performance in our vertical-flow system (86.9-90%) was consistent with other saline aquaculture effluent treatment wetland systems with a mean removal efficiency of 98% TN (Brown et al., 1999), and 98.2% TDIN (Webb et al., 2012) (**Table 3**). On the contrary, TN retention was not observed in FTW mesocosms over the experimental period as outflow concentrations exceeded the inflow concentration of effluent (**Table 2)**, leading to a negative retention rate, consistent with a previous aquaculture wastewater treatment study showing outflow concentration of TKN exceeded feed water TKN (Lin et al., 2010). However, the presence of PCL significantly

increased the TN retention, even though a negative retention occurred in this system, a greater negative TN retention was observed without the use of PCL beads in our control condition ($p < 0.0001$, Tukey-Kramer pairwise). This finding was not identified in the VFTW system (**Table 2**). A comparison study in China found that floating treatment systems had a lower TN removal efficiency compared with vertical-flow systems (Zhang et al., 2015). Newly constructed or newly restored wetlands are found to have a low C:N ratio, therefore the addition of an external carbon can enhance denitrification in these wetland systems (Bachand and Horne, 1999), as evidenced in the FTW system in our study. Overall, these lines of evidence indicate the strict carbon limitation in the FTW than the other treatment wetland systems (Zhang et al., 2015).

Inflow TN was composed of over 98% inorganic nitrogen, mainly in the form of nitrate and nitrite (**Table 1**). In the VFTW system nitrate plus nitrite ratio decreased to be 17.6% in the control and 16.2% with embedded PCL, along with a decrease of the TN:TP ratio from 8.9 to 4.7 and 4.5 respectively, indicating microbial nitrogen removal processes (i.e. denitrification or DNRA [dissimilatory nitrate reduction to ammonium]) (**Fig. 3a**). Our ammonia measurements showed the depletion of ammonia from 9.2% in inflow to 7.1% in the control and 5.7% with embedded PCL, with an increase in organic nitrogen to 75.3% and 78.2%, respectively. Based on these findings we concluded that denitrification, not DNRA, was the major process in the removal of nitrate plus nitrite pool in the VFTW. In the FTW system nitrate plus nitrite ratio decreased from 94.2% to be 40.5% in the FTW control and 10.7% in FTW with PCL condition, with an increased removal efficiency with presence of PCL (**Fig. 3a**). Additionally, we found a decrease of ammonia in outflow water from 5.8% to 3.2% in control and 4.3% with PCL conditions. Organic nitrogen increased to 56.3% in control and 85.0% in FTW mesocosms with embedded PCL, a greater proportion of organic nitrogen with presence of PCL. Aquaculture

effluent contains low organic nitrogen and phosphorus in the water column with 7-32% of nitrogen found in suspended solids (Turcios and Papenbrock, 2014). As previously discussed, TN retention was not observed in FTW mesocosms due to the production of organic nitrogen from the system not present in inflow waters leading to the negative retention of TN as production of nitrogen occurred. However, there was a decrease in inorganic nitrogen concentrations with greater decreases observed with the presence of PCL, suggesting the use of PCL as a carbon source for denitrification (**Fig. 3a**). In contrast with the VFTW system, the TN:TP ratio in outflow water increased from 13.2 to 27 in control and 17 with PCL medium supporting our finding of greater nitrogen concentration found without presence of PCL in the FTW system (**Fig. 3b**). The accumulation of organic nitrogen within the FTW system was attributed to the release of organic nitrogen from plant pot soil within the floating mats which was supported by the anomaly of water column microbial community dominated by soil bacteria, which will be discussed further in later sections. The production of organic nitrogen in wetlands can also partially be attributed to nitrogen-fixing bacteria which fix N_2 from the atmosphere leading to production of organic nitrogen reducing the overall nitrogen removal efficiency (Mitsch and Gosselink, 2015; Zhang et al., 2017b).

TP retention was significantly higher in the VFTW system than for the FTW system ($p < 0.0032$, *t*-test), however, no significance was found with the presence of PCL in both mesocosm systems (**Table 2**). The TP retention in the VFTW mesocosms had a mean retention of 74 - 81.1%, which was lower than similar studies, 99% (Brown et al., 1999) and 88% (Lymbery et al., 2006) (**Table 3**). Zhang et al. (2015) found TP removal efficiency ranged from 26-70% and was more variable than nitrogen in constructed wetland systems. The mean TP retention in the

306 FTW system was 17.4-39.5%, consistent with findings by Lin et al. (2010) with 2-18% removal
307 and Pavlineri et al. (2017) of 18.2% removal efficiency, with an increase over time (**Table 3**).

308 *3.3 Growth of Spartina in vertical-flow and floating treatment systems*

309 Change in plant height ranged from 1.7 to 17.3 mm day^{-1} with the highest growth rate
310 occurring in the FTW mesocosms with PCL medium (**Fig. 4**). Even though there seemed to be an
311 increased growth rate associated with use of PCL medium in the FTW system, the range of
312 measurements overlapped when looking at mean growth rate. Due to a low number of replicates
313 in this study (n = 2), no statistical comparison was made.

314 *3.4 Bacterial abundance*

315 Total bacterial abundance of wetland water columns generally range from 10^5 to 10^6 cells
316 mL^{-1} (Urakawa and Bernhard, 2017), which was similar with our findings. No significant
317 differences were found in the outflow bacterial abundance with the following distribution;
318 VFTW control ($3.7 \times 10^6 \pm 1.8 \times 10^6$ cells mL^{-1}), VFTW with embedded PCL ($3.5 \times 10^6 \pm 1.7 \times$
319 10^6 cells mL^{-1}), FTW control ($1.0 \times 10^6 \pm 6.3 \times 10^5$ cells mL^{-1}), and FTW with PCL medium (3.2
320 $\times 10^6 \pm 2.2 \times 10^6$ cells mL^{-1}).

321 *3.5 High-throughput sequencing of 16S rRNA gene*

322 *3.5.1 Taxonomic overview of dominant phyla*

323 A total of 86,547 sequences were analyzed and resulted in 2346 operational taxonomic
324 units (OTUs) (**Table 4**). Shannon index indicated significant differences between sample means
325 ($p = 0.02$, one-way ANOVA) with the lowest diversity in water samples and the highest diversity
326 in root samples. There were significant differences found between VFTW root and FTW water
327 samples ($p = 0.03$, Tukey-pairwise). The highest diversity found in soil samples was consistent

with previous reports (Urakawa and Bernhard, 2017). The taxonomic analysis identified 29 phyla from all samples: 12-21 collected from PCL plastic biofilm, 15-24 in root samples, 17-20 in soil samples, and 8-11 in water samples. No statistical difference was found in PCL biofilm samples ($p = 0.06$, *t*-test) and soil samples ($p = 0.5$, *t*-test). Root samples were significantly different ($p = 0.03$, $n = 2$, one-way ANOVA) between VFTW control and FTW with PCL ($p = 0.03$, Tukey-pairwise). Water samples in floating treatment system conditions having PCL bioreactor medium were significantly lower than the control samples ($p = 0.0002$, *t*-test).

The three predominant phylum present in all samples were *Proteobacteria* (2-44%), *Cyanobacteria* (0.04-51%) and *Bacteroidetes* (0.02-30%) (**Fig. 5**). These results were consistent with previous studies of wetland microbial communities (Bai et al., 2014; Liu et al., 2016; Urakawa and Bernhard, 2017). Members of *Proteobacteria* are important in wetlands because of their strong involvement in biogeochemical cycling (Liu et al., 2016) and they dominated in a majority of samples except for water column samples from FTW. The two most abundant phyla in the water column samples were *Firmicutes* (59-90%) and *Actinobacteria* (6-17%) (**Fig. 5**). Unexpectedly, the most dominant member of *Firmicutes* was identified as *Bacillus* (57-88%), this trend agreed between four samples assuring good reproducibility of the method used. We attributed this finding to the presence of soil within plant pots (**Fig. 2**). *Bacillus* is recognized as a representative degrader of biodegradable plastics. For example, *Bacillus pumilus*, isolated from a freshwater pond and river were shown to degrade poly (e-caprolactone) hydrolytically (Tezuka et al., 2004). However, presence of *Bacillus* was found regardless of PCL medium indicating that *Bacillus* was not directly enriched by the biodegradable plastics (**Table 5**).

Soil microbial communities in VFTW were dominated by *Proteobacteria* (40-85%), *Cyanobacteria* (5-40%), *Bacteroidetes* (2-10%), and *Planctomycetes* (2-7%) (**Fig. 5**). The soil

was covered with approximately 5-10 cm of water layer (**Fig. 2**). *Cyanobacteria* is a typical phylum found in freshwater sediment and water column communities (Paerl, 2014; Urakawa and Bernhard, 2017; Paerl, 2018). Thus, the observed microbial community might resemble a typical freshwater sediment community rather than a typical soil community (Zhang et al., 2013b). A steep oxic-anoxic gradient contributes to maintain high microbial diversity and functionally diverse organisms (Urakawa et al., 2017). Our results supported this finding by having the highest diversity found in soil samples of our wetland.

3.5.2 Comparison of rhizosphere communities in soil and water

In wetland plants, the rhizosphere acts as an interface between the surface of roots and the surrounding soil, which transports oxygen and other minerals to the roots which results in unique microbial communities distinct from surrounding soil and water column in a case of floating macrophytes (Mitsch and Gosselink, 2015; Urakawa et al., 2017).

The nitrogen cycle plays an important role in wetland plant metabolisms through the transformation of nitrogen species (i.e. ammonia and nitrate). *Mesorhizobium* and *Rhizobium* are essential diazotrophs and plant growth-promoting rhizosphere bacteria found in wetland systems (Zhang et al., 2013b; Urakawa et al., 2017). *Mesorhizobium* was identified in root, soil, water, and PCL biofilm samples while *Rhizobium* was identified only in vertical-flow root samples (**Table 5**). Nitrogen-fixing bacteria were more abundant in the VFTW than FTW mesocosms. The only nitrifying bacterium identified was *Nitrospira*, in root, soil, and VFTW PCL biofilm samples.

Methanogenesis is an important process in wetlands through which methane is naturally produced by methanogens and methane oxidation occurs from methanotrophic bacteria to

convert methane to carbon dioxide (Mitsch and Gosselink, 2015). Archaea are important methanogens in wetland sediments contributing to methane production (Madigan et al., 2012; Urakawa and Bernhard, 2017), three genera of methanogenic archaea found were *Methanobacterium*, *Methanoregula*, and *Methanosarcina*. Six methanotrophic genera were also found, *Methylocystis*, *Methylobacter*, *Methylococcus*, *Methylosoma*, *Methylocella* and *Hyphomicrobium*. These methanogens and methanotrophs were more abundant in the VFTW than in the FTW mesocosms (**Table 5**). *Hyphomicrobium* belonging to *Alphaproteobacteria* and *Methylibium* belonging to *Betaproteobacteria* were the two most abundant facultative methylotrophic genera and widely distributed in our constructed wetland systems, which supported a previous wetland study (Zhang et al., 2013b). *Methylocella* was the most widespread methanotroph found in this study. Coexistence of methanogens and methane oxidizers suggests the existence of the methane cycle and the skewed relative abundance of these microorganisms indicated more imperative role of this process in the vertical-flow system than in the floating wetland system.

Sulfate-reducing bacteria (SRB) were the predominant sulfur cycling microorganisms found in root samples and PCL biofilm. Although SRB were found in both systems, the vertical-flow system contained a greater diversity of organisms (i.e. *Desulfobulbus*, *Desulfatitalea*, *Desulfobacterium*, *Desulfonema*, *Desulfocapsa*, *Desulfopila*, *Desulfomicrobium*, and *Desulfovibrio*) than were found in the floating treatment system (i.e. *Desulfovibrio* and *Desulfobulbus*) (**Table 5**). The floating treatment system contained very minor amount of SRB in contrast to Urakawa et al (2017) which found a very rich SRB community in floating treatment rhizosphere. SRB communities in rhizosphere and soil in a *Phragmites australis* planted wetland

(Zhang et al., 2013b) and wetland soils (Faulwetter et al., 2009; Wang et al., 2012) were very diverse and consistent with our findings.

3.5.3 Denitrification in vertical-flow and floating treatment systems

Denitrification is the main nitrogen removal process in treatment wetland systems as discussed previously and paired with nitrification, a process in which nitrate is produced from ammonium, can fully remove nitrogen microbially from wastewater systems (Faulwetter et al., 2009). Predominant denitrifiers found in our study were *Bacillus* in water column samples, *Nitratireductor*, a marine denitrifier (Labbè et al., 2004) represented in all samples in minor amount, and *Pseudomonas* (0.2%) in soil samples with embedded biodegradable plastics (**Table 5**). *Pseudomonas* has been found to degrade plastic particles in an urban river environment (McCormick et al., 2014), soil environments (Emadian et al., 2017), and the deep-sea (Sekiguchi et al., 2011). The presence of *Pseudomonas* only in soil samples with PCL may indicate the possibility of PCL use as a substratum or degradability, as indicated by similar findings of *Pseudomonas* on plastic pot biofilm from a floating treatment wetland (Urakawa et al., 2017). These findings support our observation of increased denitrification activity in the VFTW and FTW construction with the presence of PCL.

3.5.4 PCL degradation in a vertical-flow and floating treatment constructed wetland

The most abundant genera found in PCL biofilm samples collected from VFTW sediment were identified as *Oscillatoria* (7%) and *Leptolyngbya* (6%) and from FTW bioreactors were *Brasilonema* (8%) and *Trichormus* (9%) belonging to the phylum *Cyanobacteria* (**Table 5**). Additionally, *Leptolyngbya* was identified in VFTW sediment with embedded PCL. The localization of *Cyanobacteria* in VFTW plastics was attributed to a partial exposure of plastics to

the surface (**Fig. 2**). We identified many *Cyanobacteria* within our study in presence of PCL plastic, consistent with previous marine plastic debris research (Bryant et al., 2016; Debroas et al., 2017; Quero and Luna, 2017). *Cyanobacteria* were identified as the key species in the microbial network which is formed on the surface of plastics (Debroas et al., 2017). However, none of these studies confirmed if *Cyanobacteria* are actively involved in the biodegradation of the plastics (Debroas et al., 2017; Quero and Luna, 2017). Bryant et al. (2016) and Debroas et al. (2017) identified *Leptolyngbya* on the surface of plastics collected from the surface water of the North Atlantic. It should be noted that *Cyanobacteria* are able to synthesize polyhydroxybutryate, an intracellular storage compound and bioplastic, under photoautotrophic or chemoheterotrophic conditions (Balaji et al., 2013; Singh et al., 2017). Additionally, several genera can synthesize polyhydroxyalkanoate (PHA) and contain PHA biosynthesis genes (e.g. *Oscillatoria limosa*, *Anabaena cylindrica*, *Synechoccocus* spp.), these findings can lead to the speculation they are also able to degrade these bioplastic storage compounds for intracellular use.

3.6 Economic impact of plastic-embedded constructed wetlands

As previously discussed, constructed wetlands are beneficial in terms of nutrient retention. Our approach will potentially enhance the performance of nutrient retention processes and increase the value of constructed wetlands. We used 1 kg m^{-2} of PCL within the VFTW system. If we assume to construct a 1 ha vertical-flow wetland embedded with PCL biodegradable plastics, it would cost approximately $56,500 only considering the price of plastics. Boley et al. (2000) estimated the consumption of plastic substrate per kg $N\text{-}NO_3^-$ and cost of denitrification per kg $N\text{-}NO_3^-$ in a study of an aquaculture bioreactor system with an approximate 0.64 kg of $N\text{-}NO_3^-$ removal by PCL per kg. A study by Batson et al. (2012) estimated the nitrogen removal from a constructed riparian wetland as 0.0164 kg m^{-2} yr^{-1}.

Therefore, if we assume all embedded PCL is used for denitrification, the constructed wetland has atleast a 39 times performance increase than regular constructed wetlands to remove nitrogen, suggesting a great potential for use in cityscape and other high-priced areas. In the future, we anticipate that the use of biodegradable plastics will increase due to the current plastic pollution problems. A part of used biodegradable plastics can be embedded in soil and used as a carbon source by wetland microbes. In this scenario, the cost of used biodegradable plastics can be negligible. Our study showed the potential use of this system however, much longer-term monitoring and more expanded field experiments are required in the future applications.

4. CONCLUSIONS

Wetlands play a vital role in water purification and nutrient cycling which can be utilized to treat agricultural runoff and aquaculture discharges in a sustainable fashion (Headley and Tanner, 2011; Mitsch and Gosselink, 2015). Comparison of wetland construction performance in this study between a vertical-flow treatment wetland and a floating treatment wetland showed there was an increased nutrient retention for both TN and TP with a vertical-flow system. The use of a biodegradable plastic, PCL, was utilized as a novel approach in this study as an external carbon source to enhance microbial activity. PCL was shown to increase the TN nutrient retention in the FTW system, however, this system exhibited a negative retention during our study period due to the release of organic nitrogen from soil in plant pots, which was inferred from the dominance of *Firmicutes* (59-90%) (e.g. *Bacillus*) in the water column of FTW. Presence of PCL in the FTW system allowed for a greater production of organic nitrogen and a greater removal of inorganic nitrogen, suggesting PCL enhanced nitrogen cycling within this system. Microbial community composition was shown to be altered with the presence of PCL,

community selection for cyanobacterial genera and other bioplastic-degrading microorganisms was found from high-throughput sequencing analysis. Further long-term studies are needed at this point to have a greater understanding of microbial plastic degradation and associated nutrient cycling in constructed wetland systems. A general cost analysis of utilizing a biodegradable plastic for enhanced microbial activity and nutrient removal was conducted, it was seen that the upfront cost is high, however, compared to the potential $N\text{-}NO_3^-$ removal efficiency in the system this cost is negligible over time. We believe that the potential for use of a biodegradable plastic to enhance nutrient removal within a constructed wetland can be a promising approach in wetland engineering for increased nutrient cycling efficiency.

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694 **Figure Legends**

695

696 **Fig. 1. Design of two wetland systems a)** Overview of experimental setup **b)** schematic of
697 vertical-flow treatment wetland (VFTW) mesocosm and **c)** schematic for floating
698 treatment wetland (FTW) mesocosm**. b)** and **c)** schematic denoting length (1.33 m) x
699 width (0.47 m) x height (0.61 m) with different design depth and width of mesocosms
700 with PCL, polycaprolactone, identical design was used for controls without PCL aspect.

701 **Fig. 2. Mesocosms used in this study. a)** Overview of VFTW mesocosms in setup, **b)** *Spartina*
702 *patens* location within mesocosm relative to edge of tub, **c)** VFTW mesocosm containing
703 PCL plastic beads which had the ability to float when flooded, **d)** overview of FTW
704 mesocosm with bioreactor setup (rear row), **e)** FTW mesocosm containing PCL plastic as
705 reactor medium, **f)** configuration of floating mat with 18 planting holes, **g)** view of
706 aerator pot and plant root, and **h)** overview setup of upstream aquaculture tank with
707 double filtration system that housed Pinfish (*Lagodon rhomboides*).

708 **Fig. 3. Change in inflow and outflow nutrient concentrations. a)** percent composition of
709 nitrogen in inflow and outflow and **b)** TN:TP ratio change over time, with (-) denoting
710 control and (+) presence of PCL in construction. A solid horizontal line indicates mg-
711 based Redfield ratio between N and P (8.9).

712 **Fig. 4. Change in stem height of *Spartina patens*.** Measured in mm day^{-1} with the same naming
713 scheme as previous. Data are shown as mean ± range (n = 2).

714 **Fig. 5. Relative bacterial and archaeal abundance at the phylum level.** Percent relative
715 abundance distribution after normalization to 10,000 reads per sample. *Proteobacteria*

716 are shown at the class level. Sample naming uses 1 and 2 showing replication and (-) and

717 (+) denoting presence of PCL.

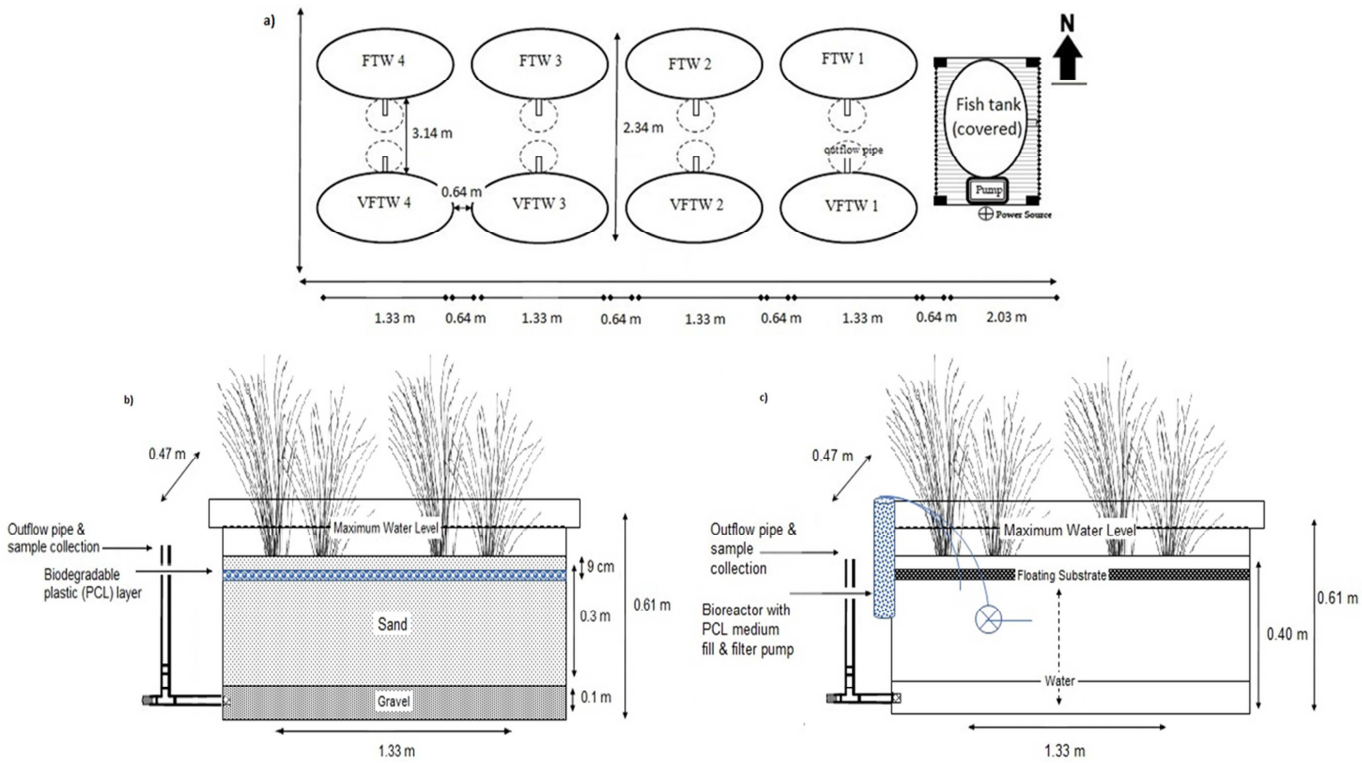

Figure 1 Design of two wetland systems. a) Overview of experimental setup **b)** schematic of vertical-flow treatment wetland (VFTW) mesocosm and **c)** schematic for floating treatment wetland (FTW) mesocosm. **b)** and **c)** schematic denoting length (1.33 m) x width (0.47 m) x height (0.61 m) with different design depth and width of mesocosms with PCL, polycaprolactone, identical design was used for controls without PCL aspect.

Figure 2 Mesocosms used in this study. a) Overview of VFTW mesocosms in setup, **b)** *Spartina patens* location within mesocosm relative to edge of tub, **c)** VFTW mesocosm containing PCL plastic beads which had the ability to float when flooded, **d)** overview of FTW mesocosm with bioreactor setup (rear row), **e)** FTW mesocosm containing PCL plastic as reactor medium, **f)** configuration of floating mat with 18 planting holes, **g)** view of aerator pot and plant root, and **h)** overview setup of upstream aquaculture tank with double filtration system that housed Pinfish (*Lagodon rhomboides*).

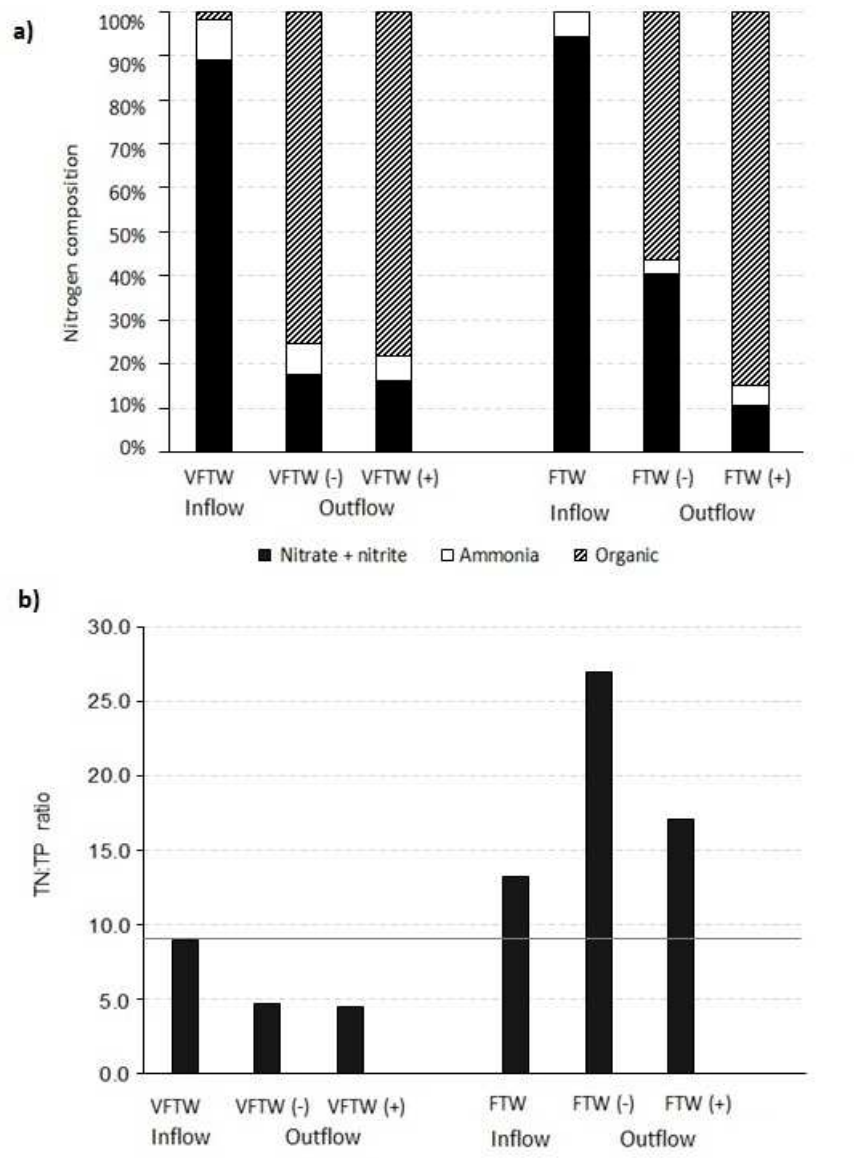

Figure 3 Change in inflow and outflow nutrient concentrations. a) percent composition of nitrogen in inflow and outflow and **b)** TN:TP ratio change over time, with (-) denoting control and (+) presence of PCL in construction. A solid horizontal line indicates mg-based Redfield ratio between N and P (8.9).

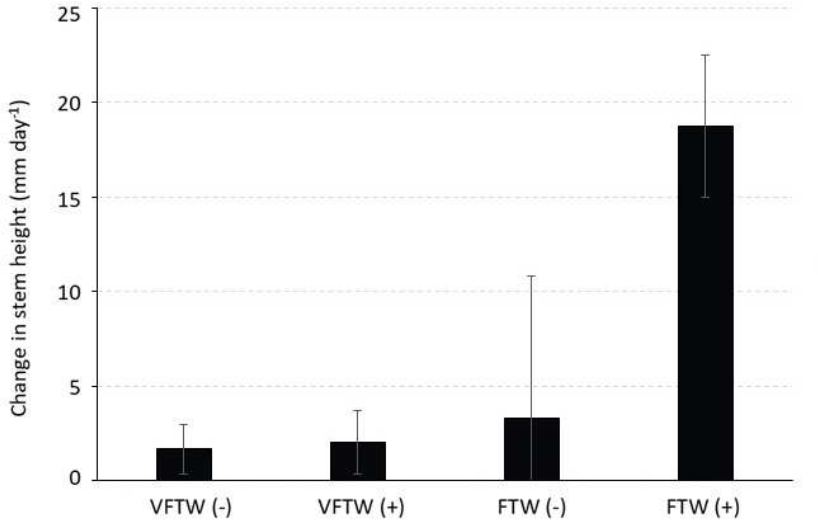

Figure 4 Change in stem height of *Spartina patens*. Measured in mm day^{-1} over the month testing period with (-) denoting control and (+) presence of PCL in construction. Data are shown as mean ± range (n = 2).

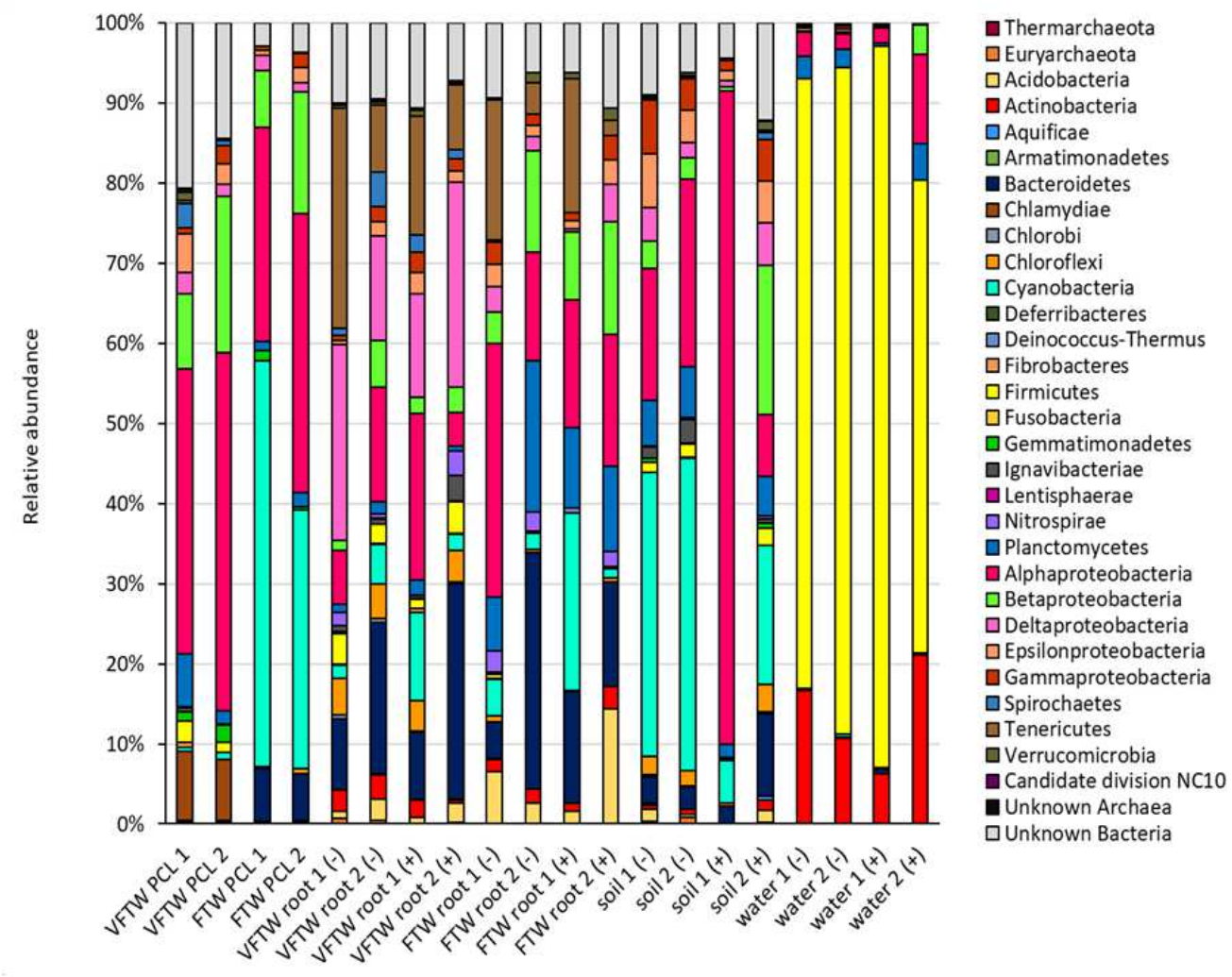

Figure 5 Relative bacterial and archaeal abundance at the phylum level. Percent relative abundance distribution after normalization to 10,000 reads per sample. *Proteobacteria* are shown at the class level. Sample naming uses 1 and 2 showing replication and (-) and (+) denoting presence of PCL.

Table 1 Inflow water quality parameters.

Wetland type	NO_3-NO_2 (mg L^{-1})	NH_4^+ (mg L^{-1})	Organic N (mg L^{-1})	TN (mg L^{-1})	TP (mg L^{-1})	DO (mg L^{-1})	Salinity (ppt)	Temp (°C)
FTW	4.35 ± 0.82	0.27 ± 0.08	0.0 ± 0.0	4.62 ± 1.10	0.35 ± 0.20	7.04 ± 0.27	16.2 ± 0.84	29.8 ± 4.25
VFTW	15.8 ± 3.34	1.64 ± 0.31	0.31 ± 0.50	17.8 ± 0.31	1.99 ± 0.41	6.60 ± 0.16	16.6 ± 0.40	29.5 ± 1.05

Data are mean ± standard error of upstream tank effluent before loading to the FTW system (n = 4) and the VFTW system (n = 4) mesocosms.

Table 2 Nutrient flux of two constructed wetland systems.

		TN (mg m^{-2} day^{-1})				TP (mg m^{-2} day^{-1})			
Wetland	PCL	Inflow	Outflow	Removal efficiency	Reduction (%)	Inflow	Outflow	Removal efficiency	Reduction (%)
VFTW	(-)	86.04 ± 0.19	8.63 ± 0.26	77.41 ± 0.45[a]	90.0	9.64 ± 0.02	1.82 ± 0.09	7.82 ± 0.11[a]	81.1
	(+)		11.29 ± 0.53	74.75 ± 0.72[b]	86.9		2.51 ± 0.19	7.13 ± 0.63[a]	74.0
FTW	(-)	22.39 ± 0.13	27.48 ± 0.59	(-5.09) ± 0.72[c]	0	1.67 ± 0.04	1.01 ± 0.06	0.66 ± 0.1[b]	39.5
	(+)		23.95 ± 0.65	(-1.56) ± 0.78[d]	0		1.38 ± 0.05	0.29 ± 0.09[b]	17.4

*Data are mean ± standard error (n = 8) for all experimental conditions with percent retention quantified from ((inflow concentration – outflow concentration) (inflow concentration)) x 100 (Olguín et al., 2017). The letter next to the monthly retention denotes statistical significance from completing, one-way ANOVA for TN, and significance shown for TP retention, *t*-test.

Table 3 Removal efficiency of various constructed wetlands for aquaculture effluent treatment.

Study	Scale	Construction type	Plant species used	Removal efficiency (%)*	Salinity (ppt)	Temperature (°C)	Location
Brown et al., 1999	Mesocosm	Subsurface flow	*Suaeda eseroa, Salicornia bigelovii, Altriplex barclayana*	TN:98, TIN:94, TP:99	10	22.6 - 37.4	Tucson, AZ USA
Lymbery et al., 2006	Mesocosm	Horizontal subsurface flow	*Juncus kraussii*	TN:69, TP:88	6.6 - 24.8	N/A	Australia
Li et al., 2007	Pilot-scale	Vertical - flow	*Canna indica, Typha latifolia, Acorus calamus, Arave sisalana*	TN:54.6 TP:80.1	0	23.6 - 24.0	China
Zhang et al., 2010	Pilot-scale	Vertical - downflow vertical - upflow hybrid	*Canna indica, Typha latifolia, Acorus calamus*	TN:48, TP:17	N/A Freshwater	N/A	China
Webb et al., 2012	Pilot-scale	Subsurface flow	*Salicornia europaea*	TDIN:98.2, DIP:36 - 89	22	23.1	North Wales, UK
Lin et al., 2003	Pilot-scale	Free water surface – subsurface flow hybrid	*Phragmites australis*	TIN:68.2, PO4-P:5.4	N/A *Litopenaeus vannamei* culture	23.5	Taiwan
Lin et al., 2010	Pilot-scale	Floating macrophyte – subsurface flow hybrid	*Eichhornia crassipes, Pistia stratiotes, Typha angustifolia, Phragmites communis, Canna generalis, Cyperus alternifalius*	TN:0 - 18, TP:2 - 18	0	N/A	Taiwan
De Stefani et al., 2011	in-stream	Floating treatment wetland	*Chrysopogon zizaniodes, Typha latifolia, Sparganium erectum*	TN:13 - 29, TP:65	N/A Freshwater	10.0 - 14.0	Italy
Li and Li, 2009	Pilot-scale	Floating treatment wetland	*Ipomenea aquatica*	TN:30.6, TP:18.2	N/A Freshwater	24.4	China
This study	Mesocosm	Vertical - flow	*Spartina patens*	TN: 86.9 - 90.7 TP: 74 - 81.1	7.4	28.8	FL, USA
	Mesocosm	Floating treatment wetland	*Spartina patens*	TN: 0 TP: 17.4 - 39.5	5.1	29.9	FL, USA

*Removal efficiencies are denoted TN (total nitrogen), TIN (total inorganic nitrogen), TDIN (total dissolved inorganic nitrogen), TP (total phosphorous), DIP (dissolved inorganic phosphorous), and phosphate. Temperature is denoted as air temperature for subsurface flow and water temperature for floating treatment systems. N/A shows data not measured.

Table 4 Summary of DNA sequencing and diversity indices.

Samples	PCL presence	Sequences	OTU	Mean sequence length	Pielou evenness	Menhinick richness index	Shannon index
PCL Biofilm							
$VFTW_1$		21,519	776	411 ± 19	0.42	2.53	2.32
$VFTW_2$		13,069	504	412 ± 6	0.41	2.04	2.18
FTW_1		13,118	211	411 ± 2	0.62	1.09	2.91
FTW_2		13,496	278	412 ± 8	0.53	1.35	2.62
Root							
$VFTW_1$	(-)	22,640	622	412 ± 8	0.39	2.5	2.14
$VFTW_2$	(-)	23,988	871	411 ± 13	0.48	2.98	2.73
$VFTW_1$	(+)	23,930	764	412 ± 6	0.48	2.53	2.14
$VFTW_2$	(+)	11,796	525	411 ± 15	0.48	1.93	2.43
FTW_1	(-)	27,026	472	411 ± 6	0.47	1.86	2.46
FTW_2	(-)	19,676	316	411 ± 6	0.51	1.33	2.48
FTW_1	(+)	15,924	194	411 ± 4	0.48	0.89	2.14
FTW_2	(+)	17,078	355	411 ± 3	0.48	1.56	2.43
Soil							
$VFTW_1$	(-)	18,851	646	412 ± 8	0.6	2.26	3.23
$VFTW_2$	(-)	17,544	529	412 ± 8	0.6	2.15	3.24
$VFTW_1$	(+)	20,381	208	411 ± 14	0.29	1.04	1.37
$VFTW_2$	(+)	22,693	827	412 ± 8	0.55	2.57	3.07
Water							
FTW_1	(-)	20,580	143	412 ± 5	0.29	0.75	1.24
FTW_2	(-)	21,014	135	412 ± 4	0.22	0.76	0.95
FTW_1	(+)	25,287	113	412 ± 4	0.17	0.64	0.69
FTW_2	(+)	26,658	128	412 ± 3	0.35	0.71	1.5

Diversity indices were calculated after normalization to 10,000 reads per sample. Samples 1 and 2 denote replicates and (-) and (+) denotes presence of PCL in construction when applicable.

Table 5 Functional groups at the genus level.

		PCL biofilm		Roots				Soil		Water	
Nitrogen - fixing bacteria		VFTW	FTW	VFTW (+)	VFTW (-)	FTW (+)	FTW (-)	VFTW (+)	VFTW (-)	FTW (+)	FTW (-)
Alphaproteobacteria	*Nitrospirillium*	77	1	15	9	0	0	20	2	0	0
	Rhizobium	1	0	33	4	0	0	4	3	0	0
	Bradyrhizobium	0	0	11	5	4	0	0	0	1	0
	Mesorhizobium	83	15	7	9	20	6	12	13	2	4
	Azospirilium	9	0	5	0	0	0	8	0	0	0
Betaproteobacteria	*Azohydromonas*	18	2	8	1	4	11	15	51	0	0
	Azonexus	2	0	13	4	0	0	1	1	0	0
	Derxia	39	0	10	14	0	0	7	115	0	0
Cyanobacteria	*Anabaena*	12	0	0	0	0	0	6	0	0	0
	Nostoc	79	115	43	19	2	1	1063	92	0	2
	Calothrix	84	6	9	30	0	0	51	30	0	0
	Cylindrospermum	36	0	79	36	0	0	526	56	0	0
Nitrifying bacteria											
Nitrospira	*Nitrospira*	1	0	1	0	265	88	0	8	0	0
Gammaproteobacteria	*Nitrosococcus*	5	1	23	59	114	63	14	21	0	0
Denitrifying bacteria											
Bacilli	*Bacillus*	3	0	7	6	0	0	26	24	7589	7245
Alphaproteobacteria	*Nitratereductor*	3	1	24	16	4	1	51	7	0	0
Gammaproteobacteria	*Pseudomonas*	27	2	4	0	1	0	0	19	0	0
Sulfate-reducing bacteria											
Deltaproteobacteria	*Desulfobulbus*	25	1	29	140	0	2	1	22	0	0
	Desulfatitalea	302	0	0	76	0	0	0	0	0	0
	Desulfobacterium	0	0	0	19	0	0	0	0	0	0
	Desulfonema	28	0	5	11	0	0	0	0	0	0
	Desulfocapsa	24	0	59	69	0	0	4	3	0	0
	Desulfopila	1	0	11	9	0	0	2	0	0	0
	Desulfomicrobium	11	0	5	12	0	0	15	6	0	0
	Desulfovibrio	40	24	137	138	6	7	44	12	0	0
Sulfur-oxidizing bacteria											
Chlorobia	*Chlorobium*	9	0	13	8	0	0	4	2	0	0
Betaproteobacteria	*Thiobacillus*	74	4	15	11	73	99	17	40	1	1
	Thiobacter	2	0	5	1	0	0	0	1	0	0
Gammaproteobacteria	*Thiothrix*	0	2	0	0	1	32	0	0	0	0
Methanogenic archaea											
Methanobacteria	*Methanobacterium*	19	0	43	13	0	0	51	11	0	0
Methanomicrobia	*Methanoregula*	0	0	3	0	0	0	0	0	0	0
	Methanosarcina	1	0	2	0	0	0	2	0	0	0

Methanotrophic bacteria											
Alphaproteobacteria	*Methylocystis*	0	0	7	0	0	0	0	0	0	0
	Methylobacter	0	0	0	0	0	2	0	0	0	0
	Methylocella	0	3	0	1	26	41	0	0	0	2
Gammaproteobacteria	*Methylococcus*	0	0	4	0	0	0	0	0	0	0
	Methylosoma	0	0	0	0	9	3	0	0	0	0
Methylotrophic bacteria											
Alphaproteobacteria	*Methylobacterium*	0	0	1	1	5	8	0	0	0	0
	Methylobacillus	0	0	5	5	0	0	0	2	0	0
	Hyphomicrobium	4	3	16	6	89	80	14	8	8	9
Betaproteobacteria	*Methylophilus*	0	0	0	0	15	4	0	0	0	0
	Methylibium	200	5	13	53	7	4	171	550	0	0

Shown are average bacterial and archaeal relative abundance of sample distribution (n = 2) after normalization to 10,000 reads per sample. *Cyanobacteria* are shown at the phylum level as class level was unidentified.