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Impact of a Natural Disturbance on the Performance and Microbial Communities of a Constructed Wetland for Industrial Wastewater Treatment

By

Marielle Hollstein

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APPROVED, THESIS COMMITTEE

Lauren Stadler (Apr 19, 2022 14:48 CDT)

Lauren Stadler
Chair
Assistant Professor, Civil and Environmental Engineering

Qilin Li

Qilin Li
Professor, Civil and Environmental Engineering

Scott Egan (Apr 19, 2022 14:58 CDT)

Scott Egan
Professor, Biosciences

HOUSTON, TEXAS

April 2022

ABSTRACT

Impact of a Natural Disturbance on the Performance and Microbial Communities in a Full-Scale Constructed Wetland for Industrial Wastewater Treatment

by

Marielle Hollstein

Constructed Wetlands (CWs) are a sustainable choice for tertiary wastewater treatment. In these environments, microbial communities play a significant role in pollutant removal. However, little is known about how microbial communities in full-scale CWs contribute to maintaining water quality or how their dynamics change in response to pulse disturbances. We characterized the microbial communities in a full-scale CW that provides tertiary wastewater treatment to a chemical production plant. The CW sampling campaign was conducted over a 12-month period that included a 100-year freeze event. Analysis of 16S rRNA gene amplicon sequences revealed that the microbial communities experienced a temporal shift. Six months after the freeze the removal of water quality constituents began to return to their former removal trends in the CW. This suggests CW functional resilience despite the shift in microbial community structure in the wetland.

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Nomenclature

CW	Constructed Wetland
TSS	Total Suspended Solids
VSS	Volatile Suspended Solids
COD	Chemical Oxygen Demand

Chapter 1

1.0 Introduction

Alternative wastewater treatment technologies to conventional activated sludge should be considered that are more energy efficient, less chemically-intensive, and have lower environmental impacts. Constructed wetlands (CWs) have been used for decades to treat industrial and municipal wastewater. CWs are also a good design alternative for tertiary wastewater treatment, since they act as biological sinks for many chemicals and nutrients (Zhu et al., 2021; West et al., 2017). Microbial communities are vital for CW performance, as they drive nutrient cycling within the system (Shirdashtzadeh et al, 2022; Ping et al, 2019; Rajan et al, 2018., Stottmeister et al, 2003)**.** Another important function of free water surface CWs and their microbial communities is to prevent eutrophication and mitigate algal blooms by regulating nutrient concentrations (Xia et al., 2020; West et al., 2017; Vymazal, 2006; Dunne et al., 2012).

This study focuses a 110-acre free water surface CW that provides tertiary wastewater treatment for a plastics manufacturing plant. The limited number of microbial community experiments and studies conducted on full-scale CWs makes this study site a good model to assess biological diversity and community dynamics for similar tertiary wastewater treatment systems utilized for full-scale industrial applications (Vymazal et al, 2021).

The objectives of this study were to (1) assess the relationship between the performance of the CW and its microbial communities, both over time and space; and (2) evaluate the short-term impact of the freeze on performance and the microbial communities in the CW. We hypothesized that (1) we would observe both spatial and temporal shifts in the microbial community across the CW; (2) shifts in the microbial community would be associated with changes in water quality

parameters; and we could identify taxonomic groups strongly associated with changes in water quality parameters, and thus CW performance. Elucidating mechanisms the CW uses for water quality improvement and predicting the fluctuating relative abundances of specific taxa can also provide insights on how other heterotrophic bacteria mitigate eutrophication in other fresh-water environments.

2.0 Literature Review

2.1 CWs' Cost and Energy Savings Makes Them an Attractive Tertiary Wastewater Treatment Alternative

CWs are a cost-effective, versatile and sustainable choice for wastewater treatment (Vymazal, 2011; Wu et al, 2014). CWs can be configured in a variety of ways to leverage specific applications and operate within a designated land footprint (Vymazal, 2001). Free water surface CWs use planted systems that closely resemble natural wetlands and are predominantly used to treat tertiary wastewater. This type of CW allows the wastewater to come in contact with air and supports the growth of diverse microbial communities in the leaf-litter layer, making this technology a less resource intensive method to remove pollutants (Kadlec and Wallace, 2009; Tang et al., 2020; Truu et al., 2009).

Operation and management costs are also much lower relative to conventional activated sludge treatment (Zhang et al., 2015). In a life cycle assessment study comparing a free water surface CW to a more traditional sequence batch reactor for tertiary treatment, the free water surface CW had significant cost, energy, and material savings relative to its alternative; estimating a net percent value savings of $282 million throughout the wetland's life cycle (Dimuro et al, 2014). Researchers also determined that CWs have much lower GHG emissions

throughout their lifetime, relative to conventional activated sludge systems (Garfi et al., 2017; Casas Ledón et al., 2017; Mander et al., 2014). This is because CWs are dependent on renewable sources of energy (e.g., gravity and photosynthesis), while sequencing batch reactors require a constant electrical expenditure (Merlin and Lissolo, 2010).

While free water surface CWs may require a large land footprint, other more conventional alternatives can also take up a similar amount of space for electrical and chemical storage (Dimuro et al, 2014; Merlin and Lissolo, 2010**).** Furthermore, an economic comparison between CWs and other conventional technologies revealed that land costs were determined to have little impact on overall costs (Firth et al., 2020). The space allocated for CWs also provides important ecological and social services. For example, CWs, provide habitats for native and endangered species, mitigate flooding, and sequester carbon (Zhang et al., 2020; Knight et al., 1997; de Klein and van der Werf, 2014). CWs also boost biodiversity, an ecological characteristic demonstrated to be linked to water quality (Hsu et al., 2011; Cardinale, 2011).

2.2 The Importance of Microbial Communities in CWs

Microbial communities are vital for CW performance, helping drive the biogeochemical cycling within these environments (Shirdashtzadeh et al, 2022; Ping et al, 2019; Rajan et al, 2018 Stottmeister et al, 2003)**.** CW microbial communities contribute to the removal of organic pollutants and nutrients, as well as the removal of inorganic substances like heavy metals, pesticides, and pharmaceuticals. (Wang et al, 2022; Guo et al, 2020; Lv et al, 2017; Yan et al, 2018). Microbial communities found in different CW zones are capable of mediating different processes (Semenov et al, 2020). For instance, microbial communities found the leaf-litter layer region contain large amounts of plant debris and other organic matter, creating an optimal environment for methanotrophs, nitrifiers, and sulfur-reducing bacteria (Lv et al, 2017; Vyamzal

et al, 2007; Chen et al, 2015; DeJournett et al, 2007). Bacteria in anaerobic zones of the sediment layer can also contribute to denitrification (Vyamzal et al, 2007)**.** Phytoplankton and other organisms found throughout the water-column are typically exposed to ample amounts of sunlight and oxygen (Kadlec and Wallace, 2009). These photosynthetic organisms supply other heterotrophic bacteria with vitamins and a source of organic carbon during their growth and subsequent decomposition (Kazamia et al., 2012). CW water quality standards are maintained because the microbial communities that inhabit these different wetland zones (i.e., different nutrient gradients) provide complementary functions to remove pollutants (Kadlec and Wallace, 2009; Horton et al., 2019)**.**

There have been few mesocosm studies and virtually no full-scale studies on the correlation between pollutant removal and microbial community structure (Rajan et al, 2018; Vymazal et al., 2021). Full-scale studies on CWs are very difficult to accomplish as plant diversity, substrate composition, hydrology, and climate all exert huge influences on microbial community dynamics (Vymazal et al, 2021). While mesocosms and other lab-scale studies may focus on an individual microbial interaction, it is difficult to identify other environmental factors that may possess indirect effects on said interaction. A combination of full-scale and lab-scale studies are required to capture more realistic microbial interactions and other mechanisms utilized for water quality enhancement.

2.3 A Valuable CW Application in the Face of Climate Change– Eutrophication mitigation

CW's have been established as a promising treatment alternative for eutrophication and algal bloom mitigation (Calero et al, 2015). The coupling of plants and beneficial microorganisms in CWs advances the removal of nutrients such as ammonia, nitrate, and phosphate (Tang et al, 2020; Stottmeister et al, 2003). Increased anthropogenic nutrient loading contributes to a

phenomenon referred to as cultural eutrophication (Rast and Thornton, 1996). Agricultural runoff is typically a primary contributor to cultural eutrophication, as the runoff is loaded with nutrient-rich fertilizers (Schindler, 2006). However, other point sources can increase eutrophication potential in a surrounding waterbody. Industrial wastewater retention ponds have also been shown to promote eutrophication as these shallow, lentic ponds offer a prime environment for nutrients to accumulate and primary production to occur (Horne, 1995; Erbas et al., 2021).

If left untreated, algae and other phytoplankton can proliferate due to the abundance of nutrients available. When the phytoplankton decompose, oxygen is consumed, which produces dead zones. Algal biomass can also block sunlight and prevent oxygen access to other organisms; creating mass fish kills and lowering biodiversity within multiple taxonomic scales (Mishra et al, 2021; Murray et al, 2015; Calero et al., 2015). While algal blooms are responsible for millions of dollars of damage annually in the U.S. alone, cyanobacterial blooms also create an immediate human and animal health danger (Anderson, 2000; Backer, 2002). Cyanobacteria, gram-negative, autotrophic bacteria, also inhabit the same aquatic environments as many eukaryotic algae. As photosynthetic organisms, cyanobacteria compete for the same resources as the green algae. However, cyanobacteria can be more problematic during bloom events because they are known to release lethal, intracellular metabolites, known as cyanotoxins (Carmichael, 1989). These toxins are responsible for many human and animal fatalities world-wide; and rates of exposure have accelerated in recent years (Wood, 2016). Increased nutrient loadings as well as other anthropogenic effects in natural water bodies have also elevated the risk of harmful cyanobacterial blooms (Paerl et al., 2016). As temperatures increase cyanobacteria possess a higher optimum growth rate relative to eukaryotic algae (Lürling et al, 2012). In the face of

climate change, faster growth rates give toxic cyanobacteria a competitive advantage over non-toxic eukaryotic algae.

Eutrophication prevention and bloom mitigation are major objectives for free water surface CWs (Xia et al, 2020). Previous studies have assessed how CWs can control phytoplankton accumulation and limit unwanted cyanobacterial species (Zhong et al, 2011). Furthermore, Calero et al asserts that free water surface CWs have the capability to reduce eutrophication and bloom intensity by both altering the taxonomic composition and increasing the richness of the residing phytoplankton species (2015). Large free water surface CWs have been shown to be exceptionally good at removing phosphorus (Kadlec 2016), which is a key nutrient in driving the toxic cyanobacterial blooms that ultimately reduce microbial community diversity (Gu et al, 2020)**.** Wetland rhizospheres and leaf-litter layers also host many microorganisms that play a role in the completion of the N-cycling and chemical degradation which mitigate eutrophic conditions before the onset of a phytoplankton bloom (Tang et al, 2020). However, bacterial community dynamics are rarely addressed as a bioindicator or an attenuating factor for cyanobacterial blooms in these CW environments.

2.4 Resiliency of Microbial Communities in CWs after Pulse Disturbances

A pulse disturbance is a temporary event that has the potential to disrupt the afflicted region's ecosystem. Pulse disturbances can be natural or man-made, such as flooding, freezes, chemical exposure, and controlled burns. The frequency and severity of pulse events are expected to increase as a result of climate change (McDowell et al., 2018). The pulse disturbances brought on by more intense storms could potentially alter a region's biodiversity. Scientists are attempting to discern whether pulse disturbances have long-term effects on the environment's

microbial communities, which could have lasting impacts on the region's overall ecological health (Jacquet and Altermatt, 2020).

There are various metrics for measuring how microbial communities cope with ecological disturbances. For instance, in the event of a disturbance, resistance measures the extent to which microbial community structure remains unchanged and resilience evaluates the rate at which a community returns to its original composition (Allison and Martiny, 2008). In several studies, a region's ecosystem will still demonstrate long-term functional resilience despite an alteration in microbial community structure after a pulse disturbance (Bao et al., 2022; Zhu et al., 2022; Li et al., 2021**)**. This is known as microbial community redundancy (Allison and Martiny, 2008). Microbial community functional redundancy is important for the durability of CW performance (Ma et al., 2018).

Unfortunately, many microbial communities within natural environments are not functionally redundant after pulse disturbances, leading to issues like decreased biogeochemical cycling and soil erosion (Sjøgaard et al., 2018; García-Carmona et al., 2021). It has also been determined that many types of pulse disturbances decrease species richness and diversity, which could also impact community function (Sousa, 1984). The prospect of increasing storm severity becomes more threatening as these events may decimate keystone species essential to CW performance. (Wang et al., 2020). Furthermore**,** freezes paired with other extreme weather events are shown to increase eutrophication potential in downstream environments (Inander et al., 2018). More research is needed to understand how CW microbial communities are linked to eutrophication potential and if pulse disturbances, such as freezes, affect this connection.

Conclusion

CWs are a cost-effective and environmentally friendly alternative to conventional activated sludge technology. Furthermore, they are an advantageous treatment technology for eutrophication and phytoplankton bloom mitigation. The microbial communities found in CWs are vital to overall wetland performance. In many studies CWs have been determined to be resilient to pulse disturbances. However, the long-term impact of pulse disturbances on microbial communities in CWs is poorly understood. These disturbances may hinder microbially-mediated biogeochemical cycling; increasing eutrophication potential in these environments. Yet, CWs may also provide conditions that limit the growth of unfavorable cyanobacteria over other species of benign phytoplankton to combat the toxic cyanobacterial bloom situation. To fully understand these mechanisms, we have to look at overarching microbial community dynamics within a full-scale CW, as well as isolate individual species to understand specific modes of interaction.

Chapter 2

3.0 Impact of a Natural Disturbance on the Performance and Microbial Communities in a Full-Scale Constructed Wetland for Industrial Wastewater Treatment

Authors: Marielle Hollstein[a], Mattheau Comerford[b], Michael Uhl[c], Michael Abel[c], Scott Egan[b], Lauren Stadler[a]*

Author affiliations:

[a] Department of Civil and Environmental Engineering, Rice University, 6100 Main Street Houston, Texas 77005, United States

[b] Department of Biosciences, Rice University, 6100 Main Street Houston, Texas 77005, United States

[c] Dow Chemical Company, 2211 H.H. Dow Way Midland, MI 48674, United States

*Corresponding author contact info: Lauren.Stadler@rice.edu

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Abstract

Constructed Wetlands (CWs) are a cost-effective, versatile and sustainable choice for tertiary wastewater treatment. In these environments, microbial communities play a significant role in pollutant removal. However, little is known about how microbial communities in full-scale CWs contribute to maintaining water quality or how their dynamics change in response to pulse disturbances such as fire or freezes. Furthermore, few studies have examined the relationship between CW microbial community structure and performance in full-scale industrial operations. We characterized the water-column and leaf-litter layer microbial communities in the a 110-acre

free water surface CW that provides tertiary wastewater treatment to a plastics manufacturing plant. The CW's sampling campaign was conducted over a 12-month period that included Winter Storm Uri, a 100-year freeze event. Analysis of 16S rRNA gene amplicon sequences revealed that the bacterial communities experienced a temporal shift. There was also a shift in microbial community structure between the influent and the first segment of the CW. However, no differences in microbial community structure were observed in the second segment of the CW. There was a negative association between microbial community diversity and chlorophyll *a,* as well as microbial community diversity and TSS; demonstrating an increase in microbial biodiversity as water quality improved throughout the CW. Six months after the freeze CW performance in terms of removal of water quality constituents began to return to their former removal trends. Yet, there was still a significant difference in microbial community structure within the CW relative to the previous year. This suggests CW functional resilience despite a shift in microbial community structure in the wetland.

3.1 Introduction

Constructed wetlands (CWs) are a cost-effective, versatile, and sustainable alternative to conventional activated sludge wastewater treatment (Vymazal, 2011). CWs are implemented as tertiary treatment for industrial and municipal wastewaters, as a technology for nutrient removal (Wu et al., 2015; Dimuro et al., 2014; Zhu et al., 2021; West et al., 2017). CWs also provide habitats for native and endangered species and are shown to boost the region's biodiversity, an ecological characteristic linked to water quality (Knight et al., 1997; Hsu et al., 2011; Cardinale, 2011). Moreover, microbial communities are vital for CW performance, as they drive nutrient cycling within the system (Shirdashtzadeh et al, 2022; Ping et al, 2019; Rajan et al, 2019 Stottmeister et al, 2003)**.** CW microbial communities contribute to the reduction of organic pollutants, as well as the removal of inorganic substances such as heavy metals, pesticides, and pharmaceuticals (Wang et al, 2021; Guo et al, 2020; Lv et al, 2017; Yan et al, 2018). Another important function of free water surface CWs and their microbial communities is to prevent eutrophication and mitigate algal blooms by regulating said nutrient concentrations (Xia et al., 2020; West et al., 2017; Vymazal, 2007; Dunne et al., 2012). Previous studies have also assessed how CWs can control phytoplankton accumulation and limit unwanted cyanobacterial species (Zhong et al, 2011). Mesocosm-scale studies have evaluated microbial community correlations with treatment of eutrophic waters and have addressed the role of bioremediation in overall water quality (Liu et al., 2020; Xu et al., 2018). Yet, few studies have been conducted on microbial community dynamics in full-scale CWs in relation to eutrophication mitigation (Vymazal et al., 2021).

This study focuses on a 110-acre free water surface CW that provides tertiary wastewater treatment for a plastics manufacturing plant, located in Seadrift, Texas. Upstream of the CW, the

wastewater is conveyed through two facultative holding ponds before entering the wetland (Dimuro et al, 2014). Due to the long retention time in these holding ponds, algae and other phytoplankton proliferate. Before the CW was built the total suspended solids (TSS) concentration in the effluent exceeded the EPA's 40 mg/L TSS limit (EPA, 2022). The phytoplankton bloom conditions also influenced the carbon dioxide concentration in the water, which resulted in a pH imbalance The wastewater then required chemical treatment to neutralize the pH before discharge. After the CW was constructed, chemical treatment for TSS removal and pH neutralization were no longer necessary. In a life cycle assessment study comparing this free water surface CW to a more traditional sequence batch reactor for tertiary treatment, the free water surface CW had significant cost, energy, and material savings relative to its conventional alternative. The net percent value saving was estimated to be $282 million throughout the CW's life cycle (Dimuro et al, 2014).

The CW has been successfully treating the plastics manufacturing plant's wastewater for 26 years, despite several notable storm events and other pulse disturbances. During August, 2017, Hurricane Harvey inundated the surrounding area, leaving large levels of plant debris behind. In March, 2018 operators conducted a prescribed burn on the two eastern cells of the CW to remove this debris (Fig. 1). It is hypothesized that the burn may have altered microbial community functioning and wetland performance in this section of the CW. In February, 2021 the freeze caused by Winter Storm Uri was yet another unprecedented pulse disturbance in this CW's history. Seadrift, Texas experienced subzero temperatures for a period of over 48 hours, which caused a massive plant die-off throughout the wetland and, for several months afterwards, plant detritus traveled through the CW. A freeze's influence on the microbial community structure within a subtropical constructed wetland of this scale has previously never been studied.

Consequently, this CW is a good model to assess biological diversity and community dynamics for similar tertiary wastewater treatment systems utilized for large industrial applications.

There is limited knowledge of how pulse disturbances impact CW microbial community structure and alter the performance of full-scale CWs. Many CWs are designed to mitigate bloom conditions generated by preceding waste stabilization ponds. Yet, there is little research on how these pulse disturbances affect downstream eutrophication potential or the organisms that balance ecosystem functioning to reduce blooms. Moreover, climate change has enhanced the severity of storms and variability of weather patterns. Scientists are attempting to discern whether the pulse events brought on by these storms have long-term effects on CW microbial communities (Jacquet and Altermatt, 2020). Studies indicate that severe storm disturbances can impact short-term plant and microbial biogeochemical cycling within estuarine, wetland, and other aquatic environments (Huang et al, 2021). More specifically, the Winter Storm Uri freeze's impact on the CW's pollutant removal trends and microbial community structure also presents an opportunity to assess how CW performance correlates to CW microbial communities.

In this study we performed a year-long characterization of the microbial communities across a full-scale, 110-acre CW. This was achieved by sampling the water-column and leaf-litter layer from six locations in the CW system between August 25th, 2020 – August 3rd, 2021 and using 16S rRNA gene sequencing to characterize the microbial communities. The objectives of this study were to (1) assess the relationship between the performance of the CW and its microbial communities, both over time and space; and (2) evaluate the short-term impact of the freeze on performance and the microbial communities in the CW. We hypothesized that (1) we would observe both spatial and temporal shifts in the microbial community across the CW; (2) shifts in the microbial community would be associated with changes in water quality parameters

(suspended solids, pH, COD, NH_4-N, chlorophyll *a*); and we could identify taxonomic groups strongly associated with changes in water quality parameters, and thus CW performance; (3) the Winter Storm Uri freeze impacted the CW's microbial community structure, also influencing performance.

3.2. Materials and Methods

3.2.1 Site Location and Sampling Procedure

The CW is located directly southwest of a plastics manufacturing plant in Seadrift, Texas. As shown in Fig. 1, influent wastewater from a facultative holding pond is conveyed to Control Box 4 where it is then distributed to Cell 2 and Cell 3 from Control Box 4 West and Control Box 4 East outlet pipes. Cell 2 and 3 make up the first segment of the CW, otherwise known as the first set of wetlands in the CW. Afterwards, the wastewater is combined in Control Box 5 and is distributed to Cell 1 and 4 through Control Box 5 West and Control Box 5 East outlet pipes. Cell 1 and 4 make up the second segment of the CW, or the second set of wetlands in the CW. Wastewater from Cell 1 and Cell 4 is consolidated again in Control Box 7, the end point of the CW. Samples were collected near the outlet pipes directly east and west of Control Box 4. Samples were also collected near the inlet pipes directly east and west of Control Box 5 and 7. Samples for Control Box 4 East and Control Box 4 West were combined since they represent an average of the CW's influent. Samples taken represent a total of 5 locations throughout the CW; the influent, as well of the ends of Cell 2, Cell 3, Cell 1, and Cell 4 (Fig 1). This was done to assess beginning, middle, and end points of the entire CW, while also isolating unburned and burned wetland cells (Cell 2 vs Cell 3 and Cell1 vs Cell 4).

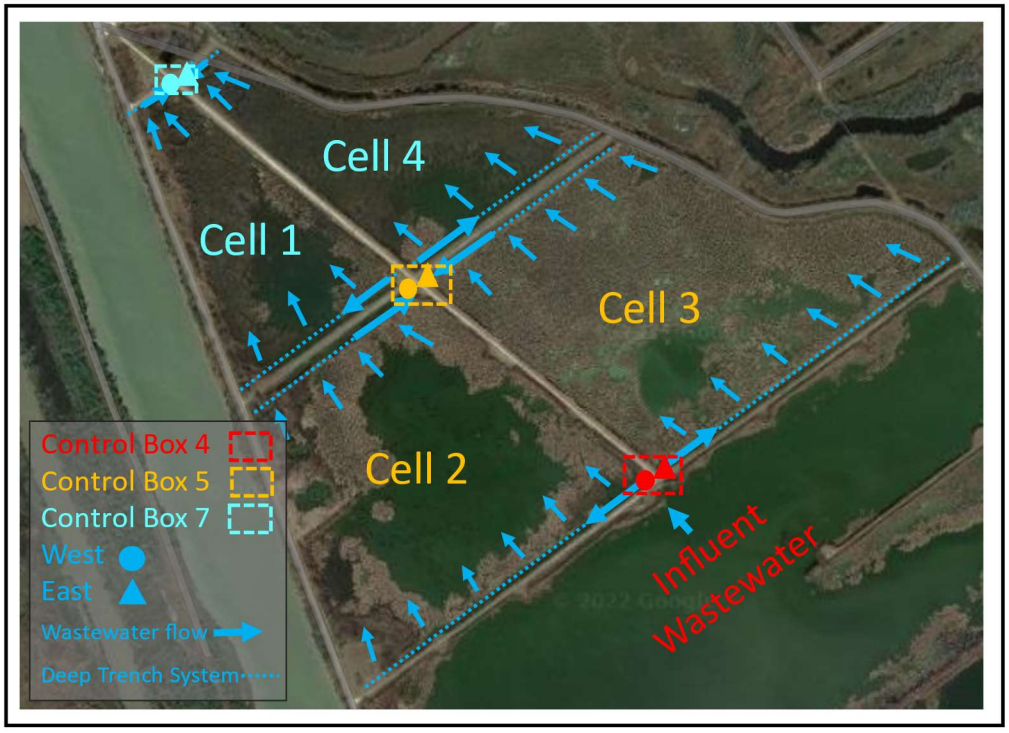

Figure 1 - A) Layout of the CW (Cell 2, Cell 3, Cell 1, Cell 4,), where Cell 2 and Cell 3 are the first set of wetlands and Cell 1 and Cell 4 are the second set of wetlands

Eight rounds of water-column grab samples were taken from August 25th, 2020 to August 3rd, 2021 (Table S1). Sample aliquots for chlorophyll *a* determination were stored in opaque amber HDPE bottles. To study the microbial communities found in the wetland's leaf-litter layer, seven rounds of passive samplers were deployed on the leaf litter layer and retrieved approximately one month afterwards (Table S1). Passive samplers were created by filling fine-mesh aquarium filter bags with granular activated carbon. Passive samplers were retrieved, stored in Whirl-Pak bags, and placed on ice. All samples were shipped within 24 hours on ice via FedEx overnight to Rice University for further processing and analysis.

2.2 Water Chemistry and Environmental Parameter Measurements

Water temperature and pH were measured onsite by CW operators. TSS and VSS were measured following Standard Method 2540 and NH_4-N was measured using Standard Method

4500 (*APHA, 2005*). COD was measured using low range CHEMetric COD vial kits with a potassium hydrogen phthalate blank standard curve (CHEMetrics, 2022). All water samples designated for chlorophyll *a* extraction were filtered and processed in low-light conditions. 150 mL of water from the CW water-column was filtered through 0.45-micron filters via vacuum pump and stored at -20˚ C for no longer than 20 days to preserve samples for chlorophyll *a* extraction. Chlorophyll *a* samples were extracted and measured spectrophotometrically following EPA method 446.0 (*EPA,* 2015).

2.3 DNA extraction and 16S rDNA gene sequencing

50 mL of water-column samples were filtered through a 0.22-micron filter and stored at -80˚ C until extraction. DNA was extracted from water-column samples representing 5 locations in the CW for 8 time periods; a total of 40 samples. Biomass was scraped from passive samplers and decanted from passive sampler collection Whirl-Pak bags into 15 mL centrifuge tubes. Solids were spun down, collected in 0.25 to 0.50-gram pellets and stored in a -80˚ C freezer until extraction. DNA was extracted from leaf-litter layer samples representing 5 locations in the CW for 7 time periods. However, three passive samplers were lost after deployment due to the location's wildlife. DNA was extracted from a total of 32 leaf-litter samples. The Aug 25th, 2020 water samples' genomic DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, France), following the manufacturer's protocol. To increase through-put all other water-column and passive sampler biofilm sample's genomic DNA was extracted using Promega's Maxwell® RSC PureFood GMO and Authentication Kit. Concentrations of DNA were measured using a DNA HS Assay kit on a Qubit fluorometer. To pass quality assurance sample DNA extracts must have contained 30 ng of DNA template. The October 20th, 2020 leaf-litter biofilm sample extract from Cell 4 did not pass quality assurance (QA) / quality control

(QC). DNA extracts from the 71 samples were shipped to BGI genomics for PCR amplification and sequencing. Technicians at BGI genomics included DNA and 16S rRNA fusion primers for PCR and amplified the V4 hypervariable region of the bacterial 16S rRNA gene using F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′- GGACTACHVGGGTWTCTAAT-3′) primers (BGI genomics). PCR products were purified by Agencourt AMPure XP beads and the purified PCR products were dissolved in an elution buffer and labeled for library construction (BGI genomics). After library QC, qualified libraries were sequenced on the HiSeq 2500 platform.

2.4 Sequencing Analysis

Microbial communities were analyzed using the Schloss lab MiSeq standard operating procedure using Mothur v. 1.43.0 (Kozich et al., 2013). The taxonomy was determined for each OTU once sequences were clustered into OTUs. All sample groups were subsampled to the smallest sample size for downstream alpha and beta diversity analysis. Relative abundances were determined for the top seven dominant phyla from the taxonomy summary files generated in Mothur. Rarefaction curves were generated to visually assess the number of OTUs identified per sample. Species richness (Chao1) indices and species evenness (Shannon) indices were calculated using Mothur for an alpha-diversity analysis. For beta-diversity, a non-metric multidimensional scaling (NMDS) paired with an analysis of molecular variance (AMOVA) test was employed to determine significant differences in sample bacterial community structure through separate CW sampling locations and sampling periods. The sequence distance matrices generated were visualized using the NMDS plots. Samples were grouped by location (i.e., Influent, Cell 2, Cell 3, Cell 1, Cell 4) to determine spatial changes and by sampling period to determine temporal changes. The AMOVA results for different sampling periods was determined with and without

influent samples included in the analysis to determine if any significant differences in influent community structure would impact p-values for samples grouped by different sampling rounds. Correlation coefficients for each OTU were calculated in Mothur using the corr.axis command to identify individual OTUs associated with shifts in microbial community structure (Kozich et al, 2013). These correlation coefficients were overlaid onto the original sample NMDS plot as an ordination biplot to visualize specific OTUs. Code for these analyses is shown in App. B.

2.5 Data Analysis

Percent removal for each environmental parameter during the first segment of the CW was calculated by subtracting averaged concentrations of the first set of wetlands from averaged influent concentrations. Averaged concentrations of the second set of wetlands were subtracted from averaged concentrations of the first set of wetlands to determine percent removal in the second segment of the CW. T-tests were used to determine significant differences in environmental parameter reduction rates between the first half versus the second half of the CW, as well as any significant differences between parallel CW cells (Cell 1 versus Cell 4 and Cell 2 versus Cell 3).

A Spearman correlation analysis was performed using the 'Hmisc' package in R to determine the degree of correlation between the relative abundance of the seven most dominant phyla and measured environmental parameters (TSS, VSS, COD, NH_4-N, Chlorophyll *a*, pH, and Temp) within samples. A heatmap was generated in R to demonstrate the correlation matrix among the different environmental parameters. Spearman correlation coefficients were also determined to assess the relationship between environmental parameters and the relative abundance of genera belonging to the OTU's most responsible for shifting microbial community structure.

3. Results and Discussion

3.1 CW wastewater treatment performance

Removal rates throughout the CW were largely dependent on influent concentrations of solids, COD, and chlorophyll *a*, which fluctuated based on seasonal changes. Table S3 shows lower removal rates typically corresponded to influent concentrations that were already within or near EPA standards (EPA, 2022). The CW had the greatest removal efficiencies on days that corresponded with some of the greatest pollutant concentrations in the influent. Studies have shown that removal efficiencies usually decrease if influent environmental parameter concentrations are especially low (Rousseau et al., 2004; Kadlec et al., 1995; Shin et al., 2010). This study's CW had the lowest removal efficiencies in the first half of the March 12th, 2021 sampling period. During this sampling period plant detritus associated with the freeze was navigating through the CW. The additional organic material elevated TSS, VSS, COD, chlorophyll *a*, NH_4-N, and pH levels at the mid and end points of the CW. Average water quality measurements for all sampling periods are presented in Table S2. Percent reductions of environmental parameters throughout the CW are presented in Table S3.

NH_4-N levels increased from the influent to the end of second set of wetlands during most of the sampling occasions. This is not unexpected because NH_4-N concentrations were relatively low within the influent (< 1 mg/L) for all but one sampling round. The natural decay of plant biomass and other organic matter in this study's CW may have been responsible for the slight uptick in NH_4-N levels in the middle and end sampling points (Yamanaka, 1995). A strong negative correlation was evident between temperature and NH_4-N (ρ = -0.7109), confirming that nitrification rates are temperature sensitive, and increase as temperature increases. It was previously determined that nitrification rates can decrease up to 82% when nitrifying biomass in a sequencing batch reactor was exposed to a cold temperature shock (Head and Oleszkiewicz,

2004). This may also explain why NH_4-N concentration tripled in value during the sampling period following the Winter Storm Uri freeze.

Cell 2 achieved significantly greater reductions in pH relative to Cell 3 ($p = 0.043$). These differences in pH reduction may be the result of suspected short-circuiting in Cell 3. For all other water chemistry parameters, parallel cells (Cell 2 vs Cell 3 and Cell 1 vs Cell 4) experienced no significant differences in percent removal ($p \geq 0.05$) There were also no significant differences in pollutant removal rates between the first and second segment of the CW. During several sampling periods, the bulk of the pollutants were removed in the first set of wetlands (Table S3). However, at other times the majority of the pollutants were removed from the second set of wetlands, which underscores how vital the entire CW is for ensuring that water quality parameters remain within EPA limits.

3.2 CW Microbial community structure

The total number of 4,177,818 sequences was determined after filtering the data and removing undesired reads. 55,625 OTUs were identified. 553 of the OTUs belonged to the archaea kingdom and the other 55,072 were recognized as bacteria. The sample with the smallest number of sequences contained 41,960; therefore, all other samples were subsampled to this size for alpha and beta diversity analyses.

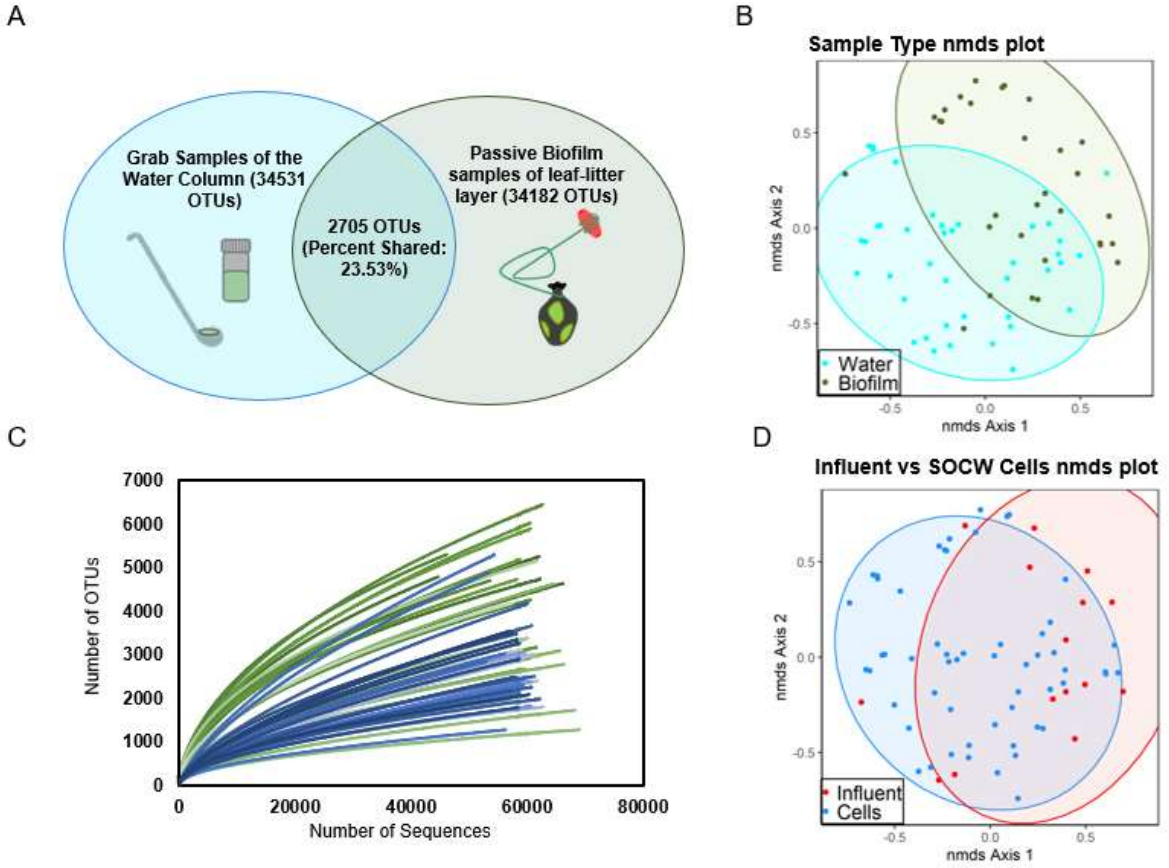

Fig. 2. Observed OTUs clustered at 97% sequence similarity; A) Venn diagrams of distinct and shared species in water-column and leaf-litter layer biofilm samples; and NMDS analysis plot of all samples grouped by B) water column versus leaf-litter layer biofilm sample type (AMOVA, $p < 0.001$) and C) rarefaction curves of leaf-litter layer (green) and water-column (blue) samples D) influent versus the rest wetland cells (AMOVA, $p = 0.009$)

The CW's leaf-litter layer microbial communities were found to be distinct from the water-column communities ($p < 0.001$) (Fig. 2A-B). Furthermore, the leaf-litter layer contained, on average, more diverse microbial communities than the water-column (Tables S4 and S5). Leaf-litter layer Shannon's diversity has previously been shown to increase in wetlands with more planted regions (Li et al., 2021). The plant matter and root exudates found within this sediment interface zone of this CW may have introduced greater substrate complexity relative to the water-column, thereby contributing to more diverse microbial communities (Rafieenia et al.,

2022). Moreover, the CW's leaf-litter layer microbial communities had fewer significant structural shifts over different sampling periods relative to the water-column communities (Table S7). Previous studies have established that substrate variability is shown to drive microbial community shift in other CWs (Vymazal et al., 2021; Wang et al., 2022; Feng et al., 2021). Thus, substantial differences in the CW's substrate composition may account for greater litter layer community stability. Microbial community succession occurs more slowly in the leaf-litter layer region relative to the water-column, since microbial communities in this region come in contact with wastewater through diffusion, which is a relatively slow process (Horne, 2000). This is consistent with our finding that this study's CW's leaf-litter layer microbial communities were more stable than water-column communities with respect to time.

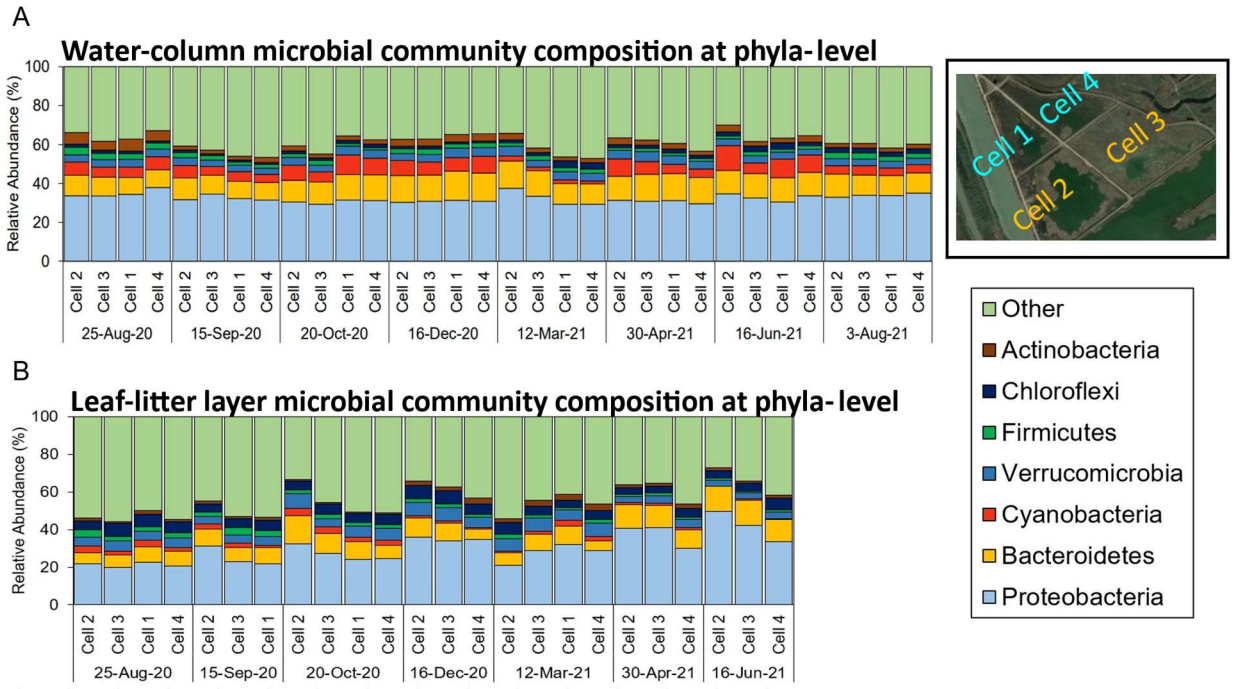

Fig. 3. Relative abundance (%) of the phylum-level taxonomic diversity of different 16S samples collected from the ends of the CW's Cell 2, Cell 3, Cell 1, Cell 4, and the influent through A) grab samples of the water column and B) passive sampler biofilms

The phyla compositions found throughout the CW water-column were similar to 16S rRNA gene studies performed on other eutrophic water bodies as well as other free water surface CWs that treat wastewater containing high nutrient concentrations**.** In the 16S rRNA gene studies *Proteobacteria* dominated, followed by *Bacteroidetes* and *Cyanobacteria* (Sanchez, 2017; Li et al., 2019; Jeong and Ham, 2017; Parulekar et al., 2017). Fig. 3 shows the relative abundances of dominant phyla throughout the CW for each sampling period within A) the water-column and B) the leaf-litter layer biofilm. The most dominant phyla in the CW water-column and the leaf-litter layer include *Proteobacteria, Bacteroidetes*, *Cyanobacteria, Verrucomicrobia, Firmicutes, Chloroflexi,* and *Actinobacteria.* In a study on a eutrophic lake environment, these phyla compositions were linked to organic matter substrate composition in the water-column (Zhang et al., 2019). Other researchers have observed that the dominance of specific cyanobacteria genera influenced phyla-level bacterial compositions in a eutrophic reservoir (Guedes et al., 2018). Furthermore, the CW's influent cyanobacterial bloom intensity and the dominant cyanobacteria genera in this bloom may be linked to the rest of CWs taxonomic composition.

3.3 16S rRNA gene analysis reveals a temporal microbial community shift, rather than a spatial shift throughout the second segment of the CW

The microbial communities in the CW's water-column experienced a significant temporal shift throughout almost all sampling periods (AMOVA, $p < 0.05$). The Aug 25th, 2020 and Sep 15th, 2020 sampling periods were the only times when no significant microbial community differences were observed. That said, the time between these sampling periods may have been too brief for a significant structural shift to occur. Lin et al. asserts that microbial community shifts can be reasonably attributed to environmental changes that occur over a period of months

rather than days (Lin et al., 2012). Seasonal changes in water temperature may drive the temporal microbial community shift in the CW. Several studies have also observed that seasonally-dependent factors, such as substrate and nutrient availability, also influence other CW microbial community structure (Koranda et al., 2013; Xu et al., 2021; Zhao et al., 2020; Koranda et al., 2013; Vymazal, 2007).

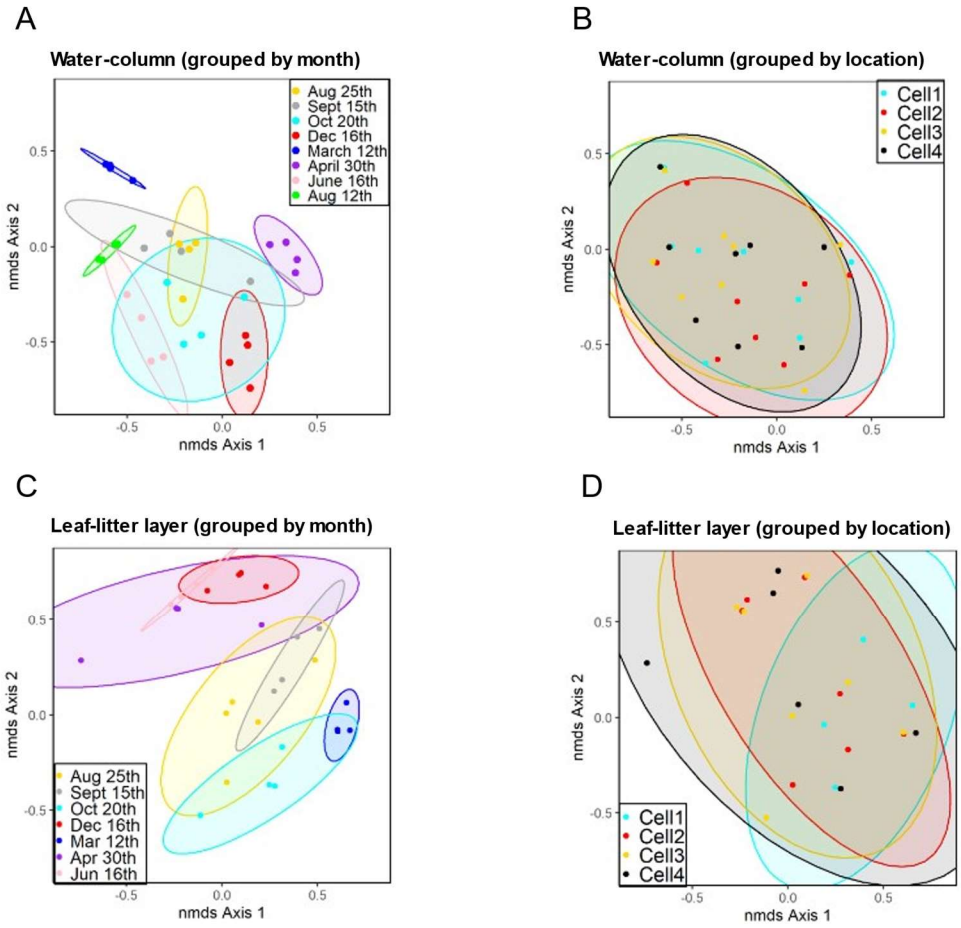

Fig 4. A) 2-D NMDS analysis plot with 90% confidence ellipses of water-column samples for CW cells grouped by sampling location; B) all water-column samples for CW cells grouped by sampling period; C) 2-D NMDS analysis plot with 90% confidence ellipses of leaf-litter layer samples for CW cells grouped by sampling location; D) leaf-litter samples for CW cells grouped by sampling period

No spatial distinctions in microbial community structure were observed in the second segment of the CW for both the water-column and leaf-litter layer within the same sampling periods (AMOVA $p > 0.05$). The absence of spatial changes in the second segment of the CW indicates that the microbial communities in this environment are more sensitive to temporal and seasonal changes. The shift in microbial community structure within the first segment may also result from substrate changes in the wastewater from the secondary and tertiary holding ponds. Moreover, no significant differences were detected in microbial communities when comparing community structure in unburned and burned cells (Cell 2 vs Cell 3 and Cell 1 vs Cell 4) (AMOVA $p > 0.05$). This indicates that the prescribed burn had no long-term impact on the microbial communities in Cells 3 and 4.

The influent wastewater's substrate composition may affect eutrophication potential throughout the CW, imposing temporal changes on microbial community structure. This is because eutrophication is heavily impacted by seasonal parameters (e.g., temperature and precipitation) and often results in large-scale microbial community shifts (Tromas et al., 2017; Xu et al., 2021; Zhao et al., 2020). Furthermore, spatial shifts in microbial communities were shown to be minor, while temporal shifts dominated community dynamics in a eutrophic bay receiving industrial wastewater (Zhang et al., 2016). It was also observed that bacterial communities had two distinct community states based on temperature during a five-year study assessing the seasonal dynamics of wastewater bacteria (LaMartina et al., 2021). Microbial community shifts in the CW may be connected to the wetland's phytoplankton bloom intensity from season to season. Substrate composition may alter the waste stabilization ponds' eutrophication potential and seasonal weather patterns can influence the severity of the ensuing phytoplankton blooms and community structure in the CW.

3.4 Microbial community diversity correlates to water quality and phytoplankton bloom conditions within the CW

Microbial community richness and diversity increased between the influent and the effluents of Cells 2 and 3 as measured water quality parameter concentrations declined. Many of these parameters, such as TSS, VSS, COD and pH, are also associated with phytoplankton bloom conditions. More importantly, the structural shift that occurred in the microbial communities within this segment of the CW is viewed as a bioindicator of healthy ecosystem functioning since the microbial community shift happened as water quality was concurrently being improved (AMOVA, $p < 0.001$). Previous research has linked other CWs' performance to microbial community structure and diversity. The greatest wetland TN and TOC removal efficiencies were achieved when another study's CW bacterial richness and diversity were also at their greatest levels (Zhu et al., 2021). Researchers found a correlation between Shannon's diversity index and BOD_5, NH_4-N, and NO_3-N in a CW built to treat eutrophic lake water; asserting that these diversity indices can be utilized as bioindicators for pollutant removal rates in this environment (Zhang et al., 2015). While the shift in this study's CW microbial community structure occurs in the first segment of the CW for all sampling periods, the period of time with the greatest microbial diversity occurred during the March 12, 2021 and April 30, 2021 sampling periods in Cell 1 and 4, the second segment of the CW (Fig. 5 A-B). During these time periods overallCW operations heavily relied on the performance of Cell 1 and 4 since removal of water quality constituents primarily occurred in these cells.

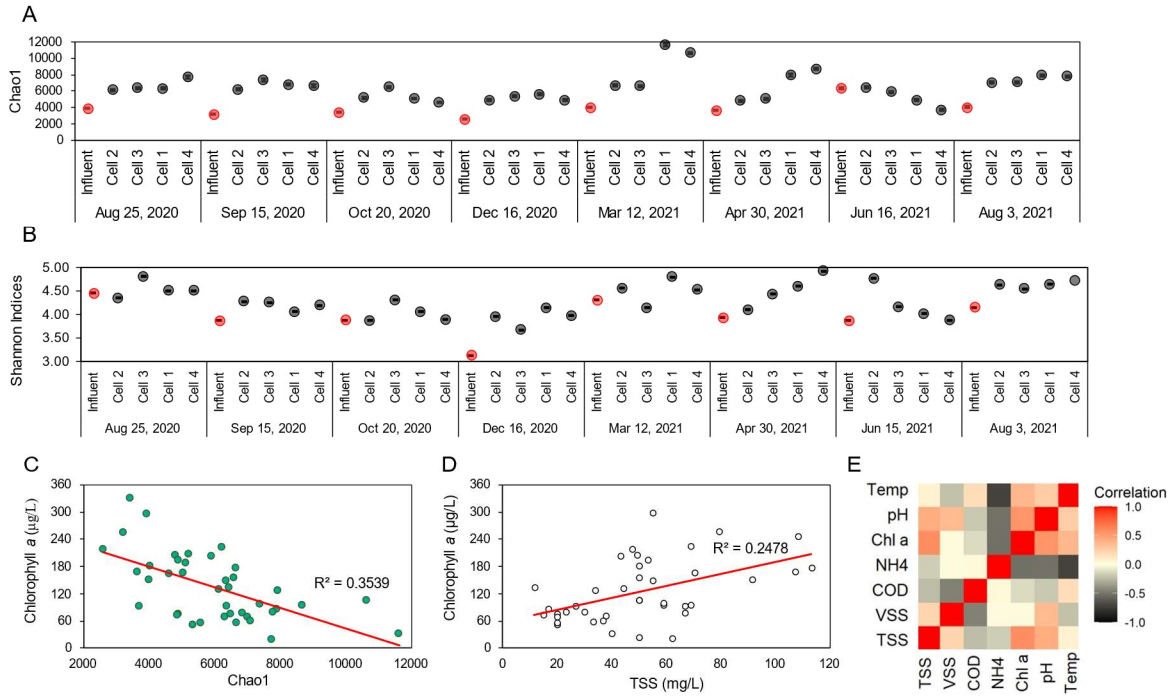

Figure. 5. A) Chao1 and B) Shannon diversity indices plotted for water-column samples in every location throughout the CW during each sampling round – with red points demonstrating influent sample indices; C) Chlorophyll *a* concentration plotted with respect to Chao1 indices with trendline and coefficient of determination; D) Chlorophyll *a* concentration plotted with respect to TSS concentration with trendline and coefficient of determination E) Spearman correlation analysis heatmap of environmental parameters (TSS, VSS, COD, NH4-N, Chlorophyll *a*, pH, and temperature) within the CW cells (left), demonstrating positive correlation between chlorophyll *a* and TSS (p= 0.526) within CW cells

Chlorophyll *a,* a proxy measurement for viable phytoplankton biomass (LaBaugh et al., 1995), was negatively correlated with Chao1 and Shannon indices, which suggests that microbial diversity is diminished in regions of the CW where phytoplankton bloom conditions prevail. Throughout the CW, parameters that were linked to elevated bloom conditions, pH and temperature, were also positively associated with chlorophyll *a* (Fig. 5. C-D, Spearman ρ >= 0.362). Moreover, chlorophyll *a* concentrations were correlated to TSS concentrations

(Spearman, $\rho = 0.575$) in the CW. This confirms that TSS, the primary environmental parameter the CW is designed to remove, largely takes the form of phytoplankton biomass. The CW thus effectively mitigated the phytoplankton blooms generated in the preceding holding ponds. In other studies, alpha diversity was also shown to decrease in environments experiencing seasonal phytoplankton blooms (Angeler et al., 2013; Su et al., 2017). When phytoplankton bloom conditions in the water-column declined near the second segment of the CW, microbial diversity increased and overall water quality improved.

More specifically, the cyanobacterial population within the phytoplankton bloom may have influenced the abundance of other CW bacteria and may have affected other water quality metrics. Cyanobacterial abundance also served as an indicator of elevated nutrient concentrations in the holding ponds and the CW. As primary producers, cyanobacteria are dependent on sources of nitrogen and phosphorus. Cyanobacterial growth demonstrates the environment's eutrophication potential (Pearl et al., 2016). The rise in cyanobacterial relative abundance during the December sampling period corresponded to greater than average NH_4-N concentrations at all CW sampling locations. Nevertheless, the high relative abundance of cyanobacteria in the CW may, then, affect overall microbial diversity. Numerous studies have shown cyanobacterial bloom occurrences are correlated with sharp changes in diversity indices, such as Shannon's (Tromas et al., 2017; Yang et al., 2021, Zhu et al., 2021). In the CW, *Microcystis,* one of the more dominant cyanobacteria genera, peaked during the sampling period with the lowest recorded Shannon and Chao1 indices (Fig 5 A-B and Fig 7 A). Other researchers have also found that *Microcystis* blooms correspond to lower diversity and evenness indices in bacterial populations throughout seasonal algal blooms (Su et al., 2017).

3.5 The Winter Storm Uri freeze altered pollutant removal patterns and microbial community structure throughout the CW cells

The CW appears to be functionally resilient, despite the Winter Storm Uri freeze's prolonged impact on microbial community dynamics. Pollutant removal trends were restored approximately six months after the freeze event. Winter Storm Uri brought on temporary changes in pollutant removal between the first and second segment of the CW (Fig. 6). Significantly greater pollutant removal percentages were reported during the second segment of the CW for VSS ($p = 0.007$) and COD ($p = 0.047$) when assessing removal rates from sampling periods that occurred after the freeze (Mar 12, 2021 – Aug 3, 2021). During this time, plant debris contributed to the water-column's organic matter content within the first segment of the CW. The freeze may have altered pH reduction trends within the first and second segment of the CW. Many irregularities in water quality parameter reduction trends were also recorded during the March 12th, 2021 sampling date, the time period closest to the freeze. When this sampling date was excluded from the analysis, there were significantly greater pH reductions in the first half of the CW ($p = 0.037$). During the March 12th sampling period NH_4-N levels sharply increased from 0.757 to 3.1412 mg/L throughout the CW (Fig. 5). Likewise, all water quality parameters had greater reduction percentages in the second segment of the CW. During the March 12th and April 30th, 2021 sampling periods, it was evident that the freeze took a toll on the CW's performance throughout the first segment of the wetland. VSS, COD, NH_4-N, and chlorophyll *a* concentration increased between the first segment of the CW, rather than being removed as they had been historically.

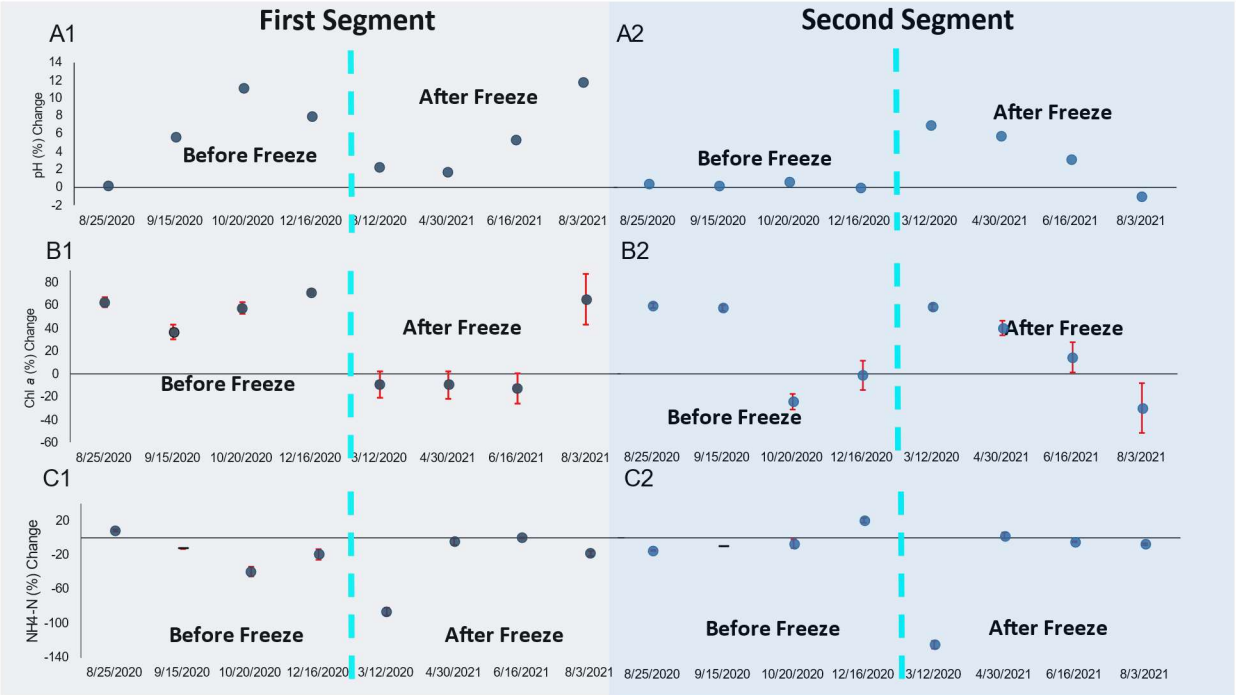

Fig 6 – Percent changes in A) pH, B) Chlorophyll *a,* and C) NH_4-N for (A1-C1) the first segment of the CW and for (A2-C2) the second segment of the CW; dashed blue lines demarcate sampling periods before (left) and after (right) the Winter Storm Uri Freeze.

The freeze shifted the CW's microbial communities, but only had a temporary impact on its performance; demonstrating the CW's ecological resilience after a pulse disturbance. The microbial communities in the water-column and leaf-litter layer biofilm samples collected closest to the freeze (March 12, 2021) were distinct from samples collected from other time periods (AMOVA $p < 0.05$). Figure 7 also reveals that in the time period following the Winter Storm Uri freeze the shift in microbial communities was driven by the decline of dominant cyanobacteria genera, *Microcystis, Synechococcus* and *Prochlorococcus*. Most notably, *Microcystis* drastically decreased in relative abundance in all CW cells during the March 12th, 2021 sampling period. *Synechococcus* and *Prochlorococcus* then increased in relative abundance during the subsequent sampling period, but fluctuated in subsequent samples. It has been witnessed that cyanobacteria, like *Microcystis*, are shown overwinter in an aquatic body's sediment layer, supplying the water-

column with an “inoculum of colonies” during warmer months (Preston et al., 1980). Moreover, the collapse of cyanobacteria after the freeze was demonstrated by the increase in relative abundance of cyanobacteria in the leaf-litter layer for all sampling locations during the March 12th, 2021 sampling period. The most significant OTUs responsible for CW microbial community shifts were also negatively associated with the microbial communities March 12, 2021 in the water-column (Fig. 7B).

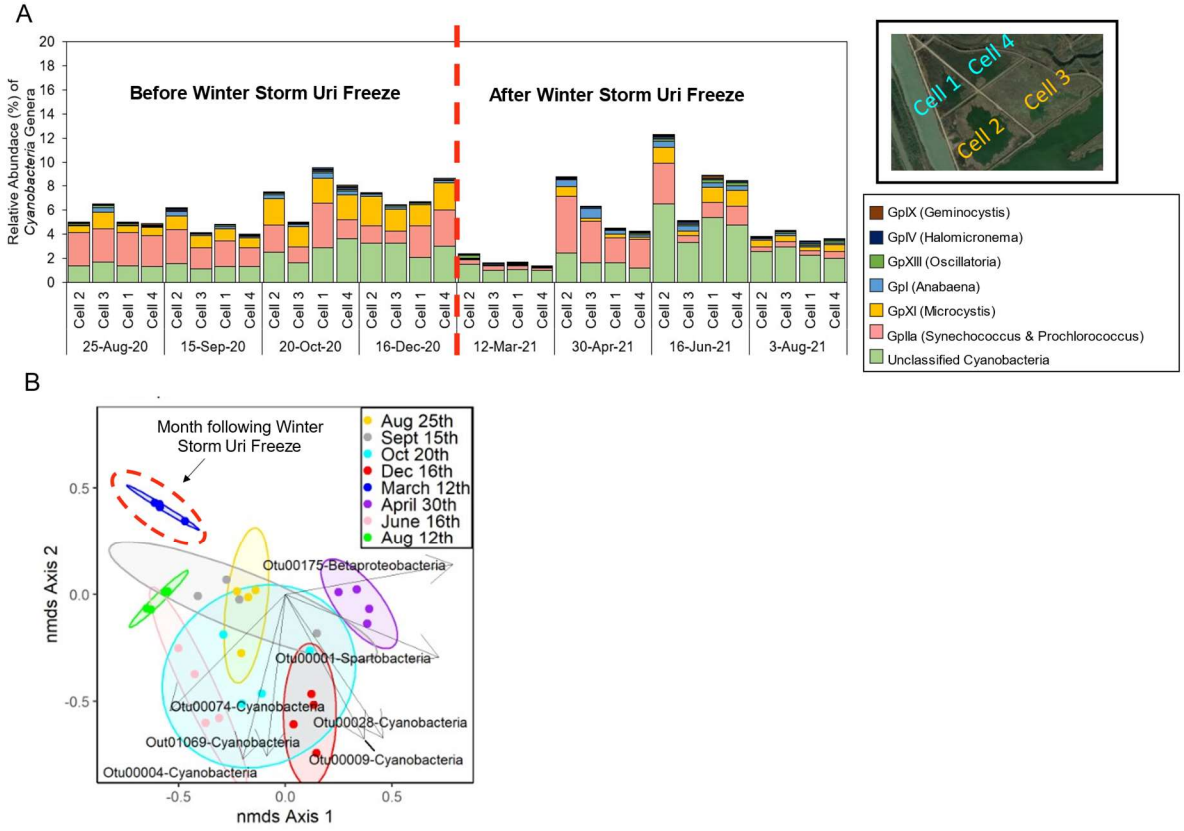

Fig 7. A) Relative abundance of cyanobacteria genera with dashed red divide representing before (left) and after (right) the Winter Storm Uri Freeze B) NMDs plot of with ordination biplot overlayed with genera of the significant OTU’s correlated with driving the shift in the CW’s microbial community composition: GpIIa (*Synechococcus* and *Prochlorococcus*), GpXI (*Microcystis*), GpXIII (*Oscillatoria*), and other unclassified *Cyanobacteria*, *Spartobacteria*, and *Betaproteobacteria* genera)

Microbial community contributions to constructed wetland performance are poorly understood, particularly in response to pulse disturbances from extreme weather events that are expected to increase in intensity and frequency with climate change (McDowell et al., 2018; Allison and Martiny, 2008; Ma et al., 2018). This study's CW microbial communities may have been both directly and indirectly affected by the freeze. After a pulse event like a freeze, wetland ecology is impacted at multiple taxonomic scales, compounding the initial disturbance's impact on the microbial communities (Means et al., 2017; Ross et al., 2009; Adams et al, 2012**).** Various cyanobacteria genera, such as *Synechococcus*, were observed to rapidly die at temperatures below 15 ℃ (Shilo and Abeliovich, 1972). Moreover, in CWs in regions with freeze-thaw cycles, the freeze process results in the release of excess nutrients from plant biomass (Sauer et al., 2017) Excess nutrients may induce future algal and cyanobacterial blooms (Pearl, 2016). Within this study's CW, this could potentially explain the sharp decline in the relative abundance of cyanobacteria during the March 12th 2021 sampling period, followed by a steady recovery thereafter. Ultimately, the CW microbial communities' dynamics after the freeze demonstrated the system's resilience in response to major pulse disturbance.

4.0 Conclusion

A 16S rRNA gene analysis on the microbial communities in this study's CW revealed a temporal shift in community structure. This demonstrated that the CW's microbial communities were potentially affected by substrate composition and seasonal changes. We did not observe any long-term impact on the CW's water-column or leaf-litter layer microbial communities from the previous controlled burn. As the CW removes TSS, the wetland's microbial diversity increases, indicating that healthy ecosystem functioning is tied to improved water quality. The freeze brought on by Winter Storm Uri was considered an unprecedented pulse disturbance during the

26-years the CW has been in operation. The freeze created a massive plant die off, raising the VSS, NH_4-N, COD and pH levels within the first segment of the CW for several months after the initial storm. Analyzing water quality parameters and microbial communities before and after the event revealed that the CW experienced an overall shift in microbial community structure. While environmental pollutant reduction trends recovered to their previous rates approximately six months after the freeze, microbial community structure remained altered. This supports the conclusion that the CW is capable of supporting functionally redundant communities in the context of tertiary wastewater treatment. The shift in pollutant removal trends after the freeze confirmed that the CW was able to rebound after a pulse disturbance.

Conclusion and Future Work

This research explored the microbial community dynamics and their association with water quality parameters in a field-scale study of a free water surface CW. We observed a positive association between microbial diversity and certain water quality parameters, such as TSS and chlorophyll *a*. However, specific interactions among phytoplankton and bacterioplankton are still poorly understood in free water CWs. Future work should focus on understanding the specific mechanisms other bacteria employ to limit unwanted cyanobacteria growth; thereby shedding light on the degree of severity toxic cyanobacteria possess in CWs.

Microcystis, a ubiquitous cyanobacteria known for emitting cyanotoxins, was shown to be a dominant player in this study's CW. *Microcystis* has also been shown to inhibit another dominant, yet benign green-alga, *Chlorella* (Song et al., 2017). However, it is unknown how prevalent this inhibition is in aquatic environments and it is unclear if other microorganisms found in CWs can alleviate said inhibition (Aswasthi et al., 2018; Kim et al., 2007). Isolating

these organisms and assessing interaction mechanisms allows for a better understanding of cyanobacteria-green algae competition and if green algae inhibition is a realistic threat in the face of climate change.

Ultimately, CWs can be viewed as both an effective wastewater treatment technology and ideal setting to explore microbial community interactions. This research will help clarify the relationship between microbial communities and water quality. Continuing efforts should emphasize the mechanisms microbial communities utilize to remove pollutants and maintain healthy ecosystem functioning in CW environments. Doing so will help optimize this sustainable technology.

Appendix – A Tables

Table S1. Sampling Campaign Dates

Dates Water Samples Collected	Dates Leaf-Litter Samplers Deployed	Dates Leaf-Litter Samplers Retrieved
Aug 25th, 2020	Aug 25th, 2020	--
Sep 15th, 2020	Sept 15th, 2020	Sept 15th, 2020
Oct 20th, 2020	Oct 20th, 2020	Oct 20th, 2020
Dec 16th, 2020	Dec 16th, 2020	Dec 16th, 2020
Mar 12th, 2021	Mar 12th, 2021	Mar 12th, 2021
Apr 30th, 2021	Apr 30th, 2021	Apr 30th, 2021
Jun 16th, 2021	Jun 16th, 2021	Jun 16th, 2021
Aug 3rd, 2021	--	Aug 3rd, 2021

Table S2. Average Concentration of Environmental Parameters in CW

Date	Location	TSS (mg/L)	TSS Standard Deviation	VSS (mg/L)	VSS Standard Deviation	COD (mg/L)	COD Standard Deviation	Chlorophyll α (µg/L)	Chlorophyll α Standard Deviation	NH_4-N (mg/L)	NH4-N Standard Deviation	pH	Temperature (°C)
Aug 25, 2020	Influent	55.00	30.64	23.33	1.67	215.31	57.98	297.26	39.35	0.90	0.02	8.17	28.60
	Cell 3	58.89	5.09	16.67	9.43	148.47	33.92	93.75	16.01	0.86	0.01	8.13	27.30
	Cell 2	44.44	7.70	38.89	4.19	178.55	28.03	129.94	34.36	0.78	0.00	8.18	27.80
	Cell 4	62.22	3.85	22.22	2.55	158.49	67.59	19.45	0.61	1.03	0.02	8.11	26.40
	Cell 1	37.78	5.09	11.67	5.89	176.32	14.57	70.61	21.77	0.85	0.02	8.13	26.80
Sep 15, 2020	Influent	79.44	17.48	70.56	13.33	52.66	7.55	254.99	2.67	-	-	8.42	27.00
	Cell 3	58.89	7.70	40.00	24.04	38.17	3.34	98.79	22.34	-	-	7.88	26.80
	Cell 2	68.89	3.85	47.78	3.85	31.49	22.57	224.28	48.03	-	-	7.99	26.80
	Cell 4	33.33	4.71	26.67	0.00	0.00	0.00	57.85	5.50	-	-	7.91	26.70
	Cell 1	30.00	5.09	22.22	6.94	10.97	11.82	78.32	6.87	-	-	7.92	26.80
Oct 20, 2020	Influent	108.33	19.25	66.67	9.62	263.77	20.05	245.00	33.36	0.73	0.01	8.97	26.85
	Cell 3	50.00	19.25	33.33	0.00	185.23	52.21	23.31	10.17	1.29	0.19	7.88	26.90
	Cell 2	50.00	19.25	33.33	19.25	218.65	65.75	105.36	13.50	0.74	0.00	8.06	26.90
	Cell 4	66.67	19.25	50.00	19.25	195.26	26.10	76.61	14.75	1.10	0.03	7.92	27.10
	Cell 1	66.67	0.00	50.00	19.25	175.20	46.79	91.56	3.85	1.04	0.01	7.91	27.20
Dec 16, 2020	Influent	47.50	2.89	37.50	2.89	142.34	55.97	217.46	52.86	1.76	0.13	8.27	12.00
	Cell 3	20.00	0.00	10.00	5.77	63.80	34.14	51.03	2.72	2.01	0.15	7.56	11.20
	Cell 2	20.00	0.00	10.00	5.77	126.18	30.14	75.35	20.78	2.19	0.31	7.65	11.50
	Cell 4	15.00	5.77	10.00	0.00	112.82	44.88	72.98	6.42	1.68	0.03	7.60	11.50
	Cell 1	20.00	5.77	10.00	0.00	85.61	76.80	55.18	14.57	1.66	0.12	7.61	11.30
Mar 12, 2021	Influent	91.67	12.64	28.33	7.89	55.27	26.56	151.00	24.47	0.76	0.02	8.82	24.35
	Cell 3	50.00	10.00	16.67	5.77	58.65	11.05	155.16	17.17	0.89	0.03	8.60	26.00
	Cell 2	113.33	76.38	56.67	5.77	70.37	12.15	176.22	51.06	1.93	0.18	8.63	26.30
	Cell 4	50.00	17.32	20.00	41.63	45.37	5.52	105.32	4.48	2.45	0.46	7.93	26.40
	Cell 1	40.00	20.00	16.67	0.00	61.78	4.42	32.04	5.44	3.84	0.27	8.01	27.90
Apr 28, 2021	Influent	107.17	26.96	25.00	26.19	46.93	26.56	167.62	19.72	0.64	0.02	8.83	27.25
	Cell 3	70.33	5.51	50.00	26.00	61.78	8.60	164.95	33.41	0.67	0.04	8.68	31.40
	Cell 2	49.33	6.66	16.67	20.00	52.40	8.60	202.92	60.02	0.66	0.05	8.66	30.30
	Cell 4	69.00	31.00	13.33	21.11	36.78	3.93	93.75	8.41	0.64	0.00	7.89	29.30
	Cell 1	34.00	15.59	33.33	25.00	53.44	3.12	126.97	2.72	0.66	0.01	8.44	34.10
Jun 16, 2021	Influent	55.00	10.52	18.33	10.77	137.19	1.69	147.74	54.89	0.67	0.01	8.77	30.75
	Cell 3	43.33	15.28	26.67	20.82	148.91	1.69	201.14	20.99	0.67	0.01	8.03	33.60
	Cell 2	11.67	7.64	13.33	7.64	160.63	4.48	131.72	24.66	0.66	0.02	8.56	32.20
	Cell 4	26.67	11.55	13.33	11.55	160.63	0.00	90.78	6.42	0.68	0.01	7.98	31.10
	Cell 1	53.33	35.12	23.33	20.82	149.88	0.00	194.02	114.42	0.71	0.02	8.03	31.80
Aug 3, 2021	Influent	50.00	5.77	38.33	19.88	219.29	14.82	179.34	25.84	0.84	0.03	8.51	29.85
	Cell 3	20.00	17.32	20.00	14.67	214.88	18.66	66.75	32.04	1.02	0.09	7.50	29.10
	Cell 2	36.67	5.77	30.00	13.33	216.84	14.69	59.63	30.36	0.97	0.07	7.51	29.10
	Cell 4	16.67	5.77	10.00	7.78	196.28	30.15	85.44	19.25	1.08	0.16	7.58	28.50
	Cell 1	23.33	5.77	6.67	6.67	219.78	19.11	79.21	21.58	1.05	0.05	7.58	28.50

*NH_4-N (mg/L) concentrations are not available for the September 15th, 2020 sampling period

Table S3. Percent Reduction of Environmental Parameters throughout the CW

Date	Location	TSS (mg/L)	VSS (mg/L)	COD (mg/L)	NH4-N (mg/L)	pH	Temperature (°C)	Chlorophyll α (µg/L)
25-Aug-2020	1st Half	6.06	19.47	24.06	8.58	0.18	3.67	62.38
	2nd Half	26.88	39.56	-2.38	-14.63	0.43	3.45	59.74
	Total	31.31	51.33	22.25	-4.79	0.61	6.99	84.85
15-Sep-2020	1st Half	30.77	50.00	33.85	NA	5.70	0.74	36.65
	2nd Half	46.47	-50.00	76.39	NA	0.25	0.19	57.85
	Total	62.94	25.00	84.38	NA	5.94	0.93	73.30
20-Oct-2020	1st Half	53.85	73.33	23.44	-39.60	11.15	-0.19	57.26
	2nd Half	-33.33	0.00	8.28	-6.50	0.69	-1.12	-24.52
	Total	38.46	73.33	29.78	-48.68	11.76	-1.30	46.78
16-Dec-2020	1st Half	57.89	-29.41	33.26	-19.71	7.99	5.42	70.94
	2nd Half	12.50	50.00	-40.46	20.47	0.00	-0.44	-1.41
	Total	63.16	35.29	6.26	4.80	7.99	5.00	70.53
12-Mar-2021	1st Half	10.91	-29.41	-16.73	-86.28	2.32	-7.39	-9.72
	2nd Half	44.90	50.00	16.95	-123.03	7.02	-3.82	58.55
	Total	50.91	35.29	3.06	-315.47	9.18	-11.50	54.52
30-Apr-2021	1st Half	44.17	-33.33	-21.64	-4.62	1.76	-13.21	-9.73
	2nd Half	13.93	30.00	20.98	2.74	5.82	-2.76	40.00
	Total	51.94	6.67	3.88	-1.75	7.48	-16.33	34.16
17-Jun-2021	1st Half	50.00	-9.09	-12.73	0.00	5.36	-6.99	-12.65
	2nd Half	-45.45	8.33	-0.31	-4.83	3.19	4.41	14.44
	Total	27.27	0.00	-13.08	-4.83	8.39	-2.28	3.61
3-Aug-2021	1st Half	26.09	47.83	1.56	-17.93	11.81	2.51	64.76
	2nd Half	47.06	66.67	3.63	-7.34	-1.00	2.06	-30.28
	Total	60.87	82.61	5.14	-26.58	10.93	4.52	54.09

Table S4. Comparison of Environmental Parameter Reduction throughout the CW

Location Comparison	Parameter	P-value	Significant Difference?
Cell 2 vs Cell 3	TSS (mg/L)	0.498	No
	VSS (mg/L)	0.336	No
	COD (mg/L)	0.132	No
	NH4-N (mg/L)	0.470	No
	pH	0.043	Yes
	Temperature (°C)	0.341	No
	Chlorophyll a (µg/L)	0.117	No
Cell 4 vs Cell 1	TSS (mg/L)	0.374	No
	VSS (mg/L)	0.265	No
	COD (mg/L)	0.215	No
	NH4-N (mg/L)	0.297	No
	pH	0.161	No
	Temperature (°C)	0.089	No
	Chlorophyll a (µg/L)	0.190	No
First half vs Second Half	TSS (mg/L)	0.144	No
	VSS (mg/L)	0.299	No
	COD (mg/L)	0.439	No
	NH4-N (mg/L)	0.351	No
	pH	0.075	No
	Temperature (°C)	0.169	No
	Chlorophyll a (µg/L)	0.325	No
First half vs Second Half (without March sampling date)	TSS (mg/L)	0.090	No
	VSS (mg/L)	0.444	No
	COD (mg/L)	0.445	No
	NH4-N (mg/L)	0.147	No
	pH	0.037	Yes
	Temperature (°C)	0.320	No
	Chlorophyll a (µg/L)	0.226	No

Table S5. Coverage, Chao, Shannon, and sobs indices for water -column samples from the CW

Sample Type	Date	Location	coverage	Chao	Shannon	sobs
Water	25-Aug-2020	Influent	0.980	3899.9	4.465	1478.2
		Cell 2	0.965	6112.2	4.358	2332.8
		Cell 3	0.964	6362.0	4.814	2463.4
		Cell 1	0.965	6292.6	4.512	2290.3
		Cell 4	0.960	7718.3	4.512	2499.4
	15-Sep-2020	Influent	0.981	3198.8	3.880	1443.5
		Cell 2	0.964	6189.4	4.281	2364.7
		Cell 3	0.960	7353.7	4.261	2480.7
		Cell 1	0.963	6813.8	4.061	2279.9
		Cell 4	0.962	6638.8	4.203	2382.5
	20-Oct-2020	Influent	0.981	3416.1	3.889	1474.3
		Cell 2	0.972	5201.9	3.874	1880.2
		Cell 3	0.962	6483.3	4.318	2440.5
		Cell 1	0.971	5095.4	4.058	1995.1
		Cell 4	0.972	4587.2	3.896	1973.2
	16-Dec-2020	Influent	0.986	2594.7	3.139	1077.3
		Cell 2	0.974	4889.1	3.961	1750.8
		Cell 3	0.971	5353.7	3.683	1821.2
		Cell 1	0.971	5583.8	4.151	1879.4
		Cell 4	0.974	4875.3	3.975	1704.9
	12-Mar-2021	Influent	0.977	4002.2	4.310	1663.7
		Cell 2	0.963	6660.5	4.560	2438.5
		Cell 3	0.962	6601.2	4.150	2504.9
		Cell 1	0.931	11621.0	4.807	4471.3
		Cell 4	0.935	10629.9	4.532	4172.4
	30-Apr-2021	Influent	0.979	3638.3	3.943	1592.8
		Cell 2	0.974	4799.9	4.112	1787.6
		Cell 3	0.972	5033.4	4.441	1996.0
		Cell 1	0.955	7936.8	4.609	2873.7
		Cell 4	0.949	8684.8	4.930	3306.0
	16-Jun-2021	Influent	0.964	6344.6	3.884	2330.0
		Cell 2	0.961	6428.7	4.770	2884.6
		Cell 3	0.968	5909.8	4.165	2004.9
		Cell 1	0.973	4885.7	4.023	1783.7
		Cell 4	0.980	3711.1	3.889	1462.7
	3rd-Apr-2021	Influent	0.978	4045.5	4.157	1692.6
		Cell 2	0.959	7002.4	4.639	2717.4
		Cell 3	0.960	7093.0	4.557	2630.2
		Cell 1	0.955	7892.8	4.646	2864.6
		Cell 4	0.955	7788.9	4.735	2901.5

Table S6. Coverage, Chao, Shannon, and sobs indices for passive biofilm samples of leaf litter layer from the CW

Sample Type	Date	Location	coverage	Chao	Shannon	Sobs
Biofilm	Aug 25th – Sep 15th (2020)	Influent	0.954	7172.5	5.903	3880.8
		Cell 2	0.952	7478.4	4.541	3577.1
		Cell 3	0.955	7207.1	6.227	4120.0
		Cell 1	0.944	8855.2	5.505	4200.2
		Cell 4	0.944	8741.5	6.023	4598.4
	Sep 15th – Oct 20th (2020)	Influent	0.951	7938.0	6.186	4127.4
		Cell 2	0.955	7395.5	5.476	3448.6
		Cell 3	0.934	10317.0	6.163	5188.1
		Cell 1	0.942	8968.7	6.667	5026.6
	Oct 20th – Dec 16th (2020)	Influent	0.951	7860.0	6.001	3965.9
		Cell 2	0.964	6133.9	4.464	2492.4
		Cell 3	0.944	9083.6	5.494	4276.2
		Cell 1	0.935	10511.6	5.946	4890.2
		Cell 4	0.939	9549.5	6.094	4832.4
	Dec 16th, 2020 – Mar 12 th, 2021	Influent	0.980	3316.9	4.582	1624.9
		Cell 2	0.970	5137.7	4.552	2153.5
		Cell 3	0.969	5309.8	4.850	2349.6
		Cell 4	0.966	5756.8	4.496	2472.0
	Mar 12 th – Apr 30 th (2021)	Influent	0.988	2005.2	2.227	948.5
		Cell 2	0.951	7679.7	4.496	3603.7
		Cell 3	0.972	4422.8	3.946	2149.8
		Cell 1	0.966	5572.6	3.779	2333.9
		Cell 4	0.982	3064.4	2.822	1266.7
	Apr 30 th – Jun 16th (2021)	Influent	0.977	3792.7	4.678	1951.7
		Cell 2	0.964	5910.8	4.680	2724.8
		Cell 3	0.964	5861.6	4.655	2751.7
		Cell 4	0.950	8089.3	6.661	4242.6
	Jun 16th – Aug 3rd (2021)	Influent	0.980	3500.7	4.637	1658.4
		Cell 2	0.977	3777.1	3.696	1621.3
		Cell 3	0.966	5633.0	4.294	2540.2
		Cell 4	0.955	7298.3	5.657	3508.5

Table S7. P-values for Analysis of molecular variance (AMOVA) on water-column samples grouped by sampling round – analysis performed without influent samples

Sample Type	Sample Comparison	p-value	Significant Difference?
Water	Round 1 vs Round 2	0.109	No
	Round 1 vs Round 3	0.03	Yes
	Round 1 vs Round 4	0.015	Yes
	Round 1 vs Round 5	0.022	Yes
	Round 1 vs Round 6	0.02	Yes
	Round 1 vs Round 7	0.029	Yes
	Round 1 vs Round 8	0.034	Yes
	Round 2 vs Round 4	<0.001	Yes
	Round 2 vs Round5	0.001	Yes
	Round 2 vs Round 6	0.001	Yes
	Round 2 vs Round 7	0.002	Yes
	Round 2 vs Round 8	0.001	Yes
	Round 3 vs Round 4	0.038	Yes
	Round 3 vs Round 5	0.036	Yes
	Round 3 vs Round 6	0.031	Yes
	Round 3 vs Round 7	0.031	Yes
	Round 3 vs Round 8	0.019	Yes
	Round 4 vs Round 5	0.008	Yes
	Round 4 vs Round 6	0.028	Yes
	Round 4 vs Round 7	0.013	Yes
	Round 4 vs Round 8	0.014	Yes
	Round 5 vs Round 6	0.04	Yes
	Round 5 vs Round 7	0.027	Yes
	Round 5 vs Round8	0.031	Yes
	Round 6 vs Round 7	0.031	Yes
	Round 6 vs Round 8	0.02	Yes
	Round 7 vs Round 8	0.021	Yes

Table S8. P-values for Analysis of molecular variance (AMOVA) on leaf-litter layer samples grouped by sampling round – analysis performed without influent samples

Sample Type	Sample Comparison	p-value	Significant Difference?
Biofilm	Round 1 vs Round 2	0.03	Yes
	Round 1 vs Round 3	0.03	Yes
	Round 1 vs Round 4	0.023	Yes
	Round 1 vs Round 5	0.035	Yes
	Round 1 vs Round 6	0.025	Yes
	Round 1 vs Round 7	0.024	Yes
	Round 2 vs Round 3	0.016	Yes
	Round 2 vs Round 4	0.104	No
	Round 2 vs Round 5	0.032	Yes
	Round 2 vs Round 6	0.095	No
	Round 2 vs Round 7	0.094	No
	Round 3 vs Round 4	0.029	Yes
	Round 3 vs Round 5	0.033	Yes
	Round 3 vs Round 6	0.026	Yes
	Round 3 vs Round 7	0.017	Yes
	Round 4 vs Round 5	0.013	Yes
	Round 4 vs Round 6	0.064	No
	Round 4 vs Round 7	0.093	No
	Round 5 vs Round 6	0.022	Yes
	Round 5 vs Round 7	0.032	Yes
	Round 6 vs Round 7	0.705	No

Table S9. P-values for Analysis of molecular variance (AMOVA) on samples grouped by sampling location and sample type

Sample Type	Sample Comparison	p-value	Significant Difference?
Total	Influent vs Cells	0.009	Yes
	Biofilm vs Water	>0.001	Yes
Water	Cell 1 vs Cell 2	0.914	No
	Cell 1 vs Cell 3	0.997	No
	Cell 1 vs Cell 4	0.408	No
	Cell 2 vs Cell 3	0.761	No
	Cell 3 vs Cell 4	0.952	No
Biofilm	Cell 1 vs Cell 2	0.334	No
	Cell 1 vs Cell 3	0.412	No
	Cell 1 vs Cell 4	0.448	No
	Cell 2 vs Cell 3	0.984	No
	Cell 3 vs Cell 4	0.947	No

Table S10. Spearman's rank coefficients between measured environmental parameters (Temperature, pH, chlorophyll a, COD, VSS and TSS) and dominant phyla (*Proteobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia, Firmicutes, Chloroflexi, Actinobacteria,*and other) in water column samples from CW cells only

	Proteobacteria	Bacteroidetes	Cyanobacteria	Verrucomicrobia	Firmicutes	Chloroflexi	Actinobacteria	Other	TSS	VSS	COD	NH4	Chl a	pH	Temp
Proteobacteria	1.000	-0.413	-0.133	-0.303	0.255	-0.048	0.188	-0.294	-0.016	0.207	0.234	-0.134	0.065	0.103	0.064
Bacteroidetes	-0.413	1.000	0.400	0.581	-0.317	0.003	0.201	-0.451	0.034	-0.095	-0.066	-0.023	0.208	0.060	0.015
Cyanobacteria	-0.133	0.400	1.000	0.237	-0.194	-0.181	0.490	-0.719	0.011	-0.073	0.237	-0.313	0.299	0.097	0.156
Verrucomicrobia	-0.303	0.581	0.237	1.000	-0.223	-0.058	0.284	-0.411	0.279	0.035	-0.109	0.024	0.158	0.379	-0.112
Firmicutes	0.255	-0.317	-0.194	-0.223	1.000	-0.125	0.328	-0.072	-0.382	0.162	0.287	0.262	-0.507	-0.254	-0.335
Chloroflexi	-0.048	0.003	-0.181	-0.058	-0.125	1.000	-0.235	0.125	-0.060	-0.410	0.357	-0.149	0.151	-0.006	0.648
Actinobacteria	0.188	0.201	0.490	0.284	0.328	-0.235	1.000	-0.768	-0.214	0.285	0.187	-0.133	-0.108	0.318	-0.127
Other	-0.294	-0.451	-0.719	-0.411	-0.072	0.125	-0.768	1.000	0.036	-0.128	-0.311	0.163	-0.126	-0.239	-0.060
TSS	-0.016	0.034	0.011	0.279	-0.382	-0.060	-0.214	0.036	1.000	0.219	-0.237	-0.173	0.575	0.430	0.067
VSS	0.207	-0.095	-0.073	0.035	0.162	-0.410	0.285	-0.128	0.219	1.000	-0.525	-0.011	-0.010	0.358	-0.214
COD	0.234	-0.066	0.237	-0.109	0.287	0.357	0.187	-0.311	-0.237	-0.525	1.000	0.022	-0.062	-0.229	0.234
NH4	-0.134	-0.023	-0.313	0.024	0.262	-0.149	-0.133	0.163	-0.173	-0.011	0.022	1.000	-0.525	-0.520	-0.711
Chl a	0.065	0.208	0.299	0.158	-0.507	0.151	-0.108	-0.126	0.575	-0.010	-0.062	-0.525	1.000	0.542	0.375
pH	0.103	0.060	0.097	0.379	-0.254	-0.006	0.318	-0.239	0.430	0.358	-0.229	-0.520	0.542	1.000	0.261
Temp	0.064	0.015	0.156	-0.112	-0.335	0.648	-0.127	-0.060	0.067	-0.214	0.234	-0.711	0.375	0.261	1.000

Table S11. Spearman's rank coefficients between measured environmental parameters (Temperature, pH, chlorophyll a, COD, VSS and TSS) and dominant phyla (*Proteobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia, Firmicutes, Chloroflexi, Actinobacteria,*and other) in water column samples from CW influent only

	Proteobacteria	Bacteroidetes	Cyanobacteria	Verrucomicrobia	Firmicutes	Chloroflexi	Actinobacteria	Other	TSS	VSS	COD	NH4	Chl a	pH	Temp
Proteobacteria	1.000	-0.643	-0.524	-0.310	-0.262	0.429	-0.405	0.595	-0.467	0.275	0.262	0.048	-0.190	-0.238	0.810
Bacteroidetes	-0.643	1.000	0.190	0.238	0.738	-0.048	-0.214	0.000	0.515	-0.216	-0.690	-0.357	-0.238	0.452	-0.571
Cyanobacteria	-0.524	0.190	1.000	-0.524	-0.238	-0.357	0.071	-0.238	0.096	-0.144	0.071	0.000	0.238	0.143	-0.429
Verrucomicrobia	-0.310	0.238	-0.524	1.000	0.357	-0.452	0.429	-0.571	0.515	0.263	-0.143	0.024	0.333	0.024	-0.333
Firmicutes	-0.262	0.738	-0.238	0.357	1.000	0.143	-0.167	0.048	0.419	-0.036	-0.238	-0.286	-0.167	0.571	-0.357
Chloroflexi	0.429	-0.048	-0.357	-0.452	0.143	1.000	-0.452	0.786	-0.503	-0.515	-0.167	-0.190	-0.810	0.048	0.548
Actinobacteria	-0.405	-0.214	0.071	0.429	-0.167	-0.452	1.000	-0.857	-0.060	0.347	0.429	0.524	0.738	-0.357	-0.286
Other	0.595	0.000	-0.238	-0.571	0.048	0.786	-0.857	1.000	-0.407	-0.323	-0.333	-0.238	-0.833	0.024	0.500
TSS	-0.467	0.515	0.096	0.515	0.419	-0.503	-0.060	-0.407	1.000	-0.145	-0.263	-0.683	0.168	0.743	-0.311
VSS	0.275	-0.216	-0.144	0.263	-0.036	-0.515	0.347	-0.323	-0.145	1.000	0.287	0.575	0.731	-0.479	-0.060
COD	0.262	-0.690	0.071	-0.143	-0.238	-0.167	0.429	-0.333	-0.263	0.287	1.000	0.405	0.524	-0.071	0.071
NH4	0.048	-0.357	0.000	0.024	-0.286	-0.190	0.524	-0.238	-0.683	0.575	0.405	1.000	0.429	-0.833	-0.286
Chl a	-0.190	-0.238	0.238	0.333	-0.167	-0.810	0.738	-0.833	0.168	0.731	0.524	0.429	1.000	-0.214	-0.286
pH	-0.238	0.452	0.143	0.024	0.571	0.048	-0.357	0.024	0.743	-0.479	-0.071	-0.833	-0.214	1.000	-0.071
Temp	0.810	-0.571	-0.429	-0.333	-0.357	0.548	-0.286	0.500	-0.311	-0.060	0.071	-0.286	-0.286	-0.071	1.000

Table S11. Spearman's rank coefficients between measured environmental parameters (Temperature, pH, chlorophyll a, COD, VSS and TSS) and dominant phyla (*Proteobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia, Firmicutes, Chloroflexi, Actinobacteria,* and other) in biofilm samples from CW cells only

	Proteobacteria	*Bacteroidetes*	*Cyanobacteria*	*Verrucomicrobia*	*Firmicutes*	*Chloroflexi*	*Actinobacteria*	Other	TSS	VSS	COD	NH4	Chl a	pH	Temp
Proteobacteria	1.000	0.687	-0.474	-0.297	-0.792	-0.516	0.361	-0.950	-0.351	-0.270	-0.068	-0.296	0.215	-0.031	0.316
Bacteroidetes	0.687	1.000	-0.197	-0.370	-0.613	-0.406	-0.141	-0.770	-0.157	-0.363	0.115	-0.526	0.262	0.109	0.543
Cyanobacteria	-0.474	-0.197	1.000	0.275	0.520	-0.051	-0.432	0.315	0.315	-0.211	0.304	0.205	0.009	-0.057	-0.081
Verrucomicrobia	-0.297	-0.370	0.275	1.000	0.238	0.457	0.155	0.151	0.048	-0.015	0.095	0.721	-0.203	-0.306	-0.722
Firmicutes	-0.792	-0.613	0.520	0.238	1.000	0.377	-0.379	0.732	0.205	0.419	0.008	0.280	-0.137	-0.066	-0.455
Chloroflexi	-0.516	-0.406	-0.051	0.457	0.377	1.000	-0.063	0.473	-0.231	0.263	0.264	0.421	-0.559	-0.295	-0.553
Actinobacteria	0.361	-0.141	-0.432	0.155	-0.379	-0.063	1.000	-0.312	-0.353	0.161	-0.460	0.232	-0.273	-0.014	-0.279
Other	-0.950	-0.770	0.315	0.151	0.732	0.473	-0.312	1.000	0.383	0.321	-0.036	0.251	-0.206	-0.009	-0.260
TSS	-0.351	-0.157	0.315	0.048	0.205	-0.231	-0.353	0.383	1.000	0.002	-0.128	-0.099	0.546	0.205	0.161
VSS	-0.270	-0.363	-0.211	-0.015	0.419	0.263	0.161	0.321	0.002	1.000	-0.391	0.124	-0.123	0.237	-0.243
COD	-0.068	0.115	0.304	0.095	0.008	0.264	-0.460	-0.036	-0.128	-0.391	1.000	-0.030	-0.017	0.083	0.183
NH4	-0.296	-0.526	0.205	0.721	0.280	0.421	0.232	0.251	-0.099	0.124	-0.030	1.000	-0.477	-0.498	-0.693
Chl a	0.215	0.262	0.009	-0.203	-0.137	-0.559	-0.273	-0.206	0.546	-0.123	-0.017	-0.477	1.000	0.406	0.362
pH	-0.031	0.109	-0.057	-0.306	-0.066	-0.295	-0.014	-0.009	0.205	0.237	0.083	-0.498	0.406	1.000	0.410
Temp	0.316	0.543	-0.081	-0.722	-0.455	-0.553	-0.279	-0.260	0.161	-0.243	0.183	-0.693	0.362	0.410	1.000

Table S12. Spearman's rank coefficients between measured environmental parameters (Temperature, pH, chlorophyll a, COD, VSS and TSS) and dominant phyla (*Proteobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia, Firmicutes, Chloroflexi, Actinobacteria,* and other) in biofilm samples from CW influent only

	Proteobacteria	*Bacteroidetes*	*Cyanobacteria*	*Verrucomicrobia*	*Firmicutes*	*Chloroflexi*	*Actinobacteria*	Other	TSS	VSS	COD	NH4	Chl a	pH	Temp
Proteobacteria	1.000	0.536	-0.857	-0.500	-0.714	-0.821	0.286	-0.857	-0.324	-0.649	-0.286	-0.286	-0.536	0.071	0.107
Bacteroidetes	0.536	1.000	-0.214	-0.643	-0.536	-0.643	-0.036	-0.607	0.559	-0.559	-0.464	-0.929	-0.357	0.750	0.357
Cyanobacteria	-0.857	-0.214	1.000	0.607	0.536	0.500	-0.071	0.607	0.649	0.342	0.179	-0.036	0.357	0.393	-0.321
Verrucomicrobia	-0.500	-0.643	0.607	1.000	0.500	0.357	0.321	0.429	0.018	0.306	0.357	0.571	0.250	-0.036	-0.857
Firmicutes	-0.714	-0.536	0.536	0.500	1.000	0.821	-0.107	0.714	0.144	0.847	-0.036	0.500	0.429	-0.214	-0.357
Chloroflexi	-0.821	-0.643	0.500	0.357	0.821	1.000	-0.536	0.964	0.072	0.955	0.357	0.571	0.786	-0.357	-0.107
Actinobacteria	0.286	-0.036	-0.071	0.321	-0.107	-0.536	1.000	-0.607	-0.252	-0.523	-0.536	0.036	-0.750	-0.036	-0.429
Other	-0.857	-0.607	0.607	0.429	0.714	0.964	-0.607	1.000	0.180	0.883	0.536	0.500	0.857	-0.214	-0.143
TSS	-0.324	0.559	0.649	0.018	0.144	0.072	-0.252	0.180	1.000	0.055	-0.144	-0.649	0.234	0.883	-0.054
VSS	-0.649	-0.559	0.342	0.306	0.847	0.955	-0.523	0.883	0.055	1.000	0.252	0.595	0.793	-0.342	-0.198
COD	-0.286	-0.464	0.179	0.357	-0.036	0.357	-0.536	0.536	-0.144	0.252	1.000	0.393	0.571	-0.143	-0.107
NH4	-0.286	-0.929	-0.036	0.571	0.500	0.571	0.036	0.500	-0.649	0.595	0.393	1.000	0.393	-0.786	-0.464
Chl a	-0.536	-0.357	0.357	0.250	0.429	0.786	-0.750	0.857	0.234	0.793	0.571	0.393	1.000	-0.071	-0.179
pH	0.071	0.750	0.393	-0.036	-0.214	-0.357	-0.036	-0.214	0.883	-0.342	-0.143	-0.786	-0.071	1.000	0.107
Temp	0.107	0.357	-0.321	-0.857	-0.357	-0.107	-0.429	-0.143	-0.054	-0.198	-0.107	-0.464	-0.179	-0.107	1.000

Table S13 Top 50 OTUs with the greatest correlation coefficients along the NMDS axes

OTU	Correlation coefficients fo NMDS axis 1	p-value	Correlation coefficients fo NMDS axis 2	p-value	Correlation coefficients axes 1 and 2 lengt	Taxa	Relative Abundance (%) of Taxa
Otu00028	0.457	6.00E-05	-0.670	0.00E+00	0.811	GpIIa	1.231
Otu00175	0.785	0.00E+00	0.139	2.43E-01	0.797	Betaproteobacteria_unclassified	5.481
Otu00004	-0.199	9.59E-02	-0.770	0.00E+00	0.796	Cyanobacteria_order_incertae_sedis_unclassifi	1.656
Otu00001	0.718	0.00E+00	-0.295	1.26E-02	0.776	Spartobacteria_genera_incertae_sedis	0.644
Otu00009	0.368	1.59E-03	-0.676	0.00E+00	0.770	GpIIa	1.231
Otu00074	-0.541	1.00E-06	-0.543	1.00E-06	0.766	GpXIII	0.059
Otu01069	-0.087	4.65E-01	-0.755	0.00E+00	0.760	GpXI	0.600
Otu00378	0.726	0.00E+00	-0.206	8.49E-02	0.755	Rhodopirellula	0.067
Otu00313	-0.051	6.68E-01	-0.750	0.00E+00	0.752	GpXI	0.600
Otu00101	0.543	1.00E-06	-0.511	5.00E-06	0.746	Rhodobacteraceae_unclassified	0.910
Otu00232	-0.397	6.11E-04	-0.630	0.00E+00	0.744	Cyanobacteria_order_incertae_sedis_unclassifi	1.656
Otu00073	0.516	4.00E-06	-0.524	3.00E-06	0.735	Bacteria_unclassified	37.609
Otu00162	-0.126	2.94E-01	-0.720	0.00E+00	0.730	GpIIa	1.231
Otu00218	-0.037	7.59E-01	-0.727	0.00E+00	0.727	Opitutus	0.137
Otu01095	-0.083	4.87E-01	-0.721	0.00E+00	0.726	Cyanobacteria_order_incertae_sedis_unclassifi	1.656
Otu01896	-0.153	2.01E-01	-0.708	0.00E+00	0.724	GpXI	0.600
Otu00195	-0.459	5.80E-05	-0.553	1.00E-06	0.718	Bacteroidetes_unclassified	3.662
Otu02670	-0.690	0.00E+00	0.186	1.19E-01	0.715	Burkholderiales_unclassified	2.918
Otu00003	-0.227	5.69E-02	-0.676	0.00E+00	0.713	GpXI	0.600
Otu00005	0.416	3.13E-04	-0.579	0.00E+00	0.713	GpIIa	1.231
Otu00029	0.364	1.81E-03	-0.598	0.00E+00	0.700	GpIIa	1.231
Otu01384	-0.104	3.83E-01	-0.688	0.00E+00	0.696	GpXI	0.600
Otu00008	0.205	8.63E-02	0.664	0.00E+00	0.695	Rhodobacteraceae_unclassified	0.910
Otu00205	-0.003	9.79E-01	-0.693	0.00E+00	0.693	Bacteria_unclassified	37.609
Otu00296	-0.155	1.94E-01	-0.675	0.00E+00	0.692	3_genus_incertae_sedis	0.622
Otu00331	-0.219	6.60E-02	-0.652	0.00E+00	0.688	Bacteroidetes_unclassified	3.662
Otu00053	-0.001	9.96E-01	0.687	0.00E+00	0.687	Rhodobacteraceae_unclassified	0.910
Otu02177	-0.186	1.19E-01	-0.661	0.00E+00	0.687	GpXI	0.600
Otu00792	-0.108	3.65E-01	-0.677	0.00E+00	0.686	Cyanobacteria_order_incertae_sedis_unclassifi	1.656
Otu00080	-0.197	9.97E-02	-0.656	0.00E+00	0.685	Cyanobacteria_Chloroplast_unclassified	0.200
Otu00077	0.322	6.18E-03	0.601	0.00E+00	0.682	Rhodobacter	0.047
Otu00180	0.505	7.00E-06	-0.450	8.30E-05	0.676	GpIIa	1.231
Otu01766	-0.144	2.29E-01	-0.659	0.00E+00	0.675	Cyanobacteria_order_incertae_sedis_unclassifi	1.656
Otu00408	-0.613	0.00E+00	0.280	1.80E-02	0.674	Opitutaceae_unclassified	0.058
Otu00066	0.081	5.00E-01	-0.665	0.00E+00	0.670	Bacteria_unclassified	37.609
Otu00081	-0.215	7.18E-02	-0.633	0.00E+00	0.669	Cyanobacteria_order_incertae_sedis_unclassifi	1.656
Otu02261	-0.221	6.37E-02	0.630	0.00E+00	0.668	Cyanobacteria_Chloroplast_unclassified	0.200
Otu01290	-0.205	8.60E-02	-0.634	0.00E+00	0.667	GpXI	0.600
Otu00174	-0.172	1.49E-01	-0.643	0.00E+00	0.666	Cyanobacteria_Chloroplast_unclassified	0.200
Otu00837	0.665	0.00E+00	0.033	7.84E-01	0.665	Gammaproteobacteria_unclassified	3.193
Otu00068	-0.562	0.00E+00	-0.351	2.71E-03	0.663	Microbacteriaceae_unclassified	0.142
Otu00002	-0.576	0.00E+00	0.325	5.74E-03	0.661	Hydrogenophaga	0.196
Otu00153	0.633	0.00E+00	-0.185	1.22E-01	0.660	Bacteria_unclassified	37.609
Otu00019	0.056	6.37E-01	-0.654	0.00E+00	0.657	GpI	0.137
Otu00276	-0.586	0.00E+00	-0.297	1.20E-02	0.657	Betaproteobacteria_unclassified	5.481
Otu02321	-0.610	0.00E+00	0.243	4.13E-02	0.656	Burkholderiales_unclassified	2.918
Otu00634	-0.515	4.00E-06	-0.406	4.44E-04	0.656	Bacteria_unclassified	37.609
Otu00931	-0.162	1.74E-01	0.635	0.00E+00	0.655	Rhodocyclaceae_unclassified	0.442
Otu00014	-0.003	9.82E-01	-0.653	0.00E+00	0.653	Acetobacteraceae_unclassified	0.257
Otu02912	-0.123	3.04E-01	-0.639	0.00E+00	0.651	Cyanobacteria_order_incertae_sedis_unclassifi	1.656

Appendix B – Implementation of Sequencing Analysis Code in Mothur

```
make.file(inputdir=., type=fastq, prefix=stability)
make.contigs(file=stability.files, processors=8)
screen.seqs(fasta=stability.trim.contigs.fasta, group=stability.contigs.groups, maxambig=0, maxlength=275)
unique.seqs(fasta=stability.trim.contigs.good.fasta)
count.seqs(name=stability.trim.contigs.good.names, group=stability.contigs.good.groups)
summary.seqs(count=stability.trim.contigs.good.count_table)
align.seqs(fasta=stability.trim.contigs.good.unique.fasta, reference=silva.v4.fasta)
screen.seqs(fasta=stability.trim.contigs.good.unique.align, count=stability.trim.contigs.good.count_table,
summary=stability.trim.contigs.good.unique.summary, start=1968, end=11550, maxhomop=8)
filter.seqs(fasta=stability.trim.contigs.good.unique.good.align, vertical=T, trump=.)
unique.seqs(fasta=stability.trim.contigs.good.unique.good.filter.fasta,
count=stability.trim.contigs.good.good.count_table)
pre.cluster(fasta=stability.trim.contigs.good.unique.good.filter.unique.fasta,
count=stability.trim.contigs.good.unique.good.filter.count_table, diffs=2)
chimera.vsearch(fasta=stability.trim.contigs.good.unique.good.filter.unique.precluster.fasta,
count=stability.trim.contigs.good.unique.good.filter.unique.precluster.count_table, dereplicate=t)
remove.seqs(fasta=stability.trim.contigs.good.unique.good.filter.unique.precluster.fasta,
accnos=stability.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.accnos)
classify.seqs(fasta=stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta,
count=stability.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table,
reference=trainset9_032012.pds.fasta, taxonomy=trainset9_032012.pds.tax, cutoff=80)
remove.lineage(fasta=stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta,
count=stability.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table,
taxonomy=stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.pds.wang.taxonomy,
taxon=Chloroplast-Mitochondria-unknown-Eukaryota)
summary.tax(taxonomy=current, count=current)
dist.seqs(fasta=stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.fasta,
cutoff=0.03)
cluster(column=stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.dist,
count=stability.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_tabl
e)
make.shared(list=stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.opti_mcc.list,
count=stability.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_tabl
e, label=0.03)
classify.otu(list=stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.pick.opti_mcc
.list,
count=stability.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_tabl
e, taxonomy=stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.pds.wang.pick.taxonomy,
label=0.03)
phylotype(taxonomy=stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.pds.wang.pick.pic
k.taxonomy)
make.shared(list=stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.pds.wang.pick.tx.li
st,
count=stability.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_tabl
e, label=1)
dist.seqs(fasta=stability.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.fasta,
output=lt, processors=8)
cluster(column=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.dist,
count=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_table)
make.shared(list=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.opti
_mcc.list, count=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.coun
t_table, label=0.03)
rarefaction.single(shared=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.
pick.opti_mcc.shared)
make.shared(list=final.opti_mcc.list, count=final.count_table, label=0.03)
classify.otu(list=final.opti_mcc.list, count=final.count_table, taxonomy=final.taxonomy, label=0.03)
merge.groups(shared=stability.opti_mcc.0.03.shared, design=sampletype.design)
```

```
count.groups(shared=final.opti_mcc.shared)
sub.sample(shared=final.opti_mcc.shared, size=41960)
dist.shared(shared=final.opti_mcc.shared, calc=thetayc-jclass, subsample=t)
nmds(phylip=final.opti_mcc.thetayc.0.03.lt.ave.dist)
amova(phylip=final.opti_mcc.thetayc.0.03.lt.ave.dist, design=sampletype.design)
amova(phylip=final.opti_mcc.thetayc.0.03.lt.ave.dist, design=InfluentvCells.design)
amova(phylip= final.opti_mcc.thetayc.0.03.lt.ave.dist, design=totaltimewithoutinfluent.design)
amova(final.opti_mcc.thetayc.0.03.lt.ave.dist, design=location.design)
corr.axes(axes=stability.opti_mcc.thetayc.0.03.lt.ave.nmds.axes, shared=stability.opti_mcc.0.03.subsample.
shared, method=spearman, numaxes=3)
merge.groups(shared=stability.opti_mcc.0.03.subsample.shared, design=sampletype.design)
venn(shared=stability.opti_mcc.0.03.subsample.merge.shared)
```

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