

**FATE AND EFFECTS OF PER- AND POLYFLUOROALKYL  
SUBSTANCES IN AERATED AND NON-AERATED TREATMENT  
WETLAND MESOCOSMS**

**SORT ET EFFETS DES SUBSTANCES PERFLUOROALKYLES ET  
POLYFLUOROALKYLES DANS LES MÉSOCOSMES DE ZONES  
HUMIDES DE TRAITEMENT AÉRÉES ET NON AÉRÉES**

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## Abstract

Per- and polyfluoroalkyl substances (PFAS) are anthropogenic chemicals found in a multitude of consumer and industrial products. Due to their strong carbon-fluorine bond structure, PFAS such as perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), and 6:2 fluorotelomer sulfonate (6:2 FTS) are resilient to conventional physical, chemical, and biological degradation, and are therefore recalcitrant in the environment. The characteristics of these chemicals have provided numerous benefits to many industries, particularly in the application of aqueous film-forming foam (AFFF) as a fire retardant. Large amounts of AFFF are periodically discharged on military firefighter training sites and have subsequently accumulated throughout the years. This can potentially contaminate ecosystems such as natural wetlands, surface waters, and groundwater resulting in concentrations exceeding environmental guidelines. While treatment wetland (TW) research on PFAS is growing, fundamental data on fate and effects of PFAS in aerated, mesocosm-scale subsurface TWs is limited. Furthermore, while artificial aeration becoming increasingly employed in commercial TW application, a granular characterization of aeration in TW systems is missing in the literature.

This thesis is comprised of two main studies: (1) an exploration of aeration effects on water chemistry, water performance, and microbial community structure, function, and activity, and (2) a study exploring the fate and effects of PFOS, PFOA, and 6:2 FTS in TW mesocosms. Both studies occurred in parallel and examined 12 mesocosms in a 2<sup>2</sup> factorial design with factors of aeration and PFAS in triplicate for a 12-week in-situ period. PFAS was added weekly (at a starting concentration of 1 mg/L) into the mesocosms. Following the 12-weeks, the control and PFAS systems were deconstructed to determine organic content and a PFAS mass balance within the loaded mesocosms.

The effect of aeration on water treatment performance in TW mesocosms results in higher DO water chemistry, higher pH, and ORP. Lower overall total nitrogen removal was observed given a highly aerobic environment in the aerated system, which inhibited denitrification and thus incomplete nitrogen removal. Lower overall microbial and catabolic activity as well as lower metabolic richness, function, and less diversity were expressed. Organic mass was slightly higher in aerated systems across layers pointing to an overall effect of microbial efficiency, and resiliency (over time). There were little effects of PFOA, PFOS, and 6:2 FTS on the treatment of standard water quality parameters (organics, nitrogen). More PFOS than PFOA or 6:2 FTS was removed (via adsorption) from both aerated and non-aerated systems, with aerated systems removing more PFAS than the non-aerated systems. The results of these studies will offer a significant contribution towards improving TW design and performance into the future.

## Résumé

Les substances per- et polyfluoroalkyles (PFAS) sont des produits chimiques anthropogéniques que l'on retrouve dans une multitude de produits de consommation et de produits industriels. En raison de leur forte structure de liaison carbone-fluore, les PFAS tels que l'acide perfluorooctanesulfonique (PFOS), l'acide perfluorooctanoïque (PFOA) et le sulfonate de fluorotélomère 6:2 (6:2 FTS) sont résistants à la dégradation physique, chimique et biologique conventionnelle et sont donc récalcitrants dans l'environnement. Les caractéristiques de ces produits chimiques ont apporté de nombreux avantages à de nombreuses industries, en particulier dans l'application de la mousse à formation de film aqueuse (AFFF) en tant que retardateur de flammes. De grandes quantités d'AFFF sont périodiquement déversées sur les sites d'entraînement des pompiers militaires et se sont ensuite accumulées au fil des ans. Cela peut potentiellement contaminer les écosystèmes tels que les zones humides naturelles, les eaux de surface et les eaux souterraines, entraînant des concentrations supérieures aux directives environnementales. Alors que la recherche sur les PFAS dans les zones humides de traitement (TW) est en plein essor, les données fondamentales sur le devenir et les effets des PFAS dans les TW aérés, à l'échelle du mésocosme et sous la surface, sont limitées. De plus, alors que l'aération artificielle est de plus en plus utilisée dans les applications commerciales des zones humides de traitement, la littérature manque d'une caractérisation granulaire de l'aération dans les systèmes de zones humides de traitement.

Cette thèse comprend deux études principales: (1) une exploration des effets de l'aération sur la chimie de l'eau, la performance de l'eau et la structure, la fonction et l'activité de la communauté microbienne, et (2) une étude explorant le devenir et les effets du PFOS, du PFOA et du 6:2 FTS dans des mésocosmes TW. Les deux études ont été menées en parallèle et ont porté sur 12 mésocosmes dans un plan factoriel de 2<sup>2</sup> avec des facteurs d'aération et des PFAS en trois exemplaires pendant une période in situ de 12 semaines. Des PFAS ont été ajoutés chaque semaine (à une concentration initiale de 1 mg/L) dans les mésocosmes. Après les 12 semaines, les systèmes de contrôle et de PFAS ont été déconstruits pour déterminer le contenu organique et le bilan de masse des PFAS dans les mésocosmes chargés.

L'effet de l'aération sur la performance du traitement de l'eau dans les mésocosmes TW se traduit par une chimie de l'eau plus riche en OD, un pH et un ORP plus élevés. Une élimination globale plus faible de l'azote total a été observée en raison d'un environnement très aérobie dans le système aéré, ce qui a inhibé la dénitrification et donc une élimination incomplète de l'azote. Une activité microbienne et catabolique globale plus faible ainsi qu'une richesse et une fonction métaboliques plus faibles et une diversité moindre ont été exprimées. La masse

organique était légèrement plus élevée dans les systèmes aérés, toutes couches confondues, ce qui indique un effet global de l'efficacité microbienne et de la résilience (au fil du temps). Les effets du PFOA, du PFOS et du 6:2 FTS sur le traitement des paramètres standard de la qualité de l'eau (matières organiques, azote) ont été faibles. Les systèmes aérés et non aérés ont éliminé (par adsorption) plus du PFOS que de PFOA ou de 6:2 FTS, les systèmes aérés éliminant plus du PFOS que les systèmes non aérés. Les résultats de ces études contribueront de manière significative à l'amélioration de la conception et des performances des systèmes d'aération à l'avenir.

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## Abbreviations and Acronyms

Acronym or Abbreviation	Definition
6:2 FTS	6:2 Fluorotelomer Sulfonate
AFFF	Aqueous Film-Forming Foam
APFN	Ammonium Perfluorononoate
APFO	Ammonium Perfluorooctanoate
AWCD	Average Well Colour Development
BOD	Biochemical Oxygen Demand
CLPP	Community Level Physiological Profiling
COD	Chemical Oxygen Demand
CSUP	Carbon Source Utilization Patterns
DO	Dissolved Oxygen
EC	Emerging Contaminants
FDA	Fluorescein diacetate
FEQG	Federal Environmental Quality Guidelines
FL	Fluorescein
FT	Fluorotelomer
FTOH	Fluorotelomer Alcohols
FWS	Free Water Surface
HPLC	High Performance Liquid Chromatography
HSSF	Horizontal Subsurface Flow
N-EtFOSE	N-Ethyl Perfluoro-octanesulfonamidoethanol
ORP	Oxidation-Reduction Potential
PASF	Perfluoroalkyl sulfonyl fluorides
PBS	Phosphate Buffer Solution
PCA	Principal Component Analysis
PFAA	Perfluoroalkyl Acids
PFAI	Perfluoroalkyl Iodides
PFAS	Per- and Polyfluoroalkyl Substances
PFC	Perfluorinated Compounds
PFCA	Perfluoroalkyl Carboxylic Acids
PFNA	Perfluorononanoic Acid

PFOA	Perfluorooctanoic Acid
PFOS	Perfluorooctanesulfonic Acid
PFOSA	Perfluorooctane sulfonamide
PFOSAA	Perfluorooctanesulfonamidoacetate
PFSA	Perfluoroalkane Sulfonic Acids
POSF	Perfluorooctanesulfonyl Fluoride
PTFE	Polytetrafluoroethylene
TN	Total Nitrogen
TOC	Total Organic Carbon
TW	Treatment Wetland
U.S. EPA	United States Environmental Protection Agency
VF	Vertical Subsurface Flow
WWTP	Wastewater Treatment Plant

# 1 Introduction

## 1.1 Background

Wastewater has been generated by humans throughout history. Over time, civilizational growth and increasing demand for a diverse array of consumer products has led to new (emerging) chemicals being incorporated into wastewater. These new chemicals can negatively impact ecosystems (Giesy and Kannan, 2001; Martin et al., 2004). A focus on treating wastewater that contained common and emerging contaminants became critical in order to protect the health of humans, wildlife and the surrounding environment. This presents unique challenges in the field of environmental science and engineering in optimizing wastewater treatment solutions, specifically in understanding the fate and transport of emerging or new environmental contaminants. While government regulation has been successful in reducing some persistent chemicals originating from the disposal and life-cycle of consumer and industrial applications, anthropogenic chemicals such as Per- and Polyfluoroalkyl Substances (PFAS) have and continue to emerge in the environment. PFAS appearing in ecosystems today often originate from cosmetics, surfactants, cookware, food packaging, and flame retardants such as Aqueous Film-Forming Foam (AFFF) (Houtz et al., 2013; Kissa, 2001; Paul et al., 2009; Prevedouros et al., 2006). Wastewater technologies for emerging contaminants such as PFAS are being developed, however a common thread is that these technologies can be resource-intensive and complex (Kucharzyk et al., 2017; Lenka et al., 2021).

Wastewater treatment systems utilize physical, chemical, and biological processes to facilitate removal of organic matter (Tchobanoglous, 2003). Wetlands have been used for wastewater treatment for years, and have incorporated these processes as an engineered system, in the form of treatment wetlands (TW), which have been evolving and continuously enhanced through intensification means (Brix, 1994; Kadlec and Wallace, 2009; Kennedy and Mayer, 2002; Vymazal, 2011). While various TW configurations exist, recent advances have presented intensified designs featuring artificial aeration as a tool to promote different redox conditions in order to achieve specific wastewater treatment targets (Nivala et al., 2020; Ouellet-Plamondon et al., 2006). However, a deeper understanding of aeration effects in TW is limited. Therefore, there is a need to characterize the physical and biological mechanisms that are exhibited in aerated TW when compared to non-aerated TW that will ultimately contribute to enhanced TW design and performance.

The application of TW as an alternative wastewater treatment technology has shown promising performance in degrading a variety of emerging contaminants, however there exists limited research on both the ability for TWs to treat and/or

tolerate PFAS (Ávila et al., 2014; Ávila et al., 2013; Button et al., 2019b; Kang et al., 2023; Lott et al., 2023; Weber et al., 2011; Yin et al., 2018, 2017; Zhang et al., 2021). There is a need to understand fate and effects of PFAS when introduced into aerated and non-aerated TW. These results will directly impact TW designs in developing adaptable and optimized systems for full-scale application. The goal of this thesis will be to close existing knowledge gaps and expand the current understanding of subsurface TW systems.

## 1.2 Objectives

This thesis is comprised of four overall objectives:

1. Assess the effects of aeration on water treatment performance in treatment wetland mesocosms.
2. Investigate the spatial and temporal effects of aeration on microbial community structure, function, and activity.
3. Evaluate effects of perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), and 6:2 fluorotelomer sulfonate (6:2 FTS) on water treatment performance in aerated and non-aerated treatment wetland mesocosms.
4. Examine PFOA, PFOS, and 6:2 FTS fate in aerated and non-aerated treatment wetland mesocosms.

## 1.3 Organization

This thesis is comprised of five chapters as follows:

**Chapter 1** contains a brief introduction of the thesis topic, objectives, and structure.

**Chapter 2** presents a literature review of treatment wetlands and PFAS as an emerging contaminant.

**Chapter 3** provides a study on the effects of aeration on treatment wetland mesocosms. This chapter will address objectives 1 and 2.

**Chapter 4** provides a study on PFOS, PFOA, and 6:2 FTS in treatment wetland mesocosms, specifically exploring fate and effects on water treatment performance. This chapter will address objectives 1, 2, 3, and 4.

**Chapter 5** summarizes the principal outcomes of the thesis and presents recommendations for future work.

**Appendix A** contains supplemental information for Chapter 3

**Appendix B** contains supplemental information for Chapter 4



## **2 Literature Review**

### **2.1 Treatment Wetlands Background**

#### **2.1.1 Natural Wetlands**

Wetlands are areas of land that are saturated with water due to the physical characteristics of the landscape they are situated in – incorporating vegetation and biological removal mechanisms (Kadlec and Wallace, 2009; Kennedy and Mayer, 2002). The diverse function of wetlands in the environment can be based on their physical, chemical and biological attributes. Physically, natural wetlands can mitigate adverse effects of flooding by intercepting storm runoff and storing excess surface waters. Chemically, wetlands are capable of removing wastewater contaminants and have for centuries been receivers of community wastewater. Because of their high biological activity and ability to be sources of plentiful biodiversity, wetlands have shown to not only store, but degrade and transform common pollutants into benign substances and nutrients for a variety of organisms (Brix, 1994; Vymazal, 2011). The wastewater treatment capability of natural wetlands has many benefits, and is used in Europe as an alternative to WWTP in local communities (Vymazal, 2011). However, diverting harmful wastewater effluents to natural ecosystems can be damaging and unsustainable. While natural wetlands are still used in controlled conditions, engineered wetlands were developed as a solution to address this issue of damaging natural wetlands, improve treatment effectiveness, and are now the preferred replacement technology, which will be explored in the next section.

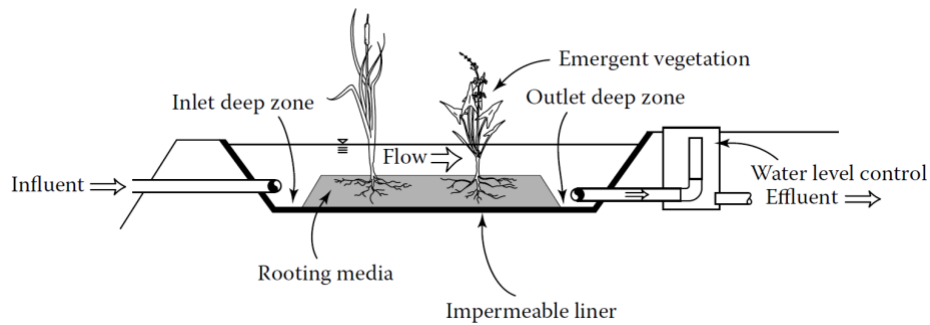
#### **2.1.2 Treatment Wetlands**

A constructed, engineered, or treatment wetland (TW) is a man-made wastewater treatment system designed to apply the physical, chemical, and biological processes of a natural wetland, but in a controlled and specific environment. As a pioneer of the initial engineered wetland applications, Dr. Käthe Seidel's work from the 1950s to the 1970s made important progress in improving treatment efficiency and designs (Vymazal, 2011). Physical processes achieved in an engineered wetland are sedimentation, filtration, and UV exposure. Chemical processes are precipitation, adsorption, and volatilization. Biological processes are microbial degradation, nutrient transformation, and plant uptake. Engineered wetlands are classified according to three main design parameters: hydrology (surface or subsurface flow), macrophytic growth (submerged, emergent, or free-standing), and flow direction (horizontal or vertical). Thus, the main configurations

of engineered wetlands are: free water surface (FWS), horizontal subsurface flow (HSSF), and vertical subsurface flow (VF) (Kadlec and Wallace, 2009). Design considerations for the application of each system depend on a multitude of variables such as pollutant characteristics, effluent results, and climate, amongst others.

#### 2.1.2.1 Free Water Surface Wetlands

The Free Water Surface (FWS) wetland configuration has an open water appearance similar to a marsh or swamp and contains floating vegetation with emergent plants. Water depth in these systems can range from 5-90 cm, with an average of 30-40 cm (Brix, 1994). As wastewater effluent flows through the system, heavy particulates and suspended solids settle while the pollutants undergo several physical, chemical, and biological processes such as filtration, adsorption, volatilization, microbial degradation, and plant uptake in an open air, oxygen-rich environment. A diagram of a FWS wetland is provided in Figure 2.1.

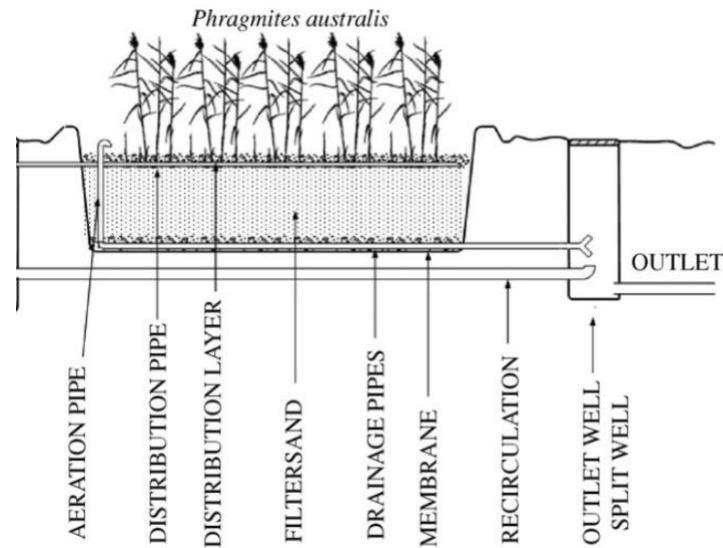


**Figure 2.1** - Diagram of a FWS wetland reproduced from Kadlec and Wallace, 2009

#### 2.1.2.2 Horizontal Subsurface Flow Wetlands

Horizontal Subsurface Flow (HSSF) wetlands are comprised of gravel or soil bed media with a planted bed of vegetation at the surface of the substrate media. Wastewater enters through the inlet and moves horizontally through the porous substrate under the plant bed towards the outlet. As it flows through the subsurface media, the contaminated water will interact with aerobic, anoxic, and anaerobic environments. During wastewater flow, contaminants will interact with biofilms on various surfaces such as plant rhizosphere and substrates as well as adsorb on these surface areas (García et al., 2010). HSSF systems typically produce anaerobic environments due to their limited oxygen transfer; however, they can produce aerobic zones in the vicinity of the rhizosphere. While clogging can be an issue for

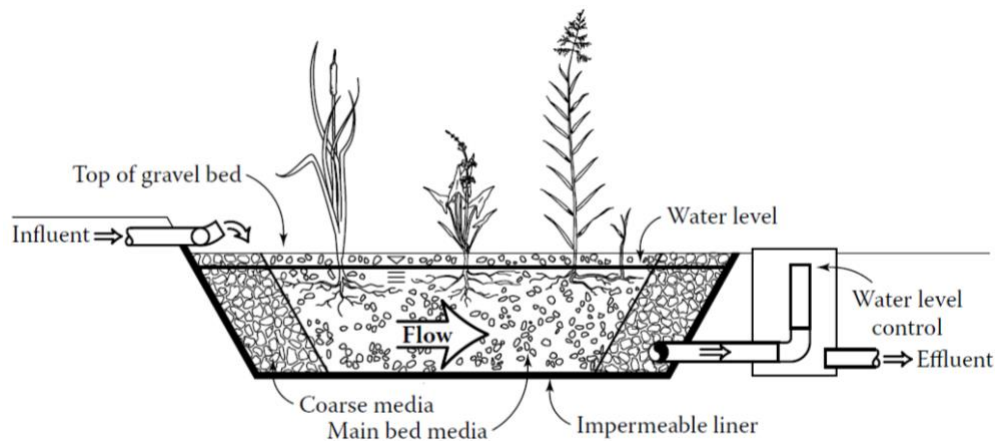
influent that has considerable suspended solid compositions, pre-treatment filtration can be a solution. A diagram of an HSSF system is illustrated in Figure 2.2:



**Figure 2.2** - Diagram of an HSSF wetland reproduced from Kadlec and Wallace, 2009

### 2.1.2.3 Vertical Subsurface Flow Wetlands

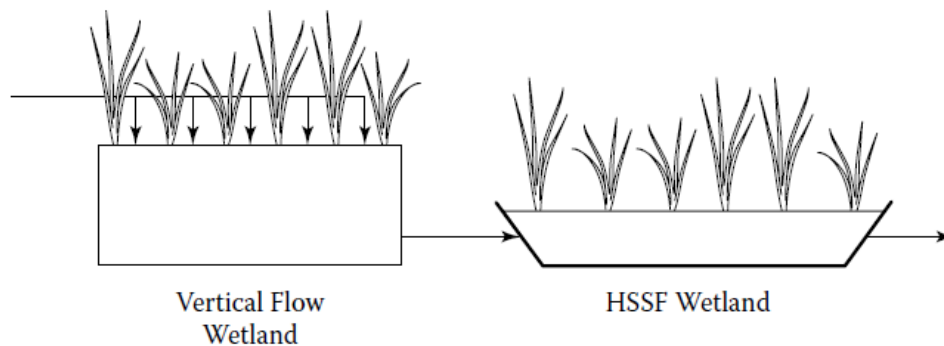
The vertical flow (VF) wetland configuration is similar to the HSSF wetland in that it incorporates a porous medium of gravel with a plant bed on top. However, VF wetlands are fed wastewater intermittently in a batch process, flooding the bed surface. Contaminated water then filters down through the plant bed and, subsequently, the substrate to a capture system at the bottom of the wetland. As the bed drains, the system is aerated – allowing air to fill the bed. The fill and drain process increases oxygen concentration in the system. Furthermore, oxygen is also transferred to wastewater during its release from the inlet pipe onto the wetland. Thus, VF wetlands often have more aerobic environments as opposed to HSSF wetlands. Moreover, a venturi effect is observed in VF wetlands, as the incoming effluent enters the substrate forming a pressure differential drawing air into the wetland system (Armstrong et al., 1992). A diagram of a VF wetland system is highlighted in Figure 2.3:



**Figure 2.3** - Diagram of a VF wetland reproduced from Kadlec and Wallace, 2009

### 2.1.3 Intensification Designs

In addition to the standard wetland configurations mentioned above, alternative designs that intensify removal efficiency have been evaluated (Ayaz et al., 2012; Foladori et al., 2013; Ouellet-Plamondon et al., 2006). A hybrid design is one that can combine different types of engineered wetlands that have specific individual strengths for a more complete treatment package and enhanced nitrogen removal. A common example of this can be pairing a VF wetland followed by an HSSF. In this case, the more aerobic environment in the VF design can act as a primary treatment promoting nitrification and solids removal before wastewater enters a secondary, more anaerobic system that undergoes denitrification. A basic diagram of a typical hybrid system is provided in Figure 2.4.



**Figure 2.4** - Basic hybrid wetland concept of VF wetland followed by HSSF wetland reproduced from Kadlec and Wallace, 2009

Another intensification design is to include artificial aeration into subsurface wetland systems. In situations where natural aeration is either insufficient or additional oxygen transfer is required in order to increase aerobic degradation, nitrification rates, or reduction in chemical oxygen demand (COD), artificial aeration can be employed (Nivala et al., 2020). With these benefits, artificial aeration can also allow wetlands to reduce in size and maintain removal efficiency in case of land constraints. However, there can also be downsides to artificial aeration. Depending on the velocity of air bubbles themselves, shear forces can remove effective biofilms on media surfaces (Rajabzadeh et al., 2015). Furthermore, the increased cost and additional maintenance associated with operating air blowers for a wetland system can be disadvantageous.

A third intensification design used commercially is the integration of an effluent recycle processes in the wetland system (Sun et al., 2003). In this concept, wastewater that exits the subsurface wetland system is then streamed to its initial entry point in the wetland for a second pass through the system. This not only allows for the organic matter to contact biofilms and bed media again, but it reintroduces oxygen transfer. While operating costs may double as a result of repeat treatment, the reduction in pollutants – considerably total nitrogen - is noteworthy along with the potential for a reduction in wetland surface area as a result of increased efficiency (Foladori et al., 2013).

#### **2.1.4 Removal Mechanisms**

In order to appreciate the extent of engineered wetland technology for future applications, it is necessary to understand the driving force behind its treatment mechanisms. Microbially mediated processes are a principal means of contaminant

treatment, with microbial transformations providing the majority of total nitrogen removal (Faulwetter et al., 2009a). Redox conditions in the wetland will determine the exact physical and chemical processes that will take place to convert organic pollutant - carbon being the primary food source. Therefore, whether the substrate is aerobic, anaerobic, or anoxic, different outcomes will be achieved. A high redox potential is indicative of a highly oxidized environment promoting aerobic processes such as aerobic respiration and nitrification, whereas lower redox potentials will normally result in reduced conditions signalling anaerobic processes such as iron reduction, methanogenesis and sulfate reduction.

### **2.1.5 Wetland Performance with Emerging Contaminants**

Remediation solutions are available to treat ECs, however they have high costs, complex processes, and persistent waste products prohibiting these technologies from mainstream application. The use of engineered wetlands as a reliable wastewater treatment system is a proven technology and can be employed to treat ECs. However, knowledge is still limited on the impact ECs have on engineered wetlands to fully understand its long-term performance and effectiveness. Prior studies have shown that wetland performance with ECs has had toxicological effects to microorganisms, however water treatment performance and resiliency were also observed in the same studies (Ávila et al., 2014; Ávila et al., 2013; Button et al., 2019b; Weber et al., 2011). It is with this understanding and motivation that a study to apply engineered wetland technology to a serious, challenging, and poorly understood EC would be of great benefit to the wider scientific community.

## **2.2 Per- and Polyfluoroalkyl Substances**

### **2.2.1 Nomenclature**

Using terminology and classifications from Buck et al., 2011, the family of PFAS can be divided into two main categories: non-polymer and polymer compounds. The polymer category incorporates substances such as fluoropolymers, perfluoropolyethers, and side-chain fluorinated polymers with varying non-fluorinated polymer bodies attached to fluorinated side chains.

The non-polymer category encompasses perfluoroalkyl and polyfluoroalkyl substances. Perfluoroalkyl substances consist of aliphatic compounds where all hydrogen species on one or more carbon atoms are replaced by fluorine atoms with exception to those in an attached functional group. The polyfluoroalkyl group is comprised also of aliphatic compounds, however, not all hydrogen species attached

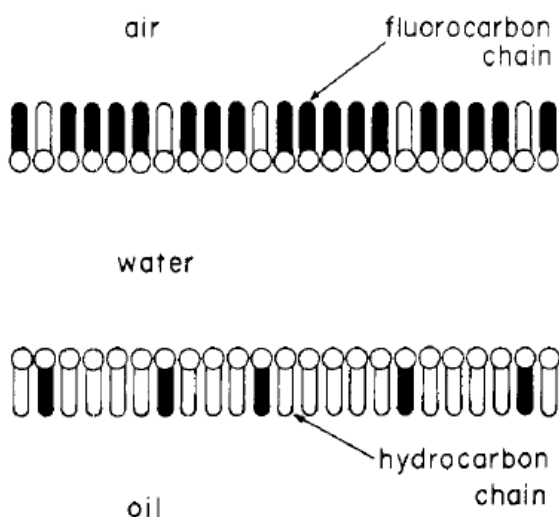
to carbon atoms have been replaced by fluorine. Perfluoroalkyl substances can be further broken down sub-categories such as perfluoroalkyl acids (PFAA), perfluoroalkyl sulfonyl fluorides (PASF), perfluoroalkyl iodides (PFAI), and others. The PFAA family (comprised of perfluoroalkyl carboxylic, sulfonic, sulfinic, phosphonic, and phosphinic acids) occupy a prominent place in PFAS studies due to their widespread use and recalcitrant nature. Furthermore, PFAA are further separated to perfluoroalkyl carboxylic acids (PFCA) (when they feature a carboxylic acid functional head group), and perfluoroalkane sulfonic acids (PFSA) (with a sulfonic acid functional head group. Buck et al., 2011 further highlights a differentiation between PFAS and what was historically called Perfluorinated compounds (PFC). While PFCs also contain carbon chains surrounded by fluorine atoms, there does not exist any functional group attached to the main carbon chain body. A summary of these groups and their associated categories is illustrated in Table 2.1.

**Table 2.1** – Summary of PFAS group and their families reproduced from Buck et al., 2011

<b>Per- and Polyfluoroalkyl Substances (PFAS)</b>	
<i>Non-polymers</i>	<i>Polymers</i>
<p><b>Perfluoroalkyl Substances</b></p> <p>Compounds for which all hydrogens on <u>all</u> carbons (except for carbons associated with functional groups) have been replaced by fluorine</p>	<p>Fluoropolymers</p> <p>Carbon-only polymer backbone with fluorine directly attached</p>
<p><b>Polyfluoroalkyl Substances</b></p> <p>Compounds for which all hydrogens on <u>at least one (but not all)</u> carbon have been replaced by fluorine</p>	<p>Perfluoropolyethers</p> <p>Carbon and oxygen polymer backbone with fluorine directly attached to carbon</p>
	<p>Side-chain fluorinated polymers</p> <p>Variable composition non-fluorinated polymer backbone with fluorinated side chains.</p>

### 2.2.2 Chemical Properties

The carbon-fluorine heat of formation is approximately 407 kJ/mol and it further increases with each carbon substituted (Kissa, 2001). For example, the heat of formation of  $\text{CH}_3\text{F}$  is 448 kJ/mol,  $\text{CH}_2\text{F}_2$  459 kJ/mol,  $\text{CHF}_3$  480 kJ/mol, and  $\text{CF}_4$  486 kJ/mol. The high electronegativity of fluorine provides strong polarity to the carbon-fluorine bonds in a PFAS compound – contributing to its stability and non-reactive characteristics in the environment. This carbon-fluorine bond is resilient to chemical, biological, and physical degradation (Rahman et al., 2014). Additionally, within the molecule, the polar head group and non-polar tail (featuring the Carbon-Fluorine backbone) provides characteristics indicative of a surfactant – a substance that can lower the surface tension of a medium by adsorption on the interface. Surfactants are used in fire-fighting foams, wetting agents, foam stabilizers, etc. In the case of PFAS, its hydrophobic properties arise from the presence of fluorine in the fluorocarbon bond. Moreover, these hydrophobic bond characteristics are accompanied by oleophobicity and provide low free-surface energy of the adsorbed surfactant. This can be of benefit in environments where conventional surfactants fail to perform. Figure 2.5 illustrates the micelle-like head/tail structure of a PFAS compound when employed in fire-fighting foams and its theoretical performance.



**Figure 2.5** - PFAS surfactant illustration featuring an air-water/oil-water interaction reproduced from Shinoda and Nomura, 1980

As Figure 2.5 highlights, the fluorocarbon chain in its hydrophobic characteristic will repel water at the air-water interface, and its polar “head” or non-fluorinated, hydrophilic functional group would attract to the water. Figure 1.6



describes an overall foam mixture comprising of a fluorocarbon and hydrocarbon chain where the hydrocarbon chain would adsorb to the oil-water interface, successfully depressing its interfacial tension. The oil can be replaced by water in a conventional sense and would form the basis of the chemistry associated with aqueous film-forming foams (AFFF) whereby the alignment of the fluorocarbon chains forms a film on the ignited surface effectively smothering it.

### **2.2.3 Types and Uses**

Once processes for the synthesis of PFAS were understood and scaled up for manufacture during the 1950s, surfactants and polymers using PFAS compounds and their derivatives have been used in many industrial and commercial applications. An overview of the main types of PFAS and their uses are explained below. The primary categories of PFAS families discussed will be PFAA comprised of PFCA and PFSA as well as those of the Fluorotelomer (FT) type.

#### *2.2.3.1 Perfluoroalkyl Acids (PFAA)*

The categorization used by Buck et al., 2011 will be employed when examining PFAA compounds, whereby PFCA and PFSA will be the only compounds highlighted. PFAA are critical substances in that they are recalcitrant in the environment, the result of precursor metabolism (discussed later) and, subsequently the subject of much effort in recent literature. Furthermore, depending on acid strength, these compounds will dissociate to their anions in aqueous media, soils, and sediments.

#### *2.2.3.2 Perfluoroalkyl Carboxylic Acids (PFCA)*

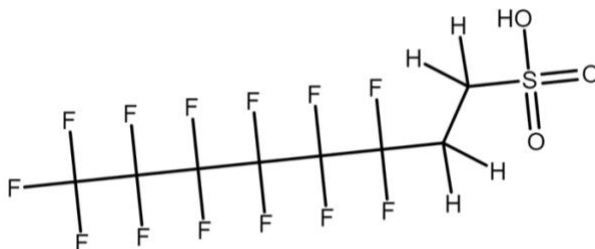
Originally synthesized in the 1940s by the ECF process and applied commercially in the 1950s, PFCA ( $C_nF_{2n+1}COOH$ ) are a major component of the PFAA family with a global industry-wide reach (Prevedouros et al., 2006). The PFCA compound with the largest distribution is perfluorooctanoic acid (PFOA), however historically, its ammonium salt: ammonium perfluorooctanoate (APFO) has been essential in the manufacture of fluoropolymers such as polytetrafluoroethylene (PTFE) and polyvinylidene fluoride, where it is used as an emulsifier for the emulsion polymerization of fluoropolymers (Frömel and Knepper, 2010; Lehmler, 2005). As well, from 1975, perfluorononanoic acid (PFNA) was also used in addition to its ammonium salt: ammonium perfluorononoate (APFN) to also produce fluoropolymers (Prevedouros et al., 2006).

### 2.2.3.3 Perfluoroalkyl Sulfonic Acids (PFSA)

The second major component to the PFAA family, also synthesized by the Perfluorooctanesulfonyl fluoride (POSF) intermediate in the ECF process beginning in the 1940s is PFSA ( $C_nF_{2n+1}SO_3H$ ) (OECD, 2018). The PFSA compound with the largest distribution is perfluorooctane sulfonic acid (PFOS) and its salts. In addition to PFOS, perfluorohexane sulfonic acid (PFHxS), perfluorodecane sulfonic acid (PFDS), and perfluorobutane sulfonic acid (PFBS) were also widely used. Historically, the applications of PFOS and its salts have been extensive throughout industry especially in the use of fire-fighting foams and will be explored in the next section.

### 2.2.3.4 Fluorotelomers

Another important family of PFAS are fluorotelomers (FT), which are produced by the telomerization process and are derivatives of the PFOA family. Telomers, or more broadly fluorotelomer-based products are a family of initial material reactants, surfactants, and polymeric products, degradation products originating from the initial fluorotelomer raw material, perfluoroalkyl iodides (PFAI). The most prominent FT-based chemicals are fluorotelomer alcohols (FTOHs) and fluorotelomer olefins, which are often used as intermediates in the fluorinated polymer process (Frömel and Knepper, 2010). These compounds follow a polyfluorinated X:Y structure where X = number of perfluorinated carbon linked to Y carbon atoms without fluorine species attached. Common FT compounds are 6:2 and 8:2 FT sulfonates as seen in Figure 2.6 or 6:2 and 8:2 FT alcohols.



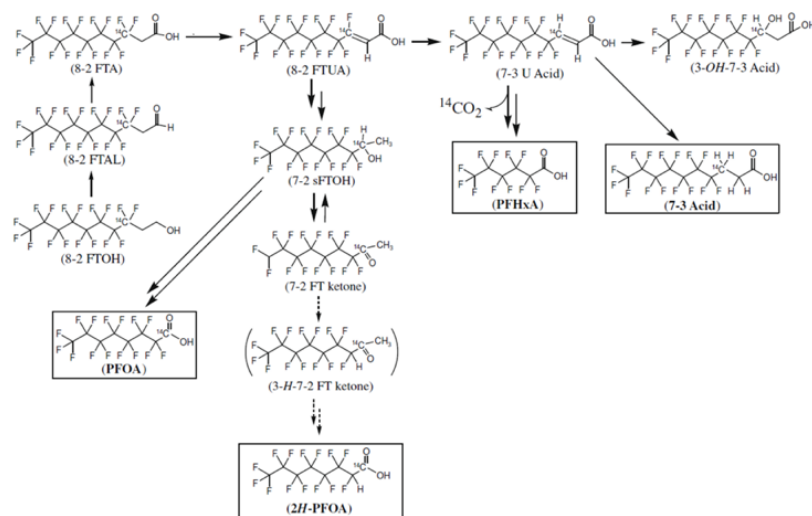
**Figure 2.6** - 6:2 Fluorotelomer sulfonate

#### 2.2.4 Precursors

Precursors are higher molecular weight derivatives of PFAS compounds that exist in the environment, are volatile (contrary to PFAS), and eventually degrade to common, shorter-chain PFAS; primarily PFOS and PFOA (Martin et al., 2010). Precursors are diverse in their composition mainly due to variance in manufactured forms and methods as well as intermediates, isomers, and impurities found in end products.

While there are a multitude of precursors, one example of this group and its degradation products can be found in perfluorooctanesulfonamidoacetate (PFOSAA) – an oxidation product and PFOS precursor of N-ethyl perfluorooctanesulfonamidoethanol (N-EtFOSE;  $C_8F_{17}SO_2N(CH_2CH_3)CH_2CH_2OH$ ). This compound is a residual found in products of the phosphate ester of N-EtFOSE, primarily used in paper and packaging protectant applications. Commercial applications of this product began in the 1960s and were introduced in human food contact paper in 1974. PFOSAA is thought to metabolize to perfluorooctane sulfonamide (PFOSA;  $C_8F_{17}SO_2NH_2$ ) and eventually PFOS ( $C_8F_{17}SO_3^-$ ) (duPont, 1948).

Although there are overall factors involved in the location and length of specific perfluoroalkyl chains in the FT alcohol, telomer precursors resulting in PFOA have been observed to degrade in the environment microbially such as that in an activated sludge treatment system (Wang et al., 2009). The paper presented an overview of 8:2 FT alcohol's biodegradation pathway (Figure xx) in an activated sludge study where the 8:2 FT alcohol not only degraded and formed PFOA but also other stable metabolites such as 2H-PFOA, PFHxA, and 7-3 acid.



**Figure 2.7** - Biodegradation pathway of 8:2 Fluorotelomer alcohol reproduced from Wang et al., 2009

## 2.2.5 Toxicity and Environmental Impacts

Understanding the applicability of PFAS throughout the global industry since the 1950s, one can predict that throughout its processes and product life cycle, release and disposal in the environment is expected. This section will explore these implications of PFAS use, primarily investigating PFOS and PFOA on the environment, humans, and wildlife. Due to the strong carbon-fluorine bonds in PFAS, its presence in the environment is recalcitrant as it can persist for years, decades, or longer, with no known natural mechanism of degradation (Lindstrom et al., 2011; Martin et al., 2010). PFAS are emitted via two main methods: direct and indirect sources. Direct sources originate from the manufacture and use of PFAS compounds such as PFCAs, PFSAs, etc. Whereas indirect sources result from degradation (e.g. precursor biodegrading into PFOS/PFOA) or chemical reaction impurities whereby certain substances degrade to form shorter-chain PFAS products.

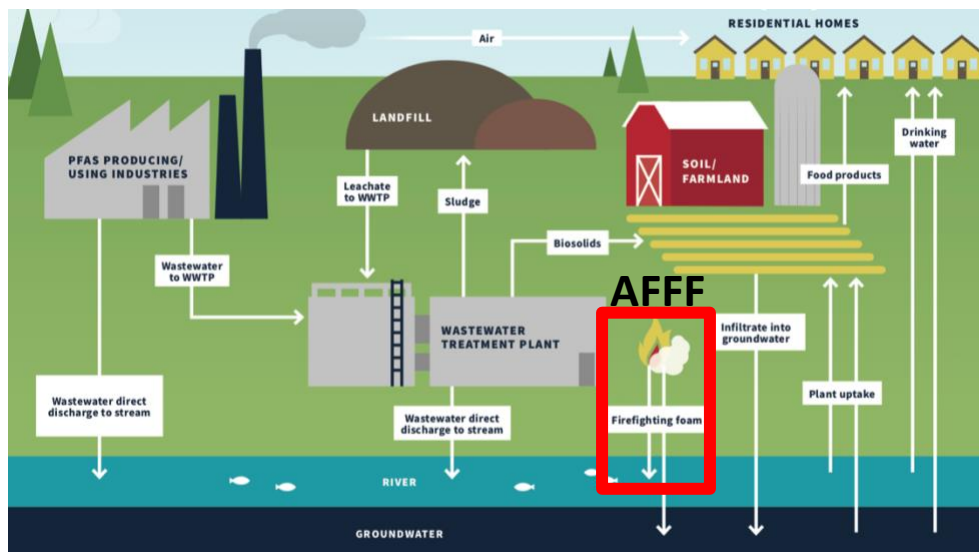
A critical example of direct and indirect PFAS emission into the environment is aqueous film-forming foam (AFFF) use. AFFF is a complex mixture containing both fluorocarbon and hydrocarbon-based surfactants used extensively in military environments during fire-fighting training activities and drills since the 1960s (Houtz et al., 2013). The result of years of AFFF use on military bases, as well as on many airports, is the contamination of spent AFFF runoff into local soils, groundwater, and surface waters (Ahrens, 2011; Kim and Kannan, 2007). While initial formulations of AFFF comprised of standard PFAS chemistry, new mixtures

of mainly PFAS precursors comprised the foams, which led to the foam degrading and biotransforming further throughout environmental media resulting in persistent short-chain PFAS (Houtz et al., 2013).

In addition to soil and groundwater contamination, PFAS have also been carried to aquatic environments (Ahrens, 2011). Their properties of high water solubility, hydrophobicity/hydrophilicity, and low volatility are major contributors to their presence in aqueous media. Sources of PFAS into aquatic environments are often rivers near industrial or municipal WWTP that are transporting PFAS compounds. Furthermore, landfill leachate comprised of spent PFAS materials and waste, and by-products of sludge treatments, are further transported to receiving waters from precipitation and soil or surface runoff (Paul, 2009). In the U.S. specifically, PFOS has been observed in surface waters downstream from a PFOS production facility in addition to WWTP and landfill leachate. An illustration highlighting the direct and indirect emission sources is presented in Figure 2.8.

Knowing that PFAS contamination pervades across many facets of the environment, it is then expected that wildlife and humans would also be receivers of these compounds. PFOS has been detected in several species of eagles, wild birds, fish, and marine mammals. The concentrations of PFOS in a range of wildlife were shown to increase from the period of 1968-2002 (Paul et al., 2009). The blood plasma of bald eagles from the U.S. contained PFOS concentrations of up to 2570 ng/mL (Giesy and Kannan, 2001).

In human populations, both PFOA and PFOS have been found in the blood serum (of U.S. humans) almost all (99%) of samples collected between 1999 and 2002 (Olsen et al., 2005). Therefore, virtually all people living in the developed world have some form of PFAS exposure in their blood. Exposure routes for humans come through the use of contact with consumer products, occupational exposure, consuming contaminated food (eg. Food wrappings, wax papers, etc.), drinking water from compromised sources as detailed above, contact with air, house dust, and other items containing PFAS chemicals. Human health impacts are primarily focused on oral ingestion and result in increased risks, such as developmental effects to fetuses, cancer, neurotoxicity, immunotoxicity, and thyroid disruption, amongst others.



**Figure 2.8** - Pathways of PFAS into the environment reproduced and modified from Michigan.gov

### 2.2.6 Regulation and Phase-out

Prior to their phase-out in 2000, 3M Company was the major global producer of POSF and its derivative products such as PFOS, although smaller manufacturers existed in Europe and Asia (Land et al., 2018). Working alongside the United States Environmental Protection Agency (U.S. EPA), 3M Company slowly discontinued PFOS related compounds from 2000 to 2002.

The Stockholm Convention on Persistent Organic Pollutants (POP) was established in 2001 as an environmental regulatory arm of the United Nations (UN). As a result of its fourth meeting in May 2009, the organization listed the production and use of PFOS, its salts, and POSF in Annex B – restricted (UNEP, 2009). This meant that the production and use of PFOS, its salts, and POSF were only for the purposes listed in the Annex: photo-imaging, etching agents, aviation hydraulic fluids, medical devices, and fire-fighting foams amongst others. At the ninth meeting of the Stockholm Convention between April and May 2019, it was decided that PFOA, its salts, and related compounds would be added to Annex A – elimination (UNEP, 2019). More consequential than Annex B, Annex A prohibits the manufacturing and use of PFOA, its salts, and related compounds other than certain exceptions.

In 2006, the U.S. EPA asked DuPont and seven other global companies to participate in a voluntary program to phase-out PFOA and other long-chain chemistry. The 2010/15 EPA PFOA Stewardship Program was designed for companies to achieve no later than 2010, a 95% reduction of emissions and product content of PFOA, precursor chemicals, and related compounds (US EPA, 2006). The end goal was to work towards the elimination of PFOA, its precursors, and derivatives by 2015. With the inception of this program, DuPont was able to cease production of PFOA by the end of 2013 (Land et al., 2018). Although, shorter chain PFAS such as perfluorobutane sulfonate (PFBS) and perfluorohexanoic acid (PFHxA) have replaced the since regulated PFOA in U.S manufacturing.

### 2.2.7 Canadian Guidelines

For Canada specifically, the Federal Environmental Quality Guidelines (FEQGs) (Environment and Climate Change Canada, 2018) are baseline values for environment quality based on toxicological or adverse effects of certain substances. The FEQGs apply to the environment and provide specific values to assist in managing environmental risk factors. The FEQG has provided data for PFOS for surface water, fish tissue, wildlife diet, bird egg, and sediment illustrated in Table 2.2.

**Table 2.2** - FEQG for PFOS (Environment and Climate Change Canada, 2018)

Water (µg/L)	Fish Tissue (mg/kg)	Wildlife Diet (µg/kg)		Bird Egg (µg/g)
		Mammalian	Avian	
6.8	9.4	4.6	8.2	1.9

Canadian drinking water guidelines for 25 types of PFAS (including PFOS, PFOA, and 6:2 FTS) have been produced (Health Canada, 2024) in order to manage human health over a lifetime’s worth of exposure with a value of 30 ng/L for the sum of detected PFAS. The U.S. EPA also published national drinking water guidelines that address PFOA and PFOS (amongst other PFAS) (U.S. EPA, 2024). The maximum contaminant level, which is defined as the highest level of contaminant allowed in drinking water, for PFOA and PFOS are 4 ppt.

## **3 Water treatment performance and microbial characterization of aerated treatment wetlands**

### **3.1 Introduction**

Treatment wetlands (TW) are engineered systems that offer an alternative wastewater treatment solution in a controlled environment. TW have been used to treat a variety of common and emerging contaminants with high efficiency and performance. While removal mechanisms in TW include adsorption, volatilization, and microbial processes, many TW design intensifications have been presented in the last decade to increase treatment efficiency. Artificial aeration has become a method to optimize pollutant removal and increase treatment effectiveness as compared to passive (non-aerated) TW configurations (Nivala et al., 2007). Aerated subsurface treatment wetlands deliver air bubbles in an otherwise anaerobic environment, often through an air compressor or blower. Bubbles travel upward through the substrate, eventually ascending to the surface. This can provide additional air-water interfaces for organics to partition to and further enhance pollutant removal from the water column (Hoff et al., 1993). Aeration also increases oxygen transfer to the interstitial water thus providing a highly aerobic redox environment for microbial communities within the pore water, and also to communities associated with the biofilms within the substrate. The enhancement of oxygen flux to microbial communities through aeration has shown to increase microbial growth and improve nitrogen and organic carbon removal through nitrification mechanisms providing high water treatment efficiencies observed in aerated TW (Cottingham et al., 1999; Faulwetter et al., 2009a; Nivala et al., 2020; Tanner and Kadlec, 2003). Aerated systems are thus categorized through their high dissolved oxygen (DO), high oxidation-reduction potential (ORP) and complete nitrification processes (Nivala et al., 2019; Sossalla et al., 2022).

Metabolic processes in TW are a significant component of pollutant degradation for wetland system contaminants (Faulwetter et al., 2009a). Microbes are largely featured on biofilms situated on gravel surfaces as well as in the interstitial water. These communities contribute to water treatment by metabolic processes that degrade organic matter. Furthermore, these metabolic actions are facilitated by enzyme-based reactions through specific functional requirements of the community thereby resulting in microbial communities in TW being structurally and functionally diverse (Weber and Legge, 2010; Weber, 2016; Weber and Gagnon, 2014). Elucidating these characteristics provide deeper understanding in determining the overall capability of TW to treat pollutants.



Previous studies have explored the efficacy of aerated TWs on emerging contaminants (Ávila et al., 2021, 2014; Nivala et al., 2019; Sossalla et al., 2022) as well as wetland resilience in an aerated configuration (Boog et al., 2018). Previous studies have also conducted structural analysis in intermittently aerated systems (Feng et al., 2021; Lai et al., 2020; J. Li et al., 2021; Yang et al., 2018), and Lai et al., 2020 assessed structural relationships with certain water treatment parameters. However there have been limited studies characterizing the function, structure, and activity of microbial communities in subsurface aerated TW environments.

While aeration is becoming increasingly common in full-scale TW applications, the current body of knowledge falls short on a detailed understanding of the advantages and mechanistic contributions of aeration to overall TW conditions and water treatment effectiveness, and is still largely unknown. Furthermore, since microbial processes contribute significantly to wetland contaminant removal, it is critical to fully understand the function and effects of microbial communities in aerated systems. This study intends to explore two main questions: (1) What are the effects of aeration on TW water treatment capability and (2) how do microbial communities respond to and vary with aeration and its relationship towards TW performance? These questions will be addressed through the following objectives using aerated and non-aerated TW: (1) investigating aeration effects on water chemistry and hydrological parameters, (2) investigating aeration effects on water treatment performance, and (3) assessing aeration effects spatially and temporally on microbial community activity, function and structure measurements. The results of this study hope to comprehensively characterize the effects of aeration technology as it pertains to wetland engineering.

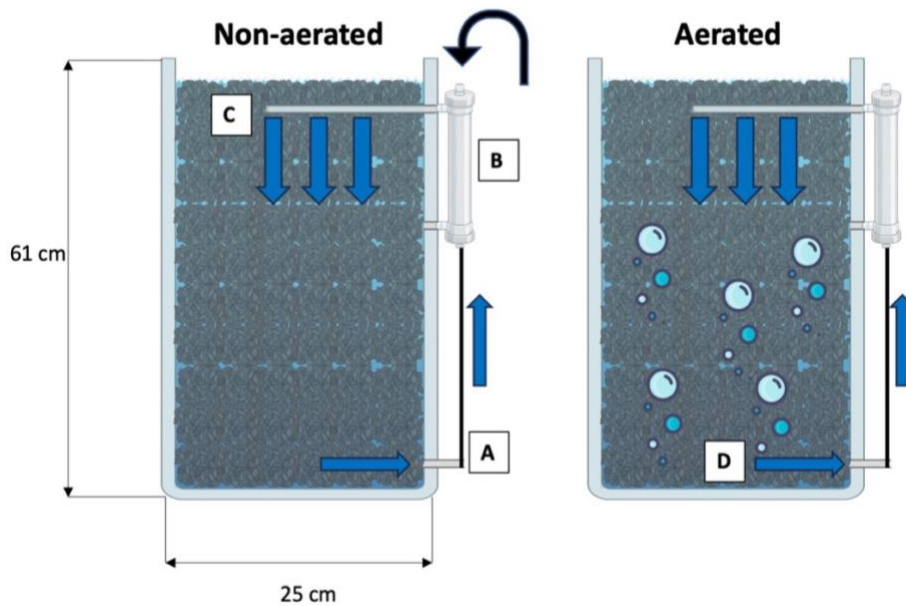
## **3.2 Materials and Methods**

### **3.2.1 Experimental Approach**

This study was accomplished through three primary phases: The first phase comprised of an approximate one-year establishment period where mesocosms were operating and sustained to ensure microbial communities were in optimal condition. This was confirmed through regular monitoring of water treatment performance, water chemistry conditions, and microbial activity. Phase two consisted of a 12-week period whereby mesocosms were examined more precisely and within temporal boundaries with the objective to glean granular effects of aeration over time. Phase three comprised of a deconstruction phase where mesocosms ceased operation and were disassembled, allowing for internal inspection and analysis at each layer providing a spatial understanding of aeration effects.

### 3.2.2 System Design

Six TW mesocosms divided in two triplicated groups (aerated and non-aerated) were used in this study. The mesocosms were in operation for approximately one year prior to the start of the presented characterization period of the study. This was to ensure microbial communities were well-established resulting in a relative equilibrium of water chemistry and performance parameters. Overall system design was adapted from (Weber et al., 2008). Mesocosms were constructed from clear PVC cylinders with a total volume of 30 L (61 cm height × 25 cm diameter) (Fig. 1). Pea gravel (1-3 cm) was filled to approximately 55 cm of cylinder length. Microbial communities were introduced in the TW through activated sludge (0.5 L/mesocosm) from a local aerobic digestion wastewater treatment plant (Cataraqui Bay Wastewater Treatment Plant, Kingston, ON). Sludge was applied during the gravel filling stage whereby layers were applied throughout the system (depths of 15, 30, and 45 cm). Once mesocosms were in operation, all systems were filled with simulated wastewater (Weber and Legge, 2011). Mesocosms were saturated and the wastewater was constantly recycled as a continuous flow. The systems recycled wastewater at an average flow rate of 3 L/min (water was circulated approximately every 3-4 minutes) with a hydraulic retention time (HRT) of seven days in both aerated and non-aerated systems. Mesocosms were subsequently drained each week of the study (seven-day HRT). Pore volumes within the mesocosms were obtained throughout the experiment using a drainable porosity method prior to nutrient solution renewal. Using this method, mesocosms were topped off with tap water just below the overflow port, drained completely from the outlet port and the drained water was subsequently weighed. Wastewater was circulated using a magnetic drive centrifugal pump (1/200 HP, 3200 rpm, 1A-MD-1/2, March Pumps, Glenview, IL). A distribution tee (1.3 cm diameter) was located approximately 5 cm below the surface of the TW which delivered wastewater at the top of the systems. Simulated wastewater comprised of ~1 g/L molasses yielding a Chemical Oxygen Demand (COD) of ~500 mg/L and a COD:N:P ratio of ~100:5:1. Evaporation of each system was measured each day following nutrient renewal by weighing the amount of water that was used to top off the overflow port at the start of each subsequent day signifying the amount of water lost per day. Figure A.1 presents a picture of the mesocosms arranged in the laboratory.



**Figure 3.1** - Schematic of the non-aerated and aerated TW mesocosms. Water is circulated by a pump located at (A), flowing vertically to the sampling port (B). The sampling port is used for contaminant and simulated wastewater loading. The water then enters a distribution tee (C) flowing vertically through the wetland. In the aerated configuration, an aeration stone is placed at (D) providing forced air throughout the mesocosm.

### 3.2.3 Water treatment analysis

Water chemistry measurements included ammonium-nitrogen ( $\text{NH}_4\text{-N}$ , mg/L), electrical conductivity ( $\mu\text{S}/\text{cm}$ ), dissolved oxygen (mg/L), oxidation-reduction potential (ORP) (mV), pH, and water temperature ( $^{\circ}\text{C}$ ) obtained from a YSI Professional Plus (YSI Inc., Yellow Springs, OH) probe. The probe was inserted into the sampling port of each TW allowing for interaction with interstitial water, as well as the simulated wastewater tank. After 2-5 min of stabilization, measurements were recorded and the process repeated at approximately the same time per day, five times a week (Table A.1 – A.3). Water treatment performance was determined using Total Organic Carbon (TOC) and Total Nitrogen (TN) analysis. Samples of interstitial water for TOC/TN removal analysis were retrieved from the sampling port (at 15, 30, 45, and 60 min) post renewal as well as the simulated wastewater tank (0 min). Additional samples were taken once per day at approximately the same time until 7 days post system renewal. All samples were maintained in a freezer (-

4°C) until thawed immediately before analysis. TOC/TN analysis was accomplished using an Analytik Jena TOC/TN analyzer (multi N/C series, Germany).

### **3.2.4 Microbial Analysis**

#### *3.2.4.1 Microbial activity*

Used in previous mesocosm studies (Weber et al., 2011; Weber and Legge, 2011), the FDA assay can indirectly determine microbial activity through the enzymatic cleavage of acetate groups in the fluorescein-diacetate (FDA) molecule via hydrolysis. The remaining fluorescein (FL) molecule produces fluorescence and can be measured photometrically. The FDA assay was performed bi-weekly three days post wastewater renewal (Farooq, 2024; Ogilvie, 2017). One mL aliquot of 5 mM FDA solution was inserted approximately 1-2 cm into the interstitial water through the sampling port of each mesocosm. Samples were taken every minute post-injection for 30 min. Each sample was immediately analyzed with a handheld fluorometer (Turner Designs, Picofluor™) using an excitation wavelength of 490 nm and emission wavelength of 520 nm. An FDA utilization rate was calculated by extracting linear slope values between 8 and 15 min (Farooq, 2024; Ogilvie, 2017). Final FDA rates for aerated and non-aerated systems were determined using average of slope values.

#### *3.2.4.2 Microbial function*

Similar to FDA, CLPP sampling and analysis was conducted bi-weekly (alternating from FDA) over the course of the study and performed three days post wastewater renewal. 50 mL of an interstitial water sample was collected from each mesocosm and promptly inoculated (within 3 h). The samples were brought to an aseptic area sterilized with 70% reagent alcohol. Each sample was first emptied into a 9 cm petri dish, then 100 µL of the sample was inoculated using a multi-channel pipette into each EcoPlate™ well with each mesocosm sampled on its own EcoPlate™. The plates were then incubated in the dark at approximately 20°C (room temperature). EcoPlates™ were then read photometrically using an EON microplate reader coupled with a Biostack plate stacker and Gen5 microplate reader software. at a regular interval (every 4 hours) until it reached an end of incubation time of 96 hours. The microplate reader would shake each plate for 3 sec to ensure sample was adequately mixed, then read each plate at an absorbance setting of 590 nm.

#### 3.2.4.3 *Microbial structure*

Supplementing the CLPP methodology used to determine microbial function, 16S rRNA metagenomic sequencing was used to elucidate microbial structure and provide a holistic understanding of microbial communities within the wetland. 50 mL interstitial water samples from each wetland were taken immediately after CLPP sample collection to ensure a similar microbial matrix was assessed where community function could be compared with its structure. Upon collection, the samples were filtered through 0.22 µm filter paper (Millipore, Bedford, MA) in a sterile vacuum filtration process. Filter papers were then stored in sterile centrifuge tubes and placed in a -80°C freezer until extraction.

Upon completion of the study, DNA was extracted and sequenced using the methodology in Silveira et al., (2022) and Ruppelt et al., (2020). Filter papers were extracted in a single batch using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) according to manufacturer protocol. DNA concentrations were then quantified using a Qubit fluorometer and Qubit dsDNA HS assay kit (Invitrogen, ON).

The variable V3 and V4 regions of the 16S rRNA gene (16S rRNA) were amplified using the Illumina 16S Metagenomic Sequencing Library Preparation guide (version B, Illumina Canada, Victoria, BC) including amplification and bead purification. Sample library concentration was measured using the Qubit fluorometer. Samples were each normalized to 4 nM, pooled together, denatured, and diluted to 4 pM before sequencing with the MiSeq Reagent v3 600 cycle kit on the MiSeq (Illumina Canada, Victoria, BC) generating 2 x 300 bp reads. A positive control 10% PhiX control library to improve sequencing quality.

De-multiplexed “fastq” files were analyzed using QIIME2 version 2021.11 (Caporaso et al., 2010) on VirtualBox (version 7.0) to analyze the 16S rRNA sequences. To ensure adequate quality control, sequences were filtered, denoised, merged, and chimeras were removed using DADA2 (Callahan et al., 2016). Sequences were classified using the Greengenes database 13\_8 (99% operational taxonomic units (OTUs) full-length sequences). Estimated alpha diversity metrics included Shannon’s diversity index, and observed OTUs.

#### 3.2.4.4 *Principal component analysis*

Principal component analysis (PCA) was employed through R Studio (R Core Team, 2020) using the covariance (n-1) matrix of CSUP data to determine differences and trends between mesocosms and over time. As per the procedure in Weber et al. (2008), data were transformed using the natural logarithm method to

ensure normality, homoscedasticity, and linear correlations were within appropriate range.

### 3.2.5 Deconstruction

Following completion of Phase two of the study, mesocosm operation was ceased and the systems were deconstructed (Figure 3.2). This provided access to the gravel substrate for spatial analysis of the TW mesocosm and granular biological characterization. Mesocosms were drained of wastewater, and gravel was deposited according to layer (Fig. B1) on a drop sheet. Microbial communities on gravel surfaces were detached using the protocol in Weber and Legge (2010). 25 g gravel samples were obtained from each layer, and shaken for 3 h at 30°C while in a 10 mM phosphate buffer solution (PBS). The PBS containing the detached biomass was then used as the microbial matrix for TOC/TN analysis using the protocol in Weber and Legge, 2010, CLPP and DNA analysis, as well as analyzing organic content on gravel surfaces.



**Figure 3.2** - Deconstructing mesocosms in the laboratory

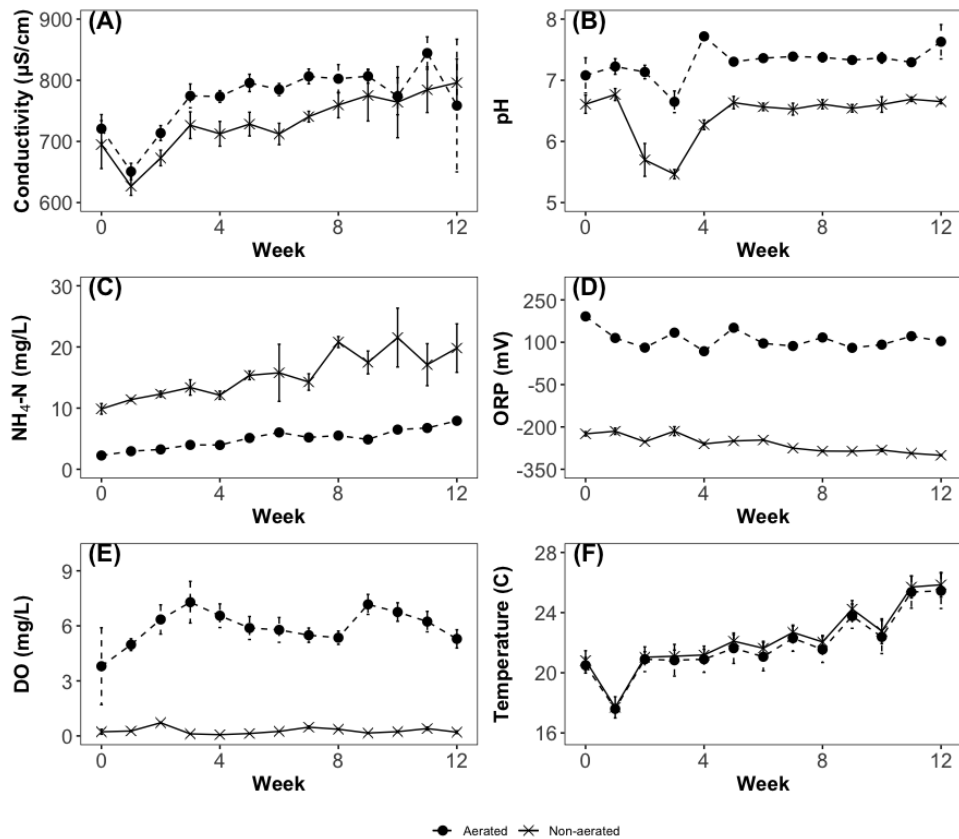
As biofilm covered gravel surfaces, organic content analysis was used to determine mass of organic content and subsequently, the amount of biofilm growth per mass of gravel when compared between aerated and non-aerated systems. The protocol from Weber and Legge, 2010 was used to determine organic content. Adapted from that study, approximately 10 g of gravel at each mesocosm layer were assessed as the basis for organic content analysis. The 10 g samples were first dried in an oven at 105°C for 24 h. The samples were then heated in a muffle furnace at 550°C for 15 min, which resulted in a mass loss from the sample, considered as the organic content (volatile solids) on each dry gravel sample.

### **3.3 Results and Discussion**

#### **3.3.1 Water chemistry parameters**

A start-up period of approximately one-year was used to establish both the aerated and non-aerated TW mesocosms to ensure a stabilized microbial function and activity. Following the establishment period, water chemistry was tracked in all TW mesocosms for 12 weeks (Figure 3.3). Data separation between aerated and non-aerated systems identified clear variation in environmental conditions. Conductivity, pH, ORP, and DO was higher in aerated systems than non-aerated, whereas ammonium-nitrogen was lower in the aerated systems. Temperature remained similar between the two system types. Although aerated systems were shown to have a slightly lower temperature, the difference between aerated and non-aerated temperatures were visually observed to be negligible. The overall trend identified both systems maintaining a relative stability throughout the 12-week study. This can point to TW mesocosms (when established) operating consistently over time. The gradual increase in temperature from week 0 to week 12 can be attributed to an increase in ambient conditions in the laboratory. The study began during the winter season (January) with the mesocosms placed in a laboratory without precise temperature control. The study ended in the Spring (April) whereby the ambient temperature naturally increased. This increasing trend was also observed in ammonium-nitrogen and conductivity and is likely related to the increase in temperature. Higher DO and ORP in the aerated systems can be attributed to aeration forcing highly aerobic conditions. This can then result in a redox environment whereby nitrification is the preferred microbial process for wastewater treatment

allowing for nitrifying bacteria to take advantage of the aerobic environment for ammonium conversion to nitrate. This therefore results in less ammonium-nitrogen concentration in the aerated systems as observed in Figure 3.3. Higher pH in the aerated systems can possibly be attributed to gas bubbles in the aerated systems purging carbon dioxide into solution as well as an overall diminished ammonium ion concentration (due to nitrifying conditions) as compared to the non-aerated systems.

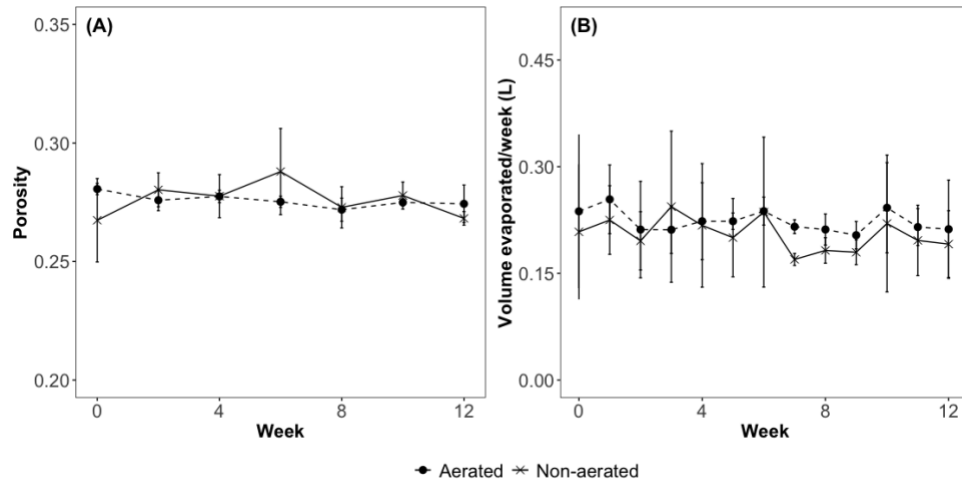


**Figure 3.3** – (A) Water chemistry parameters consisting electrical conductivity, (B) pH, (C) ammonium-nitrogen, (D) oxidation-reduction potential, (E) dissolved oxygen, and (F) temperature in degrees Celsius. Dashed lines represent aerated systems, solid line indicate non-aerated systems.

Figure 3.4 highlights the hydrological parameters where porosity and evaporation were tracked. Both parameters were consistent each week in both aerated and non-aerated systems. Evaporation was consistent, with aerated systems resulting in higher evaporation, though not statistically significant. This slight



difference can be attributed to gas exchange effects at the air/water interface caused by increased air movement against water flow resulting in greater heat loss in aerated systems and increased evaporation (Abdelrahman and Boyd, 2018; Nivala et al., 2022). As regards porosity, it is likely that aeration could have sheared biofilm repeatedly throughout the study resulting in similar porosity each week as well as similarity to the non-aerated system.



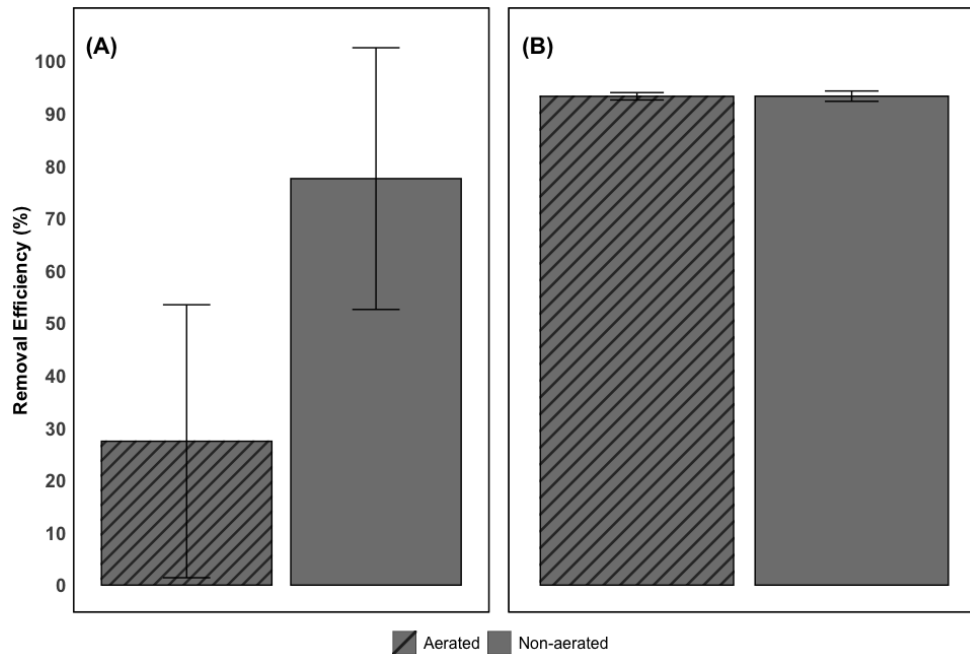
**Figure 3.4** - Porosity (A) and Evaporation (B) where the y-axis represents an average of the amount of water evaporated from the systems throughout the 7-day recycle. Aerated lines are dashed, non-aerated are solid

Overall, system parameters for both aerated and non-aerated mesocosms further point to the TW to separate environmental conditions by the introduction of artificial aeration, while simultaneously highlighting its consistency in maintaining those respective conditions for an extended period of time.

### 3.3.2 Water treatment performance

Figure 3.5 illustrates the removal efficiencies for TOC and TN as water treatment indicators, where Figure A.2 highlights TOC/TN kinetic profiles prior to the study period. No significant visual differences were observed in TOC removal between aerated and non-aerated systems. Furthermore, the non-aerated system results also emphasized data consistency and stability each week due to its minimal error bars for both TOC and TN removals alluding to reliable water treatment performance. Aerated systems showed lower removal efficiency (~25%) for TN as compared to non-aerated systems (~75%). This contrasts with Boog et al., (2014) who saw higher TN removal in aerated systems (intermittent). This can be explained whereby ammonium-nitrogen in a highly aerobic and nitrified condition in aerated

systems (producing nitrate) may not release completely from the system resulting in lower overall TN removal efficiency. This is in contrast to non-aerated systems, which are more denitrifying and thus result in nitrogen gas (thus more total nitrogen) leaving the system.

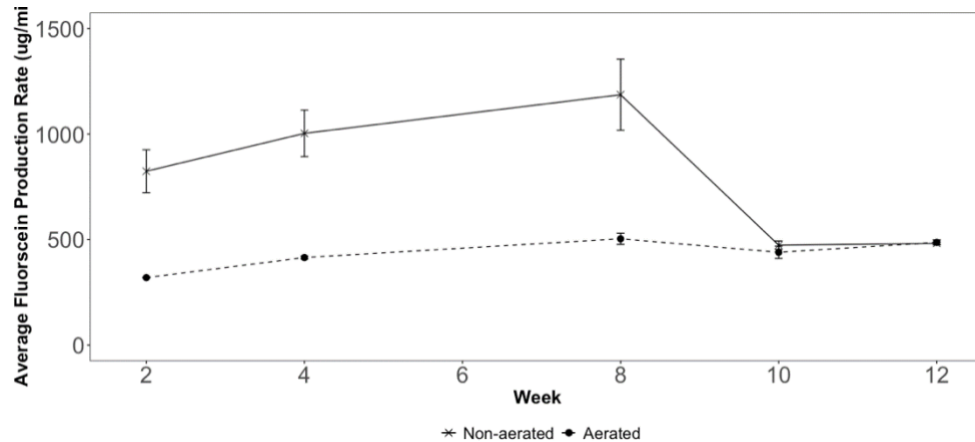


**Figure 3.5** - (A) Average removal efficiencies for Total Nitrogen and (B) Total Organic Carbon for aerated and non-aerated systems

### 3.3.3 Microbial characterization – temporal

Both interstitial and gravel-associated microbial communities were used to characterize the aerated and non-aerated TW systems. Overall microbial activity obtained through FDA hydrolysis (Figure 3.6) whereby aerated systems resulted in lower fluorescein production (~433 ug/min) over the course of the study as compared to non-aerated systems (~794 ug/min). While lower fluorescein production correlates to lower microbial activity (through enzymatic cleavage), the reality is that due to the scarcity of oxygen in the non-aerated systems, the anaerobic bacteria are conditioned to find alternative metabolic pathways. Once FDA enters the interstitial water, microbes are seen to use the molecule as an energy source much quicker based on the reduced environment as opposed to aerobic bacteria in aerated systems which are abundant in oxygen molecules. While FDA hydrolysis does still occur in aerated systems, the advantage for microbes to do so is much less than in

non-aerated systems. Both TW systems types were seen to maintain this trend of activity throughout the study emphasizing the stability of established TW systems.



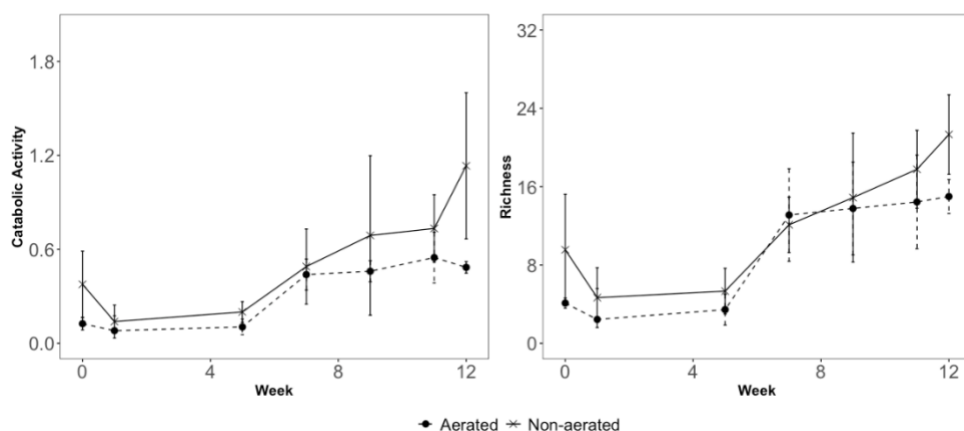
**Figure 3.6** - Average overall metabolic activity captured through FDA hydrolysis for aerated and non-aerated systems. Aerated systems are dashed, non-aerated systems are solid

Community Level Physiological Profiling (CLPP) has been used as a methodology in gravel substrate wetland studies to characterize and classify microbial communities based on carbon source utilization patterns (CSUPs)(Weber and Legge, 2010). BIOLOG™ 96-well EcoPlates™ are commonly used to perform CLPP analysis. The plates contain 31 different carbon sources and a blank in triplicate. The wells also contain tetrazolium violet, which is a redox dye indicator. Mixed microbial communities (from interstitial water and gravel-associated samples) are inoculated into each well. The production of NADH through cell respiration reduces the indicator to formazan producing a change in colour that can be measured photometrically over time. The data analysis approach used in (Weber and Legge, 2010) was applied to this study given the significant amount of data produced by the CLPP method. The first step in approaching this data was to determine a consistent time point that would provide for the greatest variance between well responses balanced with a minimal number of absorbance values above 2.0 (indicating non-linear absorbance range), thus providing a data-rich time point used to extract average metabolic activity along with a basis for follow-on multivariate analysis of CSUPs.

The average well colour development (AWCD) is used as a representative parameter of the average metabolic activity over all wells in the microplate, and calculated as the summation of all wells whereby the absorbance reading at each well is subtracted from the absorbance reading of the well without a carbon source (blank). Substrate richness can also be extracted, which measures the number of

different substrates utilized by a microbial population, and calculated as the number of wells with a corrected absorbance greater than 0.25 (Weber 2010).

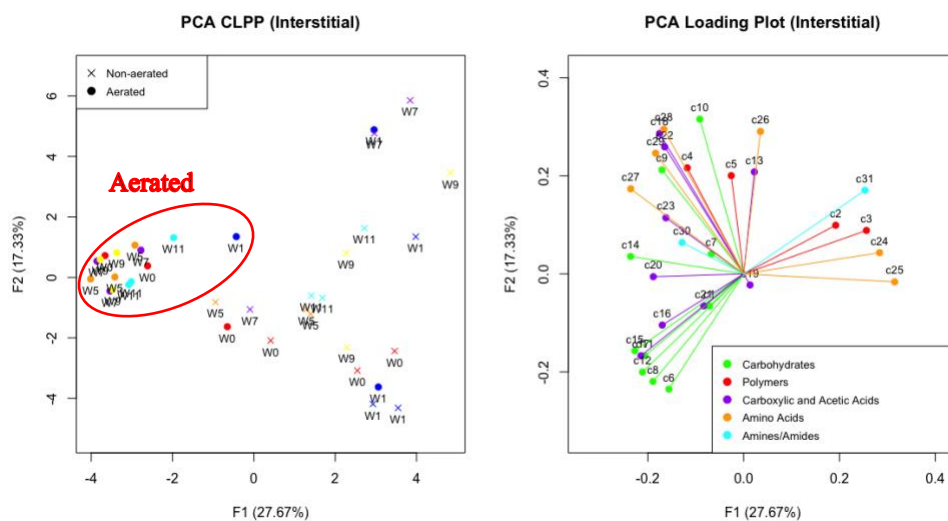
Microbial catabolic activity (AWCD) obtained through CLPP along with substrate richness (Figure 3.7) illustrated lower activity and richness in aerated systems than non-aerated systems. This can follow a similar understanding where the aerated microbes are less active due to their oxygen-abundant conditions allowing for less need to adapt metabolic pathways to changing environment. Furthermore, these aerobic bacteria could have adapted to then use less carbon sources over time thus resulting in less metabolic richness. Both AWCD and richness do increase with time over the course of the study. This could be attributed to the increase in ambient temperature as it follows a similar trend. With increased temperature, the mesocosm conditions could be in a more incubated state resulting in a more active microbial community as opposed to earlier in the study during relatively colder ambient conditions.



**Figure 3.7** - Average catabolic activity expressed through Average Well Colour Development (AWCD) and Substrate Richness. Aerated systems are dashed, non-aerated systems are solid

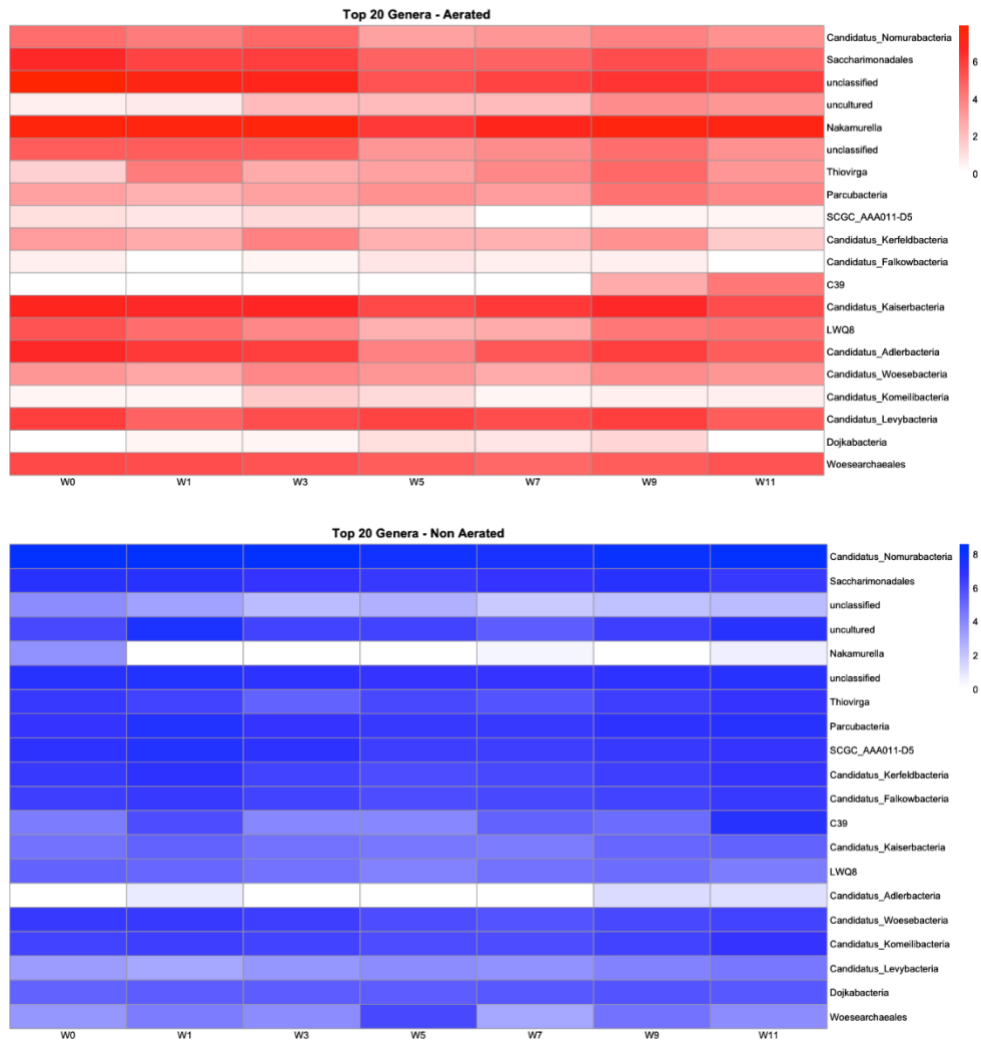
CSUPs using CLPP data from the interstitial water at multiple weeks were assessed throughout the study to elucidate trends in how specific microbial communities within the systems utilize carbon sources. F1 and F2 of the PCA ordination (Figure 3.8) account for 45% of the variation of the microbial community data. A clear separation resulted between aerated and non-aerated systems in principal component 1 where a tight group of systems (aerated) were formed towards the left side of the scores plot. The right side of the model featured the non-aerated systems with greater variation in the F1 and F2 axes. A PERMANOVA analysis on

the loadings plot quantified aerated and non-aerated groupings as statistically significant ( $p < 0.05$ ). Outliers from the aerated grouping were identified in Week 0 (W0) and Week 1 (W1) of the study pointing to an initial stabilization period, which then settles throughout the subsequent weeks. The loadings plot highlights aerated systems preferentially utilizing carbohydrates as carbon sources as well as some carboxylic and acetic acids whereas the non-aerated systems utilizing amino acids and amines. Complementing observations from the non-aerated systems earlier, it can be attributed that the non-aerated microbial communities, given their constant state of adaptation, are more diverse in their carbon source utilization with the loadings plot illustrating a spread of carbon source utilization in both F1 and F2 axes. Furthermore, given that non-aerated systems are anaerobic, it can be expected that microorganisms would be utilizing nitrogen-based carbon sources such as various amino acids and amines as electron acceptors in their metabolic processes due to the lack of oxygen in the system. This is in contrast to the aerated systems, which are highly aerobic where microorganisms easily utilize carbohydrate sources resulting in less varied function. Within mesocosm, PCA plots identify that variation between weeks is minimal in both aerated and non-aerated systems further confirming the overarching takeaway of the stability inherent in TW mesocosms (Figure A.3).



**Figure 3.8** - Principal component ordination for carbon source utilization patterns. Scores and loadings plot sourced from interstitial water microbial communities. Plot depicts samples taken from triplicated mesocosms throughout the 12-week study (each week shaded with a different colour). Ovals placed to highlight clusters. Aerated/non-aerated groupings are significantly different as performed by PERMANOVA ( $p < 0.05$ ).

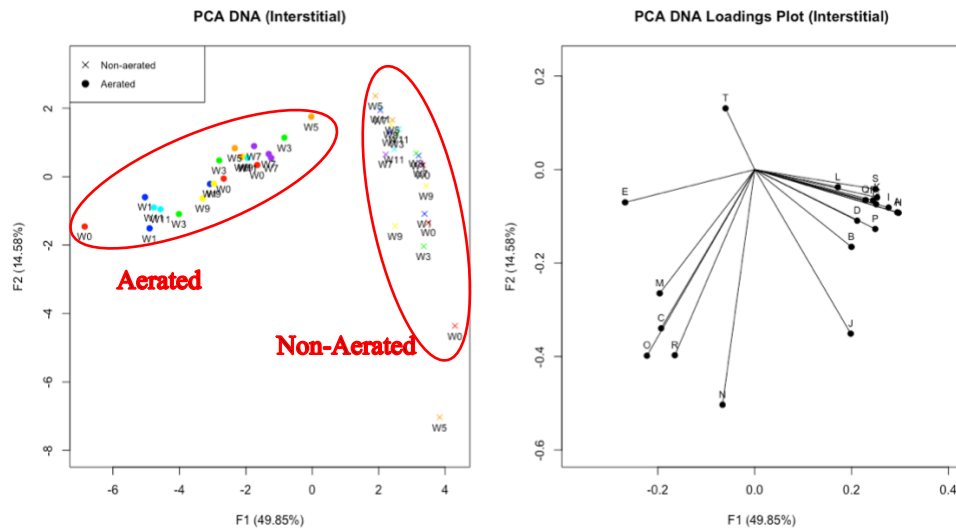
In addition to function and activity, microbial communities were further assessed through their diversity and structure. A taxonomic heat map analysis assessed the top 20 abundant interstitial microorganisms over the course of the study for non-aerated and aerated mesocosms (Figure 3.9). The most abundant taxa in the non-aerated systems were the genera *Candidatus nomurabacteria*, *Saccharimonadales*, and *C39* whereas the abundant taxa in the aerated systems were the genus *Nakamurella*, the order *Saccharimonadales*, and the genus *Candidatus kaiserbacteria*. Although microbial communities evolve and changes are dynamic throughout the weeks, the heat map illustrates no significant trends in abundance as weeks progressed in both aerated and non-aerated systems, which provides yet another line of evidence for TW conditional stability as seen through the lens of microbial diversity. The major visual difference is seen in the quantity of genera in non-aerated systems. When compared to aerated systems, the abundance in non-aerated systems is much higher than in aerated systems. This points to aeration causing a shift in development of microbial communities, where microorganisms vary based on oxygen availability.



**Figure 3.9** - Top 20 most abundant genera for aerated (red) and non-aerated (blue) systems for the duration of the study – each column representing the sampling week. Triplicated mesocosms were averaged.

PCA ordinations were also evaluated using genera abundances to further explore trends in interstitial microbial structure (Figure 3.10). F1 and F2 of the PCA ordination (Figure 3.10) account for 64% of the variation of the microbial community data, with no certain trend of communities shifting with increasing time. When separated into mesocosm specific plots, little variation occurred in both non-aerated and aerated systems. Groupings were seen to be relatively tight each week of sampling, further alluding to mesocosm stability as observed over time. Combined

with the loadings plot, it is identified that the genera at the left side of the origin strongly drive the associated correlation observed with the aerated grouping on the left-hand side of the scores plots. In contrast, the genera grouped towards the right side of the F1 axis in the loadings plot strongly drive the non-aerated variation observed in the right side of the scores plot.



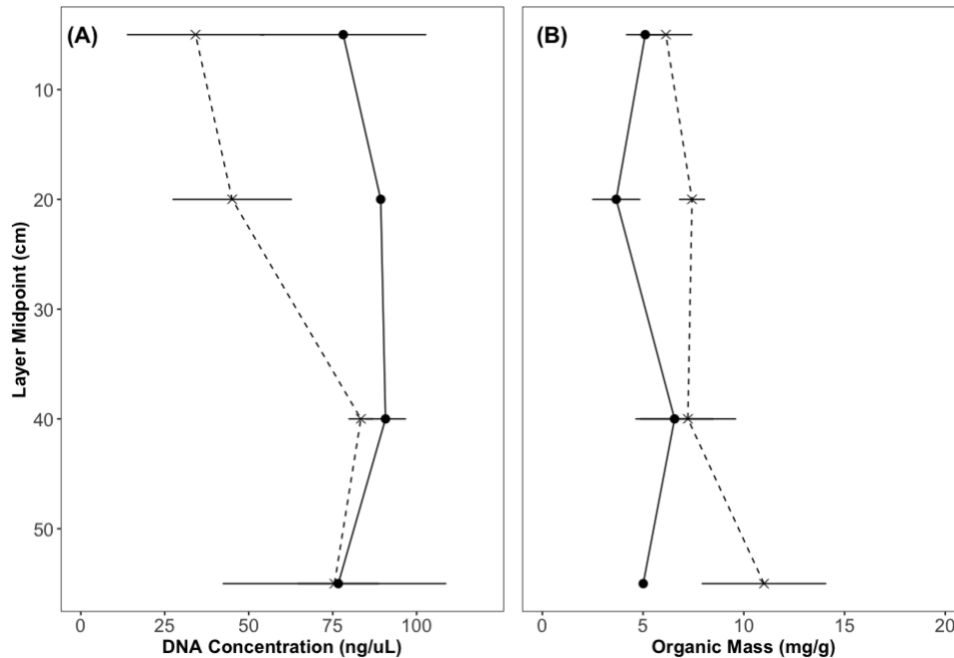
**Figure 3.10** - Principal component ordination for genera abundance data. Scores and loadings plots sourced from interstitial water microbial communities. Plot depicts samples taken from triplicated mesocosms throughout the 12-week study (each week shaded with a different colour). Genera associated by letter in the loadings plot can be retrieved at Table A.4. Ovals placed to highlight clusters. Aerated/non-aerated groupings are significantly different as performed by PERMANOVA ( $p < 0.05$ ).

### 3.3.4 Microbial characterization – spatial

Spatial analysis was conducted during deconstruction phase of the study to assess microbial community diversity, structure, function, and activity resulting from gravel-associated biofilm. DNA concentrations taken at each layer of the mesocosm are presented in Figure 3.11A, highlighting microbial density as a function of depth in both system types. Aerated systems are shown to have less DNA concentration at each depth as compared to non-aerated systems at each layer midpoint. This is correlated with earlier findings where non-aerated microbial community in the interstitial water were observed to be denser than in the aerated systems. There does exist a trend whereby the aerated DNA concentration increases with mesocosm depth. Although the systems are well mixed, this increasing



concentration can likely be attributed to settling of biomass over time resulting in higher microbial density at greater depths.

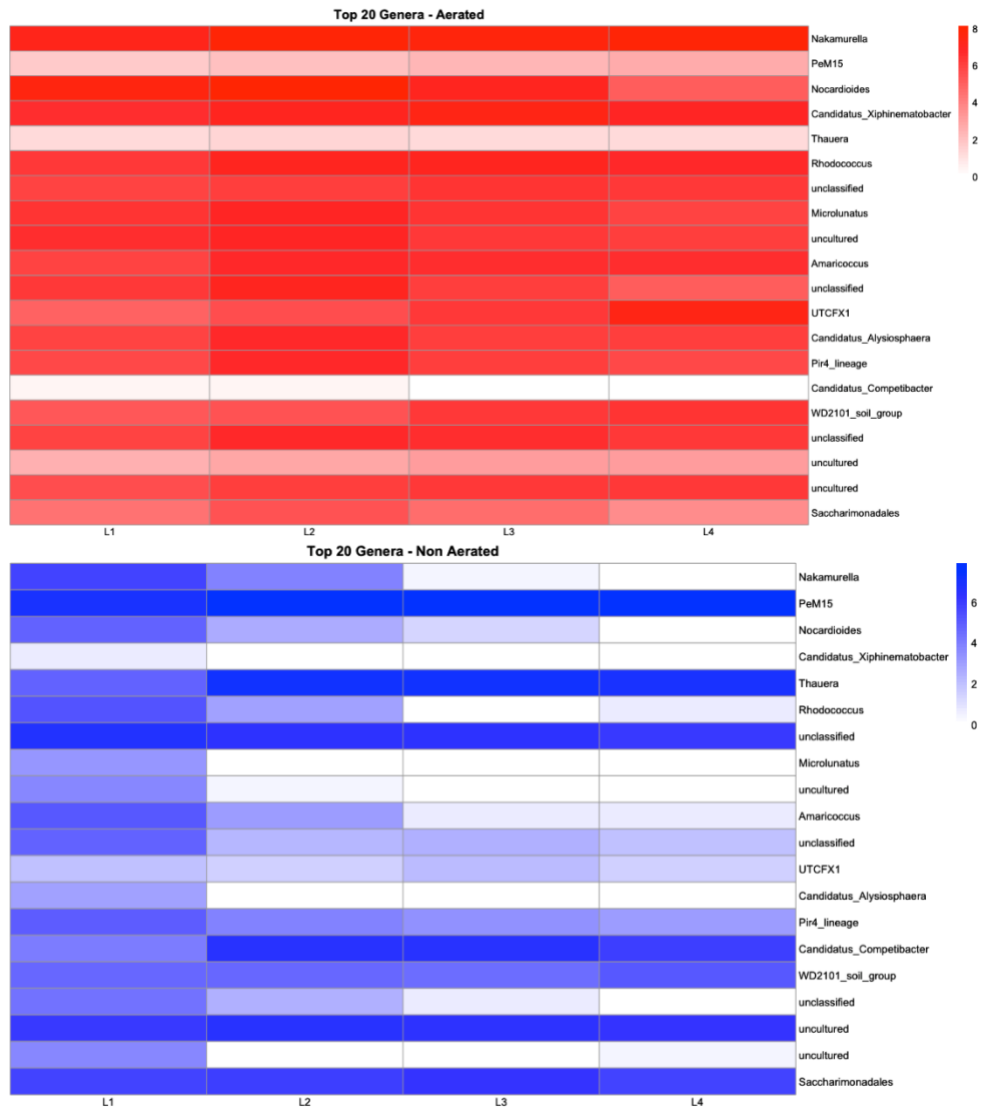


**Figure 3.11** - (A) DNA Concentration taken from gravel-associated biomass at four layers within the mesocosm during deconstruction. (B) Organic content per mass of gravel (mg/g) obtained from deconstruction analysis in aerated and non-aerated TW systems. Layer midpoints are plotted against concentration. Aerated lines are dashed, non-aerated are solid.

These results can be paired with organic content taken per mass of gravel at each depth (Figure 3.11B). Here, organic content is observed to be higher at each layer in the aerated systems, also increasing with depth. Analysing these lines of evidence, it can indicate that although aerated systems have less microbial density as compared to non-aerated systems, their metabolic functionality is much more efficient given the highly oxidative redox conditions resulting in greater organic mass. Further down the mesocosm, a combination of biomass settling as well as layer proximity to the aeration stone (as the oxygen source) provides enriched conditions for aerobic microbial communities to flourish and generate organic mass.

A taxonomic heat map assessed abundant gravel-associated microorganisms at each mesocosm layer for aerated and non-aerated mesocosms (Figure 3.12). The most abundant taxa in the non-aerated systems were the genera *PeM15*, *Thauera*,

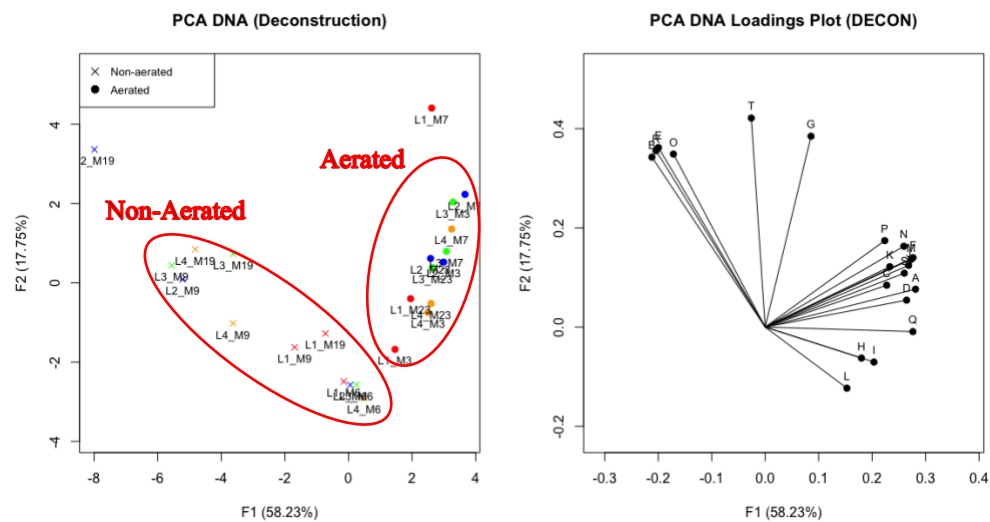
and an unknown genus whereas the abundant taxa in the aerated systems were the genera *Nakamurella*, *Nocardioides*, and *Candidatus xiphinematobacter*. The heat map illustrates no significant change in each layer for aerated systems. This relative homogeneity in microbial density could be attributed to the consistent mixing characteristics of the aerated mesocosms. As gas bubbles flow throughout the systems, it ensures a highly aerobic environment at all depths of the system thus causing a somewhat uniform distribution of microorganisms. This is in contrast to the non-aerated systems, which do not have the additional mixing caused by forced aeration. However, a greater abundance at the top layers was observed, which can be attributed to two main reasons. The first is the methodology of presenting abundance data in heatmap form is done collectively with aerated and non-aerated systems evaluated using the same genera. This is used to compare systems in a normalized method. In doing so, and with the understanding that the aerated systems are higher in density, false intensities can be observed when non-aerated systems feature areas that are more aerobic thus permitting more aerobic microorganisms to be reflected in the analysis. In the case of the non-aerated system resulting in greater microbial density at the top of the system, it is likely that the non-aerated system having its surface open to the environment results in oxygen diffusion from bulk air towards the top layers of the system. Thus, the top layer is thought to have characteristics of an aerobic environment due to more oxygen at that level in the system and therefore resulting in a higher intensity of aerobic genera near the top as opposed to the rest of the layers, deemed to be more thoroughly anaerobic. This points to aeration causing a shift in development of microbial communities, where microorganisms vary based on oxygen availability.



**Figure 3.12** - Top 20 most abundant genera at each deconstructed layer for aerated (red) and non-aerated (blue) systems for the duration of the study – each column representing the sampling week. Triplicated mesocosms were averaged.

Using abundances from gravel associated genera at each layer, F1 and F2 of the PCA ordination (Figure 3.13) account for 76% of the variation of the microbial community data. Similar to the interstitial PCA models, clear groupings were identified separating aerated and non-aerated taxa across principal component 1 and 2. In the deconstructed model, non-aerated systems were grouped towards the left

side of the plot, whereas aerated systems were grouped towards the right side. A PERMANOVA analysis on the loadings plot highlighted genera groupings between aerated and non-aerated systems that was statistically significant ( $p < 0.05$ ). While individual layers were analyzed, coherent trends were not observed between them. The results further confirms that microbial communities are varied between aerated and non-aerated systems, however from a diversity perspective, the communities seem quite varied despite layers. This can be attributed to the fact that the systems, being saturated with organics, are well-mixed in terms of pump flow dynamics preventing any layering patterns or variation in taxonomy.



**Figure 3.13** - Principal component ordination for genera abundance data. Scores and loadings plots sourced from gravel-associated microbial communities obtained during deconstruction analysis. Plot depicts samples taken from triplicated mesocosms throughout the 12-week study (each layer shaded with a different colour). Genera associated by letter in the loadings plot can be retrieved at Table A.4. Ovals placed to highlight clusters. Aerated/non-aerated groupings are significantly different as performed by PERMANOVA ( $p < 0.05$ )

### 3.4 Conclusion

This study explored fundamental questions of water chemistry, treatment performance, and spatial and temporal effects on microbial communities that characterize aeration technology in TW. Using established TW mesocosms, systems were monitored for a 12-week period with weekly wastewater loading to analyze temporal effects on water chemistry, treatment performance, and microbial

communities. Systems were subsequently deconstructed following the 12-week period to assess spatial effects of these parameters. Statistical analysis including PCA was used to elucidate deeper trends between aerated and non-aerated microbial community structure, function, and activity.

Water chemistry parameters such as conductivity, pH, ORP, and DO were higher in aerated systems than non-aerated, whereas ammonium-nitrogen was lower in the aerated systems. Higher ORP and DO are explained through the more oxygen-rich environment of aerated systems while increased pH is likely due to increased calcium bicarbonate concentration through a carbonate cycle mechanism given the presence of limestone in the gravel reacting with increased saturated carbon dioxide delivered through forced air. Temperature remained similar between the two system types, with both systems increasing over time largely attributed to ambient conditions correlating with seasonal temperature increase. A key insight was the stability in water chemistry parameters expressed in established aerated and non-aerated TW given that regular organic loading occurred throughout the 12-week study. This points to TW resiliency and consistency in water treatment performance over time. Porosity and evaporation as hydrological indicators were tracked throughout the study and remained consistent throughout the study between aerated and non-aerated systems. TOC and TN were tracked as water treatment performance indicators. Based on visual observation, no significant differences were expressed in TOC removal between aerated and non-aerated systems. Aerated systems highlighted lower removal efficiency TN as compared to non-aerated systems largely due to the highlight nitrified aerated system retaining total nitrogen concentration within the system.

Based on CLPP analysis, microbial catabolic activity, along with substrate richness, illustrated lower activity and richness in aerated systems than non-aerated systems. This can be explained by aerated microbes that are less active due to their oxygen-abundant conditions potentially resulting in less need to adapt their metabolic pathways to a changing nutrient environment as well as an adaptation to use less carbon sources over time thus resulting in less metabolic richness. Furthermore, PCA analysis of CLPP data shows that non-aerated systems, in their constant state of adaptation, are more diverse in their metabolic function with a spread of carbon source utilization. This is in contrast to aerated systems, which are less varied in function. PCA plots further expresses that variation between weeks was minimal in both aerated and non-aerated systems further confirming that established TW are stable over time. For microbial community structural data, no significant change, as visually observed through DNA abundance, was determined temporally in both aerated and non-aerated systems. When compared to aerated systems, the abundance of genera in non-aerated systems was much higher than in aerated systems pointing to an aeration effect in the development of microbial communities.

Following the spatial characterization of microbial communities and organic mass of biofilm at each layer of the system, it was observed that aerated systems are shown to have less DNA concentration at each depth as compared to non-aerated systems which aligns with (Silveira et al., 2022) where non-aerated microbial community in the interstitial water were observed to be denser than in the aerated systems. In the gravel-associated biofilm analysis, aerated DNA concentration increased with mesocosm depth, largely resulting from the settling of biomass over time. No visual significance of DNA abundance was observed in each layer for aerated systems, largely pointing to consistent mixing characteristics of the aerated mesocosms. The greater microbial density at the top of non-aerated systems likely results from surface effects whereby the system is open to the environment resulting in oxygen diffusion from bulk air towards the topmost layer of the system, thus affecting structural dynamics. PCA analysis of the deconstructed system indicated separation based on aeration and non-aeration whereas individual layers did not express coherent trends or separation. The results confirmed that microbial community structure varied between aerated and non-aerated systems, however from a diversity perspective, the communities were not varied throughout layers. This can be attributed to the fact that the systems are well-mixed preventing any layering patterns or variation in taxonomy.

## **4 PFOS, PFOA, and 6:2 FTS in Treatment Wetland Mesocosms: Fate and Effects on Water Treatment Performance**

### **4.1 Introduction**

Per- and polyfluoroalkyl substances (PFAS) have gained considerable attention as emerging contaminants due to their environmental recalcitrance, widespread detection in various media, and potential human health impacts (Ahrens and Bundschuh, 2014; Buck et al., 2011). PFAS are used in a variety of industrial and consumer applications, such as non-stick coatings, stain-resistant fabrics, food packaging, and aqueous film-forming foam (AFFF) (Høisæter et al., 2019; McGarr et al., 2023; Prevedouros et al., 2006). AFFFs have been used extensively in fire-fighting training activities on military bases and airports since the 1960s (Milley et al., 2018). PFAS-based AFFF formulations have evolved over time, initially comprised of a mixture of perfluoroalkyl acids (PFAAs) such as perfluorooctanesulfonic acid (PFOS) and some perfluorooctanoic acid (PFOA), then incorporating and migrating to the inclusion of fluorotelomer substances such as 6:2 fluorotelomer sulfonate (6:2 FTS), FTAoS X:2 telomers, and other FTBs and FTSABs (Houtz et al., 2013; Patch et al., 2024). PFAS have physiochemical properties similar to surfactants due to their amphiphilic structures, which result in a higher sorption capacity onto natural media (e.g., soil and gravel), and an affinity to partition onto air-water interfaces, which affect their transport in groundwater and surface water environments (Brusseau et al., 2019; Lyu et al., 2018). The ubiquitous use of PFAS-based AFFF applied directly into the environment has resulted in increased PFAS concentrations in soil and groundwater (Houtz et al., 2013). Furthermore, AFFF-contaminated runoff into wetland ecosystems are likely given that wetlands occupy 13% of the Canadian landscape (“Water sources: wetlands - Canada.ca”). Although many remediation strategies are being evaluated to determine their effectiveness and sustainability in addressing PFAS contamination, treatment wetlands (TW) have received limited assessment in this context.

Treatment wetlands (TW) are engineered systems designed to treat wastewater using physical, chemical, and biological processes observed in natural wetland environments (Kadlec and Wallace, 2009). Primary removal mechanisms in TW are often observed to be microbiological degradation and sorption due to a vast network of biofilms situated on gravel surfaces that contain organic matter with a high interfacial area (Brix, 1994; Vymazal, 2007; Weber and Gagnon, 2014). Intensified designs, such as artificial aeration, have also been employed in subsurface TW systems. Artificial aeration creates an aerobic environment and

controls the redox conditions, as well as increases microbial growth to increase pollutant removal rates compared to non-aerated systems (Nivala et al., 2020). While aeration has resulted in effective contaminant removal rates, the shearing of effective biofilms by forced air flow can limit performance (Nivala et al., 2019).

In addition to the treatment of conventional contaminants, the effectiveness of TW in treating emerging contaminants has been investigated with successful outcomes (Ávila et al., 2014; Button et al., 2019b; Matamoros and Bayona, 2008). However, deeper investigation into TW fate and performance as it pertains to PFAS contamination specifically is still needed. This includes having comprehensive insight into the ability of TW to remove common PFAS, and the potential for the presence of those PFAS to impair the ability of TW to treat conventional wastewater contaminants.

Recent studies of PFAS in subsurface TW support the treatment potential of full-scale TW operating with PFAS contaminated wastewater, with a majority of recent studies having investigated the use of full-scale surface and subsurface flow wetlands to treat municipal wastewater with low PFAS concentrations (ng/L to µg/L) (Lott et al., 2023; Yin et al., 2017). Subsurface TW may be well suited to the treatment of PFAS because of the important role of sorption in TW performance, and because PFAS are known to adsorb to sediments and substrates containing higher percentages of organics (Higgins and Luthy, 2006). This is supported by observations in previous TW studies where PFAS, especially longer chain PFAAs, tend to adsorb to the sediment compartment of wetland systems (Chen et al., 2012; X. qing Li et al., 2021; Qiao et al., 2021; Yin et al., 2017; Zhang et al., 2020). Therefore, subsurface TW that incorporate gravel substrates surrounded with biofilm would be expected to have even greater PFAS adsorption due to the magnitude of organic matter present on gravel surfaces (Weber and Gagnon, 2014). Research into PFAS fate and effects in subsurface TW consisting of gravel media have been identified as a potential issue requiring study.

Aeration may also be advantageous for PFAS removal. Air-water interfaces are a significant source of PFAS retention due to the affinity of PFAS to partition to air-water interfaces (Abraham et al., 2022; Brusseau and Van Glubt, 2019; Ji et al., 2021) and PFAS removal by air-sparging (i.e., air injection) has been proposed for treating contaminated groundwater (Newell et al., 2021). However, despite the increasing use of aeration in TW and the potential role of air-water interfaces on PFAS removal, an understanding of the behaviour of PFAS in a subsurface TW intensified with aeration is missing from existing wetland literature.

The current state of research on TW and PFAS suggests that there is a need for a comprehensive study to collect data needed to understand the fate, impacts, and removal mechanisms of PFAS in aerated subsurface wetlands. These prevailing



limitations can be reduced to two overarching questions: what is the fate of PFAS when introduced into an aerated TW system, and what are the effects on aerated TW functionality and water treatment capability as a result of a sustained, higher concentration PFAS load? This study approached these questions with the following objectives: (1) determine PFAS fate and fixed-film surface effects in gravel substrate aerated and non-aerated TW through adsorption kinetics and mass balance, and (2) characterize and assess TW water treatment performance and environmental conditions when exposed to PFAS concentrations to evaluate TW operational resiliency. In addition to providing insight regarding engineered systems, it is expected that this investigation of PFAS transport and wetland performance will provide valuable information that can be applied more broadly to natural environments. This includes natural wetlands, as well as soil and groundwater systems that share characteristics of the packed-bed design inherent in subsurface TW.

## **4.2 Materials and Methods**

### **4.2.1 System Design**

This study utilized a triplicated  $2^2$  factorial design with mesocosms similar to those used in Weber et al. (2008). The mesocosm-scale approach provides a replicable system designed for understanding TW functionality with insights into the physical, chemical, and biological mechanisms that affect treatment performance. Mesocosm factors were aeration (aerated and non-aerated) and PFAS (exposed and control). The mesocosm systems began operation approximately one year prior to this study resulting in them being in a mature state during the experimental phase. Clear PVC cylinders were used as the mesocosm structure, with a total volume of 30L (61 cm height  $\times$  25 cm diameter) (Figure 3.1) and filled to ~55 cm with pea gravel (grain size ~1-3 cm). In order to initiate microbial communities in the wetland ecosystem, the mesocosms were seeded with aerobic activated sludge (0.5 L/mesocosm) from a local wastewater treatment plant (Cataraqui Bay Wastewater Treatment Plant, Kingston, ON). The sludge was applied in three layers (at depths of 15, 30 and 45 cm) during the gravel filling stage. The mesocosms were drained and batch fed weekly with a simulated wastewater solution mixed with tap water based on Weber and Legge (2011). The simulated wastewater included ~1 g/L molasses yielding a COD of ~500 mg/L and a COD:N:P ratio of ~100:5:1. Mesocosms were saturated and the wastewater was constantly recycled as a continuous flow, batch operated system every 3-4 min. The systems recycled wastewater at an average flow rate of 3 L/min and a hydraulic retention time of seven days in both aerated and non-aerated systems. A magnetic drive centrifugal pump (1/200 HP, 3200 rpm, 1A-MD-1/2, March Pumps, Glenview, Illinois) distributed the

simulated wastewater approximately 5 cm below the surface of the gravel through a distribution tee made from 1.3 cm diameter clear PVC tubing.

#### **4.2.2 Experimental Approach**

The objectives of the study were addressed through three main phases: The first phase (Phase 1) encompassed an approximately one-year establishment period to facilitate the development of robust microbial communities within the mesocosms, concurrent with the monitoring of water chemistry conditions, water treatment performance (through regular wastewater renewal), and hydrological monitoring. The second phase (Phase 2) consisted of an in-situ experiment where PFAS was loaded directly into the systems for 12 weeks, and the fate and effects of PFAS throughout each mesocosm were analyzed. The goal was to determine impacts of PFAS loading on TW performance and observe any variations in environmental conditions due to this exposure. Water chemistry parameters were evaluated each week five days after simulated wastewater renewal as a representative indication of equilibrium conditions during both Phase 1 and Phase 2. The third phase (Phase 3) consisted of a deconstruction, during which the gravel substrate from each mesocosm was removed, and a mass balance was performed to determine the fate of PFAS in separate layers at different depths in the system.

During Phase 2 of the study, a characterization suite consisting of water chemistry, water treatment, and hydrological parameters was used to assess the overall wastewater treatment performance and environmental conditions of each mesocosm. Water chemistry measurements obtained using a YSI Professional Plus (YSI Inc., Yellow Springs, OH) probe were taken five times a week, including at the simulated wastewater tank used to renew the mesocosms. The probe was inserted in the mesocosm sampling port to capture recycled interstitial water. Data was recorded approximately 2-5 mins after placing the probe in the sampling port to allow for stabilization. Measurements included ammonium-nitrogen ( $\text{NH}_4\text{-N}$ , mg/L), conductivity ( $\mu\text{S/cm}$ ), dissolved oxygen (mg/L), oxidation-reduction potential (ORP) (mV), pH, and water temperature ( $^{\circ}\text{C}$ ). Water treatment capability was assessed through Total Organic Carbon (TOC)/Total Nitrogen (TN) analysis together with ammonium-nitrogen concentrations from interstitial water. A TOC/TN kinetic profile was determined based on samples taken at the simulated wastewater tank (0 h) and at the interstitial water of the sampling port at 15, 30, 45 and 60 min post-mesocosm renewal. Samples were also taken once a day at 24, 48, 72, 96 and 168 hour post-renewal. TOC/TN samples were kept in a freezer ( $-4^{\circ}\text{C}$ ) until analysis, where each vial was thawed and subsequently measured using an Analytik Jena TOC/TN analyzer (multi N/C Series, Germany). A drainable porosity measurement was taken each week immediately prior to nutrient solution renewal. Mesocosm porosity is the volume of pore space within the system substrate (initially 10 L)

relative to the total volume of the system (~30 L). The drainable porosity was measured by filling the mesocosm with water to just below the overflow port, and then draining and weighing the water from the bottom of the mesocosm (Table B.1). As the mesocosm operates, biofilm growth, due to its attachment to gravel surfaces, is expected to reduce the void volume, thereby decreasing the drainable porosity.

#### **4.2.3 PFAS Solution Preparation**

The study utilized PFOS, PFOA and 6:2 FTS powders (>97% purity) sourced from SynQuest Laboratories Inc. Stock solutions of 200 mg/L of each PFAS were prepared by dissolving in Milli-Q water. The solutions were stored in 1 L glass bottles and refrigerated (4°C) until use. PFAS loading occurred once per week, where 50 mL of each PFAS solution was pipetted into the mesocosm sampling port approximately 2-4 cm deep into the water to target a system concentration of ~1 mg/L at the beginning of each week. Variation in system concentration was due to changing pore volumes in the mesocosm (Table B.1). PFAS loading occurred one day after the mesocosms were batch fed.

#### **4.2.4 PFAS Sampling and Analysis**

After loading the systems with PFAS, water samples were collected to monitor PFAS concentrations over time (kinetic profile). Samples throughout the 12-week study were collected starting on PFAS loading days. Immediately after loading, samples were taken every 2.5 min for 15 min, every 15 min for 60 min, every 1 h for 4 h, and each day until 7 days after loading. PFAS samples were collected from the sampling port, diluted with basic (0.1% ammonium hydroxide) high performance liquid chromatography (HPLC) grade methanol (CAS 67-56-1) to a target concentration of ~50 µg/L, and refrigerated for future analysis.

Targeted PFAS analysis for PFOS, PFOA and 6:2 FTS was conducted on an Agilent 6460 MS/MS on multiple-reaction-monitoring (MRM) mode coupled to an Agilent 1260 HPLC system using a 50 mm × 2.1 mm × 3 µm Zorbax C18 Plus analytical column and paired guard column with a 5 µL injection volume. Samples were not diluted or filtered before analysis, and were not spiked with internal standards. Non-targeted PFAS analysis was conducted on a ThermoFisher Exploris 120 Orbitrap coupled to a Vanquish ultra-high-performance liquid chromatography (uHPLC) system using a 100 mm × 2.1 mm × 3.0 µm ACME C18 analytical column and paired guard column. Blanks were taken throughout each sampling event, and were all found to be below the 0.1 µg/L PFAS detection limit. Duplicate samples and internal standards (MPFAC-MXA, Wellington Laboratories) were also added throughout analysis runs for QA/QC, and to verify matrix effects.

#### 4.2.5 Deconstruction

During Phase 3, the mesocosms were deconstructed to provide access to gravel surfaces within the mesocosm substrate. During this deconstruction phase, the wastewater recirculation was turned off, the mesocosms were drained, and then immediately emptied onto a drop sheet (Fig. B.1). Gravel was deposited on the drop sheet according to four layers to provide increased resolution of the substrate (Fig. B2). Samples collected from each layer were used for PFAS analysis, a TOC/TN analysis using the detachment protocol outlined in Weber and Legge (2010), and a quantification of organic content. PFAS analysis was conducted on these gravel samples to quantify the PFAS mass adsorbed on biofilm following long-term (12 weeks) sorption, as well as to investigate possible transformation products. Triplicate samples (50 g each) were diluted with 20 mL of basic HPLC grade methanol, agitated on an end-over-end shaker at 65 RPM for 24 h, and diluted with additional basic methanol to reach a suitable concentration for analysis.

Once gravel was emptied from the mesocosms, the inner wall and base of the mesocosms (one aerated and one non-aerated) were sampled as additional sampling points for the mass balance. 5 in x 5 in Kimwipes™ were soaked in 15 mL of basic methanol. The system walls were wiped from the bottom to the top of the mesocosm as well as across the base of the system from left to right. This was to collect any PFAS mass that could have been adsorbed on the inner PVC surfaces. Kimwipes™ from each system were then placed in centrifugal tubes and filled with an additional 20 mL of basic methanol solvent. The samples were then agitated on an end-over-end shaker at 65 RPM for 30 min. The Kimwipes™ were removed from the centrifugal tubes and discarded. The samples were then centrifuged at 4000 rpm for 1 min, then passed through a 0.45 µm filter. The samples were then diluted with additional basic methanol and analyzed for PFAS mass.

#### 4.2.6 Organic and inorganic content

For gravel surfaces covered in biofilm, an organic content analysis can quantify the mass of organic content present after mesocosm operation and be used to approximate the amount of biofilm developed per unit mass of gravel. Using a protocol from Weber and Legge (2010), approximately 10 g of gravel at each deconstruction layer was used for the organic analysis. Dry weight of samples were obtained after the gravel was dried in an oven at 105°C for 24 h. Once the samples were dried, they were evaluated for organic and inorganic content. Organic content (volatile solids) was calculated as the amount of mass lost in the dry weight sample after heating in a muffle furnace at 550°C for 15 min.

## 4.3 Results and Discussion

### 4.3.1 Mesocosm environmental conditions

The TW mesocosms were in operation for approximately one year (Phase 1) before PFAS was loaded to establish vibrant and functioning microbial communities for wastewater treatment (Weber and Legge, 2011). Three weeks prior to PFAS exposure, the mesocosms were assessed based on a characterization suite consisting of water treatment and biological indicators, thus setting a baseline to evaluate PFAS effects. Control mesocosms (i.e., no PFAS loading) were then used to assess longer term variation and compare responses to PFAS contaminated mesocosms over the course of the 12-week experiment. Table 1 illustrates the state of the aerated and non-aerated mesocosms based on water chemistry parameters pre- and post-PFAS loading. Aeration provided an oxygen-rich environment, resulting in higher dissolved oxygen concentrations and a highly oxidative redox potential. The variation between aerated and non-aerated system aligns with previous mesocosm research and indicates that the systems used in this study were operating under normal conditions (Button et al., 2019b). Comparing pre-exposure conditions with post-exposure results in Table 4.1, the effect of PFAS addition on most of the water chemistry parameter was not significant in either the aerated or non-aerated systems. Exceptions were dissolved oxygen (increased) and redox potential (decreased) in the aerated systems. This can point to a possible adverse toxicity effect caused by PFAS contamination towards aerobic microorganisms, however given the lack of literature that has observed a similar result, more investigation would be needed to confirm. Overall, the lack of change in most parameters highlights that TW water treatment performance remained consistent when exposed to PFAS. While still an oxidative, aerobic environment, forced air TW could potentially experience atypical variations in redox potential and dissolved oxygen when accepting PFAS contaminated wastewater. These results show that TW health as it pertains to water chemistry indicators is resilient to PFAS exposure and TW operation can continue despite PFAS addition.

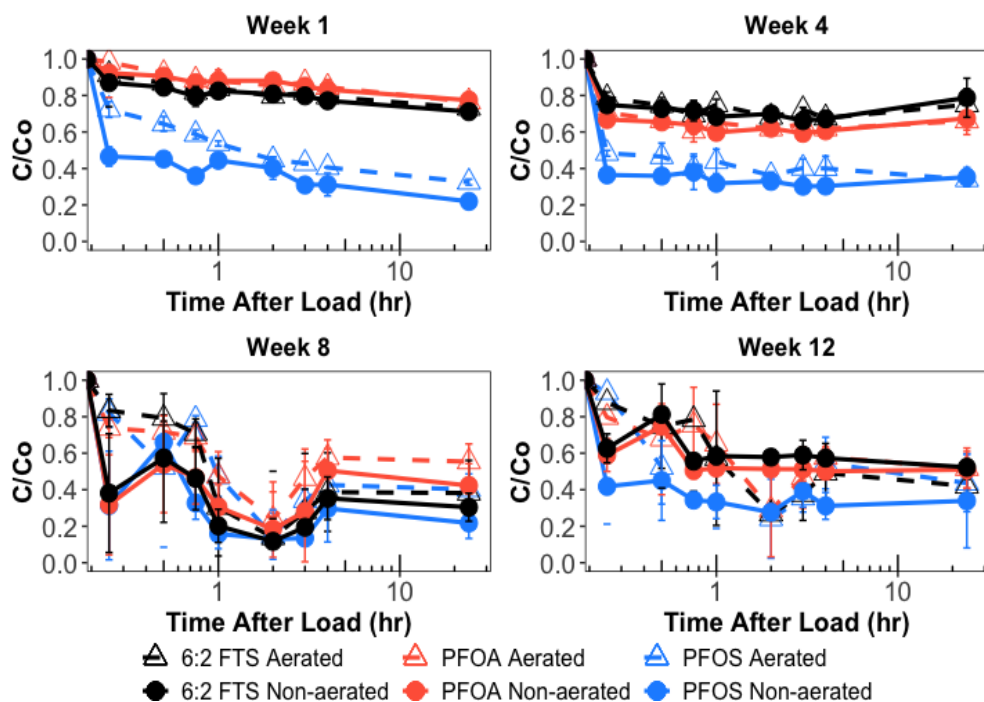
**Table 4.1** - Water chemistry parameters in non-aerated and aerated. “Pre” corresponds to TW three weeks prior to PFAS exposure. “Post” corresponds to five days post PFAS exposure averaged over the 12-week study

Parameter	Aerated		Non-aerated	
	Pre	Post	Pre	Post
Dissolved Oxygen (mg/L)	4.4 ± 0.7	6.2 ± 0.8	0.4 ± 0.1	0.3 ± 0.1
Oxidation-Reduction Potential (mV)	192 ± 1	100 ± 20	-240 ± 16	-264 ± 27
pH	7.26 ± 0.19	7.28 ± 0.18	6.77 ± 0.09	6.59 ± 0.25
Temperature (°C)	21 ± 1	22 ± 2	22 ± 1	22 ± 2
Conductivity (µS/cm)	756 ± 17	765 ± 43	714 ± 17	738 ± 41

#### 4.3.2 PFAS Removal Efficiencies and Rate

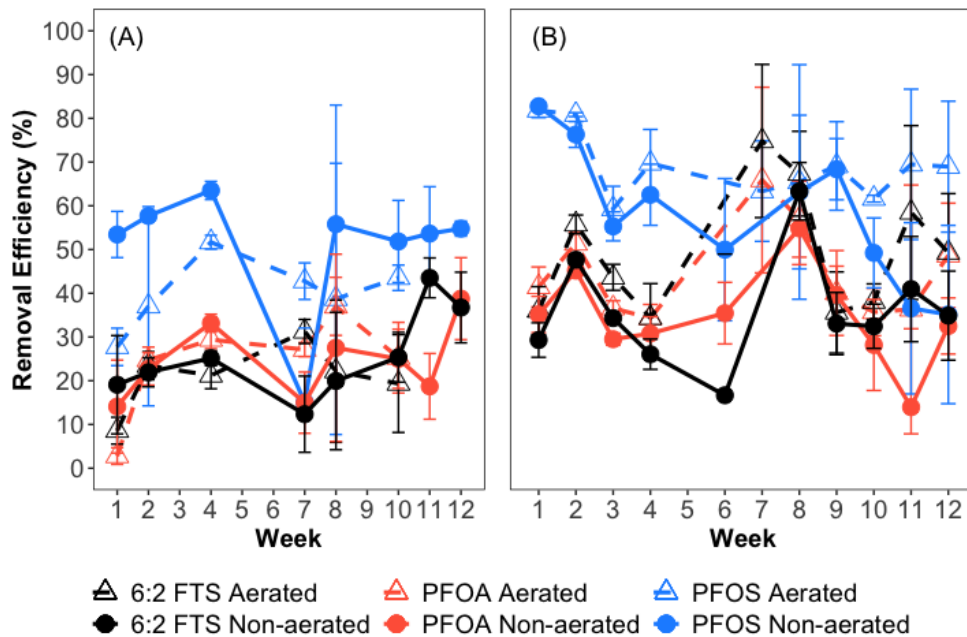
PFAS removal efficiencies in aerated and non-aerated systems were determined to evaluate the feasibility of TW to remove PFAS from contaminated wastewater as well as to discern the effectiveness of aeration as an intensified TW design factor. The concentrations of all three PFAS in the interstitial water decreased throughout representative weeks (1,4,8, and 12) (Fig. 4.1). Furthermore, that decrease was higher (i.e., concentrations were lower) in non-aerated systems than in aerated systems for all three PFAS each week within a 24 h period. (Fig. 4.1).

During all weeks and in all mesocosms, PFOS was removed to a greater degree than 6:2 FTS and PFOA. PFOA and 6:2 FTS removals were similar. The non-aerated systems showed an average of 36 ± 18 % PFOA and 6:2 FTS removal, and 67 ± 11 % PFOS removal within the first 4 h after loading over the course of the experiment (Fig. 4.1). Aerated mesocosms removed 35 ± 15 % PFOA and 6:2 FTS, and 57 ± 9 % PFOS (Fig. 4.1). This confirms that the mesocosms are capable of removing PFAS contaminated wastewater for a sustained period of time. Within a 24 h period, the largest of those removals occurred in non-aerated systems.



**Figure 4.1** - PFAS removal from interstitial water up to 24 h after PFAS loading in aerated and non-aerated mesocosms during weeks 1, 4, 8 and 12

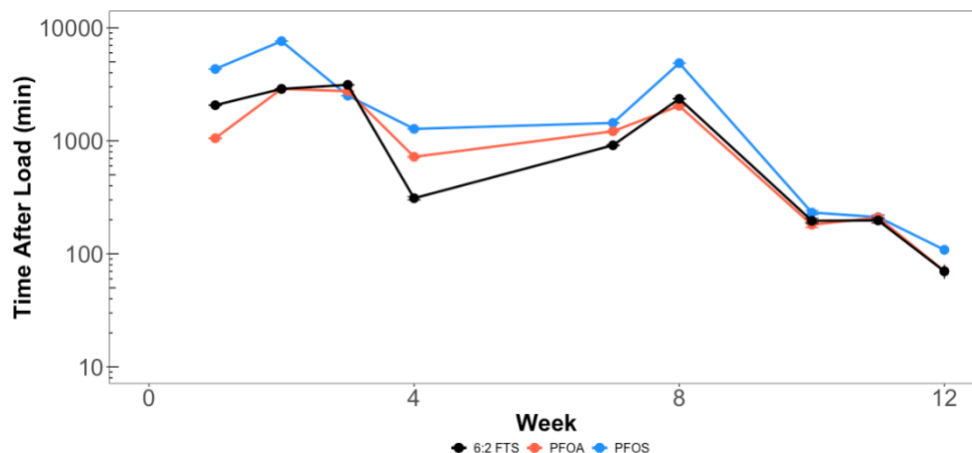
Figure 4.2 illustrates that 6:2 FTS and PFOA removal efficiency followed a similar trend each week, however PFOS means were larger in the interstitial water in both system types during the first 15 min pointing to greater removal and at a faster rate than the other PFAS. Figure 4.2-A highlights how within the first 15 min of exposure, the non-aerated mesocosms remove  $49 \pm 14$  % PFOS, and  $23 \pm 10$  % PFOA and 6:2 FTS, and the aerated mesocosms remove  $39 \pm 8$  % PFOS and  $21 \pm 10$  % PFOA and 6:2 FTS. Variation of removal between aerated and non-aerated systems were not statistically significant. Figure 4.2-B tracks removal after 7 days post exposure resulting in the non-aerated mesocosms removing  $58 \pm 16$  % PFOS and  $37 \pm 12$  % PFOA and 6:2 FTS, and in the aerated mesocosms removing  $69 \pm 7$  % PFOS and  $47 \pm 12$  % PFOA and 6:2 FTS.



**Figure 4.2** - (A) PFAS removal efficiency from interstitial water at 15 min and (B) 7 days after PFAS loading in aerated and non-aerated systems for the 12-week study period.

Overall, it was observed that the rate of PFAS removal was rapid, especially for PFOS. Non-aerated systems had higher removal averages during the initial 15 min of loading, whereas aerated systems had higher removal averages at the 7-day post loading mark. This signified a potential switch in removal efficiency whereby non-aerated systems exhibited fast removal initially (within 15 min), but aerated systems dominated removal for a longer time period (7 days). Given this switch, it was observed that there existed a point in treatment time in which aerated systems removed more PFAS than non-aerated systems. Figure 4.3 highlights the time point after PFAS loading each week where aerated systems switched from non-aerated systems resulting in higher removal efficiency to aerated systems dominating. The overall trend decreased over the course of the study signifying that aerated systems dominated removal quicker after PFAS loading as the experiment progressed. This has longer term implications whereby aeration technology can be seen as more effective with longer treatment time.



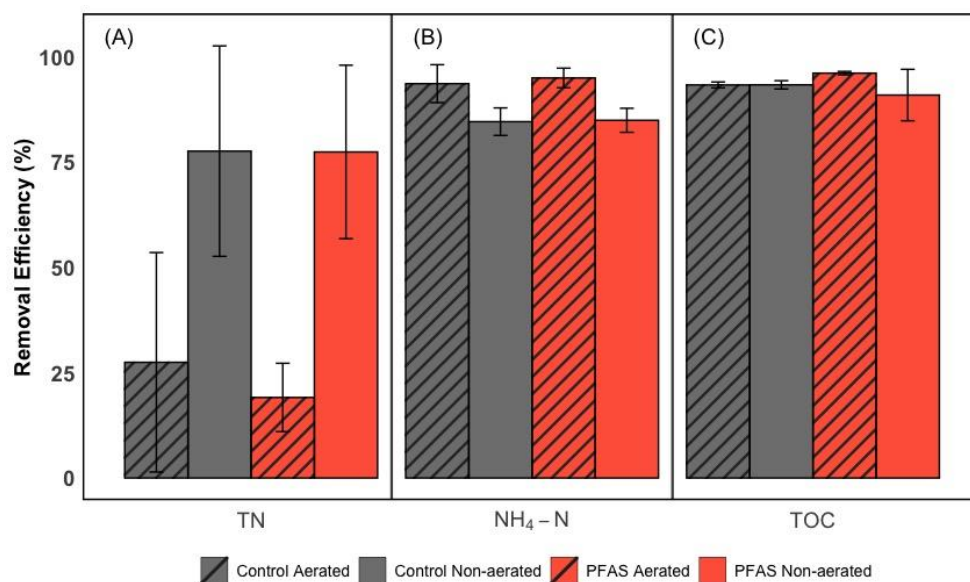


**Figure 4.3** - Time after load for each PFAS after which aerated systems showed higher removal than non-aerated systems

Rapid removal rates of PFAS in interstitial water are likely indicative of the generally strong adsorption potential for PFAS to biofilm on gravel surfaces in the mesocosms. Due to the significant amount of adsorption sites present on biofilm surfaces, the potential for PFAS removal to be driven by sorption is high upon entering the system. Furthermore, when the mesocosms were drained each week and filled with fresh simulated wastewater, it is expected that a majority of the PFAS may have desorbed into the bulk wastewater.

### 4.3.3 Nitrogen and Organics Response to PFAS

Using  $\text{NH}_4\text{-N}$ , TN and TOC removal as further benchmark indicators for water treatment performance characteristics, the results show that the non-aerated and aerated control and PFAS systems maintained removal efficiencies that are consistent with TW despite PFAS exposure (Fig. 4.4). Based on visual observation of the confidence intervals, aerated systems had slightly higher ammonium-nitrogen removal averages than non-aerated systems. Reduced TN removal in aerated compared to non-aerated systems was observed and is consistent with aerated wetland performance, where the highly aerobic environment can limit the denitrification process (Boog et al., 2014).



**Figure 4.4** - Removal efficiencies during the 12-week study of (A) Total Nitrogen (TN), (B) Ammonium-Nitrogen (NH<sub>4</sub>-N,) (C) Total Organic Carbon (TOC) in both PFAS and control TW systems.

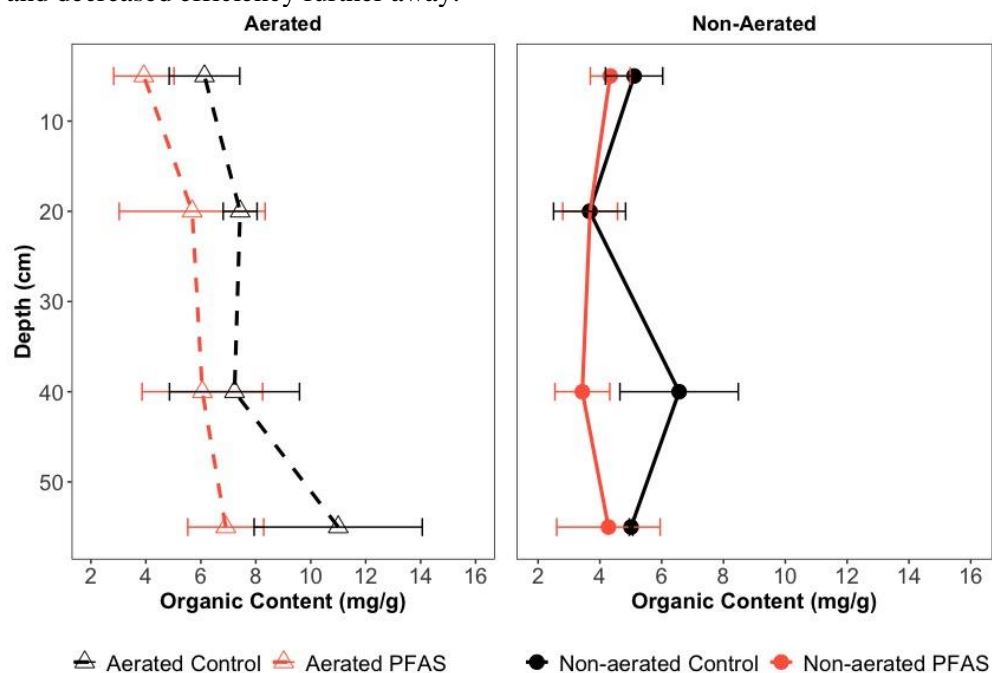
The observed robustness of the TW mesocosms in this study confirms a characteristic of resilience in established TW (based here on operation for ~1 yr prior to PFAS exposure) when treating emerging contaminants (Button et al., 2019a, 2016; Weber et al., 2011). From a water treatment perspective, the TW ecosystem, like its performance with conventional and other emerging contaminants, is resilient when exposed to the PFAS used in this experiment.

#### 4.3.4 PFAS Sorption

Organic content analysis (Fig. 4.5) conducted on gravel surfaces after PFAS was loaded for 12 weeks were obtained during Phase 3. Results highlighted more organic mass on aerated systems compared to non-aerated systems. This can be attributed to the aerobic environment in the aerated systems resulting in microorganisms contributing to more biofilm production (Faulwetter et al., 2009b). Additionally, organic content increased with depth in the aerated systems. This could be indicative of the forced air shearing biofilm throughout the system, which then settled at layer four (Fig. S1). The organic content analysis illustrated that all layers of the control systems in both the non-aerated and aerated systems had higher organic content than the PFAS systems. Although the differences between means in

each layer are not statistically significant (with exception of differences at 40 cm in the non-aerated system), a trend of reduced organic content in the presence of PFAS is apparent. This may be due to an adverse effect of PFAS, acting as a surfactant, on biofilm assembly and mass production.

Organic content increased with depth in the aerated systems but not in the non-aerated systems. Furthermore, the aerated systems were had higher overall organic content concentration than the non-aerated systems. This is likely due to the introduction of air at the bottom of the mesocosm, which contributed to efficient microbial communities flourishing in a highly oxidative zone near the aeration stone, and decreased efficiency further away.

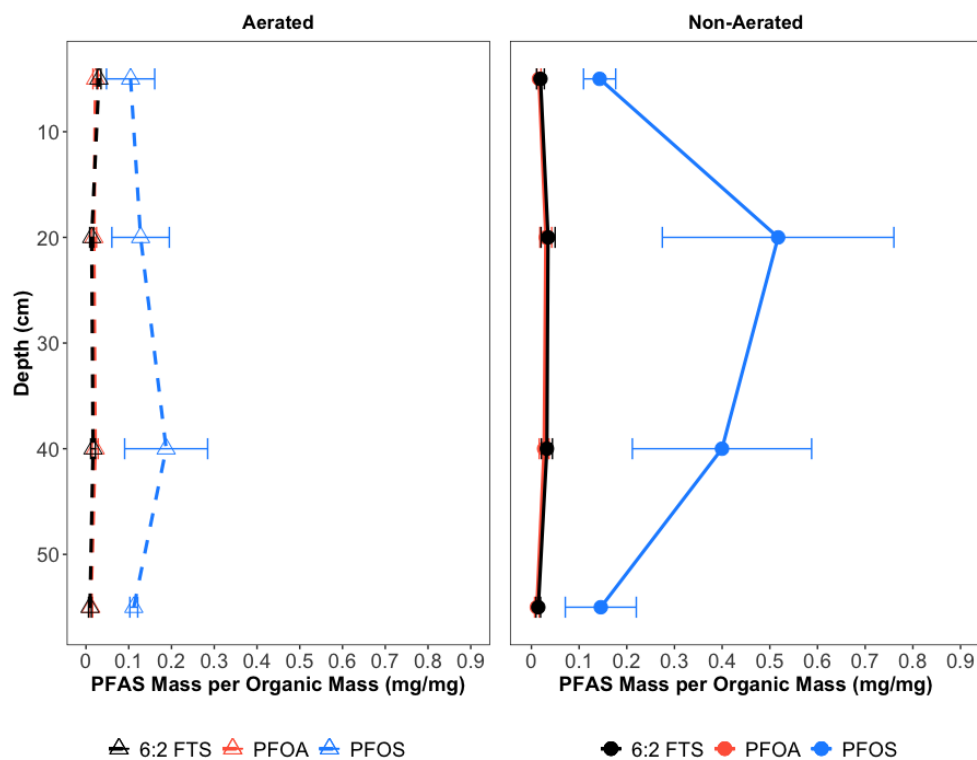


**Figure 4.5** - Organic content per mass of gravel (mg/g) for both PFAS and control mesocosms obtained from total solids analysis in aerated and non-aerated TW systems. Depth indicated corresponds to the midpoint of each mesocosm layer. Layer 1: 0-10 cm, layer 2: 10-30 cm, layer 3: 30-50 cm, and layer 4: 50-60 cm.

Organic content analysis combined with PFAS deconstruction data was used to determine the mass of PFAS per mass of organic matter at each layer for aerated and non-aerated systems (Fig. 4.6). More PFAS mass per mass of organic matter was found on non-aerated systems than aerated systems. PFOS mass was higher in the non-aerated systems than in the aerated systems. Moreover, PFAS mass was

uniformly distributed with depth in the aerated systems, but was higher in the two middle layers than in the top or the bottom layers the non-aerated systems. Adsorption, being a significant PFAS removal mechanism (Higgins and Luthy, 2006), likely resulted in the majority of removal in the TW mesocosms in this study. High surface areas of biofilm found extensively on gravel surfaces within the mesocosms are rich sources of organic matter and present numerous opportunities for PFAS to adsorb to gravel surfaces, thus causing its removal from the water. In addition, the chemistry of PFOS with its extra carbon-fluorine bond contributes to more adsorption capacity and the likely reason for its increased removal as compared to the other two PFAS.

Due to the position of the sampling port on the mesocosms (boundary of Layer 1 and 2) and direction of wastewater flow (downward), it is likely that the PFAS entered the system and adsorbed immediately within the middle layers. Although a greater amount of organic matter was found in aerated systems as compared to non-aerated systems (Fig. 4.5), perhaps a more complex removal mechanism could be occurring within the biofilm itself that could explain how the additional PFAS mass could be obtained on non-aerated systems despite the lower amount of organic matter present in those mesocosms (Fig. 4.6). Biofilm architecture is thought to have an interfacial area larger and more complex than previously known (Farooq et al., 2023). This could point to biofilm as a potential diffusive and advective transport interface for the PFAS in the non-aerated biofilms, resulting in greater adsorptive capacity of the non-aerated systems.



**Figure 4.6** - Mass of PFAS per mass of organic matter (mg/mg) for non-aerated and aerated TW. Depth indicated corresponds to the midpoint of each mesocosm layer.

The lower PFAS mass concentrations in the aerated systems may also be due to air-water partitioning. Air-water interfacial adsorption of PFAS plays a major role in contaminant transport in unsaturated porous media (Lyu et al., 2018; Schaefer et al., 2019). The absence of artificial air-water interfaces in non-aerated systems potentially increases removal efficiency due to increased opportunities for adsorption onto organic matter-rich surfaces (biofilm). Whereas aerated systems, with more air-water interfaces, could hinder PFAS adsorption onto biofilm, instead driving contaminant transport via air-water partitioning elsewhere in the mesocosm. The availability of biofilm sorption sites may also be limited by the physical effects of forced aeration, specifically due to the shearing of biofilm that makes it unavailable for PFAS removal in aerated systems.

Over the 12-week study, approximately 135 mg of PFOS, PFOA, and 6:2 FTS (totalling ~400 mg) was added to each system. The mass of PFOS, PFOA and 6:2 FTS remaining in each mesocosm at the time of deconstruction was estimated

from triplicate gravel samples at each layer (Table 4.2) combined with concentrations at the sampling port post-wastewater renewal (as a measure of mass lost via effluent). The results confirmed that more PFOS was retained on gravel biofilm than either PFOA or 6:2 FTS and support that removal efficiencies are likely indicative of increased PFOS retention on organic matter (Table 4.2). Aerated systems resulted in less PFOS mass on biofilm than in non-aerated systems. Air-water interfacial transport in the forced air systems could favour less PFAS adsorption on biofilm and instead facilitate further transport of PFAS elsewhere within the mesocosm, including at upper layers of the system as air bubbles burst at the water surface. Analytical methods included non-targeted PFAS, however no additional PFAS products were detected in any samples throughout the study (detection limit of 0.01 ppb).

**Table 4.2** - PFAS mass accounted in each layer for non-aerated and aerated systems obtained during mesocosm deconstruction.

	Aerated			Non-aerated		
	6:2 FTS	PFOA	PFOS	6:2 FTS	PFOA	PFOS
Top (0-10 cm)	1±1%	<1%	3±1%	<1%	<1%	4±1%
Upper Middle (10-30 cm)	<1%	<1%	4±2%	1%	<1%	12±3%
Lower Middle (30-50 cm)	<1%	<1%	7±3%	<1%	<1%	9±2%
Bottom (50-60 cm)	<1%	<1%	5±1%	<1%	<1%	4±1%
Mesocosm <sup>a</sup>	<1%	<1%	2%	<1%	<1%	2%
Effluent <sup>b</sup>	46±8%	50±6%	23±4%	51±8%	49±6%	63±12%
Mass Captured	49±9%	52±7%	43±12%	54±9%	52±6%	94±19%

<sup>a</sup>Inner cylinder walls and base for one aerated and one non-aerated mesocosm were evaluated to determine magnitude of adsorption on the physical mesocosm surface.

<sup>b</sup>In the absence of effluent readings during the experimental phase, a “pseudo-effluent” concentration was measured as the difference of PFAS in the interstitial water prior to and following mesocosm nutrient renewal.

The results shown in Table 4.2 do not account for the total mass added to most systems, where the mass captured in all but the non-aerated PFOS system are statistically less than 100%. Closing the mass balance was challenging likely due to the affinity for PFAS to partition to interfaces and sorb to organics, however more work and widespread sampling needs to be completed to understand these causes.

Moreover, the challenge to close PFAS mass balances has also been reported in literature as well (Duchesne et al., 2020). Weekly draining of the systems to measure porosity likely resulted in additional PFAS mass lost. Significant amounts of PFAS mass were likely adsorbed on biofilm surfaces, and were lost during drainage as biofilm sloughed off into drained water that was ultimately not captured or sampled, and is the likely culprit for the discrepancy. Weekly drainage data from a similar study by Timofee-Maberly (2024, unpublished) was used to estimate losses not accounted for in this study. The comparison showed that effluent data between both studies were virtually the same for PFOA and 6:2 FTS, however, PFOS effluent mass in both studies varied with >40% more effluent capture in this study in non-aerated systems, and >10% more effluent capture in aerated systems in this study. This could be attributed to the presence of vegetation in the Timofee-Maberly (2024) study that provided more organics for PFOS to adsorb to in those systems as opposed to leaving the systems as effluent. The inside walls of the PVC mesocosm cylinder were also wiped in both aerated and non-aerated systems and the wipes were analyzed for PFAS mass. While concentrations were present, the mass associated with the mesocosm walls was insignificant and not sufficient to close the mass balance.

#### **4.4 Conclusion**

This study sought to provide fundamental insights as to the fate, impacts, and removal mechanisms of PFAS in aerated subsurface wetlands when exposed to PFOS, PFOA, and 6:2 FTS. PFAS was loaded onto established TW mesocosms for a 12-week period, and those mesocosms were subsequently deconstructed. An organic analysis on exposed TW gravel substrate showed that organic content increased with depth in aerated systems, which was likely caused by forced air shearing biofilm throughout the system and potentially settling at lower layers. Non-aerated systems resulted in more PFAS mass in the two middle layers of the mesocosms likely due to immediate adsorption of PFAS on organic material near the location of influent. Aerated systems had less adsorbed PFAS mass indicating additional air-water interfaces forced into the systems through aeration may have caused PFAS partitioning resulting in PFAS transport elsewhere in the system or potentially vacating the mesocosm through its exposed surface. Results showed that PFAS concentrations in the interstitial water were reduced by the mesocosms, with sorption being the primary removal mechanism. More PFOS than PFOA or 6:2 FTS was removed from both aerated and non-aerated systems. Reductions in interstitial water concentration occurred rapidly (within 15 mins). Non-aerated systems dominated removal immediately upon PFAS loading, but aerated systems had greater removal at the end of the 7 day recycle period. This trend of non-aerated systems dominating early removals and aerated systems dominating later in the week was seen throughout the experiment. Furthermore, the point at which aerated systems dominated removal trended earlier in the loading week as the study progressed. PFOS had a higher affinity for adsorption than PFOA or 6:2 FTS given

its physiochemical properties resulting in it being removed the most and at a faster rate of removal. Mesocosm environmental conditions and water treatment parameters in non-aerated systems functioned normally after PFAS exposure, however aerated systems had abnormal dissolved oxygen and redox potential variations. This could indicate a potential toxicity effect to aerobic microorganisms as a result of PFAS exposure, however more biological analysis will need to be completed to achieve confirmation. The results confirmed that TW mesocosms are robust systems that can maintain their environmental conditions and wastewater treatment performance despite sustained exposure to PFAS concentrations.

## **5 Outcomes and Recommendations**

### **5.1 Conclusions**

#### **5.1.1 Assess the effects of aeration on water treatment performance in treatment wetland mesocosms.**

Conductivity, pH, ORP, and DO were higher in aerated systems than non-aerated, whereas ammonium-nitrogen was lower in the aerated systems. Higher ORP and DO were a consequence of the highly aerobic environment of aerated systems while increased pH is likely due to increased calcium bicarbonate concentration through a carbonate cycle mechanism with aeration. Water chemistry parameters as well as porosity and evaporation remained stable throughout the 12-week study in both aerated and non-aerated TW highlighting system resiliency and consistency in water treatment performance over time. No significant differences were observed (overlapping error bars at 95% confidence interval) in TOC removal between aerated and non-aerated systems. Aerated systems highlighted lower removal efficiency for TN compared to non-aerated systems largely due to the highly nitrified aerated system retaining total nitrogen concentration within the system.

Aeration therefore does have an effect on water treatment performance in TW mesocosms whereby the aerated systems featuring higher DO water chemistry, higher pH and ORP were associated with lower overall TN removal given their highly aerobic environment, which inhibited denitrification and forced incomplete nitrogen removal.



### **5.1.2 Investigate the spatial and temporal effects of aeration on microbial community structure, function, and activity.**

Established, aerated TW mesocosms were studied for 12-weeks to investigate temporal effects on microbial community structure, function and activity. Systems were subsequently deconstructed following the 12-week period to assess spatial effects of these parameters along with statistical analysis including PCA. Overall microbial activity conducted through FDA as well as catabolic activity performed through CLPP highlighted lower activity in aerated systems. CLPP analysis also showed lower substrate richness in aerated systems than non-aerated systems. Together, this lower overall and catabolic activity points to aerated microbes being less active due to living in abundantly aerobic conditions, thereby less willing to adapt their metabolic pathways which further leads to less metabolic richness. This evidence was further confirmed with PCA analysis of CLPP data illustrating that aerated systems are less varied in function. There were no significant temporal shifts based on DNA abundance. When compared to aerated systems, the abundance of genera in non-aerated systems was much higher than in aerated systems pointing to aeration affecting the development of microbial communities. Less diverse communities were present in the aerated systems, however, given the slightly higher organic mass present in the deconstructed layers, the less diverse communities may likely work more efficiently. In the gravel-associated biofilm analysis, aerated DNA concentration increased with mesocosm depth, largely resulting from the settling of biomass over time. Non-statistically significant (95% confidence interval) DNA abundance was observed between each layer for aerated systems, largely pointing to consistent mixing characteristics of the aerated mesocosms with the PCA analysis highlighting no spatial trends in structure or variation in taxonomy.

The spatial and temporal effects of aeration on microbial community function, structure, and activity are evident and were expressed through lower overall microbial and catabolic activity, lower metabolic richness and function, and less diversity. However, biofilm production in the form of organic mass was slightly higher in aerated systems across layers alluding to an overall effect of efficiency, resiliency (over time), and capability in metabolizing organics.

### **5.1.3 Evaluate effects of PFOA, PFOS, and 6:2 FTS on water treatment performance in aerated and non-aerated treatment wetland mesocosms.**

PFAS was loaded directly into three aerated and three non-aerated TW systems for 12 weeks. Six control mesocosms (aerated and non-aerated) were then used to assess longer term variation and compare responses to PFAS contaminated

mesocosms. Comparing pre-exposure water chemistry conditions with post-exposure results, dissolved oxygen increased and redox potential decreased in the aerated systems. This can point to a possible toxicity effect caused by PFAS contamination towards aerobic microorganisms that requires further investigation. Based on NH<sub>4</sub>-N, TN and TOC removal, results showed that water treatment performance in the non-aerated and aerated PFAS systems maintained removal efficiencies that are consistent with the controls.

Overall, there were little effects of PFOA, PFOS, and 6:2FTS on water treatment performance in TW. Both aerated and non-aerated systems saw a lack of change in most parameters that are used to assess TW water treatment performance. The results show that TW health as it pertains to water chemistry indicators is resilient to PFAS exposure and TW operation can continue despite PFAS addition.

#### **5.1.4 Examine PFOA, PFOS, and 6:2 FTS fate in aerated and non-aerated treatment wetland mesocosms.**

Non-aerated systems resulted in more PFAS mass in the two middle layers of mesocosms likely due to immediate adsorption of PFAS on organic material from the location of influent. Aerated systems had less adsorbed PFAS mass indicating additional air-water interfaces forced into the systems through aeration may have caused PFAS partitioning resulting in PFAS transport elsewhere in the system or potentially aggregating at the upper layers of the substrate before the air bubble breaks at the surface. It is understood then that PFAS concentrations from the interstitial water in the mesocosms were lowered, with sorption being the primary removal mechanism. More PFOS than PFOA or 6:2 FTS was removed from both aerated and non-aerated systems. Reductions in interstitial water concentration occurred rapidly (within 15 min) in non-aerated systems, with aerated systems removing more PFAS at the end of the 7-day period. Aerated systems performed better as time progressed, dominating removal over non-aerated systems earlier in the latter weeks. PFOS had a higher affinity for adsorption than PFOA and 6:2 FTS given its physiochemical properties resulting in it being removed the most and at a faster rate of removal.

## **5.2 Contributions**

This thesis provided a novel, granular characterization of aeration effects on TW along with insight on fate and effects of PFOS, PFOA, and 6:2 FTS in aerated and non-aerated TW. The results of this research determined fundamental data on TW environmental conditions (water chemistry) in both aerated and non-aerated

TW. This contributes to a better understanding of TW for wetland engineers to optimize effluent targets and life cycle design.

Data uncovered in the microbial characterization in both the interstitial water and gravel-associated biofilm can assist in further understanding critical drivers of TW pollutant removal. The spatial and temporal dynamics of microbial community structure and function in aerated wetlands in this thesis provide more detail to the field in terms of key genera present in the systems and their metabolic function. This can translate to full-scale systems and provide engineers with an understanding of microbial implications of aeration technology and the efficiencies (in organics degradation) it can offer.

Introducing PFAS in aerated wetlands was novel for the TW field, uncovering fundamental data regarding its fate and effects in both aerated and non-aerated subsurface TW, as well as its applicability to similar natural environments. The study gave insight to wetland engineers regarding the robustness of TW to accept weekly concentrations of PFAS within the system and treat organics while maintaining stable and resilient performance characteristics. Findings regarding PFAS removal dynamics were also novel and highlighted how rapid removals of PFAS (especially PFOS), likely due to adsorption, could also be observed in full-scale systems, and that aerated technology has a long-term treatment advantage over non-aerated systems when it comes to overall PFAS removal efficiency.

### **5.3 Recommendations**

Several recommendations are presented that would: (1) improve the experiments as conducted in this study, (2) generate additional fundamental data to improve TW characterization, and (3) build on current industry applications using the results of this study.

Improvements on obtaining aeration flow rate and actual volume (such as using a flow meter) in each of the aerated systems would allow for more precise aeration quantification. Challenges were experienced with nitrate sensors with possible interference from high concentrations of PFAS in the TW. More research into PFAS/nitrate sensor effects would have been useful in ensuring nitrate concentrations would be accurately analyzed and recorded to enhance water chemistry and treatment performance results. PFAS concentrations from drained water (from each TW) were missed in this study and should be recorded for more accurate mass balance calculations.

In order to provide further certainty in the fundamental data, future studies could explore different types of common PFAS to observe potential biotransformation or even higher PFAS concentrations to observe variations in treatment performance as a potential threshold limit for PFAS wastewater in TW. Given that aerated systems saw better PFAS removal with longer time in treatment, a future study could benefit from a longer study duration to fully explore the long-term effects of aeration. Due to the fact that much of TW removal mechanisms are dependent on microorganisms, future study could explore the effect of PFAS on microbial community structure, function, and activity to discern and quantify more precise contamination effects. In addition, the factorial design of future studies can explore adding systems that are abiotic to further discern biological effects. Furthermore, these studies could also elucidate the extent to which biofilms are affected by PFAS specifically and how that translates to removal effectiveness. As well, more investigation into the mechanics of biofilm diffusion would be useful in fully understanding the scope of contaminant removal occurring during PFAS loading. Furthermore, teasing out sorption isotherms for PFAS on biofilm in TWs would be useful fundamental data for wetland designers considering aerated TW applications. Future studies would benefit from also exploring similar experiments with the addition of vegetation to enhance the applicability to operational TW. An enhanced hydrological characterization can be completed in future studies by conducting tracer tests and fitting an accurate advection-dispersion model to understand dispersion effects in aerated and non-aerated TW. Finally, aerated TW systems could benefit from updated modelling studies that explore transport phenomena with additional inputs of emerging contaminants such as PFAS.

Designers and practitioners of aerated and non-aerated subsurface TW are recommended to continue using TW that receive PFAS wastewater with the understanding that the systems are resilient and that PFAS will likely adsorb to biofilm in the substrate rapidly upon contact. Aerated systems are recommended to have longer operational treatment times to improve removal efficiencies. Regulators of policy can apply the learnings from this study towards natural environments with previous studies confirming that PFAS (given its physiochemical properties) are likely to adsorb to organic components in the subsurface and partition especially easily in the presence of air-water interfaces.

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## Appendix A

Supplemental Information for Chapter 3: **Water treatment performance and microbial characterization of aerated treatment wetlands**



**Figure A.1** - Mesocosms arranged in the laboratory

**Table A.1 - Average Inlet Water Chemistry Characteristics**

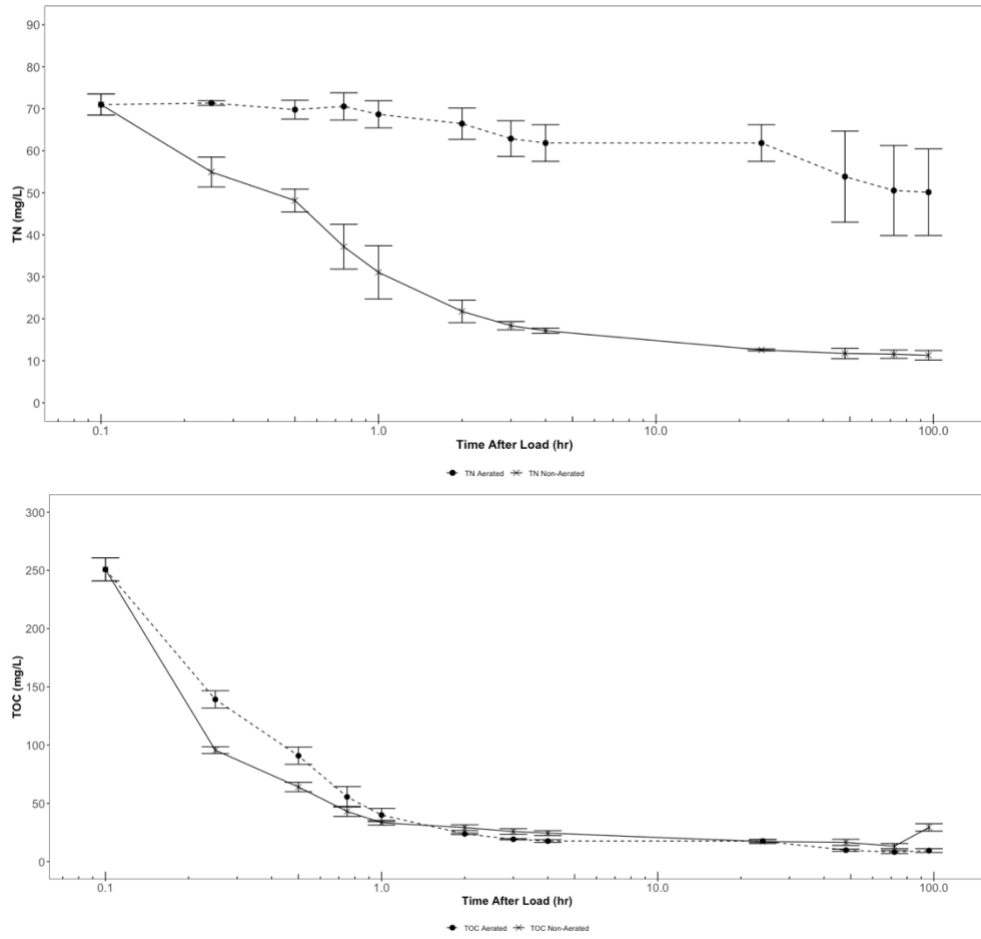
Week	Temperature		Conductivity		Dissolved Oxygen		pH		ORP		NH4	
	(°C)	(+/-)	(µS/cm)	(+/-)	(mg/L)	(+/-)	-	(+/-)	(mV)	(+/-)	(mg/L)	(+/-)
1	9.9	2.8	593.0	38.2	6.2	0.2	6.5	0.0	233.8	24.6	8.5	0.1
2	10.3	3.5	590.5	47.4	5.9	0.0	7.0	0.0	194.1	35.9	7.1	0.1
3	9.5	3.5	590.0	49.5	6.8	0.7	6.9	0.1	109.4	6.2	11.3	0.6
4	9.4	3.6	642.5	62.2	7.7	0.5	6.1	0.2	62.0	5.2	11.9	0.2
5	9.8	4.9	640.5	78.5	7.9	0.9	6.9	0.2	58.1	5.2	12.9	0.6
6	9.8	5.4	639.0	86.3	7.3	0.6	6.1	0.4	74.8	2.1	15.9	0.0
7	9.0	4.4	626.5	72.8	7.4	0.5	6.1	0.1	57.4	4.3	17.1	2.7
8	10.6	4.5	651.5	71.4	6.7	0.4	6.5	0.4	68.3	2.7	12.4	1.1
9	11.9	6.1	671.0	97.6	8.1	0.9	4.6	1.3	36.8	6.5	9.0	0.9
10	14.6	7.1	715.5	122.3	6.2	0.5	6.7	0.5	32.3	4.7	12.7	0.9
11	13.4	5.4	682.5	87.0	7.4	0.0	5.8	0.6	40.6	0.0	14.3	0.7
12	15.6	5.7	727.0	104.7	7.6	1.3	6.4	0.3	68.0	22.5	16.4	0.6
<b>Average</b>	11.1	4.8	647.5	76.5	7.1	0.5	6.3	0.3	86.3	10.0	12.5	0.7

**Table A.2 - Average (Day 4) Water Chemistry Indicators - Non-Aerated**

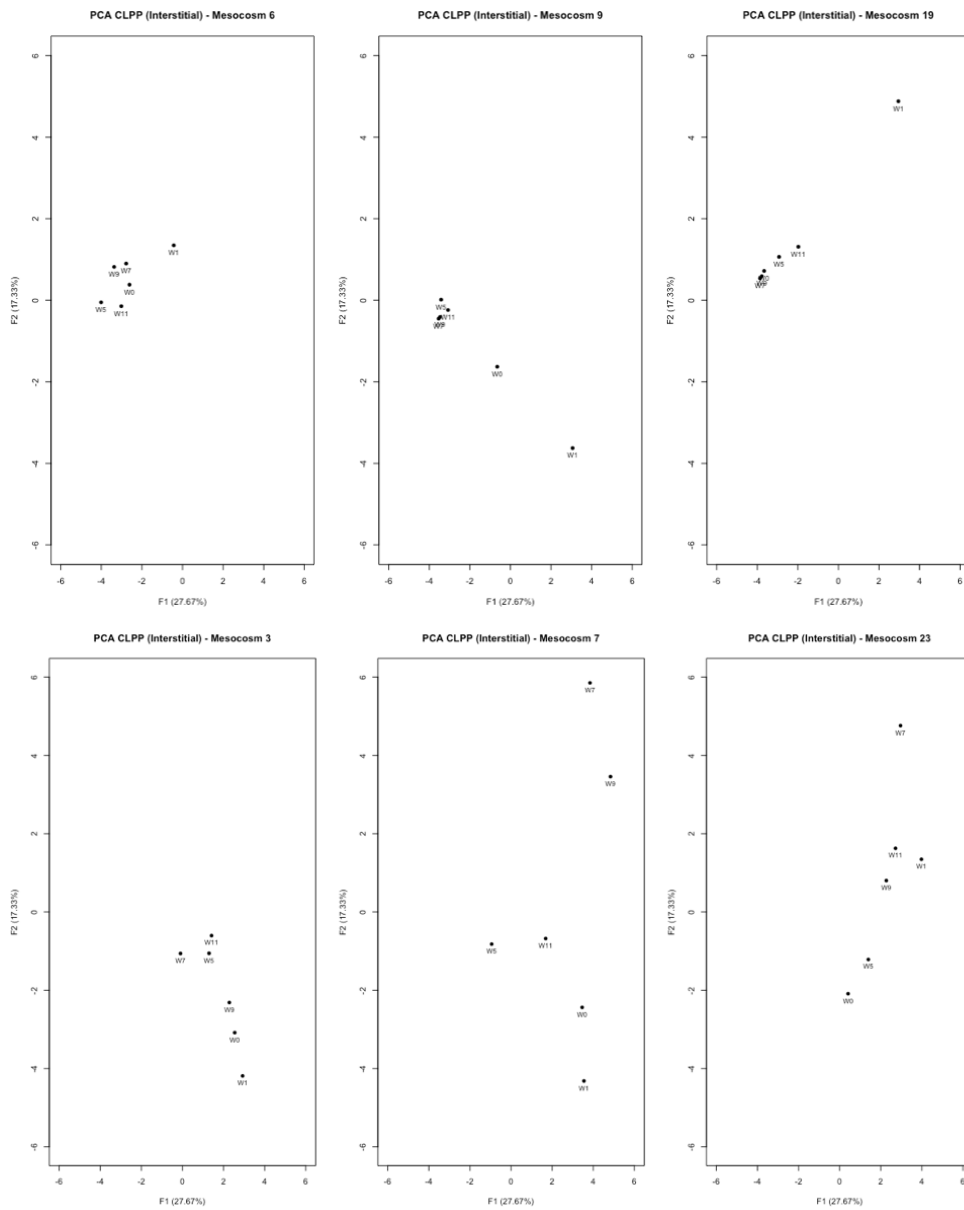
Week	Temperature		Conductivity		Dissolved Oxygen		pH		ORP		NH4	
	(°C)	(+/-)	(µS/cm)	(+/-)	(mg/L)	(+/-)	-	(+/-)	(mV)	(+/-)	(mg/L)	(+/-)
1	17.7	0.7	609.3	10.3	0.190	0.036	6.7	0.1	-231.4	7.8	10.8	0.2
2	21.3	0.4	678.0	14.7	0.693	0.064	5.9	0.6	-257.6	2.1	12.2	0.5
3	21.0	0.5	716.3	18.0	0.113	0.025	-	-	-185.8	8.7	13.3	1.0
4	21.3	0.4	709.7	21.5	0.053	0.006	6.3	0.1	-262.7	3.5	12.7	0.7
5	22.1	0.4	724.3	20.8	0.173	0.006	6.7	0.1	-251.8	2.8	16.1	0.8
6	21.3	0.4	707.0	17.4	0.263	0.006	6.6	0.1	-244.8	1.4	16.2	4.5
7	23.0	0.4	743.3	2.1	0.440	0.042	6.6	0.1	-278.2	2.9	12.4	1.2
8	21.9	0.4	756.0	21.7	0.847	0.038	6.6	0.1	-291.2	3.9	21.2	0.3
9	24.3	0.6	776.0	43.9	0.217	0.032	6.7	0.1	-294.0	2.6	18.0	1.9
10	22.6	0.6	756.3	63.3	0.263	0.055	6.7	0.1	-282.7	6.8	20.5	4.5
11	27.9	0.5	817.7	39.3	0.357	0.075	6.7	0.0	-96.3	3.8	15.0	3.0
12	25.7	0.8	790.0	41.0	0.173	0.051	6.7	0.0	-301.5	3.1	20.8	4.5
Average	22.5	0.5	732.0	26.2	0.315	0.036	6.6	0.1	-248.2	4.1	15.8	1.9

**Table A.3 - Average (Day 4) Water Chemistry Indicators – Aerated**

Week	Temperature		Conductivity		Dissolved Oxygen		pH		ORP		NH4	
	(°C)	(+/-)	(µS/cm)	(+/-)	(mg/L)	(+/-)	-	(+/-)	(mV)	(+/-)	(mg/L)	(+/-)
1	17.7	0.7	609.3	10.3	0.190	0.036	6.7	0.1	-231.4	7.8	10.8	0.2
2	21.3	0.4	678.0	14.7	0.693	0.064	5.9	0.6	-257.6	2.1	12.2	0.5
3	21.0	0.5	716.3	18.0	0.113	0.025	-	-	-185.8	8.7	13.3	1.0
4	21.3	0.4	709.7	21.5	0.053	0.006	6.3	0.1	-262.7	3.5	12.7	0.7
5	22.1	0.4	724.3	20.8	0.173	0.006	6.7	0.1	-251.8	2.8	16.1	0.8
6	21.3	0.4	707.0	17.4	0.263	0.006	6.6	0.1	-244.8	1.4	16.2	4.5
7	23.0	0.4	743.3	2.1	0.440	0.042	6.6	0.1	-278.2	2.9	12.4	1.2
8	21.9	0.4	756.0	21.7	0.847	0.038	6.6	0.1	-291.2	3.9	21.2	0.3
9	24.3	0.6	776.0	43.9	0.217	0.032	6.7	0.1	-294.0	2.6	18.0	1.9
10	22.6	0.6	756.3	63.3	0.263	0.055	6.7	0.1	-282.7	6.8	20.5	4.5
11	27.9	0.5	817.7	39.3	0.357	0.075	6.7	0.0	-96.3	3.8	15.0	3.0
12	25.7	0.8	790.0	41.0	0.173	0.051	6.7	0.0	-301.5	3.1	20.8	4.5
Average	22.5	0.5	732.0	26.2	0.315	0.036	6.6	0.1	-248.2	4.1	15.8	1.9



**Figure A.2** – TOC (top) and TN (bottom) kinetic profiles for aerated and non-aerated mesocosms from time of load to 96 h post loading.



**Figure A.3** - Principal component ordination for carbon source utilization patterns for individual mesocosms

**Table A.4** – Genera taxonomy used in interstitial and deconstructed principal coordination analysis

<b>Letter</b>	<b>Genera</b>
A	<i>Candidatus_Nomurabacteria</i>
B	<i>Saccharimonadales</i>
C	<i>unclassified</i>
D	<i>uncultured</i>
E	<i>Nakamurella</i>
F	<i>unclassified</i>
G	<i>Thiovirga</i>
H	<i>Parcubacteria</i>
I	<i>SCGC_AAA011-D5</i>
J	<i>Candidatus_Kerfeldbacteria</i>
K	<i>Candidatus_Falkowbacteria</i>
L	<i>C39</i>
M	<i>Candidatus_Kaiserbacteria</i>
N	<i>LWQ8</i>
O	<i>Candidatus_Adlerbacteria</i>
P	<i>Candidatus_Woesebacteria</i>
Q	<i>Candidatus_Komeilibacteria</i>
R	<i>Candidatus_Levybacteria</i>
S	<i>Dojkabacteria</i>
T	<i>Woesearchaeales</i>

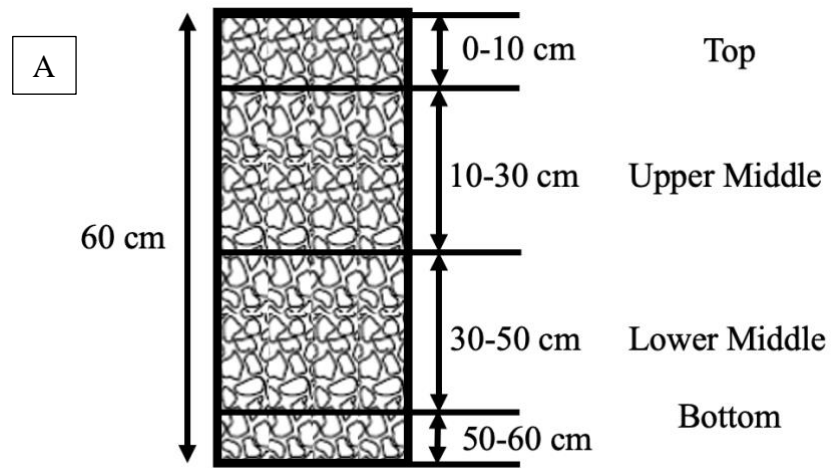


## Appendix B

### Supplemental Information for Chapter 4: PFOS, PFOA, and 6:2 FTS in Treatment Wetland Mesocosms: Fate and Effects on Water Treatment Performance

**Table B.1** - Weekly pore volumes of PFAS loaded mesocosms

<b>Week</b>	<b>Aerated (L)</b>			<b>Non-aerated (L)</b>		
<b>1</b>	8.4	8.0	7.9	8.9	8.6	8.0
<b>2</b>	8.2	8.1	7.3	9.0	8.5	8.4
<b>3</b>	7.9	7.8	7.7	9.0	8.4	8.3
<b>4</b>	8.1	8.0	7.9	8.8	8.5	8.3
<b>5</b>	7.9	7.7	8.0	8.6	8.4	7.9
<b>6</b>	8.1	8.0	8.0	9.0	8.6	8.1
<b>7</b>	7.9	7.4	8.0	9.0	8.6	8.1
<b>8</b>	8.0	7.8	8.0	9.0	8.5	7.9
<b>9</b>	7.6	7.2	7.6	8.8	8.4	8.1
<b>10</b>	8.1	7.7	7.9	8.9	8.4	7.9
<b>11</b>	7.8	7.3	7.9	8.8	8.5	7.9
<b>12</b>	8.1	7.6	7.9	8.7	8.1	8.0



**Figure B.1** – (A) Mesocosm layers, and (B) Gravel placed according to layer on drop sheet