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Faculty of Environmental Sciences

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Wastewater Treatment in Constructed Wetlands Under Various Operation Conditions

Diploma Thesis

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DIPLOMA THESIS ASSIGNMENT

B.A. Jennifer Sanchez

Landscape Planning

Thesis title:

Wastewater Treatment in Constructed Wetlands Under Various Operation Conditions

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Objectives of thesis:

This thesis aims to evaluate the effects nutrient ratios on wastewater purification in arbuscular mycorrhizal fungi-assisted constructed wetlands.

Methodology:

This study will be conducted using 8 vertical subsurface flow CWs at the Czech University of Life Sciences Prague. The 8 PVC pipes will be established to simulate the subsurface flow CWs with the dimensions of each system being 15× 55 cm (diameter ×Height). Each CW will be filled with 15 cm gravel (4-5 cm) and 25 cm sand will be used as substrates. For the AMF system, the substrates from the bottom to top are: 15 cm gravel, 10 cm sand, 10 cm sand mixed with 50 g AMF, then planted *Iris pseudacorus*, afterward add 5 cm sand. AMF inoculum will be *Rhizophagus irregularis*. The influencing factors of this study are C concentrations (low, high), N concentration (low, high). Therefore, 8 different treatments will be set. Inlet water of CWs will be simulated municipal sewage. Simulated municipal sewage with different pollutants concentrations will be fed into each CW. The experiment lasted about three months. The CWs will be protected from rain throughout the experiment.

The proposed extent of the thesis

50 pages

Keywords: C:N ratios; wastewater treatment; constructed wetlands; nitrification, denitrification

Recommended information sources:

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Author’s declaration:

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Abstract

Constructed wetlands (CWs) have been studied and have been considered as cost-effective, efficient, and sustainable wastewater treatment systems for decades. CWs use natural processes, such as nitrification and denitrification, to treat water from excessive nutrients and containments. However, their treatment performance varies depending different operation conditions and design of the system. Several studies have investigated the removal of nutrients efficiency, efficiency under different hydraulic loading rates, and the efficiency of different CW designs. Arbuscular mycorrhizal fungi (AMF) have been widely reported to play important roles in terrestrial plant resistance to abiotic stresses (e.g., heavy metals, drought, emerging pollutants, and nutrients). AMF have been known to extend the root networks of the plants which they inoculate. This study and experiment focused on the removal efficiency of CWs under different nutrient ratios, C:N with the assistance of AMF. The results of the experiment concluded that C:N ratios with the assistance of AMF were successful in the removal of ammonium, nitrate, and phosphorus. Although the CWs were inoculated with AMF, the biomass in the CWs did experience some minor stress.

Key Words: C:N ratios; wastewater treatment; constructed wetlands; nitrification; denitrification; Arbuscular mycorrhizal fungi

Abstrakt

Konstrukční mokřady (CW) jsou studovány a považovány za nákladově efektivní, účinné a udržitelné systémy čištění odpadních vod již několik desetiletí. Mokřady využívají přírodní procesy, jako je nitrifikace a denitrifikace, k čištění vody od nadměrného množství živin a kontaminantů. Jejich čisticí výkon se však liší v závislosti na různých provozních podmínkách a konstrukci systému. Několik studií zkoumalo účinnost odstraňování živin, účinnost při různých hydraulických rychlostech zatížení a účinnost různých konstrukcí CW. Všeobecně se uvádí, že arbuskulární mykorhizní houby (AMF) hrají důležitou roli v odolnosti suchozemských rostlin vůči abiotickým stresům (např. těžkým kovům, suchu, novým znečišťujícím látkám a živinám). Je známo, že AMF rozšiřují kořenovou síť rostlin, které inokulují. Tato studie a experiment se zaměřily na účinnost odstraňování KS při různých poměrech živin C:N za pomoci AMF. Výsledky

pokusu dospěly k závěru, že poměr C:N s pomocí AMF byl úspěšný při odstraňování amoniaku, dusičnanů a fosforu. Přestože byly CWs inokulovány AMF, biomasa v CWs zažívala menší stres.

Klíčová Slova: C:N poměry; čištění odpadních vod; vybudované mokřady; nitrifikace; denitrifikace; Arbuskulární mykorhizní houby

List of Abbreviations:

AA – Additional aeration
AMF – Arbuscular mycorrhizal fungi
BOD - Biological oxygen demand
C:N ratio – Carbon to nitrogen ratio
C – Carbon
COD – Chemical oxygen demand
CW – Constructed wetland
CWs – Constructed wetlands
DO – Dissolved Oxygen
Fm – Maximum fluorescence
Fo – Minimum fluorescence
FWS – Free water surface
HLR hydraulic loading rate
HM – Heavy metals
HRT - hydraulic retention time
HSSF – Horizontal subsurface flow
N – Nitrogen
NH4+ - Ammonium
NO – Nitric oxide
NO2- - Nitrite
NO3- - Nitrate
N2O - Nitrous oxide
ORP - Oxidation-reduction potential
O2 – Oxygen
PAM – Pulse Amplitude Modulation
PO4 3- - Phosphate
RE – Removal efficiency
TN – Total Nitrogen
TOC – Total organic carbon
VFCWs – Vertical flow constructed wetlands
VSSF – Vertical sub-surface flow

Table of Contents

Chapter 1: Introduction 12

Chapter 2: Aim of Diploma Thesis 13

Chapter 3: Literature Review 14

3.1 Constructed Wetlands 14
3.1.1 Types of Constructed Wetland 14
3.1.2 Vertical Subsurface Flow Wetlands (Operation) 16

3.2 Plants 17
3.2.1 Photosynthesis 18

3.3 Hydrologic Regime in Constructed Wetlands 19

3.4 Nutrient Cycling in Constructed Wetlands 20
3.4.1 Removal Mechanism 20
3.4.2 Nitrogen Cycle 21
3.4.3 Carbon Cycle 25
3.4.4 Phosphate Removal 27

3.5 Microbial Activity 28

3.6 Arbuscular Mycorrhizal Fungi (AMF) 30

4.1 Experimental Design 33
4.1.1 Location 33
4.1.2 Materials 33

4.2 Sample Analysis 36
4.2.1 Sample Testing Machinery 38

4.3 Data Analysis 39

4.4 Statistical Analysis 40

Chapter 5: Results 41

5.1 Water Loss, pH, ORP 41

5.2 Ammonium 44

5.3 Nitrite 47

5.4 Nitrate 49

5.5 Total Nitrogen 51

5.6 Total Organic Carbon 53

5.7 Total Carbon 55

5.8 Phosphate 58

5.9 Maximum quantum yield of PSII 61

5.10 Physical Observations 62

5.10.1 Biomass Weight: 63

Chapter 6: Discussion 65

6.1 C:N Ratios 65

6.2 Ammonium (NH4+), Nitrite (NO2-), and Nitrate (NO3-) 65

6.3 Total Organic Carbon (TOC) and Total Carbon (TC) 66

6.4 Phosphate 67

6.5 AMF 68

6.6 Plant Health 68

Chapter 7: Conclusion 70

Appendix A: Inflow Results 71

Appendix B: Constructed Wetlands throughout the experiment 76

References: 79

Chapter 1: Introduction

This thesis will discuss constructed wetlands (CW), their removal efficiency under various conditions and arbuscular mycorrhizal fungi (AMF). This topic was of interest because I was not familiar with CWs or AMF until recently. I had previously taken a course called water protection resources and this class sparked a curiosity to learn more about how constructed wetlands function and their benefits. The opportunity to study CW in detail arose, along with also studying about arbuscular mycorrhizal fungi. This thesis will discuss CW and AMF and how they perform together.

Constructed wetlands are known as sustainable wastewater treatment systems that have been proven to be efficient, low cost and require low maintenance. There are various types of constructed wetland designs that can support various types of macrophytes and wetland vegetations. CWs can provide different efficiency results based on their design.

Arbuscular mycorrhizal fungi (AMF) are the soil microbes that colonize majority of the plant root and establish a connection between the plant and the substrate (Zhu, 2022). Plants have a network of roots that allow them absorb nutrients and AMF is a type of fungi that extends the roots of a plant, therefore expanding the root network. However, there are limited studies on the removal efficiency of AMF in CWs.

In this thesis, the removal efficiency of constructed wetlands was analyzed. The CW's performance was tested under various conditions. The first was condition was under different carbon (C) and nitrogen (N) ratios, this was done by the creation of 8 different wastewater influent treatments. The second condition was the inoculation the *Iris pseudacorus* with AMF, *Rhizophagus irregularis.* Furthermore, this experiment looked at how nutrient ratios influenced wastewater purification in AMF assisted constructed wetlands. The experimented lasted approximately 8 weeks from October to December, therefore the experiment underwent different temperature changes and provided results from different seasons.

Chapter 2: Aim of Diploma Thesis

The aim of this thesis is to investigate how nutrient ratios will influence wastewater purification with the assistance of arbuscular mycorrhizal fungi (AMF) in constructed wetlands (CWs). Arbuscular mycorrhizal fungi (AMF) have been widely reported to play important roles in terrestrial plant resistance to abiotic stresses such as heavy metals, drought, emerging pollutants, and nutrients. However, regarding the different nutrient carbon (C), nitrogen (N) ratios in wastewater purification in AMF assistant CWs are poorly studied.

Chapter 3: Literature Review

3.1 Constructed Wetlands

Constructed wetlands (CW) are human made wetlands and systems that imitate the natural processes of natural wetlands (Kominkova, 2022). Although they are an unconventional method for wastewater treatment, their main purpose is to purify wastewater, stormwater runoff, and can be a pre-treatment before the water reaches the water treatment plant (Kominkova, 2022). The concept of using wetlands as wastewater treatment has been documented since the 1950's and overtime the study of wetlands was practiced throughout different parts of the world such as Germany, Netherlands, South Asia, North American and Australia (Vymazal, 2022). Through trial and error, these countries realized that the using natural wetlands to treat wastewater resulted in the destruction of many wetlands around the world due to uncontrolled wastewater disposal (Vymazal, 2022). As a result, the use of natural wetlands was replaced with constructed wetlands. Constructed wetlands can be built under more controlled conditions therefore making them more efficient by selecting specific substrates, vegetation, flow patterns, size of the wetland, and deciding on the specific location (Vymazal, 2022). Constructed wetlands may also be used for land reclamation after mining, refineries or other ecological disturbances that have been lost due to development (Kominkova, 2022). The main characteristics that constitute a constructed wetland are macrophyte vegetation presence that is common in natural wetlands, water-logged or substrate conditions, and inflow of contaminated waters with components that will be removed (Fonder, N et al., 2010).

3.1.1 Types of Constructed Wetland

Constructed wetlands for wastewater treatment are not limited to one design. The design and type can vary based on the type of vegetation such as free-floating plants, floating leaved plants, emergent plants and submerged plants (Kominkova, 2022). Under the emergent plants category there are surface flow and subsurface flow wetlands. Subsurface flow wetlands consist of vertical flow, horizontal flow, and hybrid. Figure 3.1 is a classification diagram, by Vymazal, that displays the different types of constructed wetlands.

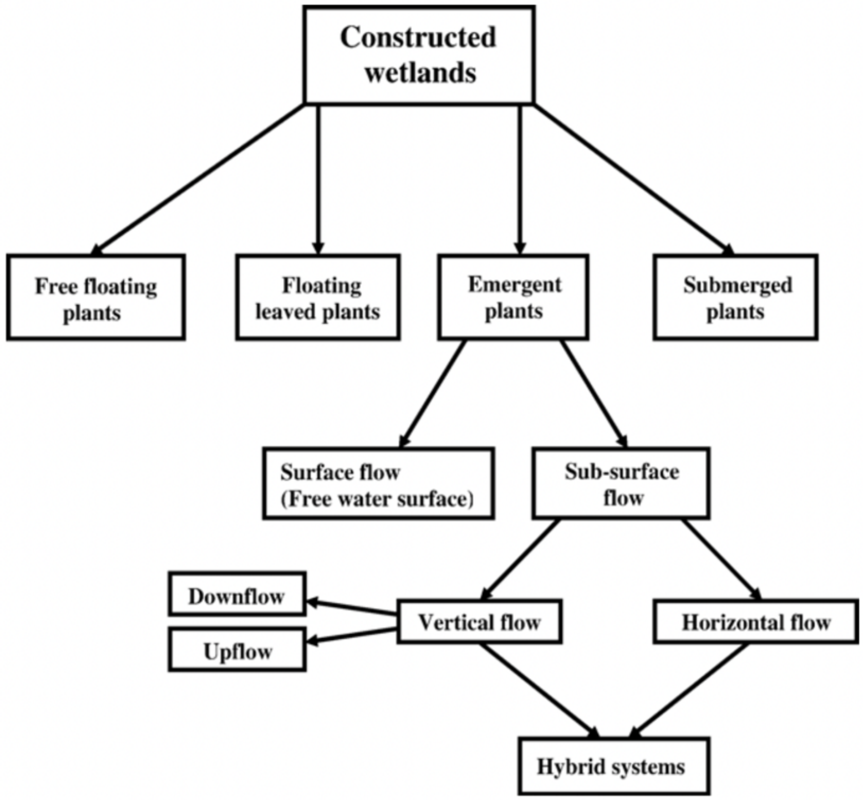

Figure 3.1 1 Classification of constructed wetlands for wastewater treatment (Vymazal, 2007).

However, the two common types of constructed wetlands are free water surface (FWS) wetlands and horizontal subsurface flow (HSSF) wetlands (Kadlec, 2009). Surface flow wetland are densely vegetated by different types of plants species and tend to have water depths of up to .3 m (Kadlec, 2009). The free water surface flow wetland has standing water exposure to the atmosphere and the subsurface flow wetland maintains water below the surface of gravel or other substates and allows for the growth of rooted wetland plants (EPA, 2000; Lui et al. 2005). In a subsurface flow wetland, the wastewater will flow horizontally through the substrate beneath the surface vegetation and will make contact with bacteria that can use dissolved oxygen (DO) living within the substrate and plant roots (Lui et al. 2005). Figure 3.1.1 displays illustrations of how the different CWs are designed and how their design impacts their operation.

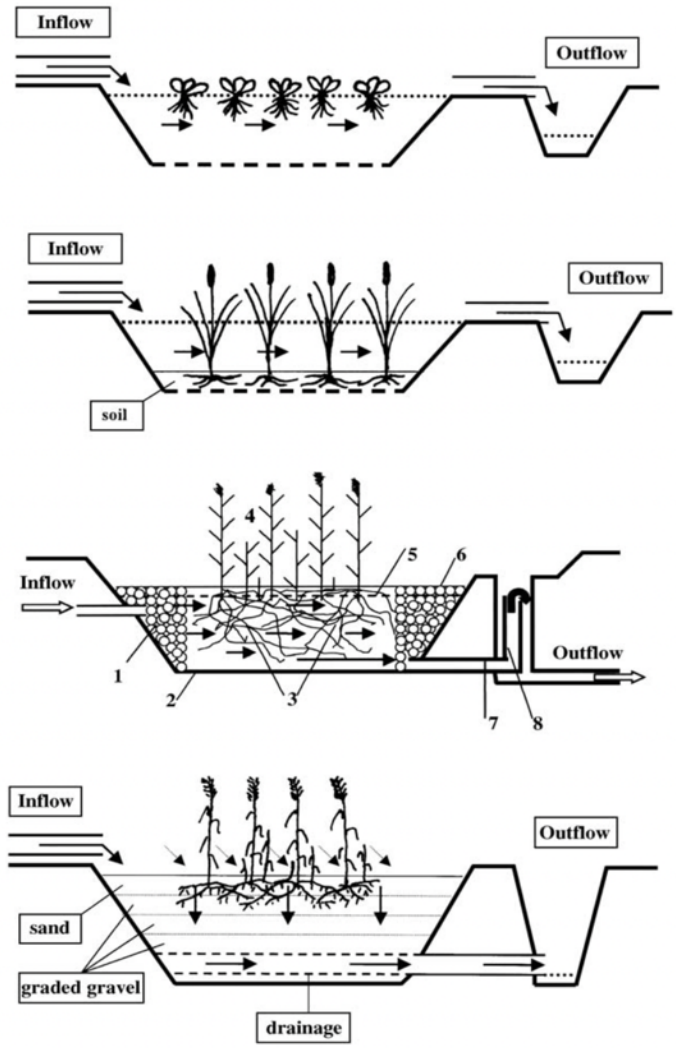

Figure 3.1 2 Constructed wetlands for wastewater treatment (from top to bottom): CW with free-floating plants (FFP), CW with free water surface and emergent macrophytes (FWS), CW with horizontal sub-surface flow (HSSF, HF), CW with vertical sub-surface flow (VSSF, VF) (Vymazal, 2006).

3.1.2 Vertical Subsurface Flow Wetlands (Operation)

Constructed wetlands' design can also be classified by their hydrological and vegetation characteristics (Fonder, N et al., 2011). In vertical flow constructed wetlands the effluent or wastewater is filled on the surface of the planted bed filter (Brix, H et. al., 2005). Pollutants are removed or transformed by the bacteria that is attached to the substrate and the root system of the plants (Brix, H. et al., 2005). Brix implies that it is important for the filter to not be saturated with water but to have aerobic conditions to secure high levels of oxygen in the filter. The treated wastewater is then collected by a system that has aerated drainage pipes placed at the bottom of the filter, half of the effluent is then recirculated to the sediment tank in order to enhance denitrification and improve the system performance (Brix, H. et al., 2005). However, Vymazal (2007) explains that ammonium adsorption is limited to constructed wetlands with subsurface flow where the contact between substrate and wastewater is efficient. Saeed (2012) states that the disadvantages to VSSF CWs are poor denitrification process, low nitrate removal, and decrease in performance especially when it comes to phosphorus removal.

3.2 Plants

Aquatic plants and microorganisms play a key role in the treatment of wastewater in in constructed wetlands (Rehman et. al., 2017). In constructed wetlands, plants provide oxygen through photosynthesis or by directly transporting it there from the atmosphere via their stems and roots (Rehman et. al., 2017). Brix (1997) stated that wetland plants play important roles in the process of wastewater purification utilizing CWs. Plants are fundamental since they play an active role in supplying of oxygen and root exudates, helping to maintain a healthy microbial life within the wetland, even during drying periods (Brix, 1997). Vyzamal (2007), explains that plant uptake is the major removal mechanism in constructed wetlands with free-floating macrophytes, influencing factors with the removal of ammonia and phosphorus. In addition, the main function of the plants is to prevent clogging of the filter and insulate the are to prevent frost during the winter (Brix, H. et al., 2005). Plants, and in particular their root systems are a huge part of wetland biomass (Huang 2010). Huang et. al. (2010) states that aquatic plants play a crucial part in the wastewater purification process, the impacts of plants can be observed in the pH, DO, and ORP of their surroundings. The presence of plants provides a huge surface area and medium for attached microbial growth (Brix, 1997). However, direct plant uptake has been observed to play a minor role in removal processes (Brix, 1997).

For this experiment, the *Iris pseudacorus* was used in the vertical flow constructed wetland simulation. The addition of this aquatic plant can help to enhance nitrogen removal (X. Gu et al., 2021). Based on the results of Gu's (2021) experiment, they concluded that the *Iris pseudacorus's* dosage provided better operation parameters regarding the weight and wastewater inflow ratio. Bragato et. al. (2006) states that macrophytes produce high biomass at fast growing rates and have high treatment potential for macronutrients and heave metals. Wetland plant are very productive organisms due to their functions regarding wastewater treatments, such as flow resistance, the trapping of particulates, nutrient uptake and insulation (Brix, 2003; Kadlec and Wallace, 2009; Shelef et al., 2013). Shelef et al. (2013) states that the physical effects of the root structure helping with particulate capture and aeration are the most significant ways that plants contribute to CW treatment operations.

3.2.1 Photosynthesis

Photosynthesis is the process by which plants use sunlight, water, and carbon dioxide to create oxygen and energy in the form of sugar. In wetlands, photosynthesis generates oxygen which is then moved from leaves to roots of plants by diffusion and convection mechanisms, enhancing the oxygen content of wetland beds (Grosse et al., 1991; Ottava et al., 1997; Armstrong et al., 2000). According to Huang, this creates an aerobic micro-environment, which then supports the decomposition reactions of root microorganisms. Seyoum concluded that dissolved oxygen in experiment water in wetlands with different plants had a significant difference (Seyoum and Marc, 2008).

The photosynthetic rate is a sensitive index that can reflect the operative status of a plant's photosynthetic structure (Huang, 2010). The ability of plants to produce and transport oxygen can be directly impacted by changes in photosynthetic rate caused by environmental and existing plant factors, which can ultimately affect how wetlands function (Huang, 2010). Huang's (2010) experiment stated that the photosynthetic rate of the wetland plants was determined at different light intensities.

Huang's experiment indicated that when the temperature was low, the photosynthetic rate increased as temperature increased, indicating that the rising temperature had a stimulating effect, however there was a blight when the temperature increased because photosynthesis rate would drop then. Huang states that the photosynthetic rate was low at low temperatures since the enzyme reactions of photosynthesis proceed slowly at these temperatures, but at the same time, photosynthetic inhibition and photosynthetic oxygenation occurred. He continues stating that the reason why the photosynthetic rate was lower at high temperatures is because high temperatures caused the destruction of chloroplast and cytoplasmic tissues, and the enzyme of chloroplast was in passivation, so photosynthesis was restrained (Huang 2010). Huang concluded that the rate of respiration was more than photosynthesis when at high temperature simultaneously, although the real photosynthesis rate was likely to improve, the net photosynthesis rate would reduce because of restriction of respiration (Huang 2010).

When doing an analysis on the chlorophyll fluorescence, light energy is absorbed by the chlorophyll molecules in a leaf (Maxwell, 2000). This analysis can have 3 outcomes: it can be used to drive photosynthesis (photochemistry), extra energy can be dissipated as heat, or it can be re-emitted as light (chlorophyll fluorescence) (Maxwell, 2000). These three outcomes occur in competition that if there is an increase in efficiency in one result there will be a decrease on efficiency in the other two outcomes. Therefore, by measuring the total chlorophyll fluorescence, information about changes in the efficiency of photochemistry and heat dissipation can be gained (Maxwell, 2000).

3.3 Hydrologic Regime in Constructed Wetlands

Hydrology is a critical factor in the performance of constructed wetlands, as it affects the water flow, retention time, and nutrient cycling within the system. Hydrology is an essential element to CWs' design, operation and maintenance because this must consider hydrological conditions such as climate/weather, evapotranspiration and groundwater exchange (Insel et al., 2007). Hydrological processes in CWs can be divided into three categories: precipitation and evapotranspiration, surface hydrological process, and subsurface flow (Jiang, et al., 2022). Meanwhile, plant interception can also change seasonally depending on the plant richness within the CW (Jiang, et al., 2022). Jiang et. al., (2022) stated that the common changes of hydrological conditions can be natural such as seasonal changes in precipitation. However prolonged flooding may cause a negative impact on vegetation and on the performance of the constructed wetland (Maltchik et al., 2007; Peterson and Baldwin, 2004). Another form of artificial practice is to change the CWs flow patterns, including artificial pulsing and tidal or intermitted operation, etc. While these do not necessarily change the three parameters above, they do change the hydrodynamic conditions within the CWs (Jiang, et al., 2022). However, there is a threshold beyond which an increase of hydraulic retention time (HRT) has minimal effects on pollutant removal efficiency (Sultana et al., 2016). Performance changes of pollutant removal can be quantified and a simple prediction of removal efficiency under changing HRT and hydraulic loading rate (HLR) is proposed. (Jiang, et al., 2022). In summary, the hydrology regime in constructed wetlands is a critical factor in ensuring effective treatment of wastewater and stormwater runoff. The design of the system, the hydraulic loading rate, the vegetation type, and the climate are all important factors that must be considered to maintain a stable water level and consistent flow of water through the wetland.

3.4 Nutrient Cycling in Constructed Wetlands

Constructed wetlands (CWs) have been successfully used for treating wastewater and are considered to be a sustainable wastewater management option around the world (Wang et. al., 2017). They are an emerging ecological engineering technology and have been widely used to treat micro-polluted water while maintaining low operation cost (Wang et. al., 2021). Part of the self-purification process in constructed wetlands has to do with nutrient cycling. Nutrient cycling in constructed wetlands involve the transformation, removal, and recycling of nutrients such as nitrogen (N) and phosphorus through the actions of plants, microbes, and other organisms (Vymazal, 2007).

3.4.1 Removal Mechanism

Constructed wetlands use their natural, chemical, and biological processes to purify and treat wastewater and these processes are known as the main contaminant removal mechanisms (Mustafa, 2017). The physical removal mechanisms taking place in CWs include sedimentation, volatilization and diffusion (Wallace and Knight, 2006). Kadlec (1992) also identified volatilization, sedimentation, sorption and biological degradation as the main processes affecting organic matter loads in wetlands.

Volatilization is a significant removal mechanism for organic compounds because of their significant vapor pressures also known as volatile organic compounds or VOCs, which vaporize and escape to the atmosphere (Hansen et al., 1998). Moshiri (1993) stated that when dissolved compounds are physically transferred from places with higher concentrations to areas with lower concentrations, the diffusion process takes place. Sorption of a chemical to soil or sediment can result from the physical or chemical attachment of molecules to solid surfaces, or from partitioning of dissolved molecules between the water phase and soil organic matter (Imfeld et. al., 2009). Biological degradation can be defined as the decay caused by organisms such as fungi and bacteria in the presence of excess moisture and air over a long period of time (Dungani, et. al., 2019). Organic matter is decomposed by bacteria and fungi (calrecycle.ca.gov).

Water quality results in constructed wetlands vary in different situations, studies of water quality indicators tend to focus on BOD/COD, ammonia (NH_4^+), nitrate, (NO_3^-) and total nitrogen (TN), phosphate (PO^{3-}), total phosphorus (TP) and total suspended solids TSS (Vymazal, 2007). The most widely studied process in CWs is the removal of nutrients (i. e., TN, NH_4^+, NO_3^- , TP, PO_4^{3-}), and the overall efficiency of nutrient removal (Vymazal, 2007).

3.4.2 Nitrogen Cycle

Constructed wetlands (CWs) have been widely used to treat micro-polluted water due to high N removal efficiency and low operation cost (Wang et. al., 2021). Nitrogen in wastewater has been a concern because it can eutrophication and effect the oxygen amount in receiving waters. (Kadlec and Wallace, 2009). In CWs, the N removal mechanisms to treat wastewater are known to include biological (e.g., ammonification, nitrification, denitrification, plant uptake) and physicochemical processes (e.g., ammonia volatilization, and adsorption) (Coleman et al., 2001; Lee et al., 2009). Per Mustafa (2017), some processes won't completely remove nitrogen from waters but rather convert them into other forms of nitrogen.

The nitrogen cycle is a complex process of chemical and biological reactions that occur as nitrogen is being circulated within plants, animals, and the atmosphere (Schipper, A. et al., 1996). This activity brings two hidden processes of the nitrogen cycle, nitrification and denitrification, out into the open, which is the transformation of ammonium to nitrate and its reduction to nitrogen gases. (Schipper, A. et al., 1996). Biological nitrification followed by denitrification is believed to be the major pathway for N removal in CWs (Saeed and Sun, 2012). The Figure 1 is a table by Vymazal that lists the processes and transformation of nitrogen within constructed wetlands.

The most important inorganic form of nitrogen in wetlands are ammonium (NH4+), nitrite (NO2−) and nitrate (NO3−). Gaseous nitrogen may exist as dinitrogen (N2), nitrous oxide (N2O), nitric oxide (NO2 and N2O4) and ammonia (NH3), (Vymazal, 2006). Figure 3 is a table by Vymazal that shows the nitrogen transformations within constructed wetlands.

Nitrogen transformations in (constructed) wetlands

Process	Transformation
Volatilization	ammonia-N (aq) → ammonia-N (g)
Ammonification	organic-N → ammonia-N
Nitrification	ammonia-N → nitrite-N → nitrate-N
Nitrate-ammonification	nitrate-N → ammonia-N
Denitrification	nitrate-N → nitrite-N → gaseous N_2, N_2O
N_2 Fixation	gaseous N_2 → ammonia-N (organic-N)
Plant/microbial uptake (assimilation)	ammonia-, nitrite-, nitrate-N → organic-N
Ammonia adsorption	
Organic nitrogen burial	
ANAMMOX (anaerobic ammonia oxidaton)	ammonia-N → gaseous N_2

Figure 3.4.2 1: Nitrogen transformations in constructed wetlands. (Vymazal, 2007)

The next stage of the nitrogen cycle is nitrification, which is carried out by certain bacterial groups known as nitrifying bacteria and use ammonium as a fuel source (Schipper, A. et al., 1996). During the process, bacterial groups (Nitrosomonas and Nitrococcus), transform ammonium to nitrite (Schipper, A. et al., 1996). Nitrite is usually a mediator nitrogen and Nitrobacter bacteria, among others, quickly convert nitrite to nitrate (Schipper, A. et al., 1996). Oxygen is required for nitrification, and warm, moist, well-aerated soils provide ideal conditions (Schipper, A. et al., 1996).

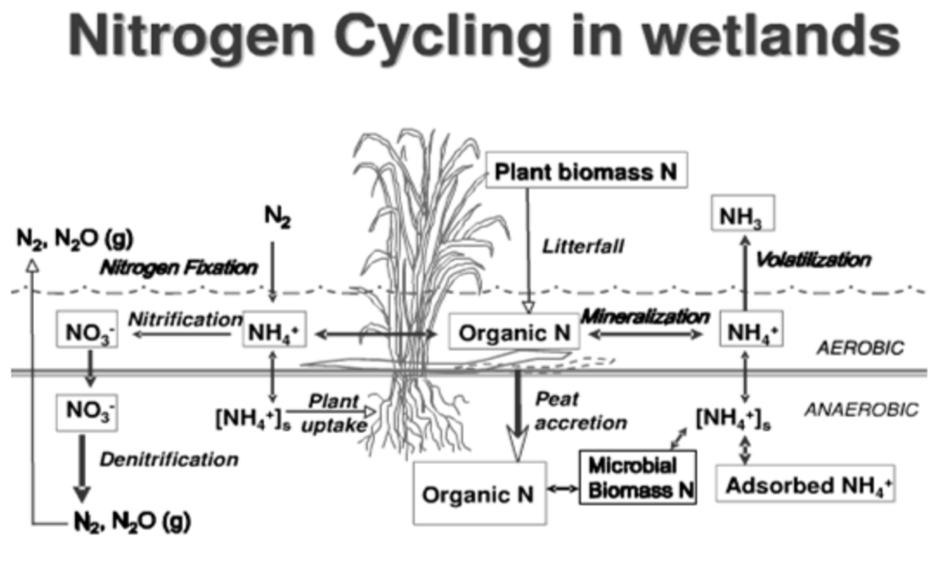

Figure 3.4.2 2 Principal components of the nitrogen cycle in wetlands (Docstoc, 2013).

At this point, nitrate is either assimilated by soil microorganisms or plants, lost to leaching, or undergoes another microbial conversion—denitrification (Schipper, A. et al., 1996). Under anaerobic conditions, which occurs in poorly drained soils, denitrifying bacteria use nitrate instead of oxygen when obtaining energy during the breakdown of organic compounds (Schipper, A. et al., 1996). During the process, nitrate is converted to nitrous oxide (N2O) and dinitrogen (N2) gases, which are released to the atmosphere (Schipper, A. et al., 1996). Figure 4 is a diagram by Docstoc which explains the nitrogen cycle within constructed wetlands.

Different forms of nitrogen are constantly involved in chemical transformations from inorganic to organic compounds and back from organic to inorganic. Some of these processes require energy, typically derived from an organic carbon source, to proceed, and others release energy, which is used by organisms for growth and survival (Schipper, A. et al., 1996). The mechanisms that will mainly remove nitrogen from wastewaters include only ammonia volatilization, denitrification, plant uptake (with biomass harvesting), ammonia adsorption, ANAMOX and organic nitrogen burial (Mustafa, 2017). While other processes, such as ammonification or nitrification, only convert nitrogen among various nitrogen forms but do not actually remove nitrogen from the wastewater. (Mustafa, 2107). A thin layer of almost saturated dissolved oxygen (DO) is created at the top of the water column as a result of the diffusion of oxygen from the atmosphere into the water (Mustafa, 2017). DO allows for aerobic decomposition and nitrification in CWs, which is essential for the survival of fish, other aquatic organisms, and for the general health of receiving water bodies (Kadlec and Wallace, 2009).

Xiong's experiment concluded that CWs have been effective for treatment of secondary effluent N, which was mainly composed of NO_3 –N (Xiong et. al., 2011). Carbon resource was a key to optimal denitrification. During Xiong's experiment, peat was used as a C source for denitrifying bacteria that can remove (NO_3 –N) as well as (NH_4 +- N). While floating beds can further remove ammonium, nitrate, and nitrite mediated by plant uptake and by rhizospheric denitrifying/nitrifying bacteria (Xiong et. al., 2011). Filter can further remove NH_4 +-N, NO_3 −-N and NO_2 −-N through nitrification and denitrification provided with longer distance, shallow water level and dissolved organic materials released from peat and plants. (Xiong et. al., 2011). Vymazal states that the magnitude of processes which ultimately remove total nitrogen from the systems is usually low, and therefore removal of TN is commonly low in single stage constructed wetlands (Vymazal, 2006). Nitrification is a limiting process for nitrogen removal from most types of constructed wetlands because ammonia is the dominant species of nitrogen in sewage and many other wastewaters (Vymazal, 2006).

Nitrification and denitrification have been recognized as the primary processes for ridding nitrogen from contaminated water (Vymazal, 2006). Denitrification is considered as a major removal mechanism for nitrogen in most types of constructed wetlands. The concentrations of nitrate, however, are usually very low in wastewater (Vymazal, 2006)

3.4.2.1 Denitrification

Denitrification is the microbial process of transforming nitrate and nitrite to gaseous forms of nitrogen, mainly of nitrous oxide (N_2O) and nitrogen (N_2) and a large range of microorganisms can denitrify (Skiba, 2008). Most denitrification is accomplished by heterotrophic bacteria (Wu et. al., 2017).

Denitrification is a response to changes in the oxygen (O_2) concentration of its immediate environment. Only when O_2 is limited, will denitrifiers switch from aerobic respiration to anaerobic respiration, using nitrate (NO_3^-) as an electron acceptor (Skiba, 2008). When N oxides it serves as a terminal for electron acceptors for respiratory electron transport and organic compounds serve as electron donors, the biochemical reaction strongly depends on carbon availability (Wu et. al., 2017).

Denitrification processes involved the initial NO_3^--N reduction to NO_2^--N, followed by further reduction to nitric oxide (NO), nitrous oxide (N_2O) and finally to molecular nitrogen (N_2). Increase in alkalinity is an indicator of denitrification resulting in an increase in effluent pH. (Xiong et al. 2011). Xiong's results showed that effluent pH was slightly higher than initial pH and in the range of 7.6–8.5. It implied that there was the possibility of denitrification in the integrated CWs. The increase in pH might be caused by the improvement of the denitrification conditions for the microbes in the sediment, which consume some acidic substances in the water (Luai et al., 2019). The NO_3^--N significantly decreased during the growth period. The decrease in NO_3^--N may be due to the filtration of suspended solids and particulates by the extensive root system as well as the biodegradation of refractory organics (Bindu et al., 2008; Achak et al., 2009).

Generally, a COD/N (chemical oxygen demand: total nitrogen in influent) ratio of 4 or more is generally considered to the best situation for microorganism reproduction and N removal in CW (Ding et al., 2012; H. Wu et al. 2017). But the characteristics of micro-polluted are the low pollutant concentrations and the low COD/N ratios, which make biological treatment more difficult. Plants can convert atmospheric CO_2 into biomass (organic C) through photosynthesis, which might eventually become available to denitrifying bacteria through a number of pathways such as the death and decomposition of plant litter and the secretion of root exudates (Zhai et al., 2013). During winter, the temperature in the water decreased from 22.3 °C to 12.6 °C, and all pollutant removal efficiencies of CWs decreased with the cold temperature (Wu et al. 2017).

3.4.3 Carbon Cycle

The organic carbon in CWs generally originates from wastewater, root exudates, and plant materials (Van Oostrom and Russell, 1994). This means that the root of macrophytes can produce and discharge organic carbons, such as amino acids, organic acids, sugars, and polysaccharides, during plant growth. The quantity of root exudates depends upon plant species, plant age, and external biotic and abiotic factors (Baetz and Martinoia, 2014). These organic carbons can serve as a carbon source in nitrogen conversion (Martin et al., 1999) and improve nitrate removal efficiency (Lin et al., 2002). Therefore, the usage of the carbon source derived from plants improves the NO3 − removal in CWs (Lin et al., 2002). Figure 5 is a representation of the inland wetland carbon cycle. Major pathways of carbon sequestration include photosynthesis and organic carbon accumulation (Bernal and Mitsch, 2012).

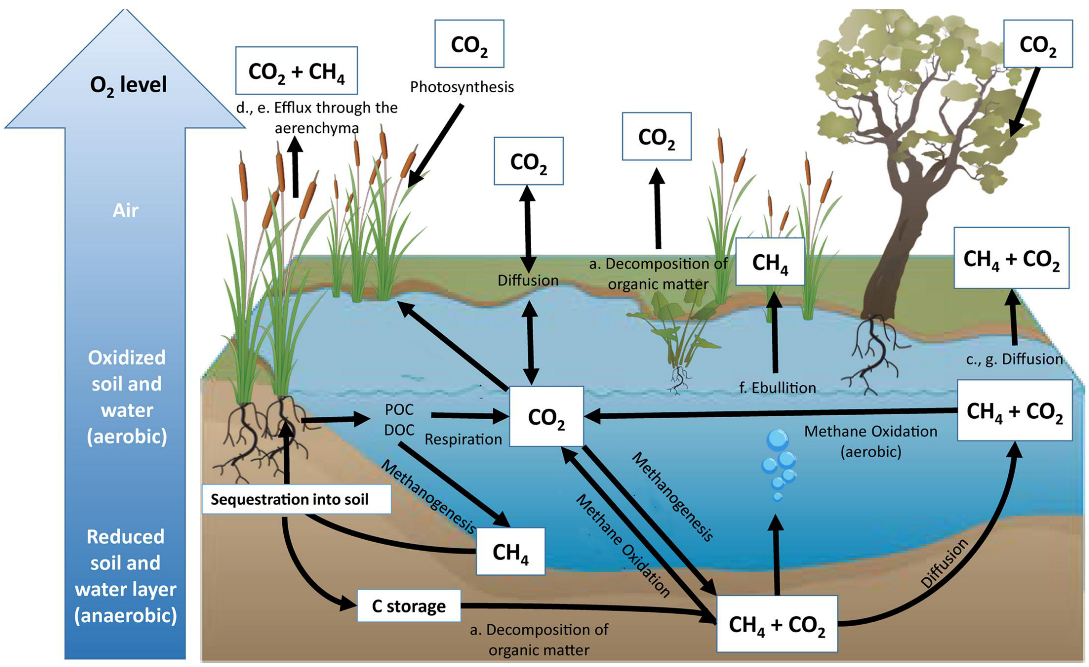

Figure 3.4.3 1 Representation of the inland wetland carbon cycle. Major pathways of carbon sequestration include photosynthesis and organic carbon accumulation (Bernal and Mitsch, 2012).

Studies have shown that the TOC of the receiving water quickly increased during the first stages of decomposition and that this rate of increase increased with higher doses of biomass, which may be caused by the leaching of some organic matter (such as sugars) from the rapidly decomposing materials in the biomass (Luai et al., 2019).

Reinhardt's (2006) study suggests that a higher TOC consumption rate was achieved after sediment addition with 0.5 g L biomass dose, this suggests that the microbes in the sediment may accelerate the denitrification process, which is supported by the lower TOC and DO observe in the later stage of the process after sediment introduction (Reinhardt et al., 2006). Organic matter in the biomass was more easily released from the biomass than nitrogen even when a different dose of biomass was added (Fan et. al., 2021). Previous studies on denitrification have suggested that high microbial activity corresponds to high nitrate removal efficiency (Khan et al., 2019). The high ratio of TOC/TN derived from the biomass decomposition implies that it can be a carbon source for denitrification (Fan et. al, 2019).

The decomposition process may contribute to nitrogen elimination in CWs because it can release some organic carbon, which may be utilized as carbon sources for denitrification processes (Park et al., 2008). Carbon sources can serve as electron donors of biological denitrification processes in bioreactors and CWs (Mateus and Pinho, 2020). This can significantly increase nitrogen elimination in treating water with low C/N ratios, such as municipal wastewater effluent and agricultural runoff (Mateus and Pinho, 2020).

3.4.4 Phosphate Removal

Phosphorus in wetlands occurs as phosphate in organic and inorganic compounds (Vymazal, 2007). Free orthophosphate is the only form of phosphorus believed to be utilized directly by algae and macrophytes and therefore represents a major link between organic and inorganic phosphorus cycling in wetlands. Wetlands provide an environment for the interconversion of all forms of phosphorus. Plants absorb soluble reactive phosphorus and transform it into tissue phosphorus. It can also sorb into the soils and sediments of wetlands (Vymazal, 2007). If the organic matrix is oxidized, the organic structural phosphorus may be released as soluble phosphorus (Vymazal, 2007). Phosphorus transformations in wetlands consist of peat/soil accretion, adsorption/desorption, precipitation/dissolution, plant/microbial uptake, fragmentation and leaching, mineralization and burial (Vymazal, 2007). Therefore, all these elements should be quantified when assessing a wetland ecosystem's ability to hold onto P (Vymazal, 2007). Sorption as well as storage in biomass are saturable processes, meaning they have a finite capacity and therefore cannot contribute to long-term sustainable removal (Dunne and Reddy, 2005).

The soil phosphorus cycle is fundamentally different from the N cycle. There are no valency changes during biotic assimilation of inorganic P or during decomposition of organic P by microorganisms (Lindsay, 1979). Soil P primarily occurs in the +5 (oxidized) valency state, because all lower oxidation states are thermodynamically unstable and readily oxidize to PO4 even in highly reduced wetland soils (Lindsay, 1979).

Like nitrogen, P is also a nutrient required for plant growth. According to IWA (2000), there are 3 main processes for P removal in CWs which includes: 1.) soil sorption, 2.) absorption by biota, including bacteria and macrophytes, and 3.) accretion. Wallace and Knight (2006) state that the settling and trapping of phosphate contributes to the accretion process. Jakubaszek (2021) stated that the main P removal processes in CWs are: 1.) sedimentation, 2.) absorption of plants, 3.) absorption of phosphorus by denitrifying phosphate accumulating organisms (DPAO), and 4.) absorption by the substrate. CWs around the world have faced many issues when dealing with N and P (Gao and Zhang, 2022). Marques (2001) experiment concluded that sand-based subsurface flow wetlands treating anaerobically treated municipal wastewater performed similarly, except for phosphate removal, between planted and unplanted cells under low loading conditions. However, plants improved treatment efficiency under high loading conditions (Marques et al., 2001). Cheesman's experiment concluded that under nutrient-rich conditions, P is sequestered by the buildup of chemicals generated from microbes and the presumed concentration of endogenous macrophyte P. Under nutrient-poor conditions, standing P pools within wetland soils appear to be independent of the heterotrophic decomposition of macrophyte leaf litter (Cheesman et. al., 2010).

3.5 Microbial Activity

A wide variety of microorganisms, including bacteria and fungi, can be found in constructed wetlands and microbial biomass is an important and storage place for organic carbon and other nutrients (Moshiri, 1993). Wallace and Knight (2006) stated that the breakdown and consumption of organic matter, such as biological oxygen demand (BOD) in influent wastewater, is carried out by microorganisms. Microorganisms also absorb and transform nutrients such as nitrogen (Wallace and Knight, 2006). Organic matter is degraded by either aerobic or anaerobic microorganism, and when bacteria convert and mineralize organic matter, rhizospheric oxygen plays a crucial part in the deterioration of the material (Rehman et. al, 2017). Rehman (2016) states that aerobic microorganisms consume oxygen in order to breakdown organic matter to CO2 and water provides the energy and biomass for microorganisms. Whereas aerobic bacteria are primarily found at the roots of wetlands and obtain their nutrition and energy through symbiosis, anaerobic bacteria break down organic matter to produce methane for nutrition and energy (Rehman 2016). Aerobic, anaerobic, and anoxic degradation are the three primary types of processes involved in the decomposition of organic matter (Stottmeister, et. al, 2003). Stottmeister et. al., stated that the most effective mechanism is aerobic degradation, in which oxygen acts as the final electron acceptor. Nitrates, sulfates, and carbonates act as the final electron acceptor in an anoxic environment, where they are converted to oxides (Stottmeister, et. al, 2003).

Previous studies on denitrification have suggested that high microbial activity corresponds to high nitrate removal efficiency (Khan et al., 2019). Microbial activity is higher in warmer conditions and slows down as the temperature cools (Schipper, A. et al., 1996). Bacterial activity nearly stops as temperatures approach 0°C and as mentioned earlier, warm, moist, well-aerated soils provide ideal conditions for nitrification (Schipper, A. et al., 1996). Climate has a direct and indirect impact on plant nutrient intake, microbial modification of wastewater components, and plant litter in wetlands. (Wittgren and Mæhlum, 1997).

Bacteria sticks to surfaces in the wetland (solid particles and/or plant roots) and form a biofilm (Watnick & Kolter, 2000). This biofilm is responsible for most of the essential transformations and decomposition of contaminants in the wastewater (Larsen & Greenway, 2004). However, little is known about the bacterial populations involved in the formation and activity of this biofilm (Tru, 2009). Ibekwe et al. (2007) examined sediments and rhizosphere from surface flow constructed wetland system and showed that the majority of obtained sequences belonged to unclassified taxa, while the second dominant group consisted of members of the proteobacteria.

Different studies have previously investigated how various wetland conditions affected the make-up of the microbial community in the wetland biofilm. Truu et al. (2007) have successfully assessed microbial community structure in different layers of planted soil wetland for domestic wastewater treatment. Regarding bacterial communities, water depth had an impact on the wetland biofilm's microbial community structure, such as ammonia-oxidizing bacteria, and archaea (Iasur-Kruh, et. al., 2010). In contrast, the presence of various substrate materials or vegetation has no impact on the makeup of the microbial community in wetlands. (Osem, et. al, 2007). Nguyen (2000) states that organic matter had an impact on the biomass and activity of distinct wetland systems' biofilms, surface properties, and depth. Therefore, the creation of the wetland's physical and chemical characteristics influences how biofilms assemble and work (Iasur-Kruh, et. al., 2010).

3.6 Arbuscular Mycorrhizal Fungi (AMF)

Arbuscular mycorrhizas (AM) are defined as by the presence of arbuscules, which is the nutrient exchange between the plant and fungi, that normally form in root cortex cell and based on their morphology of fungal branching within the roots (Brundett, M. C. et. al., 2018) Arbuscular Mycorrhizal Fungi (AMF) are soil microbes that colonize majority of the plant root and establish a connection between the plant and substrate (Sharma, et. al, 2021). They also form symbiotic associations with 80% of vascular plant species (Smith, S.E. et. al, 2008). Brundett (2019) provides additional morphological characteristics of the AMF and they are as followed in the table below:

Morphological Characters	Arbuscules present; vesicles present/absent; colonization from root surface mycelia or from neighboring cells
Plant Dependency	Mostly obligatory (survival with reduced competition)
Benefits supplied to plants	Nutrition (mineralized nutrients), limited protection
Benefits to fungi	Carbon energy, habitat, deep water from trees
Presence of cheating association	In plants (multiple groups)

Table 3.6

Although AMF studies are very common to see for terrestrial plants, several studies have also occurred for aquatic plants in wetland habitats (Calheiros, C. et al., 2019). Recent studies have demonstrated that AMF are present in the roots of many wetland plants, such as submerged aquatic plants and in various wetland types (Xu et. al, 2016). As a result, Xu states that AMF's functional roles in wetland ecosystems and the possibility of application in wastewater bioremediation technical installations should be further studied (2018).

Vegetation provides different results in organic and nutrient removal in treatment wetlands however both bacteria and fungi have an important role in the assimilation, transformation, and nutrient cycling present in wastewater (Calheiros, C. et al., 2019; Kadlec, R.H., 2009). Calheiros' experiment resulted in successful AMF colonization within the roots of the plants in the constructed wetlands, however this success was due to the water quality, the season, and plant species.

AMF has been well documented for its important effects on plant growth, resistance, and rhizospheric microbial activity in response to biotic and abiotic challenges (Camenzind et al. 2016; Huang et al. 2018). It has been observed in the past two decades, that AMF could develop mycorrhiza with wetlands plants, enabling them to carry out their functions in aquatic habitats (Hu et al. 2020; Xu et al. 2016). The extension of plant roots is also beneficial to the uptake of $NH4^{+}$-N, as well as for the assimilation activities of plants and microorganisms (Xiong et. al., 2011)

AMF entirely dependent on their plant hosts for carbon (C) therefore high $CO2$ could directly affect the C allocations to mycorrhizas (Feng et al. 2021). Xu concluded that AMF had the ability to establish under the circumstance of CWs and definite potential in phytoremediation of wastewater. Aeration is also used to raise the amount of dissolved oxygen in water bodies, which is well known as a physical way to enhance the capacity of wetlands to remove contaminants (Feng et al. 2021). These findings suggested that increased aeration (AA), particularly when heavy metal (HM) concentrations were high, improved AMF colonization in VFCWs, which was consistent with the results of Xu et al. (2021).

Huang and Wang et. al. state that one of the main reasons that AMF may not colonize roots at a high level like in terrestrial ecosystems is because wetland areas have less oxygen than other types of habitats. Aeration not only increases the amount of dissolved oxygen in wetlands but also encourages the growth of wetland plants, increasing the amount of surface area available for the growth of microorganisms and altering the diversity of microbial communities distributed in the rhizosphere (Feng et al. 2021). Ferreira et al. (2021) and Viollet et al. (2017) stated that the diverse microbial communities, such as nitrogen-fixing bacteria and plant-growth-promoting rhizobacteria, played a beneficial role in promoting AMF colonization by helping AMF obtain more nutrients and facilitating cell growth to produce spores, and suggested that this was also the case in aquatic environments. Therefore, the AA played a promising role in promoting AMF colonization in wetland plant roots.

AA accelerated the absorption and transport of water and nutrients; therefore, it improved the growth of plants, which was beneficial to increase the resistance to combined HM stress and the transfer of HMs from roots to shoots (XU, et. al. 2022). AA also enhanced AMF colonization in plant roots, leading to a greater accumulation of HMs in roots and enhancing plant physiological state (XU, et. al. 2022). Xu et. al concludes that AMF inoculation and AA were beneficial for wetland plants to resist heavy metal stresses. AA also increased oxygen supply for VFCWs because it led to the growth of microorganisms on the substrates and roots, therefore creating more biofilm. It is very important to control water content in wetland systems in order to increase AMF colonization in wetland plants. Previous studies reported that AMF colonization in wetland plants gradually decreased with the increase of water regime (Miller, 2000; Wang et al., 2011).

Physiological functions of AMF inoculated wetland plants (plant height, plant biomass, RWC, and nutrient contents, such as total nitrogen and phosphorus), can be improved under low water level or fluctuating water (Hu et. al., 2020). Therefore, physiological functions of wetland plants might be limited due to lack of water in wetland system, although high AMF colonization was confirmed. However, specific water regime conditions, such as fluctuating water might become a possible method to meet the water requirements of AMF colonization and physiological functions of wetland plants. (Hu et. al., 2020)

Chapter 4: Methodology

4.1 Experimental Design

4.1.1 Location

This experiment took place at the Czech University of Life Sciences Prague (CZU) and lasted approximately three months. The constructed wetlands were placed at the greenhouse located within the university campus. The samples taken from the constructed wetlands were then taken to the laboratory located at the FES building.

4.1.2 Materials

The constructed wetlands were placed in 8 PVC pipes and the pipes were established to simulate vertical subsurface flow CWs. The dimensions of each CW system were 15× 55 cm (diameter × height). Inside each constructed wetland there were substrates that consisted of sand and gravel:

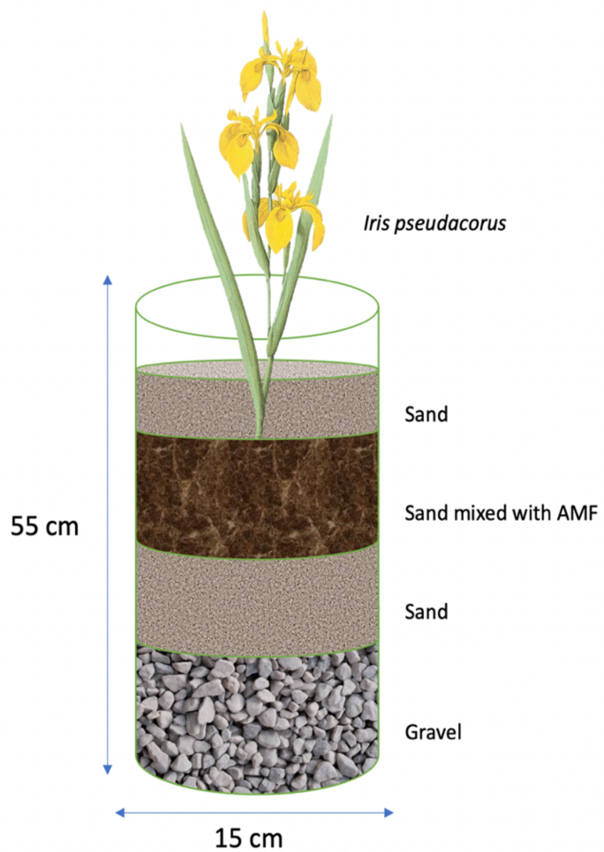

Figure 4.1.2.1 Reactor model with inside materials

Each reactor was filled with 15 cm of gravel and 25 cm of sand. In regard to the Arbuscular mycorrhizal fungi (AMF) system, the substrates from the bottom to top were: 15 cm gravel, 10 cm sand, 10 cm sand mixed with 50 g of AMF. The planted vegetation consisted of *Iris pseudacorus* (yellow iris), afterwards 5 cm of sand was be added, and the AMF organisms consisted of *Rhizophagus irregularis*.

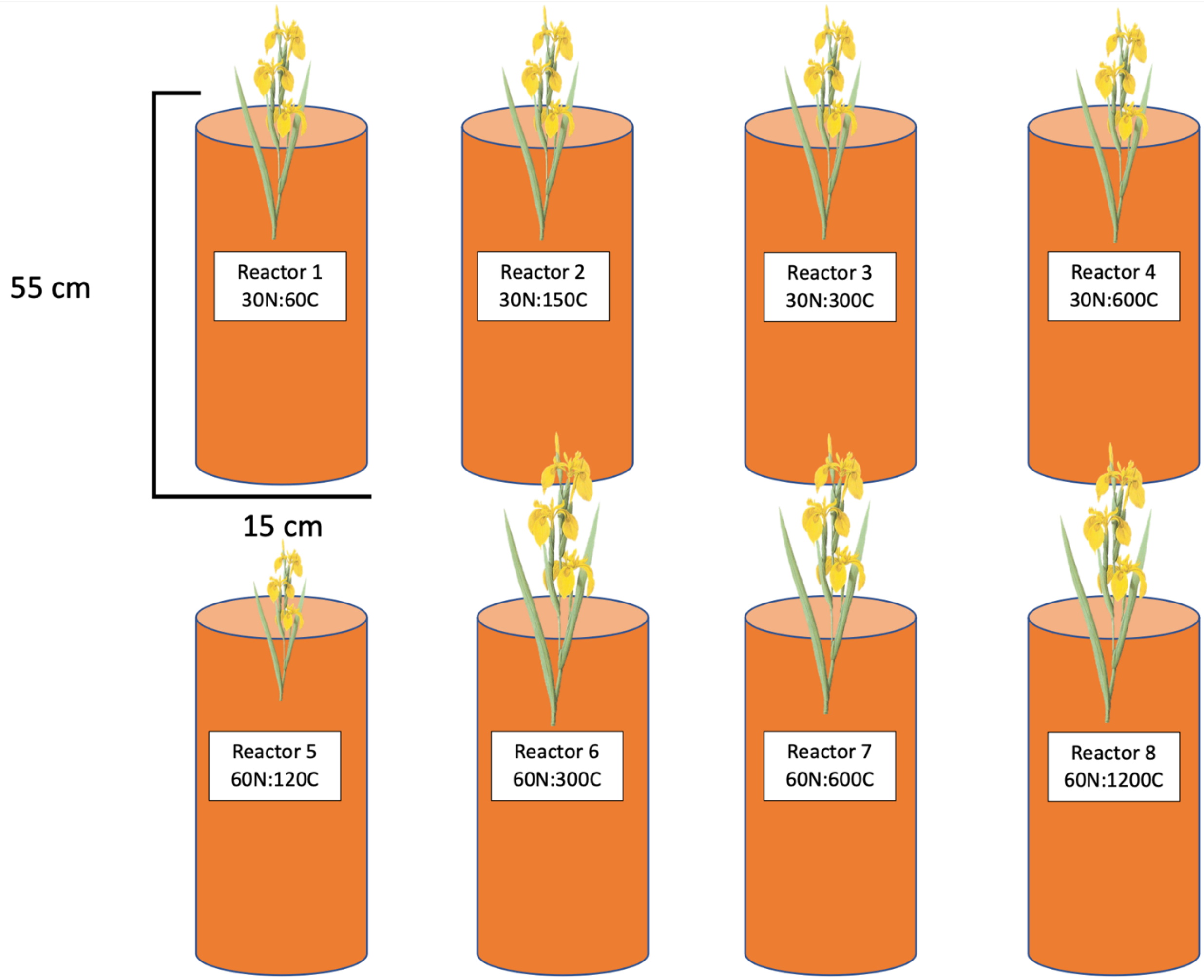

Figure 4.1.2. 2 Experiment diagram of the 8 reactors

The conditional variables of this study are the different ratios of carbon concentrations (low, high) and nitrogen concentrations (low, high). The influent wastewaters were prepared with reagents that simulate and are commonly found in municipal sewage wastewater. Therefore 8 different wastewater treatments with different ratios of nitrogen and carbon were prepared for each reactor.

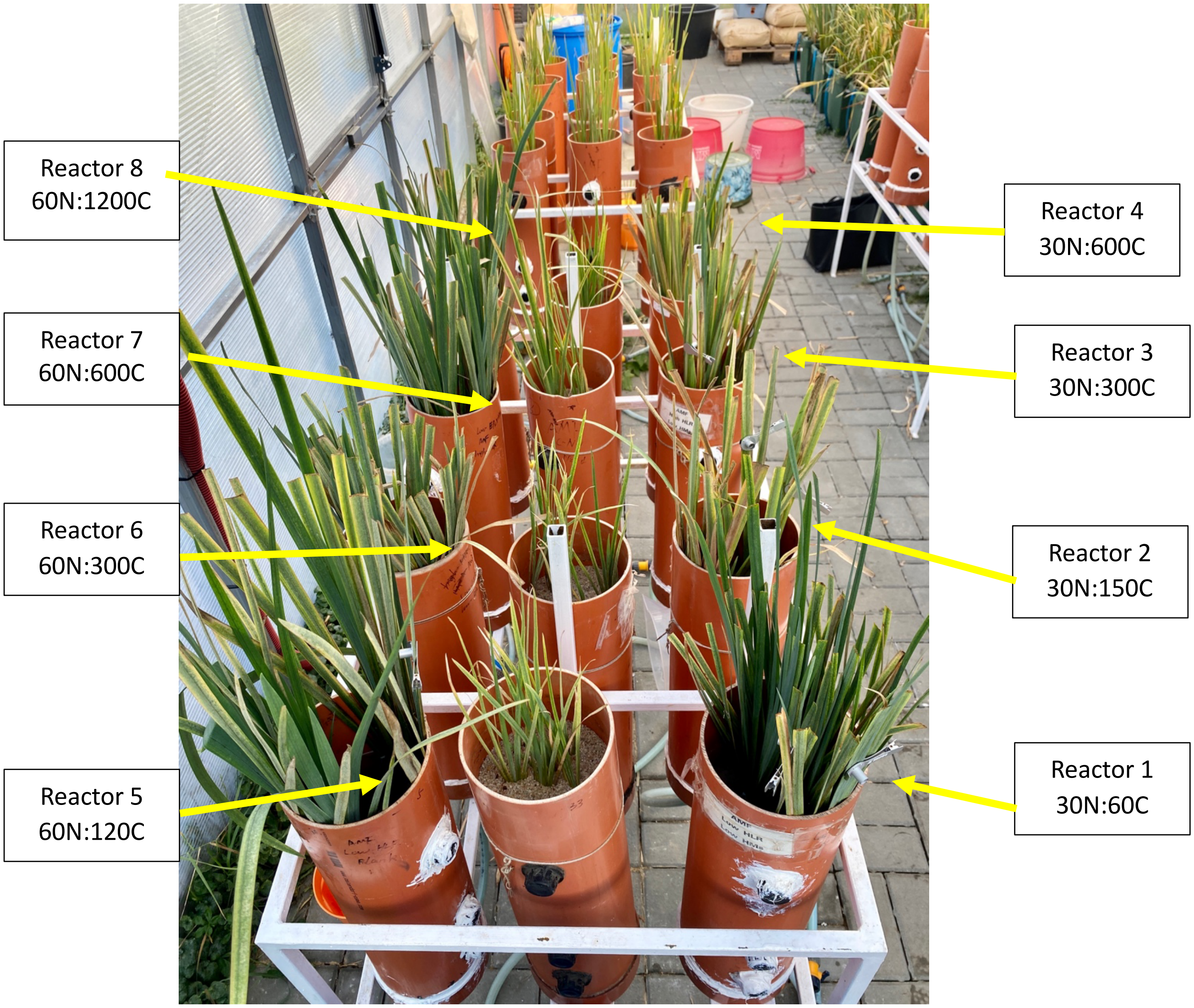

Figure 4.1.2. 3 Experiment setup in the greenhouse

The effluent that was prepared for this experiment was wastewater that consisted of urea, ammonium chloride (NH_4Cl), sodium acetate trihydrate ($CH_3COONa*3H_2O$), peptone, yeast extract, skim milk, sodium bicarbonate ($NaHCO_3$), Magnesium Chloride ($MgCl_2*6H_2O$), monopotassium phosphate (KH_2PO_4). The microelements used in the effluent reagents consisted of copper sulfate pentahydrate ($CuSO_4*5H_2O$), ferrous sulfate heptahydrate ($FeSO_4*7H_2O$), boric acid (H_3BO_3), sodium molybdate dihydrate (Na_2MoO_4 $*2\ H_2O$), chromic potassium sulfate ($KCr(SO_4)_2*12\ H_2O$). The sodium acetate trihydrate ($CH_3COONa*3H_2O$) was used as a carbon simulate and ammonium chloride (NH_4Cl) was used as the simulate for nitrogen. Table 4.1.2 provides the measurements of the different reagents (simulated wastewater) and their nitrogen and carbon ratios:

3L	C:N 2	C:N 5	C:N 10	C:N 20	C:N 2	C:N 5	C:N 10	C:N 20
Reagent (g)	30N 60C	30N 150C	30N 300C	30N 600C	60N 120C	60N 300C	60N 600C	60N 1200C
Urea	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
NH_4Cl	0.08	0.08	0.08	0.08	0.465	0.465	0.465	0.465
$CH_3COONa*3H_2O$	0.45	1.965	4.572	9.72	1.47	4.572	9.72	20.55
Peptone	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Yeast extract	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Skim milk	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
$NaHCO_3$	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
$MgCl_2*6H_2O$	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
KH_2PO_4	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
microelements (mL)								
$CuSO_4*5H_2O$	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
$FeSO_4*7H_2O$	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
H_3BO_3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
$Na_2MoO_4*2H_2O$	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
$KCr(SO_4)_2*12H_2O$	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Table 4.1.2 1: Reagents and amounts for simulated wastewater and C:N ratios

The prepared reagents were fed into each constructed wetland and were the inlet water of the CWs. The CWs were protected from the rain throughout the experiment to prevent pollution or additional nutrients from entering the CWs and to prevent any result alterations. The prepared wastewater was poured into the vertical column, after the wetland is drained, with a hydraulic load rate of 1.5 liters. The wastewater would remain in the wetland for 7 days, then drained for testing, and refilled again with the prepared effluent. This process would happen once a week.

4.2 Sample Analysis

Once a week, three liters of simulated wastewater (inflow) were prepared for each reagent ratio and 1.5 liters of the wastewater was inserted into their respective constructed wetland. The filtered water from the CWs would be drained, its volume assessed, and samples obtained on the same day. Plant water uptake (evapotranspiration) was calculated when the outflow water was drained from the wetlands and the volume was measured by liters. From those liters, approximately 50mL of the outflow was collected for sampling. For each constructed wetland, 2 samples were to be collected and sampled giving us a total of 16 samples to be analyzed each week. The 16 samples (8 outflow and 8 inflow) were then taken to the laboratory and were first analyzed for pH, temperature (C°), and oxidation-reduction potential (ORP) using the HQD Field Case (HACH). Then the samples were prepared for the ammonium (NH_4^+) analysis by taking 1 mL of the sample and adding 7.4 mL of distilled water, .8 mL of reagent A, and .8 mL of reagent B (color additive). The samples were then placed in the dark for approximately 30 minutes and were then analyzed using the Cary 60 UV-VIS spectrophotometer (Agilent Technologies). The samples were then prepared to find the concentrations of nitrate (NO_3^-), nitrite (NO_2^-), sulfate (SO_4^{2-}) and phosphate (PO_4^{3-}). Approximately 12 mL of the samples were filtered through a syringe filter and into a plastic tube. The filtered samples were then placed on the 883 Basic IC plus to determine their concentrations. The machine would take approximately 15-20 minutes per sample and the results were taken the next day. The samples were then prepared to find the concentrations of Total Nitrogen (TN) and Total Carbon (TC). Approximately 6 mL of the outflow sample was diluted with deionized water and 2 mL of the inflow sample was diluted with deionized water, once the samples were diluted, they were placed on the Formacs^{SERIES} TOC/TN analyzer (SKALAR). Dilution of the samples was necessary because when there are high amounts of TOC/TN concentrates it can potentially breakdown the machine. The machine would take approximately 15-20 minutes per sample and the results were taken the next day.

Every 2 weeks the photochemical efficiency of the wetland leaves was examined using the PAM (Pulse Amplitude Modulation) 2500 fluorometer. The leaves would have a Leaf Clip attached to them for 15 minutes to permit dark acclimation of small leaf areas. The fluorometer would then be inserted into the leaf clip and the sliding shutter of the DLC-8 can be opened so that, FO and FM level fluorescence can be measured without interference of ambient light. The measurements would then be seen on the mobile touch computer screen. This process would be done for each constructed wetland.

After the experiment was over, the biomass was removed from the PVC pipes and the roots were then cleaned and weighed. The biomass was weighed twice, the first time was right after the biomass was removed from the PVC containers and the biomass was wet.

The second time the biomass was weighed one month after the first time and the biomass was dry.

4.2.1 Sample Testing Machinery

Several machines were used during this experiment to test the water samples that were taken from the constructed wetlands. The first machine that was used after taking out the sample was the HQD Field Case (HACH) which is a portable meter with detachable probes. The probes are able to measure the pH and oxidation-reduction potential (ORP) in the water samples. The Cary 60 UV-VIS spectrophotometer (Agilent Technologies) was used to run an ammonium analysis. The spectrophotometer measures the number of photons (the intensity of light) absorbed after it passes through sample solution (chem.libretexts.org). The Formacs^{SERIES} TOC/TN analyzer (SKALAR) was used to measure the total nitrogen, total carbon and total inorganic carbon. The machine uses an injection to collect the sample and uses a high temperature catalytic combustion to analyze the sample (skalar.com). The 883 Basic IC plus machine was used to find the nitrate (NO_3^-), nitrite (NO_2^-), sulfate (SO_4^{2-}) and phosphate (PO_4^{3-}) in the samples. This machine is used to separate and identify different chemical compounds by passing a gas or solution through columns containing beads that can selectively retain or control the rate of movement of different chemical species based on molecular size (Simon, 2012). When doing an analysis on the chlorophyll fluorescence, the PAM 2500 portable machine was used, light energy is absorbed by the chlorophyll molecules in a leaf (Maxwell, 2000). This analysis can have 3 outcomes: it can be used to drive photosynthesis (photochemistry), extra energy can be dissipated as heat, or it can be re-emitted as light (chlorophyll fluorescence) (Maxwell, 2000). These three outcomes occur in competition that if there is an increase in efficiency in one result there would be a decrease on efficiency in the other two outcomes. Therefore, by measuring the total chlorophyll fluorescence, information about changes in the efficiency of photochemistry and heat dissipation can be gained (Maxwell, 2000). After the results were retrieved from the PAM 2500, a Fv/Fm test was done for the results. The Fv/Fm test is designed to allow the maximum amount of the light energy to take the fluorescence pathway. It compares the dark-adapted leaf pre-photosynthetic fluorescent state, called minimum fluorescence, or Fo, to maximum fluorescence called Fm. In maximum fluorescence, the maximum number of reaction centers have been reduced or closed by a saturating light source. In general, the greater the plant stress, the fewer open reaction centers available, and the Fv/Fm ratio is lowered (Maxwell K., Johnson G. N. 2000). Fv/Fm is a measuring tool that works for many types of plant stress. An Fv/Fm value in the range of 0.79 to 0.84 is the approximate optimal value for many plant species, with lowered values indicating plant stress (Maxwell K., Johnson G. N. 2000)

4.3 Data Analysis

Once the results were provided by the testing machines they were dated and organized on excel. In the case of missing data or 0 values provided by the results, the missing values were retrieved by taking the averages of the other reactors from the same day or, if 2 results were missing from the same reactor, the values of the same reactor with different dates were then averaged to retrieve the missing values.

Once the missing values were retrieved, the data was adjusted by doing the conversion factors for total nitrogen and by multiplying the number of times the results were diluted. The diluted results were done for Total Nitrogen (TN), Total Carbon (TC), Total Inorganic Carbon (TIC), and Ammonium (NH_4^+). The outflow results for were multiplied times 2 and the inflow results were multiplied times 6 for TN, TC, and TIC. All ammonium results were multiplied times 8. The nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+) were converted to get the nitrogen values by using the following conversion factor:

The results for (NO_3^-) were multiplied by .2259 to get the value of nitrogen within the nitrate. The results for (NO_2^-) were multiplied by .3044 to get the value of nitrogen within the nitrite. The results for (NH_4^+) were multiplied by .788 to get the value of nitrogen within the ammonium. After these conversions were completed, the removal efficiency (RE) was calculated on excel for each reactor to determine the removal of each nutrient. The following equation was used to calculate the removal efficiency:

$$R = \frac{C_0 - C_e}{C_0} \times 100\%$$

4.4 Statistical Analysis

Once the removal efficiency was retrieved, we were able to see the efficiency values within the constructed wetlands. After the removal efficiency (RE) was calculated for each nutrient, a box whisker plot was created for each nutrient. The plot displayed the averages, min, max and medium values. Afterwards, a statistical analysis was done the RE values using a T-Test to get their P-Values. The T-test was done on Graphpad.com.

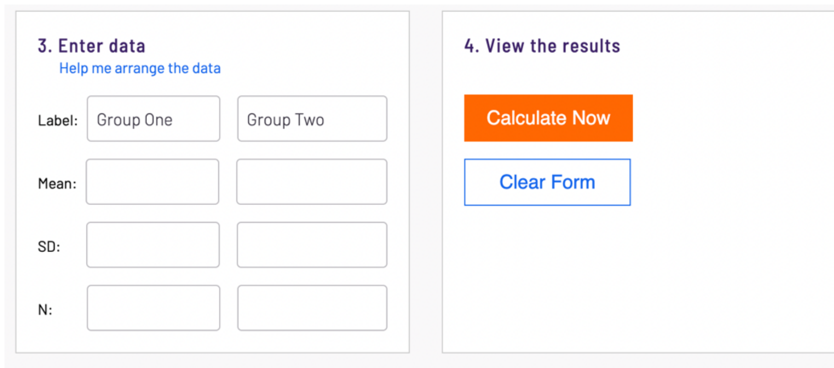

Figure 4.4 1 Graphpad.com layout for T-test

A T-test is a statistical test that is used to compare the means of two groups. It is often used in hypothesis testing to determine whether a process or treatment actually has an effect on the subject of interest, or whether two groups are different from one another. The RE values were tested with values that corresponded with the same ratio of N and C (e.g. Column C) and testing the values with the same ratio of 30N and 60N (e.g. Column B and C). The values were tested as follow:

A.	B.	C.
Reactor 1 vs Reactor 2 Reactor 1 vs Reactor 3 Reactor 1 vs Reactor 4	Reactor 5 vs Reactor 6 Reactor 5 vs Reactor 7 Reactor 5 vs Reactor 8	Reactor 1 vs Reactor 5 Reactor 2 vs Reactor 6 Reactor 3 vs Reactor 7 Reactor 4 vs Reactor 8

Table 4.1 1 T-test for reactor results

The next part of the thesis will discuss and display the different results retrieved from the machines, removal efficiencies and statistical analysis.

Chapter 5: Results

Once a week, samples were collected from each constructed wetland (reactor). In total 16 samples were collected weekly; 8 samples were from the treated wastewater (outflow) and the other 8 samples were the wastewater influent (inflow). Each collected sample was approximately 50mL, the samples were then taken to the laboratory for further analysis.

5.1 Water Loss, pH, ORP

Measuring the volume of the outflow for each reactor was the first task that was done before adding the new influent and before taking the samples to the lab. The volume of the outflow indicates any evapotranspiration or water loss that took place during the week. The Y-axis is the water loss volume in percentage. The change in volume indicates that there was more water loss during the months of October and early November. As the temperature decreased the water volume remained almost the same after one week.

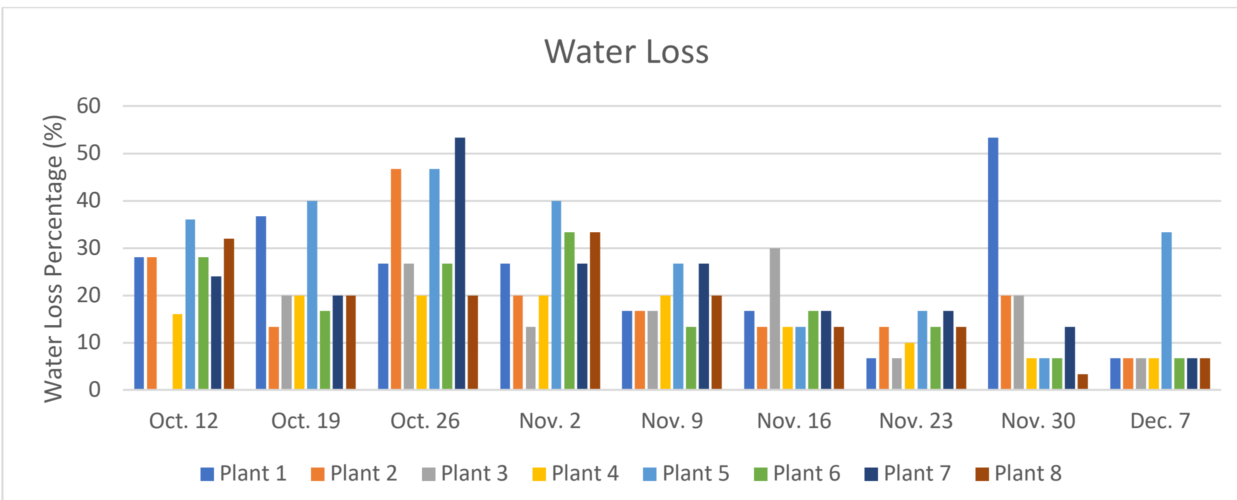

Figure 5.1 1 Water loss percentage calculated over the course of the experiment

The first results that were retrieved from the weekly samples (8 inflow and 8 outflow) were for pH and oxidation reduction potential. The pH values remained consistent during the experiment with 6.76 being the lowest, 7.327 being the median, and 7.7.35 being the highest value. The expected pH for constructed wetlands is between 6.5-7.5, which is consistent with the values from the reactors. The literature suggests that increases in pH might be caused by the improvement of the denitrification conditions for the microbes in the sediment.

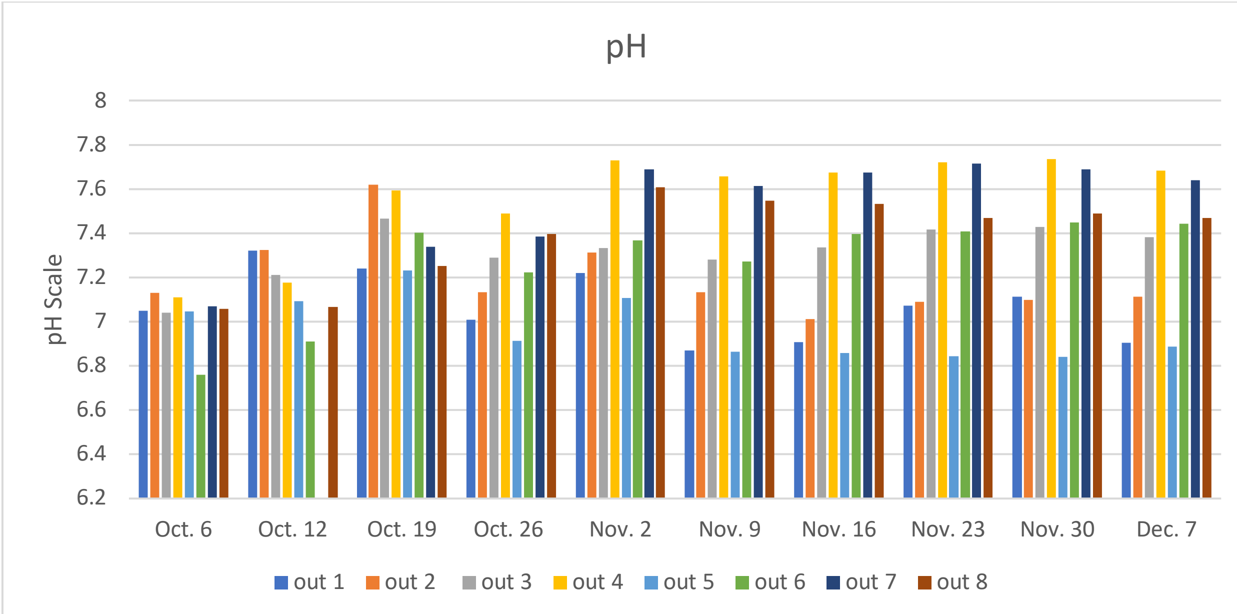

Figure 5.1 2 pH results over the course of the experiment

The oxidation reduction potential (ORP) or redox potential is used to describe a system's overall reducing or oxidizing capacity. The redox potential was measured in millivolts (mV). Søndergaard (2010), stated that in well-oxidized water, if oxygen concentrations stay above ∼1 mg O2 l−1, the redox potential will be highly positive (above 300–500 mV). The redox potential will be low (below 100 mV or even negative) in reduced environments, such as deep water in stratified lakes or silt in eutrophic lakes (Søndergaard, 2010). The redox potential can be lowered by microbially mediated redox processes to as low as -300 mV (Søndergaard, 2010).

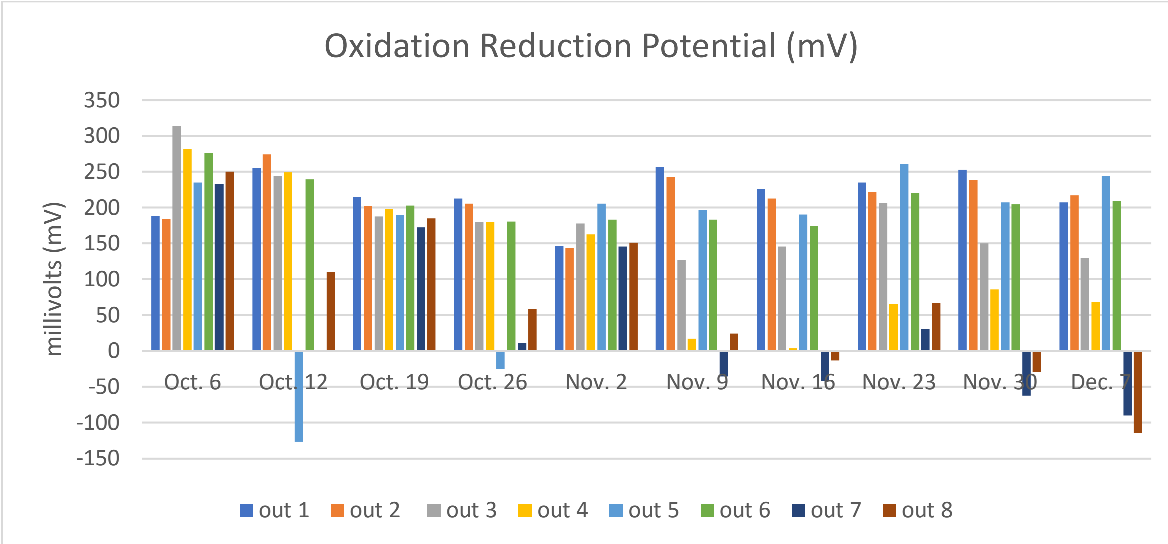

Figure 5.1 3 ORP results measured in (mV) over the course of the experiment

This part of the thesis will discuss the results that were taken from the samples over the 9 weeks that the experiment took place. When preparing the wastewater influent, the different variables in the experiment were the different concentration ratios between nitrogen and carbon. The results will show if the variables have a significant impact, if they are probable, or if the different variables were able to be efficient when treating wastewater. The discussion will focus on the removal efficiency and P-Values, and that were generated from the results. The concentrations from the resulted samples were measured as mg/L. Table 5.1 represent the different nitrogen and carbon ratios that were placed in each reactor:

Reactors	1	2	3	4	5	6	7	8
	C:N 2	C:N 5	C:N 10	C:N 20	C:N 2	C:N 5	C:N 10	C:N 20
Reagent (g)	30N 60C	30N 150C	30N 300C	30N 600C	60N 120C	60N 300C	60N 600C	60N 1200C
NH_4Cl	0.08	0.08	0.08	0.08	0.465	0.465	0.465	0.465
$CH_3COONa*3H_2O$	0.45	1.965	4.572	9.72	1.47	4.572	9.72	20.55

Table 5. 1 Different C:N ratios and nitrogen and carbon concentrations

5.2 Ammonium

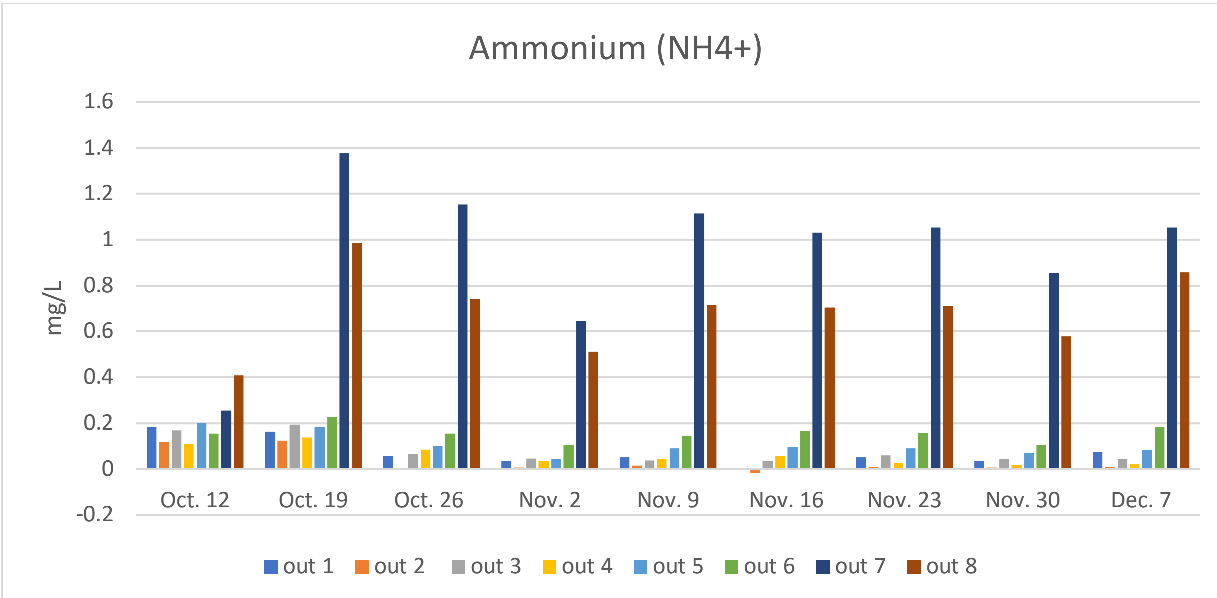

Figure 5.2 1 Ammonium (NH4+) Concentration in Outflow Samples measured in mg/L

Figure 5.2.1 shows the outflow concentrations for NH4+, it was noticed that reactor 7 and 8 (out 7 & out 8) had the highest concentrations of ammonium.

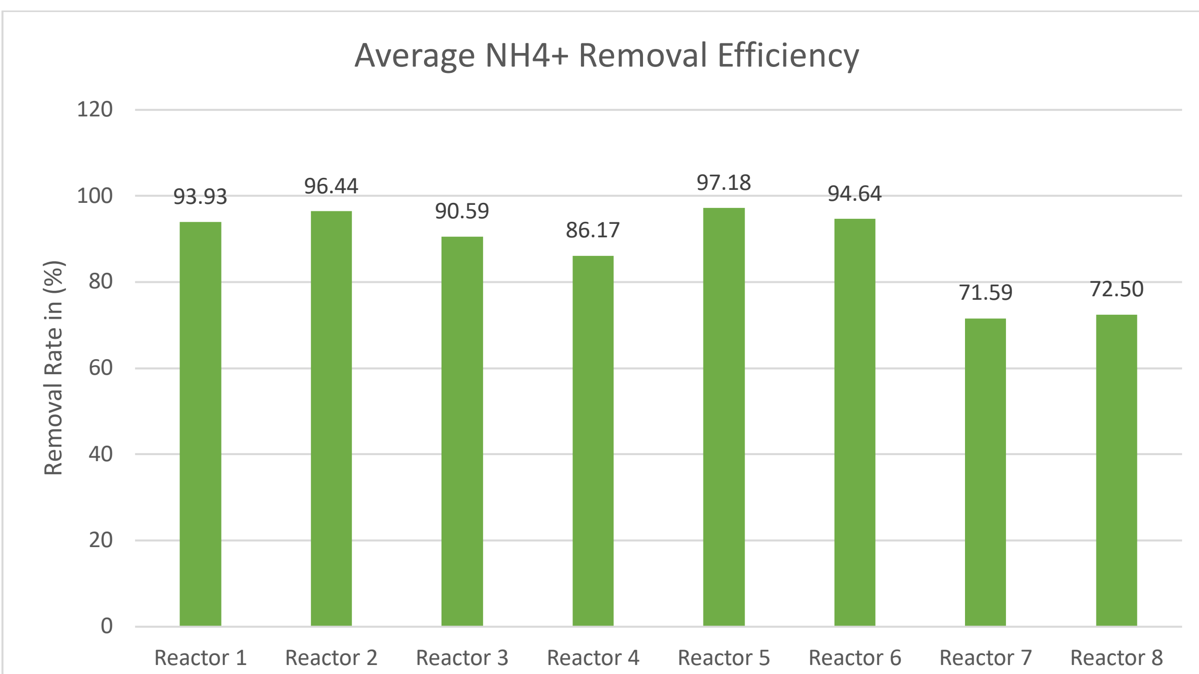

Figure 5.2 2 Removal efficiency average for ammonium

The removal efficiency is the amount of waste removed from the constructed wetland in a percentage format. The graph below shows the average removal efficiency for each reactor over the course of the experiment, 9 weeks. Although the results were positive, reactor 7 and reactor 8 had the lowest removal efficiency for ammonium. This percentage rate is consistent with the outflow results as reactors 7 and 8 had high traces of ammonium each week compare the rest of the reactor.

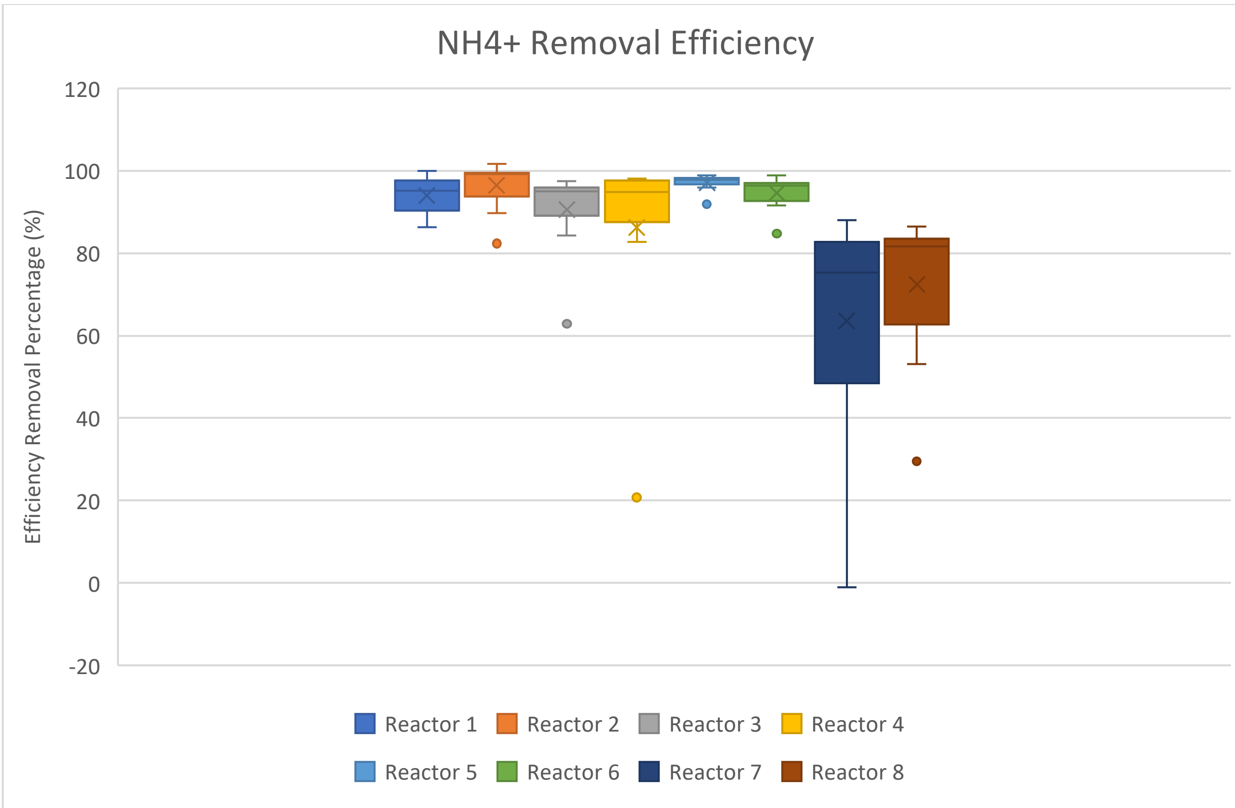

Figure 5.2 3Represents the variation in the data for ammonium.

The greater variations of data were between reactor 7 & 8. The other reactors had consistent results above 80% removal efficiency. The P-Values prove that there is a significant impact when there was low N and high C. The amounts were also x10. The probability of seeing the observed differences is 5% or less. The significant values were observed when the amounts of carbon and nitrogen ratios were high.

Statistical Results:

C:N	2	5	10	20
	1 vs 5	2 v 6	3 vs 7	4 vs 8
P – Value	0.0626	0.4873	0.0161	0.2103

Table 5.2 1 – P-values for the reactors with the same C:N ratio vs doubled counterpart.

C:N	30N		60N
p	P – Value		P – Value
1 vs 2	0.3396	5 vs 6	0.1312
1 vs 3	0.4118	5 vs 7	0.0006
1 vs 4	0.3722	5 vs 8	0.0014

Table 5.2 2 – P-values for reactors with the same N amounts and different C amounts

The t-test done for Table 5.2.1 provided a significant value within the C:N 10 ratio. The p-value was less than .05. Table 5.2.2 proved that the t-test done for 5 vs 7 and 5 vs 8 provided results which were indicated that these values are highly significant due to them being less than $p < .01$. The significant values were observed when the amounts of carbon and nitrogen ratios were high.

5.3 Nitrite

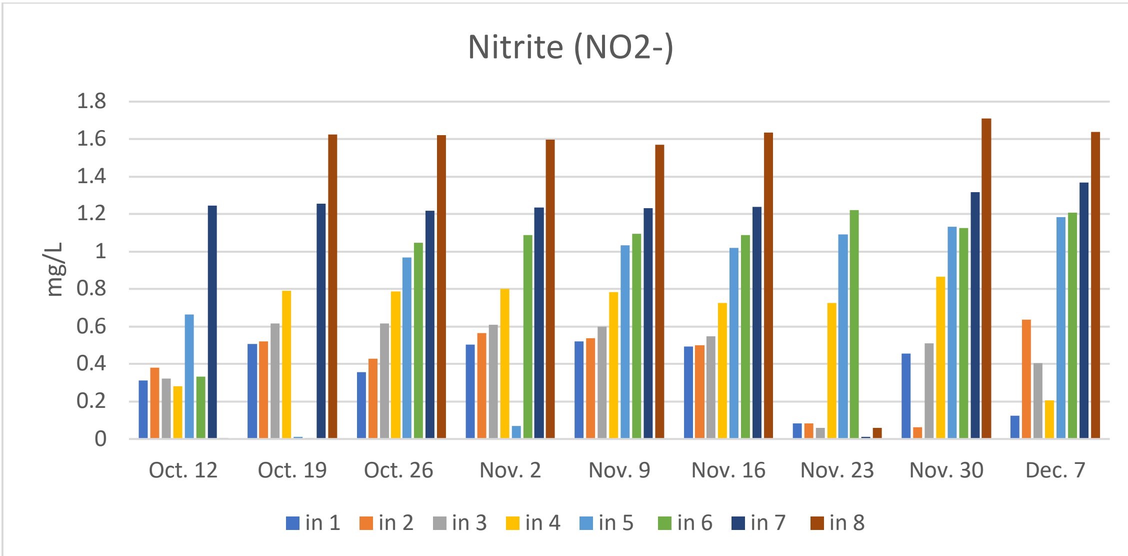

Figure 5.3 1 Nitrite (NO2-) Inflow Concentrations measured in mg/L

Figure 5.3.1 shows that the inflow concentration values for nitrite were consistent, with the highest value being approximately 1.7 mg/L for reactor 8. The nitrite outflow concentration, Figure 5.3.2 was consistent throughout the experiment, except for reactor 8 on November 9^{th}. Nitrite values appear to be higher for reactor 7 (out 7) during November 30^{th} and Dec 7^{th}.

The outflow samples had more nitrite than the inflow samples, this tells us that nitrite was accumulated in the systems. Majority of the outflow samples had concentration values that were greater than 1.7 mg/L.

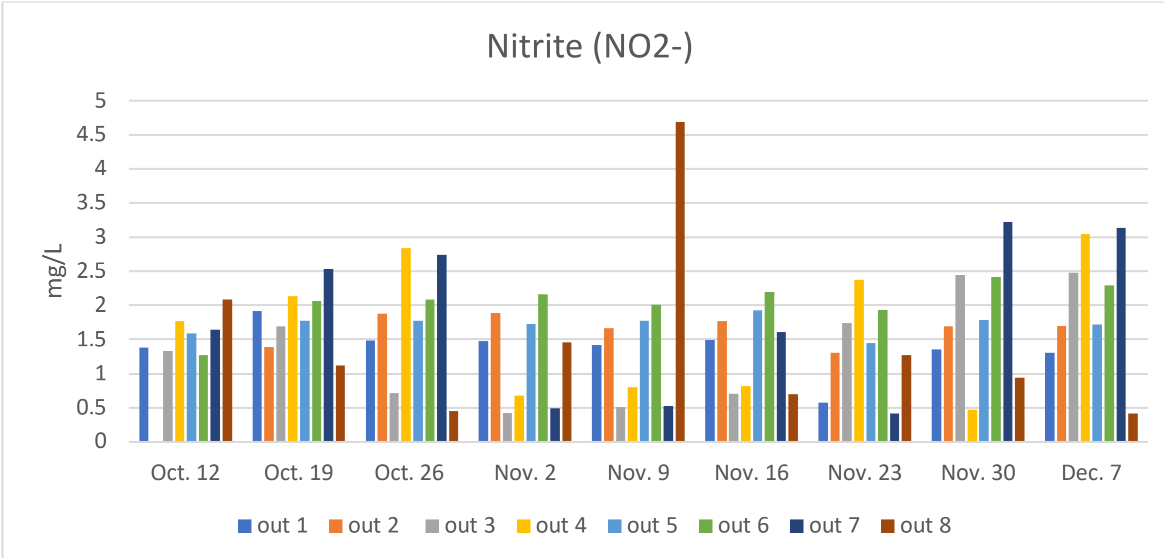

Figure 5.3 2 Nitrite (NO2-) Outflow Concentrations measured in mg/L

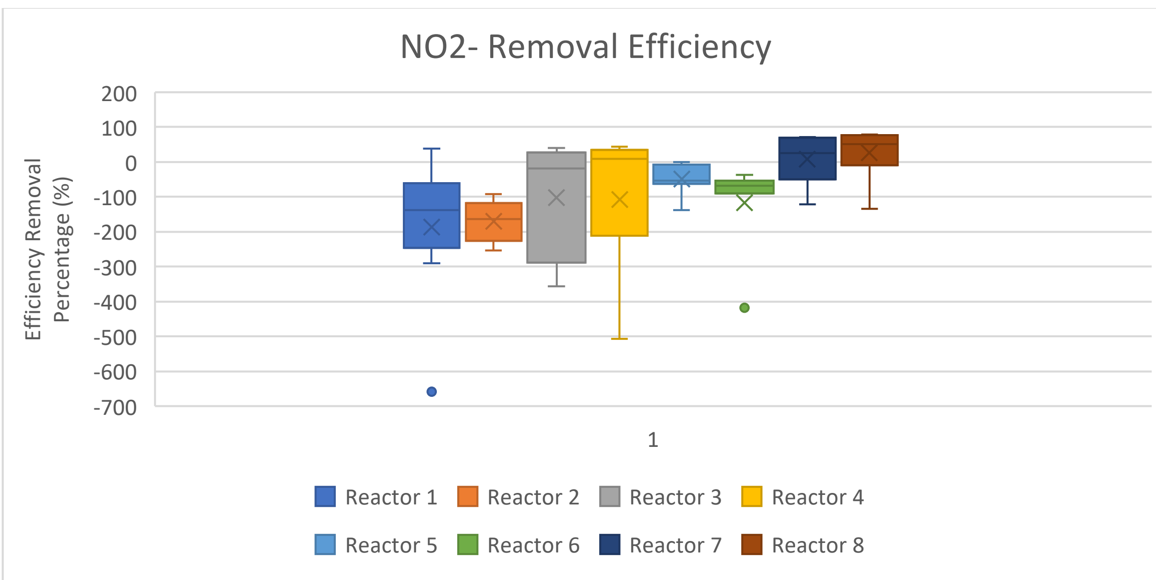

Figure 5.3 3 Represents the variation in the data for nitrite

The greater variations of data were between reactor 3 & 4 however reactors 7 and 8 had the higher removal efficiency values.

Statistical Results:

C:N	2	5	10	20
	1 vs 5	2 v 6	3 vs 7	4 vs 8
P - Value	0.1012	0.3897	0.1012	0.1091

Table 5.3.1 – P-values for the reactors with the same C:N ratio vs doubled counterpart.

C:N	30N		60N
	P - Value		P - Value
1 vs 2	0.8484	5 vs 6	0.2329
1 vs 3	0.3669	5 vs 7	0.0964
1 vs 4	0.3941	5 vs 8	0.0627

Table 5.3.2 – P-values for reactors with the same N amounts and different C amounts

The p-values suggest there are no significant impacts between C:N ratios and C and N amounts when treating nitrite.

5.4 Nitrate

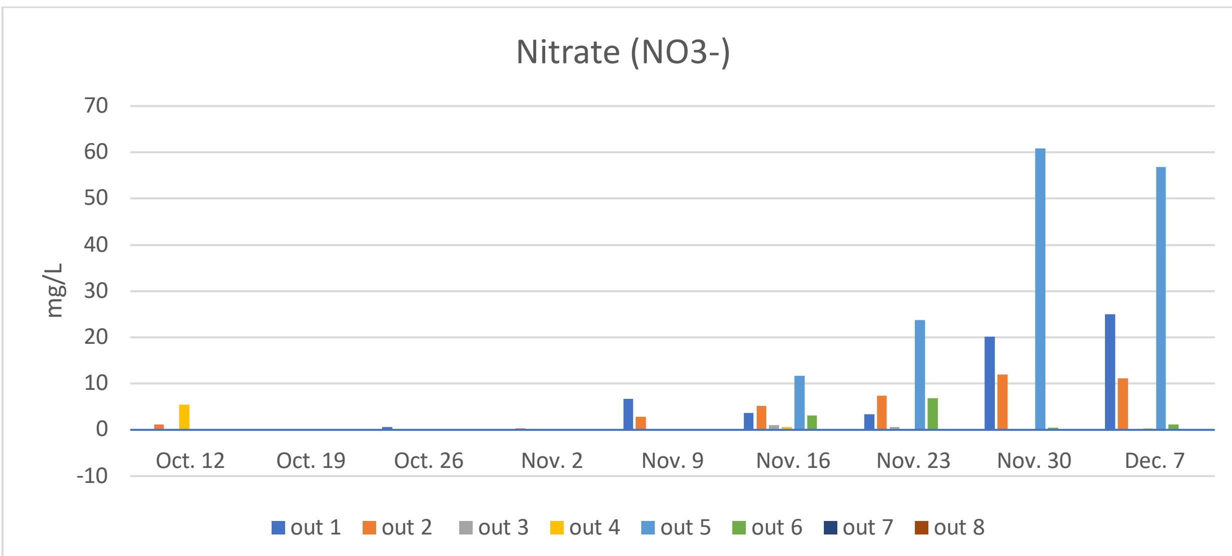

Figure 5.4.1: Nitrate (NO3-) concentration in outflow samples measured in mg/L

Figure 5.4.1 shows that nitrate concentration appeared to be consistent and almost non-existent from October 12 – November 9. Starting November 16th, the results started to vary with reactor 5 (out 5) having the highest nitrate concentration.

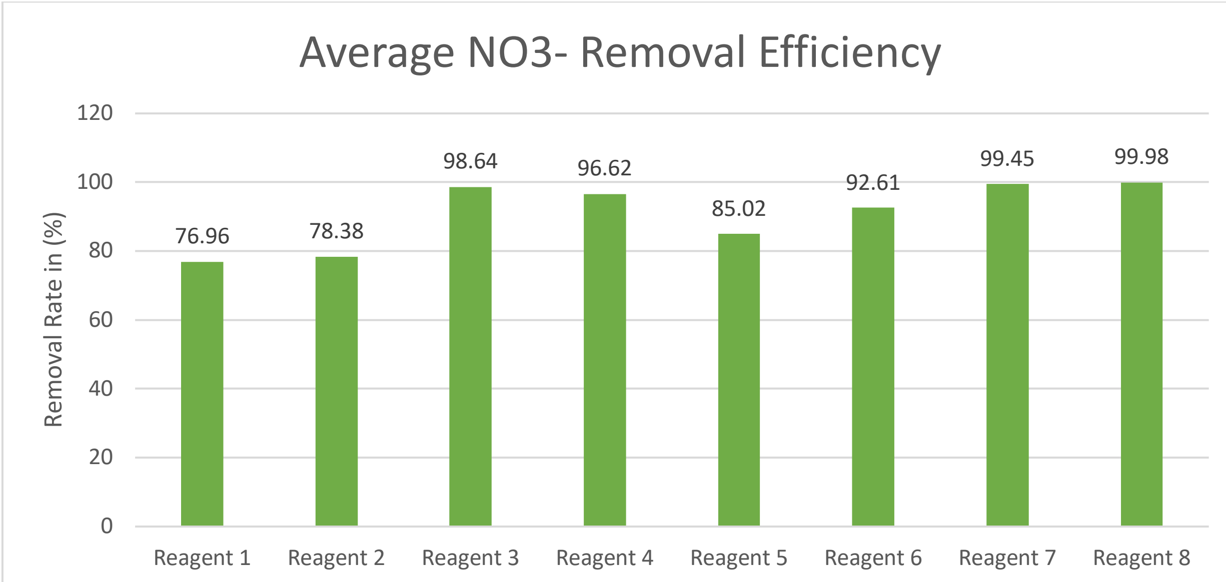

Figure 5.4.2: Average removal efficiency for Nitrate (NO3-)

The average values were consistent throughout the experiment, all the reactors had high values for efficiency removal for nitrate. However, reactors 1 and 2 had the lowest removal efficiency rate.

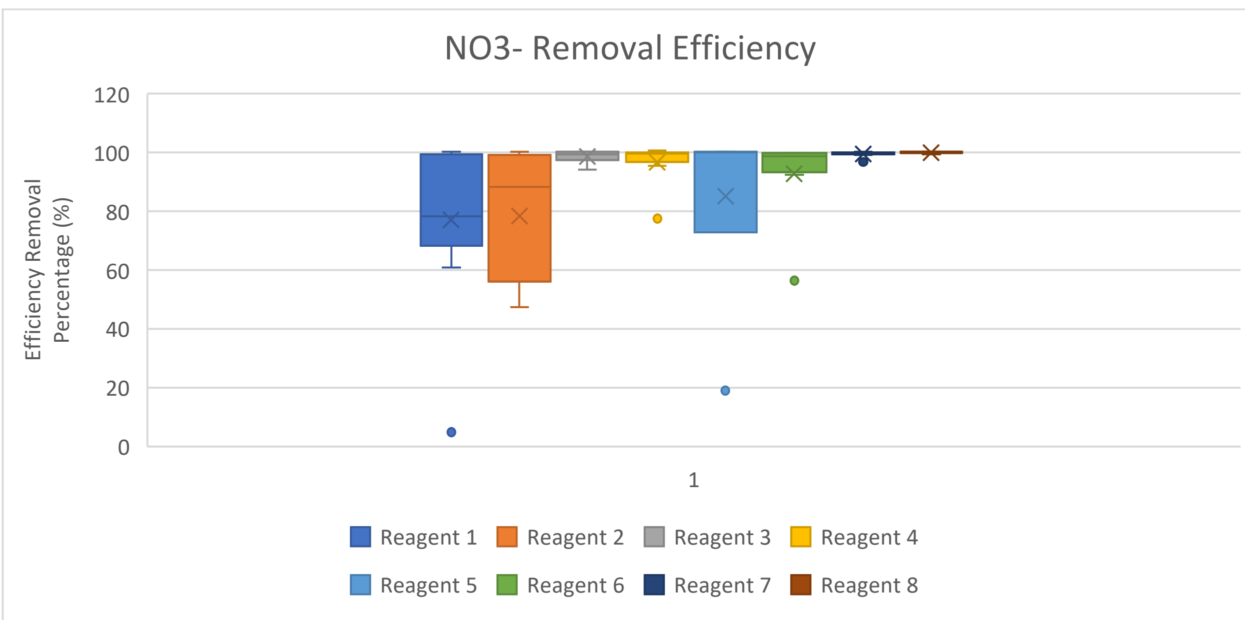

Figure 5.4.3 represents the variation in the data for Nitrate.

The greater variations of data were between reactors 1 & 2 however reactors 7 and 8 had the higher removal efficiency values. The C:N 10 and C:N 20 ratios also appeared to have the move higher removal efficiency values.

Statistical Results:

C:N	2	5	10	20
	1 vs 5	2 v 6	3 vs 7	4 vs 8
P - Value	0.6337	0.1689	0.327	0.1878

Table 5.4.1 – P-values for the reactors with the same C:N ratio vs doubled counterpart.

C:N	30N		60N
	P - Value		P - Value
1 vs 2	0.912	5 vs 6	0.5891
1 vs 3	0.0496	5 vs 7	0.1983
1 vs 4	0.0788	5 vs 8	0.1829

Table 5.4.2 – P-values for reactors with the same N amounts and different C amounts

The p-value results from table 5.4.1suggest that there are no significant impacts between C:N ratio amounts when treating nitrate. The t-test done for Table 5.4.2 provided a significant value when the N was low. The p-value was less than .05. and proved that the t-test done for 1 vs 3 had a significant value.

5.5 Total Nitrogen

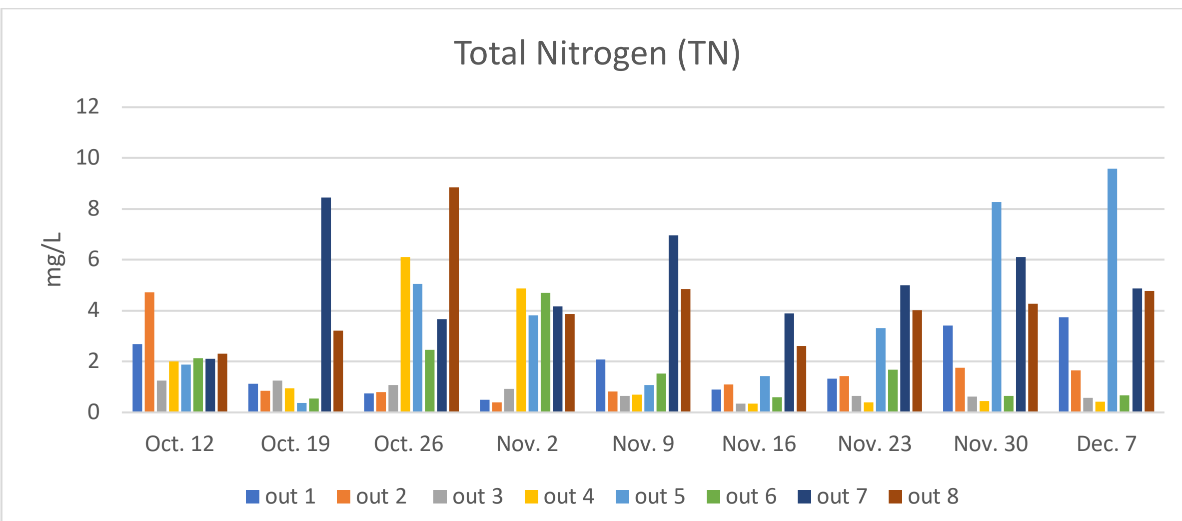

Figure 5.5.1: Total Nitrogen (TN) Concentration in Outflow Samples measured in mg/L

Figure 5.5.1 shows that total nitrogen concentration appeared to vary. Starting November 23rd, the results to increase for reactors 5, 7, and 8 (out 5, 6, 7) with reactor 5 (out 5) having the highest total nitrogen concentration.

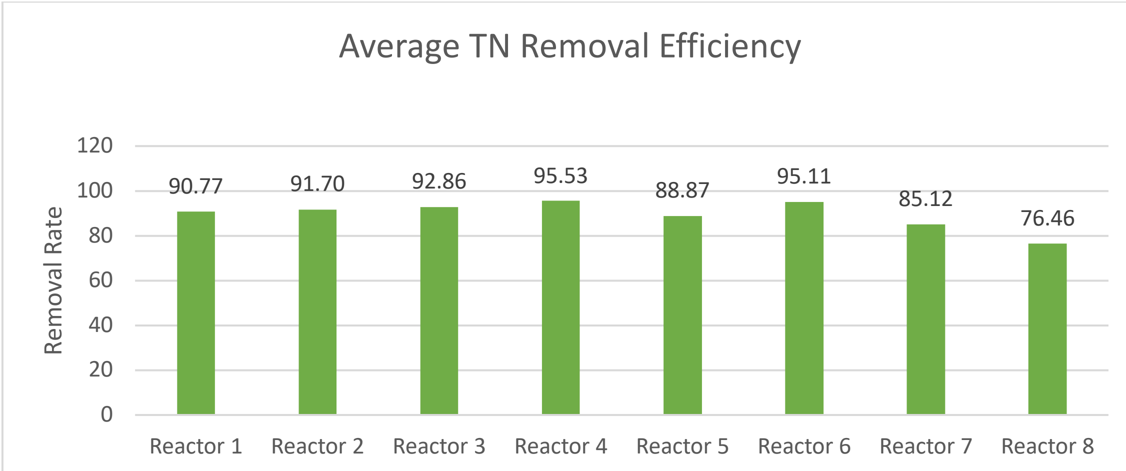

Figure 5.5.2: Average removal efficiency for total nitrogen

The average values were consistent throughout the experiment, all the reactors had high values for efficiency removal for Total Nitrogen. However, reactor 8 had the lowest removal efficiency rate.

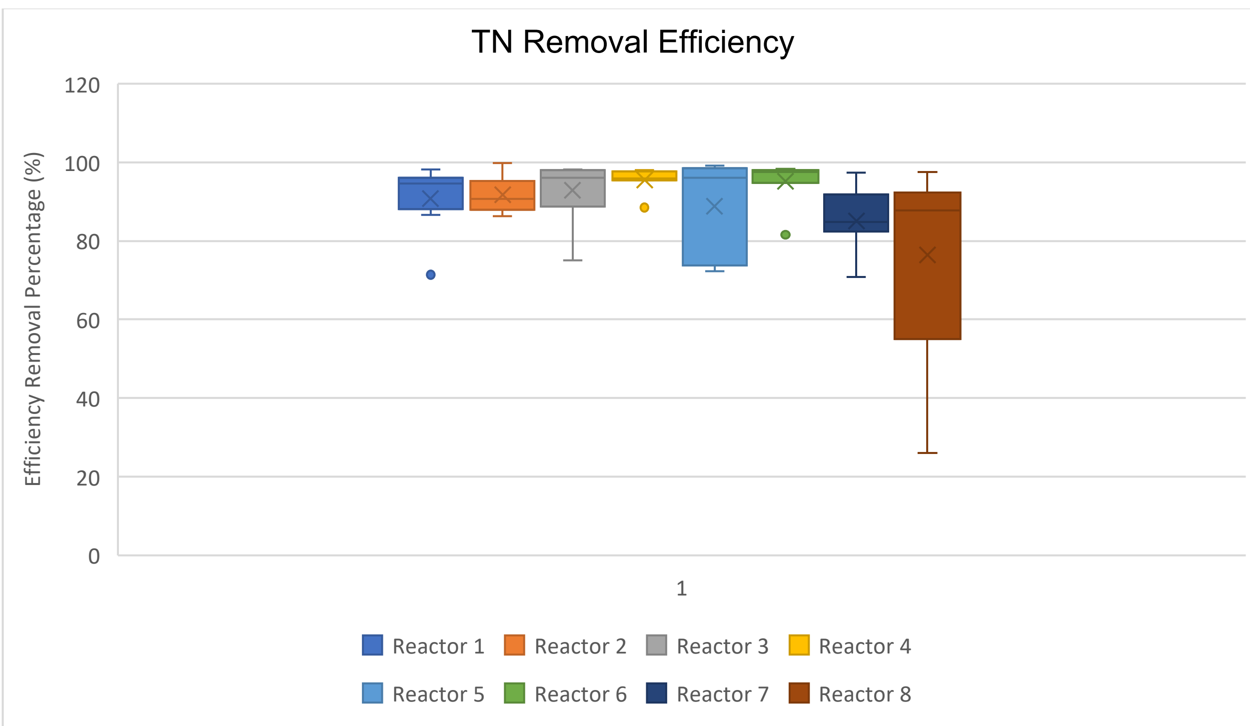

Figure 5.5.3 represents the variation in the data for total nitrogen

The greater variations of data were between reactor 5 & 8. The reactors (reactor 1-4) with low N appeared to have greater removal efficiency for N.

Statistical Results:

C:N	2	5	10	20
	1 vs 5	2 v 6	3 vs 7	4 vs 8
P - Value	0.6999	0.2183	0.0803	0.0593

Table 5.5.1 – P-values for the reactors with the same C:N ratio vs doubled counterpart

C:N	30N		60N
	P - Value		P - Value
1 vs 2	0.772	5 vs 6	0.2318
1 vs 3	0.5939	5 vs 7	0.4933
1 vs 4	0.1442	5 vs 8	0.2181

Table 5.5.2 – P-values for reactors with the same N amounts and different C amounts

The p-value results from table 5.5.1 suggest that the only statistically significant value was when the ratio was C:N 20, $p < .0593$. The t-test done for Table 5.5.2 provided no significant values when treating total nitrogen.

5.6 Total Organic Carbon

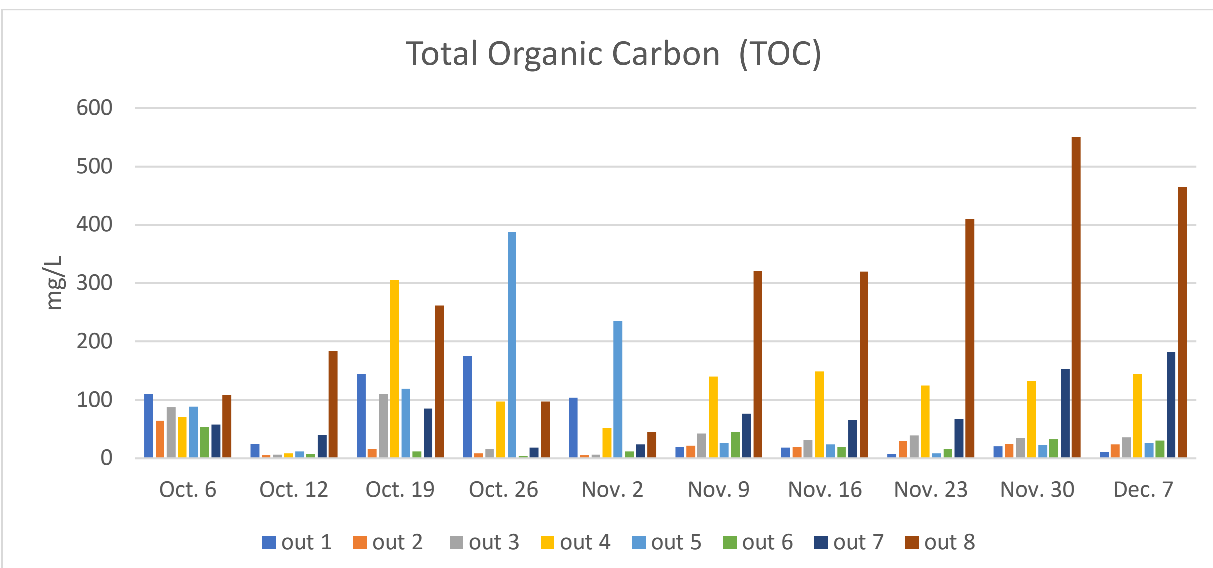

Figure 5.6.1: Total Organic Carbon (TOC) Concentration in Outflow Samples measured in mg/L

Figure 5.6.1 shows that TOC concentration appeared to vary. Starting November 9th, the results appeared to be more consistent with reactor 8 (out 8) having the highest TOC concentration.

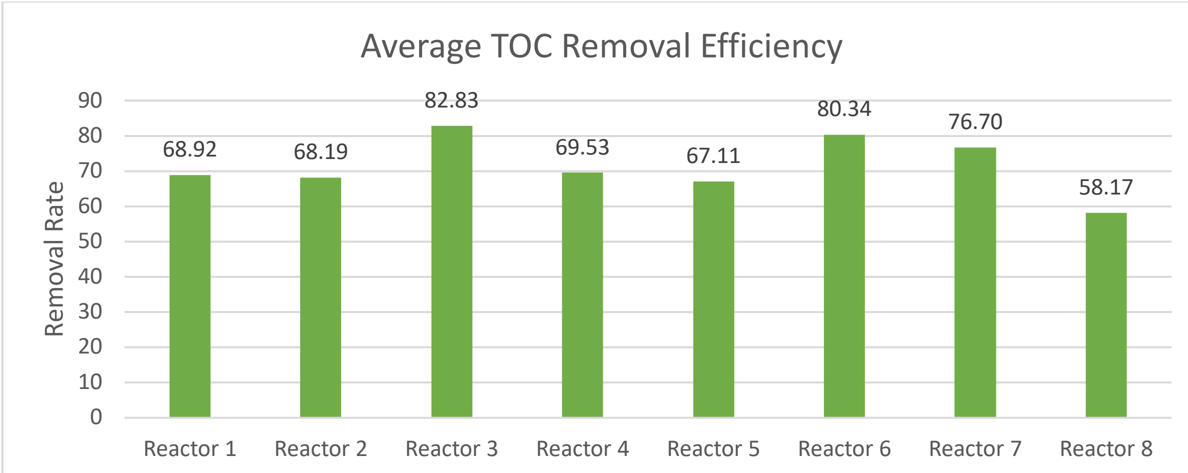

Figure 5.6.2: Average removal efficiency for Total Organic Carbon

The average values were consistent throughout the experiment with the exception of reactors 3 and 6 as they had the highest removal efficiency rate.

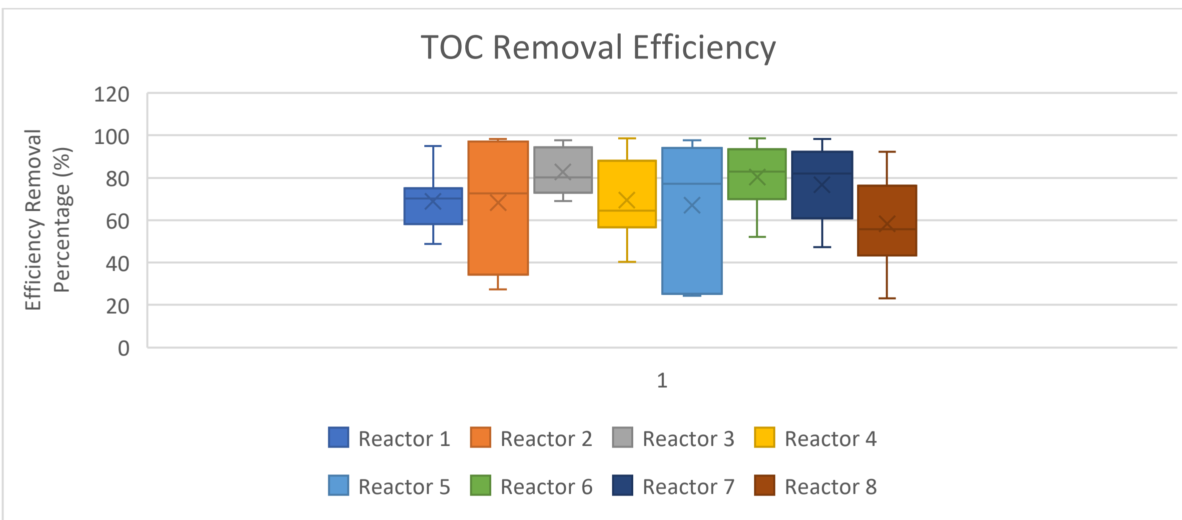

Figure 5.6.3 represents the variation in the data for Total Organic Carbon

The greater variations of data were between reactor 2 & 4. The reactors CN:10 and CN:20 appeared to have consistent results.

Statistical Results:

C:N	2	5	10	20
	1 vs 5	2 v 6	3 vs 7	4 vs 8
P - Value	0.8929	0.2943	0.423	0.2849

Table 5.6.1 – P-values for the reactors with the same C:N ratio vs doubled counterpart

C:N	30N		60N
	P - Value		P - Value
1 vs 2	0.9523	5 vs 6	0.287
1 vs 3	0.0574	5 vs 7	0.4744
1 vs 4	0.9475	5 vs 8	0.5173

Table 5.6.2 – P-values for reactors with the same N amounts and different C amounts

The p-value results from table 5.6.1 suggest that there are no significant impacts between C:N ratio amounts when treating total organic carbon. The t-test done for Table 5.6.2 provided a significant value when the N was low. The p-value was $p < .0574$, and proved that the t-test done for 1 vs 3 was statistically significant.

5.7 Total Carbon

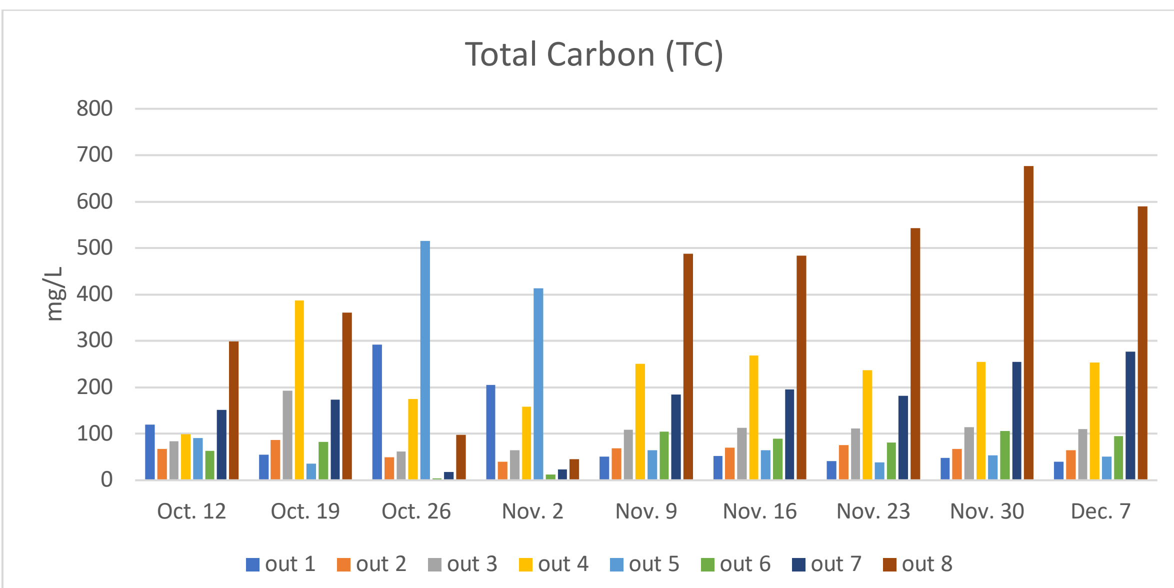

Figure 5.7.1: Total Carbon (TC) Concentration in Outflow Samples measured in mg/L

Figure 5.7.1 shows that the Total Carbon concentration appeared to be consistent throughout the experiment with the exception of October 26 and November 2. TC concentrations increased and remained high for reactor 8 (out 8).

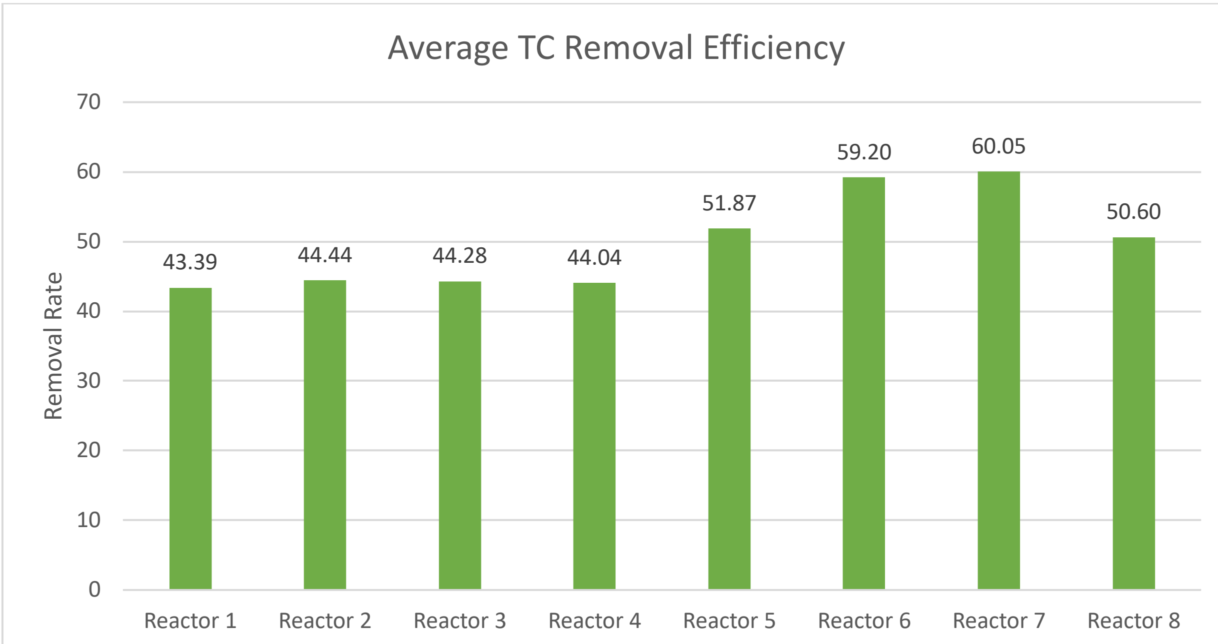

Figure 5.7.2: Average removal efficiency for total carbon

The average values varied for each reactor, however reactors 6 and 7 had the highest had the highest removal efficiency rate. Over half of the reactors did not reach or barely reached 50% of the removal efficiency.

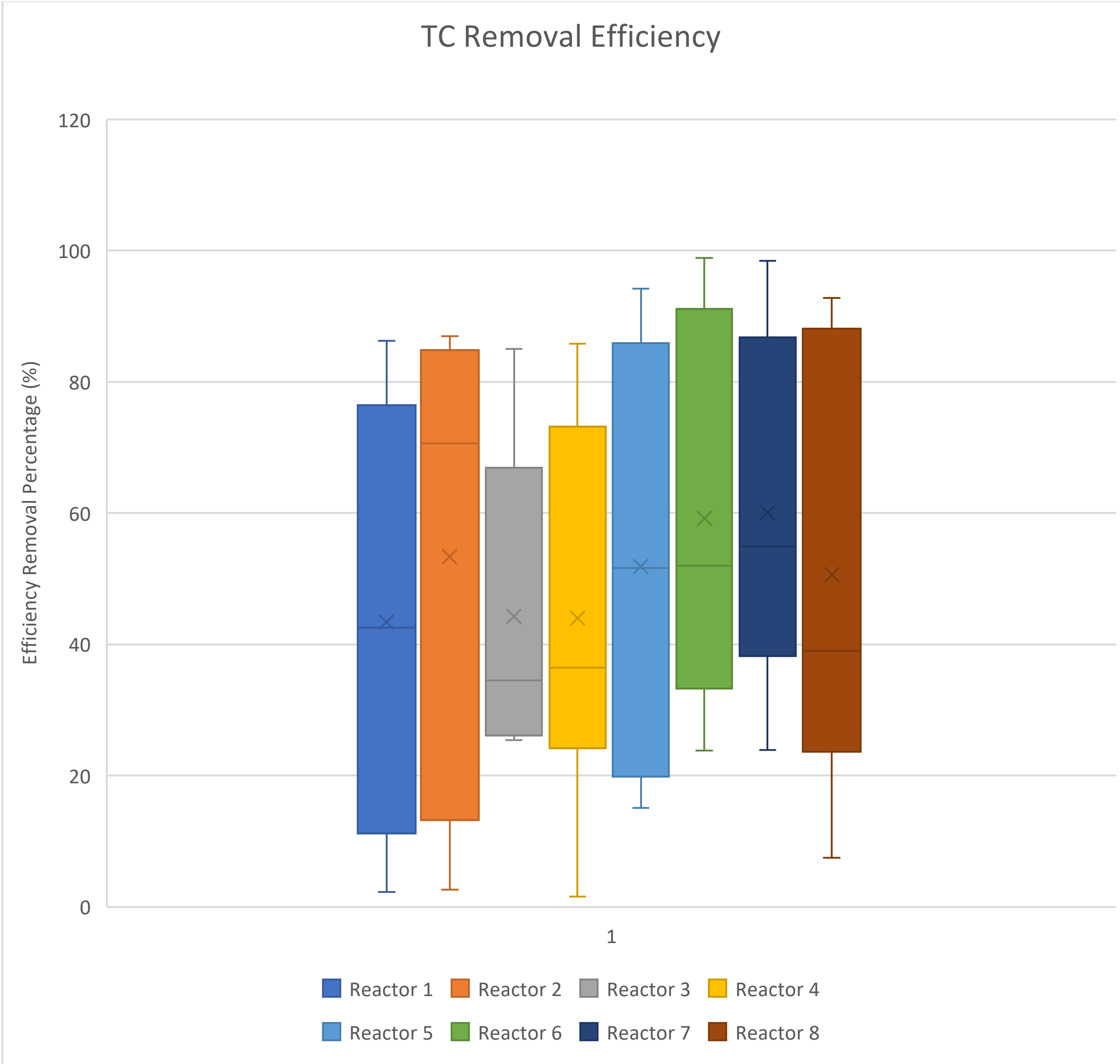

Figure 5.7.3 represents the variation in the data for total carbon

Figure 5.7.3 illustrates that the reactors had varying results. Reactors 6 and 7 had the highest removal efficiency compared to the other results.

Statistical Results:

C:N	2	5	10	20
	1 vs 5	2 v 6	3 vs 7	4 vs 8
P - Value	0.667	0.441	0.2252	0.6811

Table 5.7.1 – P-values for the reactors with the same C:N ratio vs doubled counterpart.

C:N	30N		60N
	P - Value		P - Value
1 vs 2	0.9656	5 vs 6	0.6443
1 vs 3	0.9549	5 vs 7	0.5894
1 vs 4	0.9717	5 vs 8	0.9402

Table 5.7.2 – P-values for reactors with the same N amounts and different C amounts

The t-test done for Table 5.7.1 did not provide any significant values. Table 5.7.2. did not provide any significant values as all the value were greater than $p > .05$., indicating that these values are not significant.

5.8 Phosphate

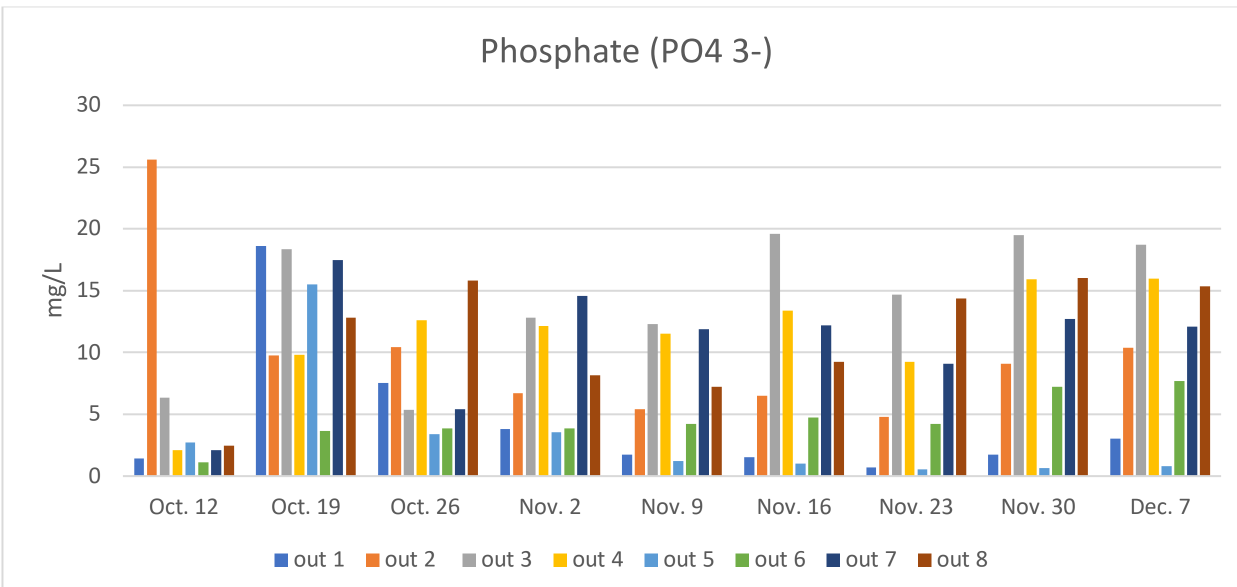

Figure 5.8.1: Phosphate (PO4 3-) Concentration in Outflow Samples measured in mg/L

Figure 5.8.1 shows that Phosphate concentration appeared to be consistent throughout the experiment with the exception of October 12 and October 19.

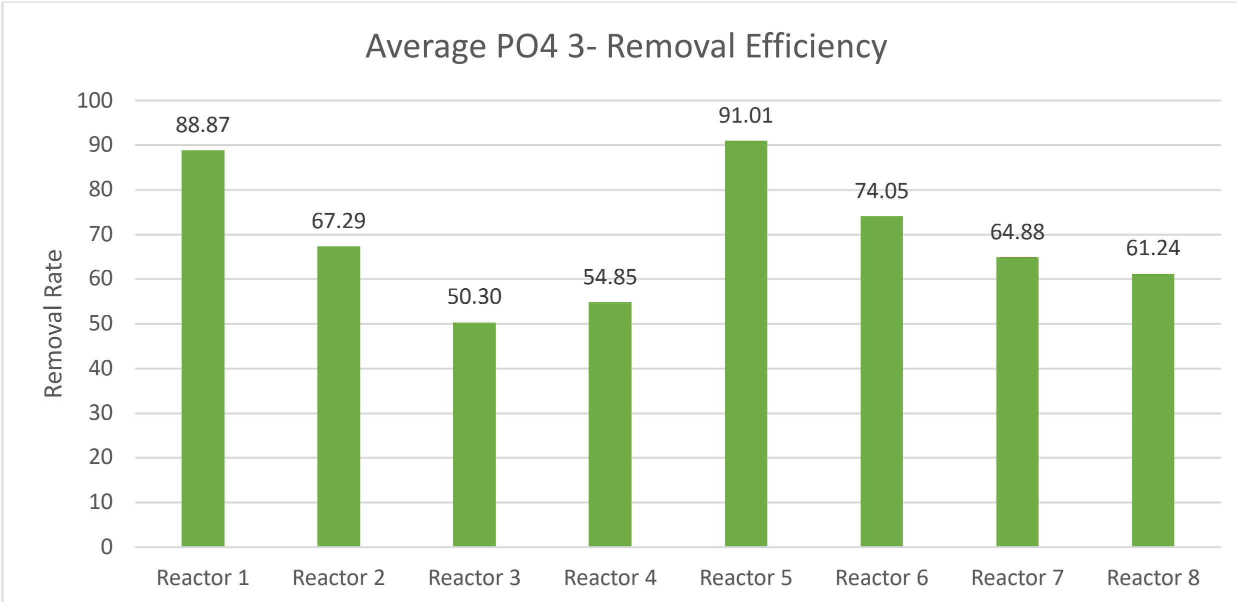

Figure 5.8.2: Average removal efficiency for Phosphate

The average values varied for each reactor, however reactors 1 and 5 had the highest had the highest removal efficiency rate.

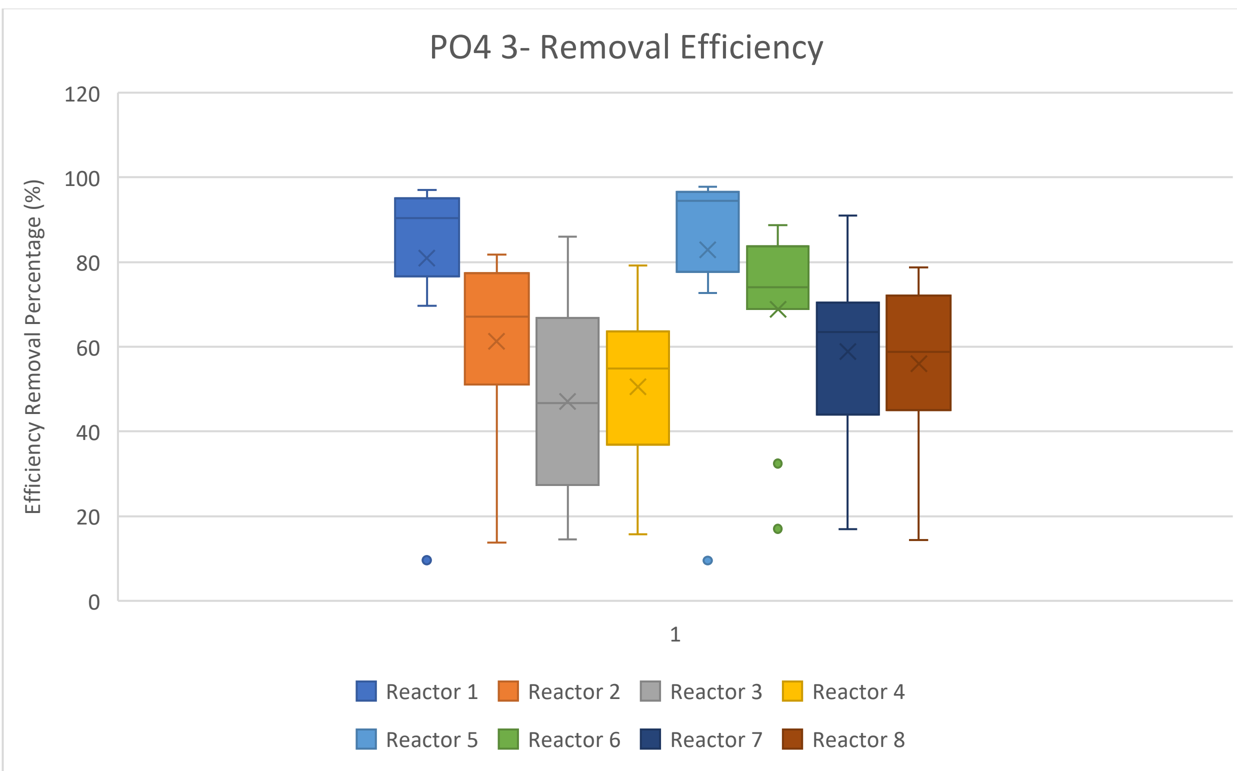

Figure 5.8.3 represents the variation in the data for Phosphate.

Figure 5.8.3, illustrate that the reactor with the C:N 2 ratio had the highest removal efficiency compare to the other results. However the results appear consistent for C:N 5, C:N 10, C:N 20 ratios.

Statistical Results:

C:N	2	5	10	20
	1 vs 5	2 v 6	3 vs 7	4 vs 8
P - Value	0.6629	0.4186	0.3087	0.4426

Table 5.8.1 – P-values for the reactors with the same C:N ratio vs doubled counterpart.

C:N	30N		60N
	P - Value		P - Value
1 vs 2	0.0036	5 vs 6	0.025
1 vs 3	0.0034	5 vs 7	0.0032
1 vs 4	0.0002	5 vs 8	0.0003

Table 5.8.2 – P-values for reactors with the same N amounts and different C amounts

The t-test done for Table 5.8.1 did not provide any significant values. However, table 5.8.2. all the results were less than p <.05., indicating that these values are highly significant with the exception of 5 vs. 6 being on significant as it is not less than 1%.

5.9 Maximum quantum yield of PSII

An Fv/Fm value in the range of 0.79 to 0.84 is the approximate optimal value for many plant species, with lowered values indicating plant stress (Maxwell K., Johnson G. N. 2000). The overall results indicate that the plants were under stress. None of the results provided a value above .79 and the lowest value being 0.176.

	Dark curve	Light curve
Reactor 1	0.715926	0.679604
Reactor 2	0.744771	0.666223
Reactor 3	0.722884	0.646505
Reactor 4	0.690137	0.689594
Reactor 5	0.755327	0.735285
Reactor 6	0.176471	0.697531
Reactor 7	0.721887	0.725603
Reactor 8	0.728796	0.724239

Table 5.9.1 Maximum quantum yield of PSII for October 13th

	Dark curve	Light curve
Reactor 1	0.705406	0.714748
Reactor 2	0.729785	0.697065
Reactor 3	0.724482	0.723657
Reactor 4	0.744169	0.741838
Reactor 5	0.715249	0.7
Reactor 6	0.726979	0.72478
Reactor 7	0.75143	0.743594
Reactor 8	0.73922	0.723268

Table 5.9.2 Maximum quantum yield of PSII for October 27th

	Dark curve	Light curve
Reactor 1	0.718851	0.725132
Reactor 2	0.765366	0.712714
Reactor 3	0.743854	0.669068
Reactor 4	0.733382	0.735294
Reactor 5	0.696324	0.700088
Reactor 6	0.721311	0.703744
Reactor 7	0.692647	0.688042
Reactor 8	0.694367	0.679759

Table 5.9.3 Maximum quantum yield of PSII for November 11th

	Dark curve	Light curve
Reactor 1	0.687288	0.673734
Reactor 2	0.663378	0.650714
Reactor 3	0.643299	0.632936
Reactor 4	0.642805	0.650906
Reactor 5	0.686358	0.678992
Reactor 6	0.653404	0.634247
Reactor 7	0.648047	0.624761
Reactor 8	0.697081	0.677771

Table 5.9.4 Maximum quantum yield of PSII for December 2nd

5.10 Physical Observations

The descriptions below are for the physical observations that took place during the course of the experiment. These observations were the most common changes that took place.

- Reactor 1 (30N:60C) had a strong smell in the treated wastewater, new plants were sprouting, and the existing biomass continued to grow. However, as the temperature decreased the smell of the treated wastewater non-existent.
- Reactor 2 (30N:150C) had a semi-strong in the treated wastewater, new plants were sprouting but the existing biomass was decomposing. As the temperature decreased the smell of the treated wastewater was also non-existing.
- Reactor 3 (30N:300C) the physical results were very similar to reactor 2.
- Reactor 4 (30N:600C) the physical results were very similar to reactor 2 and 3.
- Reactor 5 (60N:120C) the smell of the treated wastewater was present but not strong, new plants were observed sprouting and the existing biomass continued to grow.
- Reactor 6 (60N:300C) the physical results were very similar to reactor 5.
- Reactor 7 (60N:600C) had the 2nd strongest smell out of all the reactors, new plants were sprouting, and the existing biomass continued to grow but simultaneously the existing biomass was decomposing.
- Reactor 8 (60N:1200C) - had the 2nd strongest smell out of all the reactors, new plants were sprouting, and the existing biomass continued to grow but simultaneously the existing biomass was decomposing.

Figure 5.10.1 Reactors on October 5th 2023

Figure 5.10.2 Reactors on December 14, 2023

5.10.1 Biomass Weight:

The tables below represent the total mass/weight of the plants at the end of the experiment. The plants were weighed twice, the first time was when they were removed from the reactor therefore the plants were wet, and the second time was 2 weeks after they had been removed from the reactor therefore the plants were dried.

Wet weight from Dec. 16			
	Roots	Leaves	Total Weight
Reactor 1	0.6	0.08	0.68
Reactor 2	0.37	0.03	0.4
Reactor 3	0.24	0.035	0.275
Reactor 4	0.28	0.03	0.31

Reactor 5	0.58	0.13	0.71
Reactor 6	0.35	0.065	0.415
Reactor 7	0.255	0.065	0.32
Reactor 8	0.335	0.035	0.37

Table 5.10.1 Wet biomass weight in kg

Dry weight from Jan. 27			
	Roots	Leaves	Total Weight
Reactor 1	0.30857	0.017	0.32557
Reactor 2	0.13354	0.00692	0.14046
Reactor 3	0.05505	0.00794	0.06299
Reactor 4	0.0968	0.00758	0.10438
Reactor 5	0.22102	0.02489	0.24591
Reactor 6	0.12717	0.01388	0.14105
Reactor 7	0.07901	0.01643	0.09544
Reactor 8	0.07692	0.00872	0.08564

Table 5.10.2 Dry biomass weight in kg

Chapter 6: Discussion

Constructed wetlands have been proven before to be successful in treating wastewater. Based on the results from this experiment, some of the removal efficiency results were higher than other removal efficiency results. This part of the thesis will discuss the reasoning behind these results. The experiment focused on the removal efficiency of constructed wetlands based on the C:N ratios and the amount of C and N added to the influent. All the reactors provided consistent results based on the nitrification and denitrification processes going on within the CW. The results from the experiment allowed us to determine if different carbon and nitrogen ratios were able to influence wastewater purification in constructed wetlands with the assistance of arbuscular mycorrhizal fungi.

6.1 C:N Ratios

Zhu et. al. (2014) stated that the C:N ratio can affect the nitrification and denitrification functions of microorganisms because the C:N represent reasonable amount of carbon source in the system. When observing the data in box plots it was noted that when the reactors shared the same C:N ratio they appeared to have similar results. This can be said for figures 5.3.3, 5.4.3, 5.7.3. and figure 5.8.3.

6.2 Ammonium (NH4+), Nitrite (NO2-), and Nitrate (NO3-)

Studies by Vymazal (2006), Zhu et. al., (2014), and Fang et. al., (2018) have all reported that C:N ratios have an impact on the removal efficiency of nitrogen within CWs. The C:N ratio of the organic material entering the wetland influences the rate of decomposition of organic matter and this result in the transformation or immobilization of nitrogen in the system (Wooton et. al., 2014). If the organic material entering the wetland contains more nitrogen in proportion to the carbon, then nitrogen is released into the wetland from the decomposing organic material (Wooton et. al., 2014). The results from this experiment provided insight on the effects of C:N ratio when removing or transforming nitrogen within CWs.

Figures 5.2.2 and 5.2.3 exhibit that the removal efficiency of NH4+ decreased when the C:N ratios were at the highest amount. This result was also in agreement with Zhu et. al.

(2014). Zhu et. al. explains that the removal of NH4+ depends on nitrification activated by nitrifying nitrite bacteria. Zhu et. al. states that the increase of C:N ratios leads to less efficient nitrification process and interferes with the transformation of ammonium.

Figures 5.4.2 and 5.4.3 exhibit that the removal efficiency of NO3- increased when the C:N ratios were at the highest amount. Reactors 7 & 8 had a removal efficiency of 99%. This result was also in agreement with Zhu et. al. (2014). The C:N ratios can affect the change of dissolved oxygen (DO) in water, when C:N increases, the decomposing of the organic matter requires more oxygen and DO can decrease quickly in water (Zhu et. al., 2014). This outcome leads to a stronger oxygen-lacking environment or an anaerobic environment (Bernet et. al. 2001).

Figures 5.5.2 and 5.5.3 exhibit that the removal efficiency (RE) of TN increased when there was high C:N values with the greater TN RE at C:N 20, however the p-value was ($p \leq .0593$) when he t-test compared the reactors with C:N 20 (reactor 4 vs. reactor 8). This could potentially be resulted from the lack of oxygen as the ORP remained in the negative values for reactor 8. Zhu et. al. (2014) states that the degradation of organic matter in CWs is correlated with the change of DO concentrations in the system and therefore the ORP in CWs, which represents the change of the oxidation–reduction condition, is a critical and influential factor for nitrogen removal. Mateus and Pinho (2020) stated that carbon sources can serve as electron donors of biological denitrification processes in bioreactors. Followed by them stating that this can significantly increase nitrogen elimination in treating water with low C/N ratios, such as municipal wastewater effluent and agricultural runoff (Mateus and Pinho, 2020).

6.3 Total Organic Carbon (TOC) and Total Carbon (TC)

Carbon sources can serve as electron donors of biological denitrification processes in bioreactors and CWs (Mateus and Pinho, 2020). This can significantly increase nitrogen elimination in treating water with C:N ratios, such as municipal wastewater effluent and agricultural runoff (Mateus and Pinho, 2020). Based on the experiment results, high amounts carbon and total organic carbon concentrations was observed in the outflow sample for reactor 8. Reactor 8 also had the lowest RE value for TOC however reactor 8 had the highest nitrate RE value. These results can probably explain because the organic carbon source is the major electron donor for denitrification, and the lack of carbon source limits the denitrification process. With the increase of C:N ratio, more carbon source was supplied for the system, which provided plenty of electron donors for denitrification by microorganisms. Therefore, the heterotrophic denitrificans bloomed and improve the denitrification efficiency (Zhu, 2014).

Based on the results from this experiment, the C:N ratios had varying effects on the removal efficiency of TOC. When the C:N ratio was 2, the reactors provided similar but low RE results ranging from 67.11 ± 68.98. When the C:N ratio was 20, the reactors provided the lease efficient RE results ranging from 69.53 ± 58.17. This shows that when both C:N ratios are low and high they provided low RE. However, when the C:N ratio was 10, the reactors provided carbon RE results ranging from 82.83 ± 76.70. These results tell us that when N was low, and C was high the reactors provided higher RE for TOC. Further testing will need to be done to really confirm the results from this experiment. Further testing will need to be done to really confirm the results from this experiment.

Based on the results from this experiment, the C:N ratios had varying effects on the removal efficiency of TC, that differ from TOC. Reactors 1-4 all had a low N ratio and they all provided low RE of TC ranging from 43.39 ± 44.44. This tells us that low N ratios effected the RE of TC. However, the when the C:N ratios were doubled, the RE of TC improved ranging from 50.60 ± 60.05, with reactors 6 and 7 having the highest values. Further testing will need to be done to really confirm the results from this experiment.

6.4 Phosphate

Based on the results from this experiment, the C:N ratios had varying effects on the removal efficiency of phosphate. When the C:N ratio was 2, reactors 1 and 5 provided RE results ranging between 88.87 ± 91.01, meaning that the RE was higher when the C and N ratios were lower. However, when the C:N ratio was 20 the RE noticeably decreased, ranging from 54.85 ± 61.24. These results can tell us that the RE was higher when C:N ratios were lower and when C:N ratios were at the highest amount the RE noticeably decreased. Further testing will need to be done to really confirm the results from this experiment. Reactors 1 and 2 also had the largest plant mass. Vymazal (2007) states that most of the phosphorus is taken up by plant roots, absorption through leaves and shoots is restricted to submerged species but this amount is usually very low. The most significant benefits of mycorrhizae are the increase in P absorption by the plant (Barea 1991).

6.5 AMF

It was noted that some of the removal efficiency values were higher than what the literature had suggested. It is believed that AMF played a role in allowing the plants to absorb more nutrients and therefore providing greater efficiency values. The most significant benefits of mycorrhizae are the increase in P absorption by the plant (Barea 1991). This statement can be confirmed with the reactors 1 and 5 having the greatest P removal efficiency and having the greater biomass weight. AMF is known to extend the root networks of plants and the extension of plant roots is also beneficial to the uptake of NH4 +-N, as well as for the assimilation activities of plants and microorganisms (Xiong et al, 2011). Reactors 1-6, all had removal efficiency values that were above 90% which is in agreement with Xiong's et. al (2011) statement. The literature also suggests that lower oxygen content in wetland habitats may be one of the most important factors leading to AMF not to colonize roots at a high level like in the terrestrial ecosystems (Huang et al. 2021; Wang et al. 2015), which may be a reason why reactors 7 and 8 did not absorb as much of the ammonium compared to the other reactors. These presumptions are based on this experiment and their results, along with corresponding. Therefore, AMF application in constructed wetland wastewater treatment systems should be further studied.

6.6 Plant Health

Overall plant health and photosynthesis was consistent throughout the experiment. An Fv/Fm value in the range of 0.79 to 0.84 is the approximate optimal value for many plant species, with lowered values indicating plant stress (Maxwell K., Johnson G. N. 2000). The Fv/Fm values for this experiment ranged from 0.624760 ± 0.7653656. Maxwell et. al (2000) suggests that some stressor may be caused by the temperature, carbon fixation, and changes in the electron transport.

Reactors 1 and 2 both had C:N ratios of 2 and this ratio had the most effect on plant health and total biomass because these reactors had largest biomass and had new leaves coming out each week. It was also noted that reactors 6-8 had new plants sprouting each week and these reactors had C:N ratios with high amounts of carbon. The reactors with low C:N ratios, usually had the most decaying plants and new sprouts were hardly growing or not at all. Based on these experiment results C:N ratios did have an impact of the plant's health and overall growth.

Based on the overall physical observations of the plants, it did appear that AMF contributed to their overall wellbeing. The literature states that AMF assists with reducing plant stressors and because the experiment was in the winter its very likely that the plants would have been noticeably under the .79 threshold, although the plants were under the threshold the lowest value was .62.

On the last day of the experiment the temperature in Prague reached -2ºc. As the water samples, for December 14th, 2023, were about to be collected the reactors had frozen, therefore it was not possible to get a sample for that day. The literature has suggested that biomass is a way to prevent the subsurface water from freezing. However, the existing biomass in the reactors was not able to prevent the freezing due to the size of the reactor. The outflow results for each nutrient had higher concentration levels and removal efficiency decreased during the colder season. These results were in agreement with Wu et. al. (2017) experiment results determined that during winter, the temperature in the water decreased from 22.3 °C to 12.6 °C, and all pollutant removal efficiencies of CWs decreased with the cold temperature.

Chapter 7: Conclusion

Constructed wetlands play an important role in wastewater purification, including cycling of carbon, nitrogen, and other nutrients. They are sustainable, efficient, and low-cost treatment systems. The nitrogen and carbon ratios have an impact on the removal efficiency of constructed wetlands and these ratios allow for the CWs to be more efficient in their removal mechanism. The increase in C:N ratios resulted in the increased removal efficiencies of total nitrogen and nitrate. However, the removal efficiency of ammonium decreased with higher C:N ratio values. AMF and carbon assisted the plants in absorbing more of the nutrients, including phosphate. Based on the experiment and literature, this concludes that the inoculation of the AMF and the C:N ratios were successful in increasing the efficiency of constructed wetlands.

Appendix A: Inflow Results

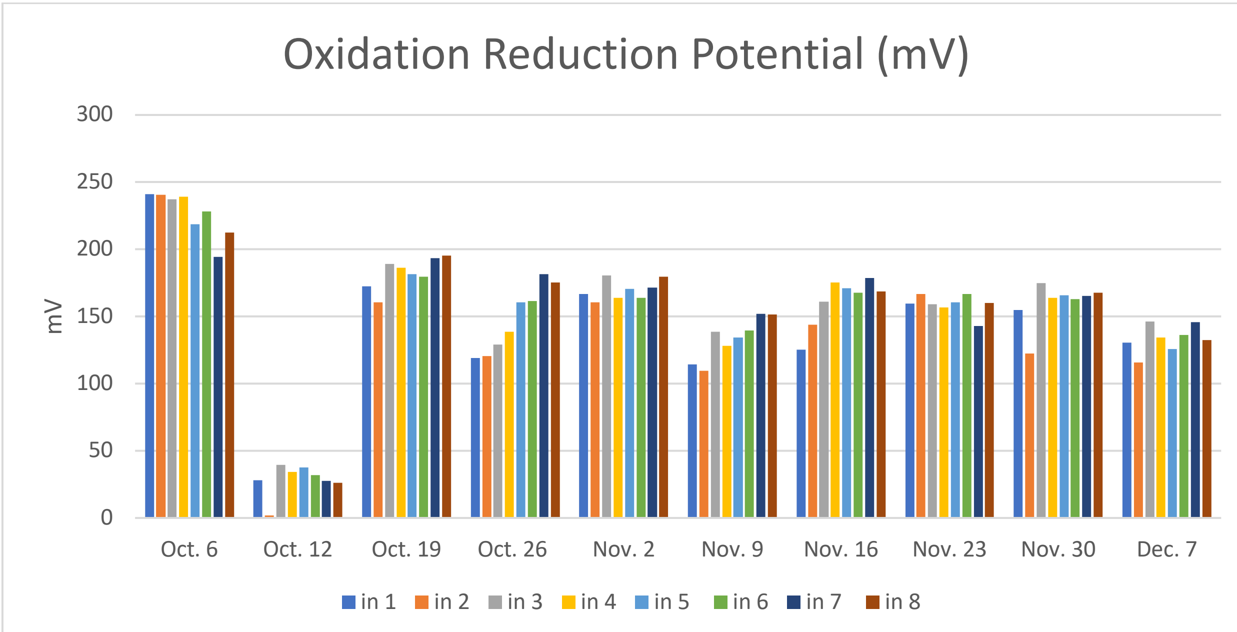

Figure 1: ORP values for inflow samples

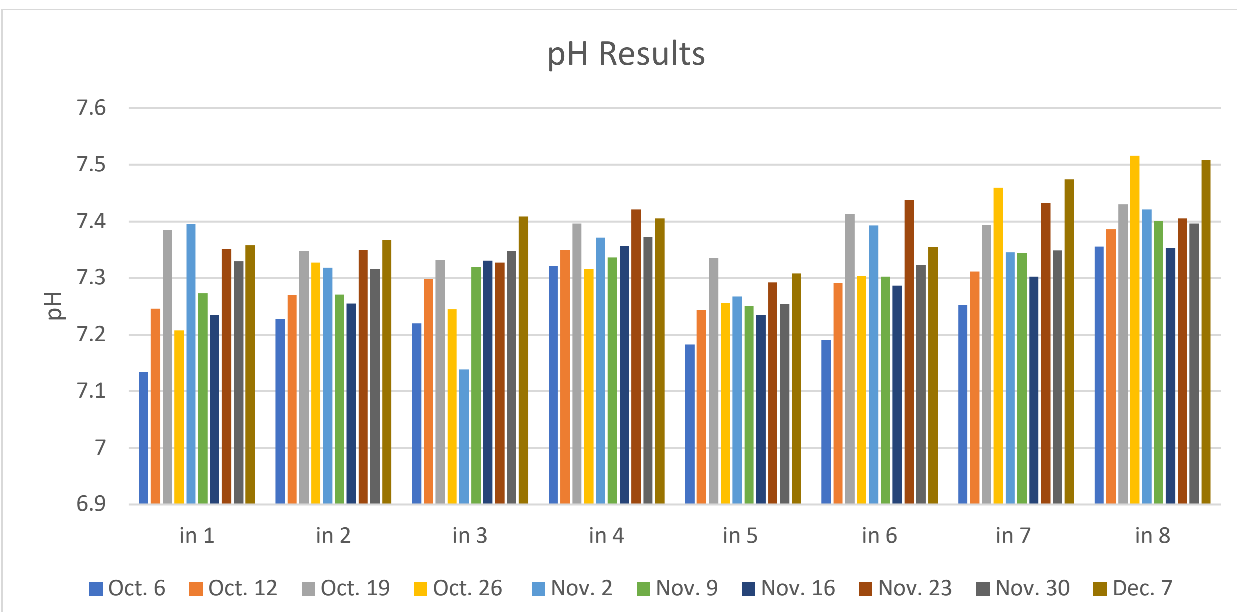

Figure 2: pH values for inflow samples

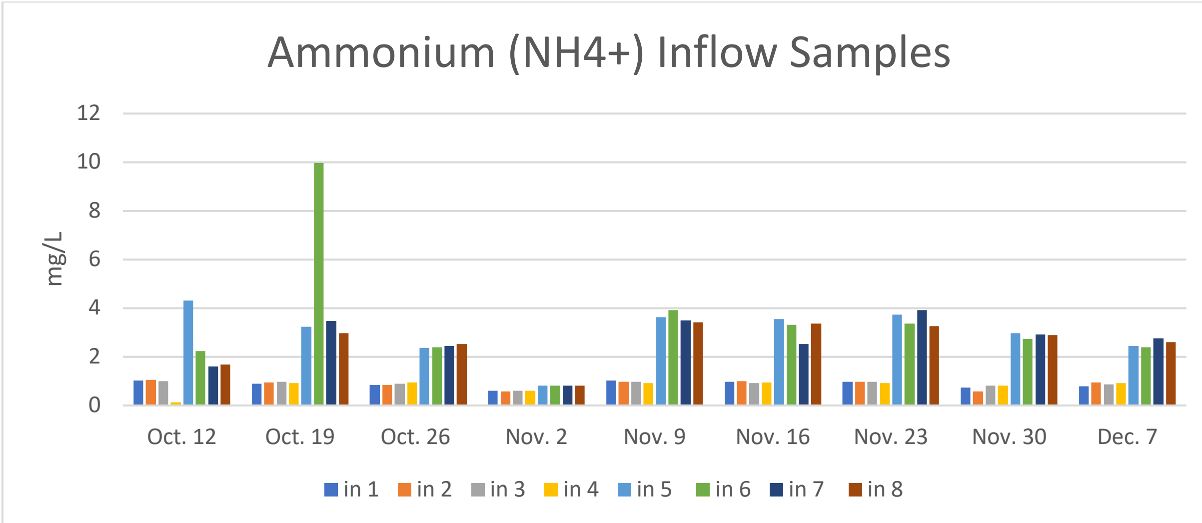

Figure 3: Ammonium concentration values for inflow samples

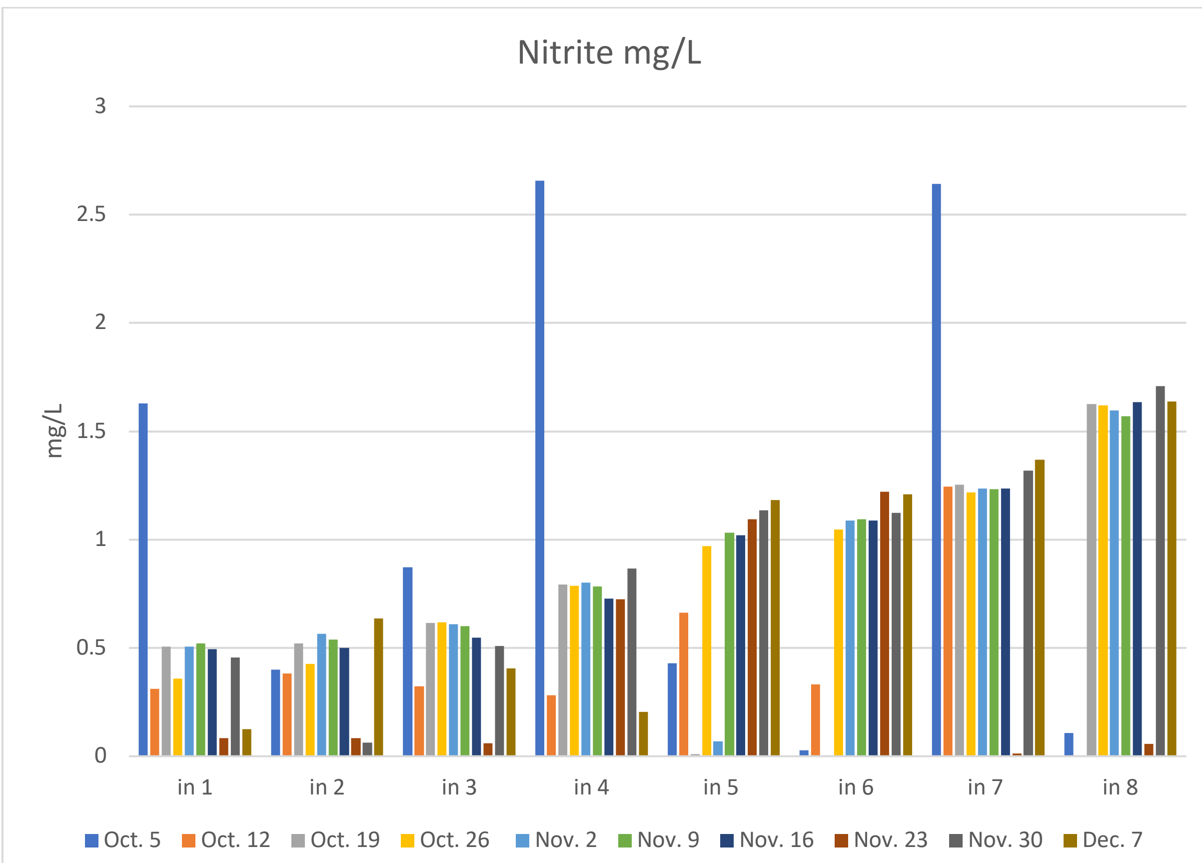

Figure 4: Nitrite concentration values for inflow samples

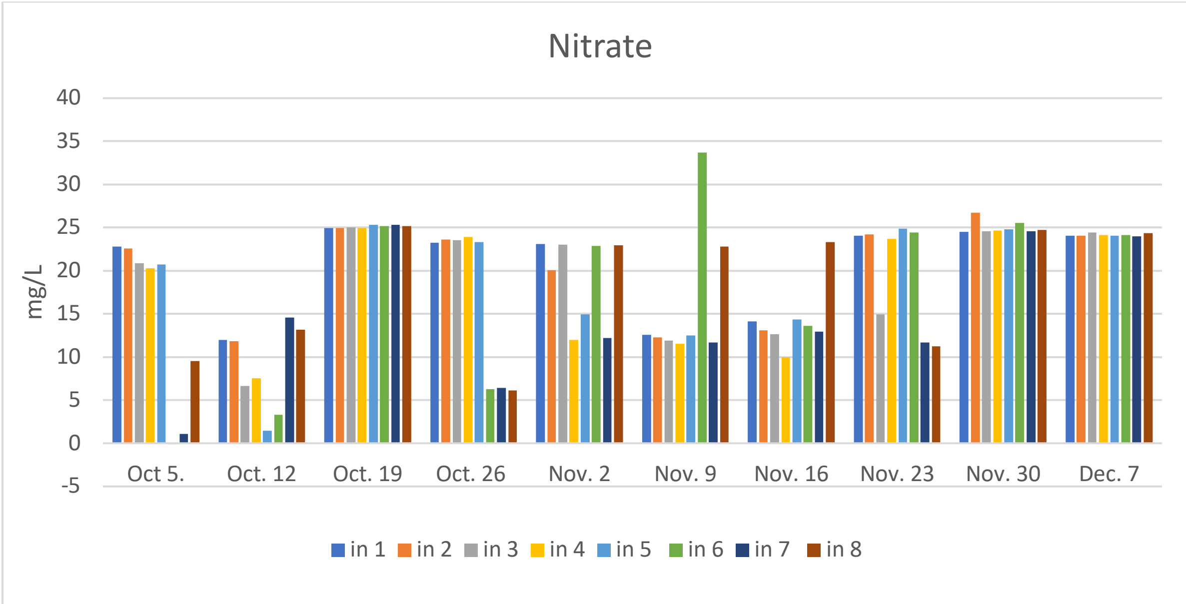

Figure 5: Nitrate concentration values for inflow samples

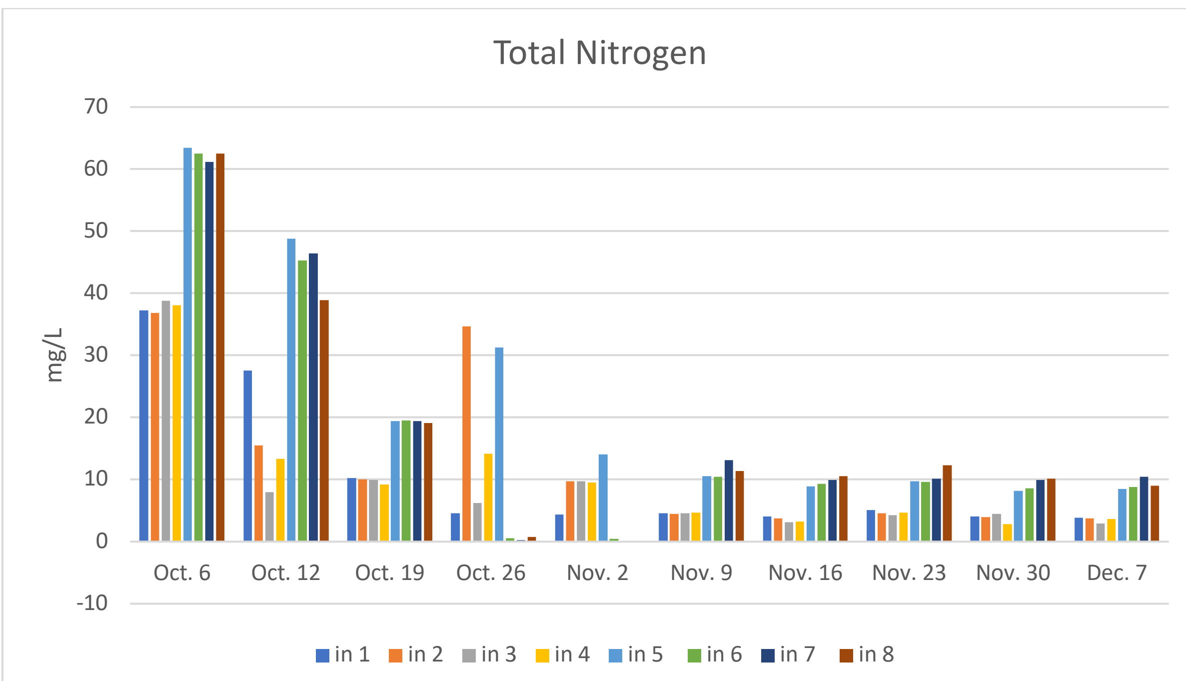

Figure 6: Total nitrogen concentration values for inflow samples

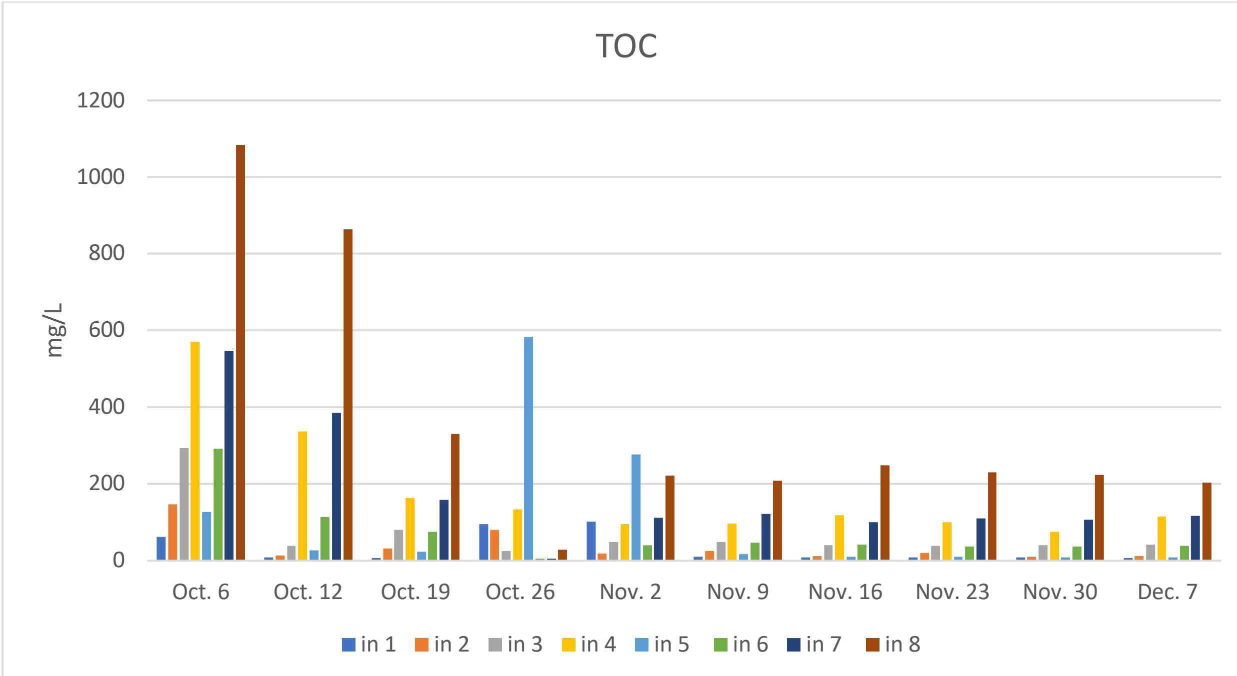

Figure 7: Total organic carbo concentration values for inflow samples

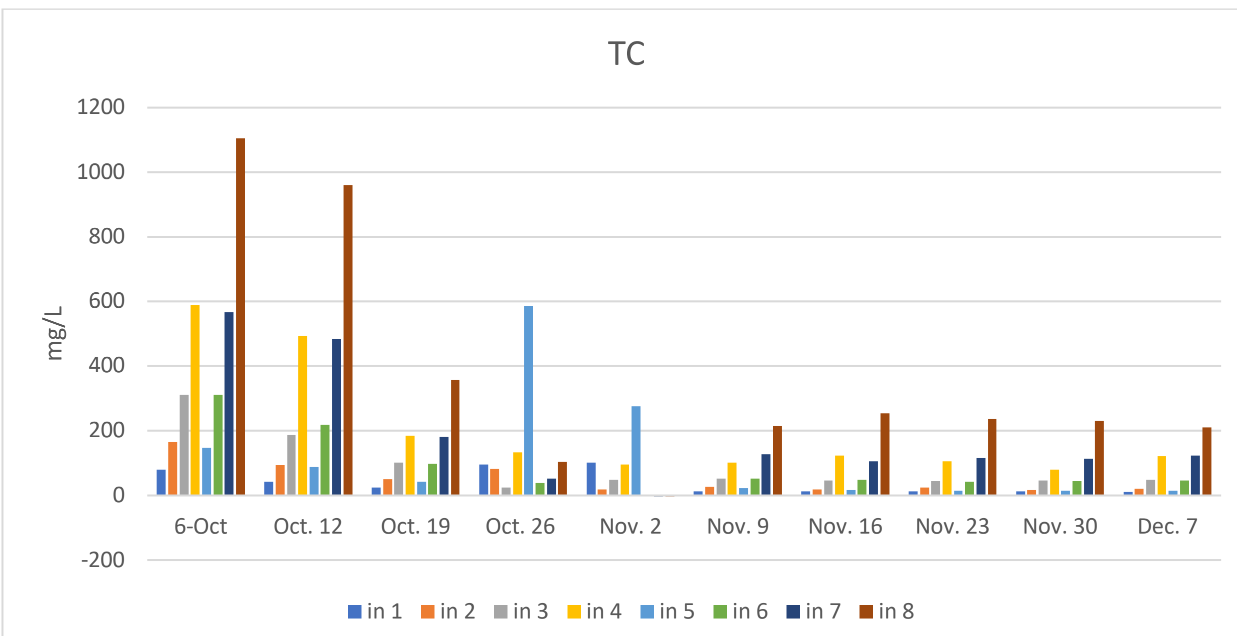

Figure 8: Total carbon concentration values for inflow samples

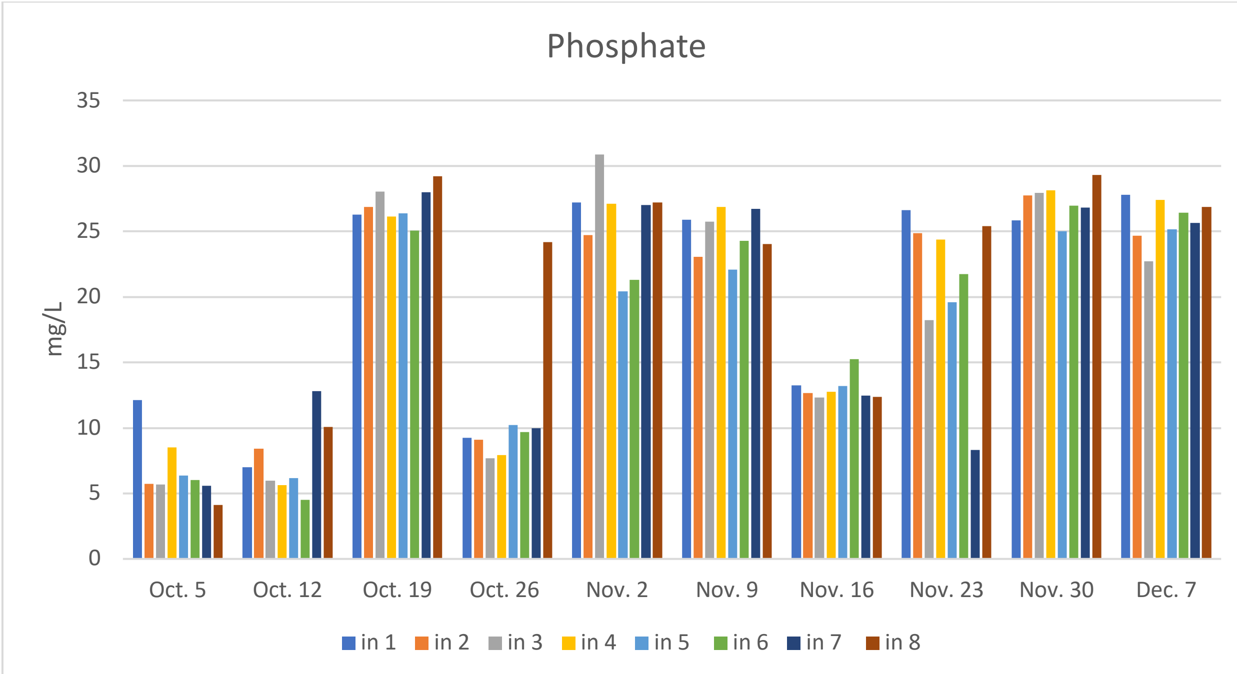

Figure 9: Phosphate concentration values for inflow samples

Appendix B: Constructed Wetlands throughout the experiment

Figure 10: Experiment on October 19, 2022

Figure 11: Experiment on October 26, 2022

Figure 12: Experiment on November 2, 2022

Figure 13: Experiment on November 9, 2022

	
Figure 14: Experiment on November 16, 2022	Figure 15: Experiment on November 23, 2022
	
Figure 16: Experiment on November 30, 2022	Figure 17: Experiment on December 7, 2022

Figure 18: Experiment on December 14, 2022

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