CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Environmental Sciences
Department of Applied Ecology

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Diploma Thesis

The effects of substrates on ibuprofen removal in constructed wetlands

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DIPLOMA THESIS TOPIC

Author of thesis:	Bc. Marko Tichý
Study programme:	Engineering Ecology
Field of study:	Nature Conservation
Thesis supervisor:	doc. Zhongbing Chen
Supervising department:	Department of Applied Ecology
Language of a thesis:	English

Thesis title:

The effects of substrates on ibuprofen removal in constructed wetlands

Objectives of thesis:

Pharmaceuticals and personal care products (PPCPs) have become an ever increasing concern as emerging pollutants due to their extensive use and incessant release into the environment. Even at trace-level concentrations, pharmaceuticals such ibuprofen may cause subtle, yet significant effects that impede natural ecosystems in the long-term, causing chronic aberrations to the delicate balance of aquatic systems. The use of artificial plant-based systems such as constructed wetland has been successfully engineered to treat wastewater, acting as a biological filter removing a range of pollutants such as PPCPs. However, research on the removal of PPCPs using constructed wetland is still limited. The aim of this thesis will be to examine the effects of using different substrates on the removal of ibuprofen in constructed wetland to determine the efficiency of such artificial systems. The study may be useful as a basis of engineering a more efficient artificial wetland systems for the removal of emerging pollutants.

Methodology:

The thesis is comprised by primary and secondary research with the former being a laboratory-scale vertical flow constructed wetlands that will assess the effectiveness of different substrates upon the removal of ibuprofen. An experiment will be built to collect data on a number of factors including presence of different substrates, plants as well arbuscular mycorrhiza. The theoretical portion of the thesis utilizes knowledge gained from academic research articles pertaining to the problematics of constructed wetland, emerging pollutants, PCPPs etc.

The proposed extent of the thesis: 50

Keywords: constructed wetland, emerging pollutants, pharmaceutical and personal care products (PPCPs), substrates, Arbuscular mycorrhiza (AMF), removal efficiency, pollutant degradation

Recommended information sources:

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Expected date of thesis defence:	2019/20 SS - FES

Electronically approved: 6. 2. 2020	Electronically approved: 11. 2. 2020
prof. Ing. Jan Vymazal, CSc.	**prof. RNDr. Vladimír Bejček, CSc.**
Head of department	Dean

Recognition

I would like to thank doc. Zhongbing Chen for the consultations necessary to begin and elaborate this thesis and for comments on the submitted work after its writing. My thanks also belong to Ph.D. candidate Bo Hu for his valuable advice, willingness and patience which he provided during the process of writing this thesis.

Declaration

I declare that I am the sole author of this master's thesis, and that I have cited all used information sources and literature. This work or its parts have not been submitted for another academic degree

In Prague, 2020

Signature:

Abstract

Pharmaceutical and personal care products (PPCPs) have become an ever-increasing concern as emerging pollutants due to their extensive use and release into the environment. Even at trace-level concentrations, pharmaceuticals such ibuprofen may cause subtle, yet significant effects which cause chronic aberrations to the balance of natural water ecosystems. The use of artificial plant-based systems such as constructed wetlands has been successfully engineered to treat wastewater, acting as a biological filter removing PPCPs and other organic compounds. Laboratory scale constructed wetlands consisting of 24 reactors has been developed to examine the effects of different substrates and symbiotic arbuscular mycorrhiza on the removal of ibuprofen and organic compounds. The experiment achieved a 65,71 – 76,48% removal efficiency for ibuprofen using a sand only substrate while 83,59 – 88,58% using perlite, 85,72 – 91,42% for vermiculite and a 99,96 – 99,98% removal efficiency using biochar. These results determine that the use of various different types of substrates not only affects removal rate of pollutants, but also influences numerous mechanical, chemical and biological processes within the CWs rhizosphere. The study may be useful as a basis of engineering a more efficient artificial wetlands systems for the removal of emerging pollutants.

Key words: constructed wetlands, emerging pollutants, pharmaceutical and personal care products, substrates, arbuscular mycorrhiza, removal efficiency

Abstrakt

Farmaceutické výrobky a výrobky pro osobní péči (PPCPs) se stávají stále více znepokojujícími látkami, které znečišťují přírodní ekosystémy. A to především kvůli jejich rozsáhlému užívání a následnému uvolňování do životního prostředí. I při nízkých koncentracích mohou tyto látky, například ibuprofen, vyvolat výrazné účinky, které dlouhodobě narušují přirozené vodní ekosystémy. Artificiální čistící systémy, jako jsou vybudované mokřady, působí jako biologický filtr a úspěšně čistí odpadní vody. Odstraňující také řadu znečišťujících látek, jako ibuprofen a jiné organické sloučeniny. V rámci této práce byly vyvinuty umělé mokřady skládající se z 24 reaktorů. Ty zkoumaly účinky různých substrátů a symbiotické arbuskulární mykorhizy pro odstranění ibuprofenu a organických sloučenin. Experiment dosáhl 65,71 - 76,48% účinnosti odstraňování ibuprofenu použitím pískového substrátu, 83,59 - 88,58% účinnosti použitím perlitu, 85,72 - 91,42% účinnosti pro vermikulit a 99,96 - 99 , 98% účinnosti při odstraňování pomocí biouhlí. Výsledky stanovují, že použití různých typů substrátu ovlivňuje nejen rychlost odstraňování znečišťujících látek, ale také ovlivňuje četné mechanické, chemické a biologické procesy v rizosféře umělých mokřadů. Tato studie může být užitečná jako základ pro konstrukci účinnějších systémů umělých mokřadů pro odstraňování znečišťujících látek.

Klíčová slova: uměle vybudované mokřady, znečišťující látky, farmaceutické a osobní výrobky, substráty, arbuskulární mykorhiza, účinnost odstraňovaní

Abbreviations

PPCP – Pharmaceutical and Personal Care Products

CW – Constructed Wetlands

EC – Emerging Pollutant

AMF – Arbuscular Mycorrhizal Fungi

H-SSF – Horizontal Subsurface Flow

V-SSF Vertical Subsurface Flow

TC – Total Carbon

TOC – Total Organic Carbon

IC – Inorganic Carbon

TN – Total Nitrogen

UV – Ultra-Violet

PS – Plant + Sand

PSA – Plant + Sand + AMF

PP – Plant + Perlite

PPA – Plant + Perlite + AMF

PV- Plant + Vermiculite

PVA – Plant + Vermiculite + AMF

PB – Plant + Biochar

PBA – Plant + Biochar + AMF

WWTPs – Wastewater Treatment Plants

Table of Contents

1. Introduction

The onset of the modern and industrialized age has seen substantial innovations at unprecedented rates in the fields of medicine as well as cosmetics, which has led to the widespread use of pharmaceuticals and personal care products also known as PPCPs. However, this has become a problem of great concern as the introduction of numerous contaminants in large quantities into the environment affects organisms including humans throughout all trophic levels (Nguyen et al. 2019). These emerging contaminants are comprised of prescription and non-prescription drugs, hormones, narcotics as well as cosmetic products and their subsequent metabolites and conjugates which undergo modifications when released into the environment (Daughton and Ternes 1999). Municipal wastewaters are among the primary sources of pollutants such as PPCPs because they are dispelled directly into the sewer system following human consumption and use (Kim et al. 2007; Hijosa-Valsero et al. 2010). However, most wastewater treatment plants (WWTPs) have not been devised for eliminating PPCPs. Consequently, numerous of these substances are released into surrounding surface waters where they can have profound and unpredictable ecotoxicological effects despite low concentrations, due to the sheer amount of compounds and biologically active combinations present (Ternes et al. 2004; Joss et al. 2006).

Constructed wetlands (CWs) have been proposed as an ecological alternative to the treatment and removal of PPCPs from the environment, but the precise mechanisms by which these emerging contaminants are eradicated remains understudied (Matamoros et al. 2008; Hijosa-Valsero et al. 2010). The simultaneous existence of many micro-environments within constructed wetlands gives rise to diverse microbiological communities, which are able to remove emerging pollutants, and offer a solution to PPCP degradation through multiple metabolic pathways depending on the physical and chemical design specifications of constructed wetland systems (Imfeld et al. 2009). Although CWs were found successful in removing some PPCPs, the most effective design of CW parameters has not been established (Matamoros and Bayona 2006). The use of different substrates is a key element that may increase or decrease CWs efficiency in removing PPCPs. The purpose of this thesis is to examine the role of substrates within constructed wetlands in the removal of ibuprofen, a pharmaceutical product of widespread international use.

2. Literature Research

2.1 Pharmaceutical and Personal Care Products (PPCPs)

Currently, more than hundreds of pharmaceutical products have been detected in surface waters all over the world (Hughes et al. 2013). As an example, the United States alone released more than seventy million prescriptions of the pharmaceutical metformin in 2014, which has led to widespread contamination of water resources that includes tap water at concentrations surpassing fifty percent of the permitted limit proposed by the Rhine River Basin agency (Trautwein et al. 2014). The rate at which PPCPs are released into the environment is rising ever faster, as the use and sales of these substances surges by more than five percent every year (Nguyen et al. 2019). Because many emerging pollutants can persist in the environment for many years following discharge, their ongoing accumulation poses a challenge in sustaining healthy ecosystems. Their presence is even more disturbing due to the fact that many of these contaminants do not appear in the environment exclusively, but as a compound complex that may have synergistic effects raising toxicological distress (Pal et al. 2010). The pervasiveness of numerous potentially poisonous pollutants in the environment demonstrates the demand for a better understanding of their persistence, fate and ecological impact (Petrie et al. 2014).

Figure 1 - Dominant pathways of pharmaceutical and personal care products (PPCPs) in the environment (Nguyen et al. 2019)

Emerging contaminants such as PPCPs have been found throughout the entire water cycle including drinking water sources, wastewater treatment plant effluents, agricultural and landfill runoff which collects in rivers and streams (Kim et al. 2007; Anawar et al. 2019) as can be seen in **Figure 1**. Although PPCPs have been found to occur in low concentrations ranging from only parts per trillion to low parts per billion (Heberer et al. 2002; Jiang et al. 2013; Ternes et al. 2004; Anawar et al. 2019) conventional wastewater treatment techniques are not effective in entirely eliminating these pollutants (Matamoros et al. 2008; Anawar et al. 2019). A number of secondary and tertiary treatment techniques have been utilized to remove emerging contaminants such as ozonation, advanced chemical oxidation as well as UV radiation (Kim et al. 2009; Liu et al. 2009; Rosal et al. 2010). However, these methods proved to be ultimately ineffective on a wide-use scale due to their high operational and maintenance costs, especially in rural and remote regions.

2.2 Ecotoxicity of PPCPs

As was previously mentioned, PPCP compounds discharged into the environment may have harmful ecotoxicological effects to organisms across the food web. Even though concentrations of specific contaminants may exist in concentrations that are too low to cause damage by themselves, most PPCPs found in ecosystems co-exist as compound mixtures, purportedly reducing biological diversity among macroinvertebrates in rivers, causing declines in fish populations, and reportedly cause physiological stress in freshwater mollusks (Matamoros et al. 2017; Gillis et al. 2014).

Specific examples of stronger toxic effects of PPCPs have been reported by a study done on *Daphnia magna* when exposed to a compound mixture of carbamazepine, diclofenac, and ibuprofen simultaneously (Cleuvers et al. 2004). Moreover, a study by Cleuvers et al. 2004, revealed significant acute toxicity for a mixture of pharmaceuticals diclofenac, ibuprofen, naproxen and aspirin at the same concentrations whilst these pollutants did not cause severe toxicity by themselves. Although chronic toxicity due to PPCPs may be more likely to consider because of the continuous discharge of these compounds into the environment, it is essential to evaluate the chronic impact of PPCP mixtures at environmentally relevant concentrations (McEachran et al. 2016). However, this has brought about its own set of challenges, as it is very problematic to determine subtle chronic toxicological effects in comparison to more acute toxicity such as mobility impairment or mortality which a more readily measurable.

Nonetheless, a growing amount of analyses that assess the chronic impact of emerging contaminant compounds have found significant effects (Petrie et al. 2014). The toxicity linked to PPCPs poses detrimental effects to prey and predators alike as it penetrates all trophic levels in the food web. For instance, the white-backed vulture's (*Gyps africanus*) decline in population by about 95% was found to be correlated to renal failure due to diclofenac, an anti-inflammatory drug that is widely used as a veterinary drug in livestock on which vultures feed upon (Oaks et al. 2004). These examples indicate that PPCPs have the potential to directly and indirectly affect species' physiology and survival.

2.2 Ibuprofen

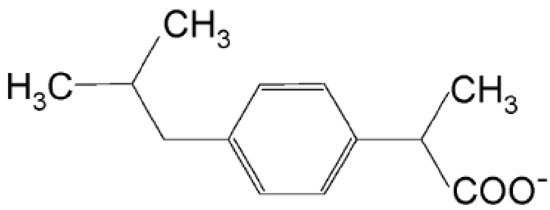


Figure 2 - Chemical structure of ibuprofen (Matamoros et al. 2005)

Ibuprofen a non-steroidal, anti-inflammatory, analgesic and antipyretic drug (**Figure 2**) has been chosen as the compound of interest in this study as it is one of the most widely used and well-studied PPCPs all across the world (Ferrando-Climent et al. 2012). Ibuprofen is able to enter the aquatic environment due to incomplete metabolization in humans. Every year, hundreds of tons of ibuprofen are discharged into ecosystems all over the world, and conventional wastewater treatment methods are able to remove the pharmaceutical with about seventy percent efficiency (Oulton et al. 2010). Furthermore, analysis of the relative contribution of the aerobic and anaerobic pathways in the degradation of ibuprofen suggests that aerobic degradation pathways play the dominant role (Matamoros et al. 2008).

Although ibuprofen may pose acute toxicity to aquatic organisms at environmentally relevant concentrations, it is suggested in relevance to other PPCPs, that damage caused by chronic, continuous exposure due to continual release into the environment plays a much more significant role and could pose long-term ecological effects (Ferrando-Climent et al. 2012). As an example, an investigation on the effects of ibuprofen on the expression of CYP360A, CYP314, and GST genes in *Daphnia magna* which are involved in the detoxification process exposed chronic ibuprofen exposure significantly deceased development and reproduction of the species, including decreases in the number of eggs produced and reductions in body length (Wang et al. 2016).

To further exemplify ibuprofen's ability to impair development in aquatic organisms, Veldhoen et al. 2014 found that ibuprofen acted as a disruptor of endocrine mediated post-embryonic development in the North American Bullfrog (*Rana catesbeiana*). David and Pancharatna 2009, conducting a study on the development of Zebrafish (*Danio rerio*) found that developing embryos tolerated lower doses of ibuprofen, but when exposed to higher doses, the fish exhibited "retarded development, decreased hatching rate and growth, cardiac anomalies, spinal curvature, pectoral fin malformation as well as behavioral alterations resulting in greater mortality of experimental embryos" (David and Pancharatna 2009). As with other PPCPs, ibuprofen metabolites such as hydroxyibuprofen and carboxyibuprofen which are products of ibuprofen degradation are also frequently detected in the environment, and have also been found to occur at higher concentrations than their parent substances with carboxyibuprofen being even more concentrated than ibuprofen in influent samples (Weigel et al. 2004).

2.3 Constructed Wetlands

Due to the costly nature of the aforementioned methods of water treatment, alternative, widespread, and low-cost technologies such as constructed wetlands (CWs) present a suitable complementary for wastewater treatment and recycling. Constructed wetlands usage has increased in recent times as they are simple to operate and maintain, have a small ecological impact producing low waste, and are able to be naturally implemented into existing landscapes (Ávila et al. 2014). The removal of emerging contaminants in CWs ensues as an effect of complex physical, chemical and microbial interactions. The rates and efficiency at which CWs remove PPCPs depends on a multitude of factors including and not limited to the design of CWs such as bed depth, choice of substrates, hydraulic and organic loading times, and aeration (Paola Verlicchi and Zambello 2014). The efficiency of CWs is often times decreased as the construction design does not take into account all of the parameters mentioned above (Ávila et al. 2013). Also, as many of the various PPCPs leeching into ecosystems undergo chemical changes with new properties, these compounds can also interact with one another as well as with biotic and abiotic factors in the environment, resulting in new metabolites, that may be bioactive or constant in the environment. PPCP metabolites resist microbial degradation and their presence is of even more concern than the parent compounds due to their toxicity and stability (Ávila et al. 2013; Ebele et al. 2017). Furthermore, additional attention should be paid to metabolites produced in the degradation pathway of emerging organic contaminants, so as to get a better insight of the major processes involved in their removal (Ávila et al. 2013; Ebele et al. 2017).

Constructed wetlands may be utilized to successfully treat wastewater influent acting as a primary, secondary or tertiary treatment step depending on design parameters as is represented by **Figure 3**. CWs were found to have the highest efficiency as secondary and tertiary treatment steps essential for water recycling, and are currently widely utilized for the treatment of wastewater and industrial effluents (Rousseau et al. 2008**;** Anawar et al. 2019)**.**

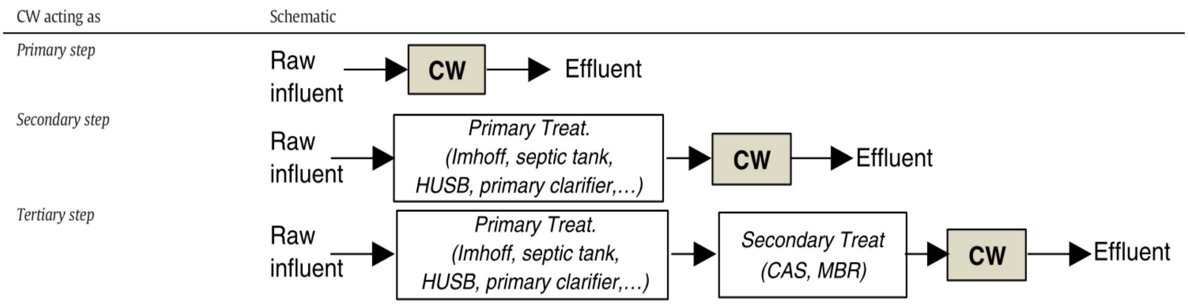


Figure 3 – Constructed wetlands as primary, secondary and tertiary treatment steps (Paola Verlicchi and Zambello 2014)

Principal constituents of CWs consist of water, a wetland plant species, choice of substrate and a natural microorganism environment. Literature describes many different CW design optimizations. Constructed wetlands have conventionally been categorized into two main types, surface flow and subsurface flow depending on the flow of water. In surface flow CWs, water flows above the surface in comparison to subsurface flow CWs in which water flows below the top layer of ground decreasing potential exposure (Halverson and Nancy 2004). Additionally, subsurface flow CWs are further divided into horizontal subsurface flow and vertical subsurface flow, contingent on the hydraulic regime as is represented by **Figure 4** (Fonder and Headley 2013; Ávila et al. 2014). A study conducted by Matamoros and Bayona conducted in 2006, revealed that horizontal subsurface flow CWs that have a shallow bed achieved better results than CWs with a deeper bed due to a higher oxidation potential, as a high redox status of CWs has been linked to an enhancement in removal efficiency of emerging contaminants (Matamoros and Bayona 2006).

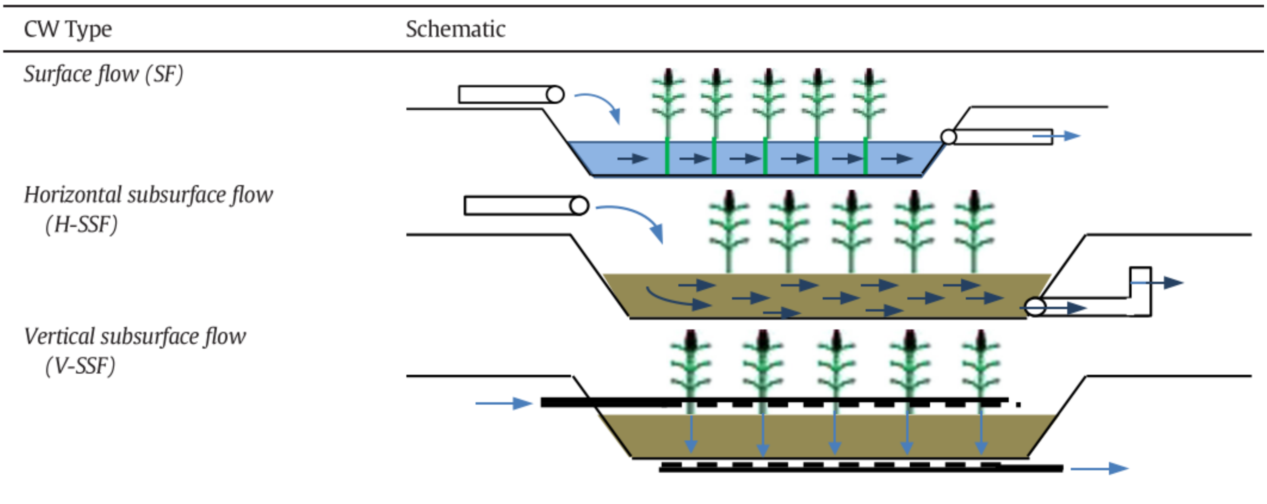


Figure 4 – Conventional types of constructed wetlands (Paola Verlicchi and Zambello 2014)

It has been suggested that the dominance of high redox potentials in wastewater treatment techniques supports aerobic respiration which has a higher rate of pollutant removal than degradation pathways relying on anaerobic pathways (Onesios et al. 2009). Limitation of subsurface oxygen availability is one of the main factors that limit conventional horizontal subsurface flow (H-SSF) wetland specifications, and thus, unsaturated vertical flow (V-SSF) wetlands have been developed to counter this limitation and increase oxygen accessibility, which is improved by the design parameters and operating conditions (Vymazal 2005; Torrens et al. 2009; Nivala et al. 2019). Another study conducted by Matamoros et al. 2007 also reported improved function of vertical flow CWs in comparison to horizontal flow CWs owing to higher redox conditions that have been promoted by the unsaturated operational mode of the vertical beds. More compact in design than horizontal flow CWs, unsaturated vertical flow CWs treating municipal sewage waters are able to absorb greater volumes of contaminant loads (Vymazal 2005; Cooper 2005). Pollutant removal efficiency depends entirely on the design of CWs, including parameters such as type of wetland configuration employed, choice of soil, and substrate matrix including its depth, and the operational mode of influent loading times whether they be batch or continuous (Zhang et al. 2014). It has been shown that alternating phases of saturation and unsaturation promoted oxygen availability compared to functioning under continuously saturated conditions, which in effect considerably increased the removal of pollutants (Ávila et al. 2013). Therefore, operation of CWs in cycles alternating between saturation and unsaturation enhances the treatment influent.

For example, use of alternating loading times may offset unavailability of a particular substrate to increase efficiency. In regions where sand substrates may not be available, employment of gravel substrates with lower hydraulic loading rates should be proficient at achieving adequate treatment (Ávila et al. 2014). Furthermore, the use of alternating loading times actually allows for the coexistence of multiple microenvironments where aerobic and anaerobic degradation pathways take place, resulting in the improved removal of variable pollutant compounds (Matamoros et al. 2008). Additionally, although the loading frequency rate of hourly vs. bi-hourly did not affect elimination of contaminants in CWs, lower redox values have been observed in the effluent from bi-hourly CWs, which in the study by Ávila et al. 2014, significantly affected the removal of contaminants diclofenac, tonalide and bisphenol. Decreasing the loading frequency resulted in a higher velocity, and pulse of the permeating water which decreased contact time and redox rates which resulted in lower treatment efficiencies of tonalide and bisphenol. On the other hand, the removal efficiency of diclofenac actually increased in less oxidized CWs (Ávila et al. 2014).

The efficiency of CWs at removing emerging contaminants also depends on the species of plants chosen for the particular design because plants and their roots play a crucial role in the uptake of nutrients, absorption of contaminants, production and release of root exudes which aid in the decontamination process, oxygenate the soil matrix and provide an ideal environment for the growth of microbial communities and symbiotic arbuscular mycorrhiza, and thus assist in elimination of PPCPs (Zhang et al. 2014). An experiment performed by Hijosa-Valsero et al. 2010 that assessed design specifications of seven different CW configurations showed that the presence of plants greatly improved the efficiency of PPCP removal including ibuprofen, which further exemplified the importance of highly oxygenated conditions in CW for the removal of ibuprofen. Seasonal variability was also detected as a factor which improved efficiency especially in summer months indicating that a biological process of removing PPCPs through microbial pathways seemed to be taking place, and these processes have been collectively deemed as the most significant pathway by which PPCPs are eliminated (Hijosa-Valsero et al. 2010).

2.4 Role and Efficacy of Substrates in CWs

Substrates in constructed wetlands have a central role in the growth and expansion of plants and microbial communities in the rhizosphere, while also offering physical support to plants and providing an environment where roots, microbes and organic matter can directly interact with PPCPs through mechanical and chemical processes. An appropriate selection of substrates is an essential aspect in the optimization of CWs as the interactions in the microenvironment of CWs directly influence the performance and efficiency of pollutant removal (Dordio et al. 2009). Therefore, finding an optimal, cost-effective substrate that not only supports development of plants and microbes, but also successfully aids in the removal of pollutants is a critical issue that may either increase or decrease the efficiency of the entire microenvironment. Physical characteristics of substrates including surface area, particle size, porosity, hydraulic retention time, electrical conductivity along with biological and chemical properties such as charge, toxicity, and chemical stability of electron donors and acceptors must be assessed to determine CW effectiveness in removing pollutants (Yang et al. 2018). Particle size of substrates is a defining factor accounting for their porosity and hydraulic characteristics and the porosity (Yang et al. 2018). Substrates may be categorized by their origin as natural, artificial, and industrial by-products. Natural and most traditionally employed substrates include gravel and sand because of their relative low-cost, accessibility, abundance and retention abilities for organic contaminants such as nitrogen and phosphorus (Arroyo et al. 2013; Yang et al. 2018). For example, a study by Allende et al. 2012 determined that substrate type was a main factor that affected the removal efficiency of As and Fe from acidic water.

However, as conventional substrates do not provide a carbon source, they limit biological degradation pathways such as denitrification. This may be remediated by the use artificial or emerging substrates such as perlite, vermiculite and biochar as they achieve an increased removal efficiency of pollutants while being cost-effective without producing waste by-products (Yang et al. 2018). Vermiculite has been assessed by studies for its properties as an adsorbent of organic compound pollutant in part for its wide particle distribution and high relative porosity resulting in high hydraulic conductivity a water retention (Abate and Masini 2005; Dordio et al. 2009). Emerging substrates may be further characterized based on the major pathways for contaminant removal, such as substrates with high P sorption, carbon rich substrates and denitrification substrates efficient in removing N (Yang et al. 2018). These characteristics are important for targeted PPCP removal as different pollutants in CWs have their own preferred removal pathways. Furthermore, the type of substrates employed in CWs have a considerable effect on microbial community structure, diversity and richness as determined by studies that used Illumina high-throughput sequencing to expose changes in microbial community structure between CWs using gravel, steel slag and zeolite substrates (Ghannad et al. 2015; Long et al. 2016).

This relationship is further exemplified in a study conducted by Ávila et al. 2014 in which unsaturated wetlands that used sand as a substrate attained considerably greater efficiency in eliminating wastewater pollutants than CWs that had gravel as its substrate. Furthermore, not only were wastewater pollutants removed, but most of the emerging organic contaminants have been removed as well including ibuprofen among other PPCPs due to increased aeration and oxygen availability, which has been shown to increase redox rates, and thus, promote elimination of substances whose removal is mainly dependent on aerobic degradation pathways (Ávila et al. 2014). The choice of a specific substrate can also influence mechanisms by which specific pollutants are removed. In the same study, sand based CWs removed hydrophobic compounds, mainly removed by sorption, more efficiently than gravel-based systems (Ávila et al. 2014)

Biochar, a carbon-rich organic constituent produced as a result of pyrolysis, a method by which materials are broken down as a result of very high temperatures in an inert environment, may be utilized as a simple, effective, and low cost substrate to treat water, remove organic compounds and toxins to provide a suitable medium for microorganisms within CWs (Gupta et al. 2016). Biochar is able to function in a number of ways which include improving soil quality through retention of fertilizers, encouraging growth of symbiotic and valuable microbes while also remediating organic and inorganic pollutants (Liang et al. 2006; Warnock et al. 2007; Marschner et al. 2013). Furthermore, as a result of its porous structure, biochar is also effective in the adsorption of heavy metals, especially in aquatic based systems (Zhengang Liu and Zhang 2009). Biochar, has been previously employed in studies as a sorbent for soil immobilization and removal of the contaminants naphthalene, nitrobenzene and m-dinitrobenzene (Chen et al. 2008). In other studies, substrates with added biochar of varying proportions in CWs were able to remove organic compounds more effectively, and shown to immobilize toxic heavy metals respectively (Rozari et al. 2015; Zhang et al. 2013).

A study by Gupta et al. 2016 evaluated the efficiency of biochar as substrate in order to test whether the combined use of biochar as a medium along with CWs improved removal of organic compounds. The study used a horizontal subsurface wetland design with biochar and gravel as a comparison, and was cultivated with *Canna* species plants. Synthetic wastewater flowed through the system, and pollutant removal performance was compared between the controlled (gravel) and experimental (biochar) CWs (Gupta et al. 2016). This study revealed that CWs with biochar were more efficient as compared to CWs with gravel alone in reducing overall organic and inorganic pollutants (Gupta et al. 2016). Also, the removal of contaminants in biochar based CWs depended on a number of factors including adsorption, precipitation, filtration, sedimentation, microbial degradation, and plant uptake as was reported in CWs using other types of substrates. Finally, this study indicated that CWs enriched with biochar are specifically successful at remediating wastewater as a secondary treatment step (Gupta et al. 2016). Although biochar has been successfully shown to be capable of remediation, research is preliminary and more investigation with a more rigorous methodology is needed. Meanwhile, biochar provides an ecological and cost-effective wastewater treatment substrate option.

2.5 Pollutant Removal Pathways

Within the microenvironment of constructed wetlands transpires a number of complex metabolic interplays, and these interactions are one of the main reasons why the design of CWs including substrate choice is a decisive parameter. **Figure 5** illustrates a number of pollutant and organic compound removal mechanisms existing within CWs, including plant uptake and phytoremediation, photodegradation, microbial degradation as well as sedimentation, adsorption and precipitation of emerging contaminants.

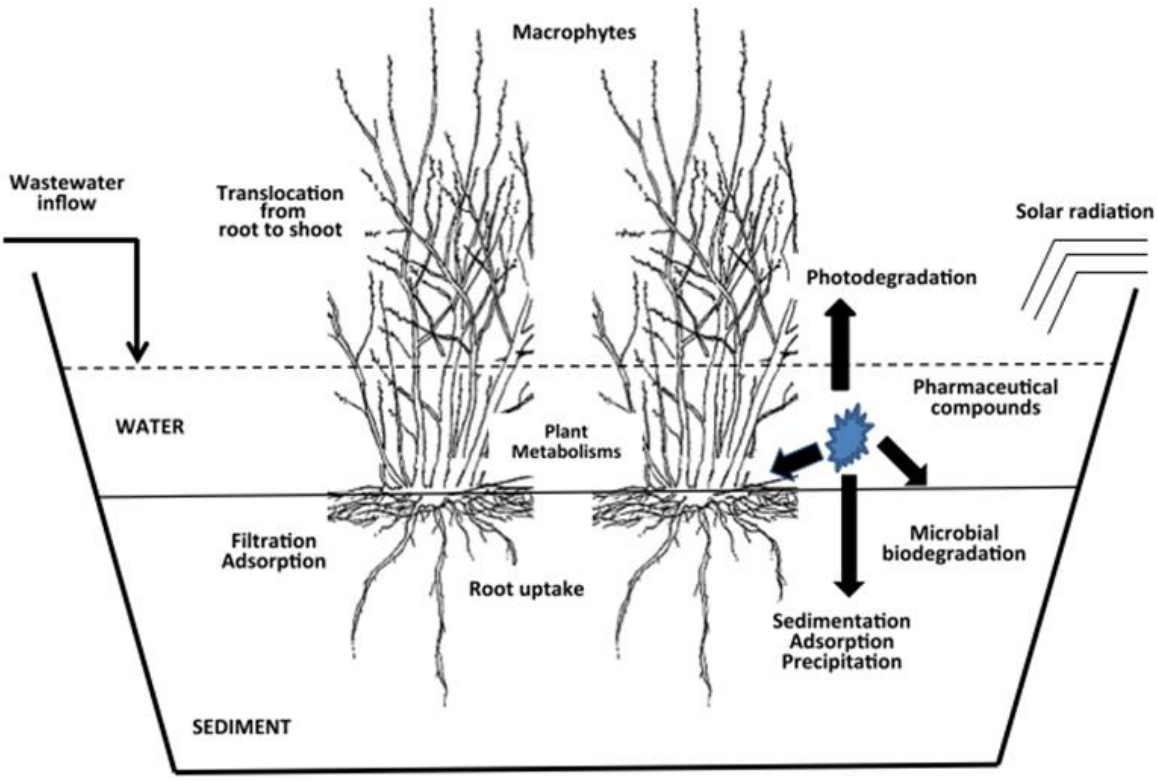


Figure 5 - Emerging contaminant removal mechanisms in constructed wetlands. (Zhang et al. 2014)

2.5.1 Photodegradation

Photolytic degradation has been described as the dominant removal pathway for certain emerging contaminants (Buser et al. 1998). The efficiency by which ECs are removed by photolytic degradation in aquatic systems depends on several factors including sunlight accessibility, which goes hand in hand with seasonal differences as sunlight, essential for photolysis, light intensity and the reduction of light caused by deeper water depth (Buser et al. 1998). For example, sunlight's interaction with the soil matrix can result in number of photochemical reactions and photodegradation pathways which include hydrolysis, oxidation and reduction to name a few (Maldonado-Torres et al. 2018). The importance of photodegradation in the removal of PPCPs is demonstrated by a study performed by Maldonado-Torres et al. 2018 which examined the photodegradation of ibuprofen. The study found that in a dark setting without a ready access to sunlight, there was about a 10% loss of the initial concentration of ibuprofen (attributed to adsorption) after more than 10 days. However, under sunlit conditions, photodegradation pathways including photolysis, ibuprofen displayed total transformation into secondary compounds, highlighting the significance of photodegradation pathways of PPCPs (Maldonado-Torres et al. 2018).

2.5.2 Sorption Processes

Because many suspended particle compounds, including pollutants from wastewater are retained in the substrate beds of CWs, a significant mechanism of pollutant removal may be through sorption of "soil, organic carbon, mineral surfaces and biofilms coating the gravel bed" (Zhang et al. 2014). PPCPs in CWs are influenced by their adsorption capacity to soil and sediment. Adsorption causes an effect on the distribution of dissolved substances between water and the solid surface, which subsequently affects their mobility in the aqueous environment, and regulates their eventual fate (Bui and Choi 2010). An understanding of a pharmaceutical compound's ability to be adsorbed by various media to solids is an important, if not a critical aspect determining pollutant's fate and their potential to be removed by CW mechanisms (Zhang et al. 2014).

Most organic chemicals have adsorption capabilities at least to some extent, and highly hydrophobic pollutants that persist may have great potential to be adsorbed in CWs (Matamoros et al. 2008; Zhang et al. 2014). Hydrophobic compounds are easily adsorbed onto organic matter present in CWs, and are thus less likely to be degraded which results in their excessive accumulation in CWs beds. In comparison, hydrophilic contaminants are removed much more easily according to their specific physio-chemical characteristics (Rosal et al. 2010). Interactions between aliphatic and aromatic groups in the structure of a contaminant with the lipophilic cell membrane of microorganisms, or the lipid fractions of the suspended solids may be some of the hydrophobic interactions that cause pollutants to be adsorbed (Verlicchi et al. 2012). Many previous studies have suggested that the relatively high level of PPCPs adsorbed to substrate in CWs may be attributed to their hydrophobic character (Zhang et al. 2014)

2.5.3 Plant uptake and Phytodegradation

The ability of plants to directly uptake, accumulate and effectively translocate contaminants is considered an important mechanism for pollutant removal known as phytoremediation (Collins et al. 2006). Phytoremediation has been employed as an effective and ecological technique that simulates the functions of natural wetlands as ecosystem remediators. Predominantly, it aims to employ the complex potential interactions among water, substrates, plants, microbes and the atmosphere to remove ECs through the constructed wetlands (Nguyen et al. 2019). Phytotoxicity in plants is countered by various cellular detoxification mechanisms. The direct uptake and absorption of PPCPs and other emerging contaminants by plants and phytoremediation processes that take place have only recently been extensively studied (Zhang et al. 2014). Available data on PPCP uptake and ensuing assimilation is limited as most studies have focused on only a few pharmaceutical compounds and species of plants. Furthermore, most studies on plant uptake of pharmaceuticals have been done in controlled hydroponic solutions, rather than more relevant field scale experiments, so the basic mechanisms involved in plant uptake of PPCPs remain poorly understood (Redshaw et al. 2008). Following plant uptake, organic contaminants may be partially or completely degraded, metabolized, or transformed to different compounds that become bound to plant tissue (Salt et al. 1998).

Complete phytodegradation through mineralization of organic pollutants has been observed in macrophyte aquatic plants (Susarla et al. 2002). Enzymes within plants interact with micro-organic compounds and are able to either degrade them completely to inorganic compounds such as CO_2, H_2O, and Cl_2, or partially degrade pollutants into stable intermediates, which become bound and stored in the plants (Susarla et al. 2002). However, because PPCP uptake by plants is relatively understudied, potential toxicity of the intermediate products which are produced by the plants' enzymes and metabolism must be considered when designing CW, and it remains an essential aspect in developing phytoremediation strategies (Zhang et al. 2014). Another characteristic to consider is that removal efficiencies of PPCPs by phytoremediation processes vary in different CWs acting as a primary, secondary, or tertiary treatment system (Verlicchi and Zambello 2014). For example, experiments that utilized constructed wetlands as a primary treatment of pollutants, 98% of several PPCPs have been successfully removed from the influent including caffeine with over 99.9% efficiency, acetaminophen with a 99.98 efficiency, ibuprofen with a 99.6% removal efficiency, naproxen at 99.4%, and finally, triclosan which was removed with a 98% efficiency (Verlicchi and Zambello 2014). These numbers demonstrate the proficiency by which CW are able to remove PPCPs among other emerging contaminants. CWs that performed as a secondary treatment step were able to remove various PPCPs such as like salicylic acid, sulfadimethoxine, and caffeine with an efficiency greater than 75%, while poor treatment performance with less than 25% efficiency was observed for other PPCPs that included clarithromycin, gemfibrozil, sotalol, sulfamethoxazole, sulfapyridine, triclocarban, and triclosan (Verlicchi and Zambello 2014). Finally, CWs operating as a tertiary treatment displayed peak removal efficiencies which were above 75% for the PPCPs diclofenac, verapamil, furosemide, and alfuzosin while other PPCPs that comprised of salicylic acid, carbamazepine, and sotalol, exhibited very low removal efficiencies below 25% (Verlicchi and Zambello 2014). These varying results in removal efficiencies suggest that CWs effectiveness at removing pollutants by phytoremediation varies among CWs acting as a primary, secondary or tertiary treatment.

2.5.4 Microbial Degradation

While phytoremediation is efficient, ecological and cost-effective in removing organic contaminants including PPCPs, the very presence of pollutants in the plants' rhizosphere has a negative effect on their growth, metabolism and consequently, their efficiency at removing pollutants as PPCPs (Carvalho et al. 2014; Gerhardt et al. 2009). This drawback may be countered by applying relevant microenvironments through microbial interactions, and the synergetic nature between plants and microbes which include symbiotic arbuscular mycorrhiza. It has been proposed that microbial degradation pathways are the dominant processes by which pollutants are removed (Vo et al. 2018). Prokaryotic and eukaryotic microorganisms including bacteria and arbuscular mycorrhiza use organic compounds such as carbon as an energy source, and are able to metabolize them in the "presence of suitable growth substrates" (Fester et al. 2014). Carbon originating from plant root exudates initiates these metabolic processes as pollutants in the soil serve as electron donors, and thus, they become oxidized under both aerobic and anaerobic circumstances. The presence of numerous electron acceptors other than oxygen in the substrate microenvironment can enable anaerobic respiration processes (Fester et al. 2014). Several aerobic and anaerobic removal pathways such as denitrification and nitrification in the rhizosphere have been identified having great potential to either transform organic compounds, or increase contaminant removal (Saeed and Sun 2012). Furthermore, microbial communities have been recognized for their efficacy in removing organic carbon (OC), along with the reduction of sulphate which may also play an important function in the removal of OC (Faulwetter et al. 2009). Microbial transformation pathways have been shown to be the dominant methods by which the greater part of total nitrogen (TN) is removed (Faulwetter et al. 2009). This demonstrates that the cycling and transformation of carbon, nitrogen and sulphur is centered around the microbial environment and processes within it. PPCP removal by microbial degradation can further be subcategorized by the mechanism by which they are removed being mineralization, conversion to hydrophobic compounds, or to more hydrophilic compounds (Onesios et al. 2009; Vo et al. 2018). Transformation of compounds including PPCPs leads to a decrease in their toxicity as well as increased and degradation can decrease toxicity and increase in their water solubility (Faulwetter et al. 2009; Vo et al. 2018).

Microbial community structure and the ensuing degradation pathways can further be enhanced by altering operational parameters of CWs such as temperature, pH, hydraulic retention time and the types microbial species present in the system. This also further signifies the importance of redox potential in CWs and the importance of utilizing various substrates with diverse properties. The simultaneous presence of aerobic and anaerobic zones within the differing substrates means that multiple microbial communities and consequently multiple degradation pathways can successfully coexist within one CW system increasing its total remediation capacity. Aerobic degradation pathways such as nitrification require an oxidized microenvironment with a high redox potential, while anaerobic processes such as denitrification and sulphate reduction need lower redox potential and reduced microenvironment (Faulwetter et al. 2009). For example, the removal efficiency of the PPCPs naproxen and diclofenac increased in an anaerobic microenvironment, while aerobic conditions increased the removal rates of bisphenol and ibuprofen (Ávila et al. 2010; Vo et al. 2018). This means that a CW system can be engineered to treat wastewater within a range of multiple redox potential favoring multiple degradation pathways and pollutants, or on the contrary, be designed to remove specific pollutants utilizing microbial respiration processes within a restricted range of redox conditions (Onesios et al. 2009; Faulwetter et al. 2009).

This methodology improves the potential for phytoremediation and increases the remediation process efficiency (Rehman et al. 2018). The symbiotic relationship between plants and microbes takes advantage of plant growth promoting traits (PGPs), and pollutant degrading genes that either rhizobacteria or endophytic bacteria possess (Gerhardt et al. 2009). In reciprocation, plants provide the microbes with nutrients, minerals and environment in which they can proliferate. The microbes improve bioavailability of nutrients within with the microenvironment of plants' rhizosphere, and they are able to mineralize a variety of organic pollutants as well (Nguyen et al. 2019). The symbiotic nature of the relationships between plants and microbes have become an important aspect in studying CWs and the mechanisms by which emerging contaminants are removed as microbes and plants supplement each other's needs. Although promising, research regarding plant-microbe relationships is still in its infancy and will require further research to not only to better understand the relationships themselves, but also the ways that these interactions support the removal of PPCPs (Nguyen et al. 2019).

2.6 Arbuscular Mycorrhizal Fungi

The presence of microorganisms in remediating CWs is increasingly regarded as an option that overcome weaknesses of plants (Fester et al. 2014). Arbuscular mycorrhiza (AMF) is a symbiotic relationship that is developed in the soil between the roots of plants and arbuscular mycorrhizal fungi which penetrate cortical cells of plants located within the roots (Sarrazin et al. 2011; Smith and Read 2008). This special relationship is very beneficial for both organisms as plants provide the fungi with nutrients in the form of carbohydrates, and in return, the fungi stabilize substrate structure and provide essential minerals for the plants creating a hospitable microenvironment which directly and indirectly increases plants' tolerance of biotic and abiotic stress factors including heavy metals and emerging contaminants (Smith et al. 2010; Fester 2013).

An investigation analyzing AMF in the roots of *Phragmites australis* in two vertical-flow CWs aimed at remediating polluted wastewater hypothesized that the fungi played a role in heavy metal removal (Xu et al. 2018). The results of the experiment showed that the fungi were tolerant of heavy metals, and that the second CW which was planted with *Phragmites australis* along with inoculating AMF "exhibited significantly higher Cd and Zn removal efficiencies than the first CW along with a different AMF community" (Xu et al. 2018). The study was limited however, due to a short time span, and more data was reported to be needed to analyze the role of AMF in enhancing heavy metal removal in CWs (Xu et al. 2018).

Arbuscular mycorrhizal fungi can influence the circulation of contaminants within plants by selective transport and distribution (Debiane et al. 2009; Langer et al. 2010). It has been reported that AMF are able to reduce the concentration of pollutants in the shoots of plants by transporting them specifically to the rhizodermis where concentrations of pollutants have been shown to increase (Huang et al. 2007; Wu et al. 2009). The selected transport of contaminants may serve to protect the plant from damage caused by pollutants. Furthermore, not only do plants reap benefits from the effects of AMF, but reportedly so do microbes, notably bacteria which have the ability to degrade organic compounds (Corgié et al. 2006; Alarcón et al. 2008). Through both of these mechanisms, AMF can indirectly stimulate the rate by organic contaminants are degraded in the soil microenvironment (Fester 2013). Studies concerning the effects AMF on biodegradation of organic pollutants have shown that the symbiosis positively affected the removal of polycyclic aromatic hydrocarbons also known as (PAHs) by italian ryegrass (*Lolium multiflorum*) as well as the dissipation of PAHs by alfalfa (*Medicago sativa*) respectively (Yu et al. 2011; Zhou et al. 2009).

A study by Fester 2013 investigated the colonization of AMF in constructed wetlands contaminated with ammonia, benzene and, methyltert-butyl (MTBE) over the course of 4 years. The parameters of this design which can be seen in **Figure 6**. Samples of the plants' roots have shown that AMF successfully inhabited the CWs even under stressful conditions created by the pollutants at the inlet part of the basin (Fester 2013). Conversely, at the outlet part of the basin, no AMF colonization has been found exemplifying the importance of a solid substrate for AMF development (Fester 2013).

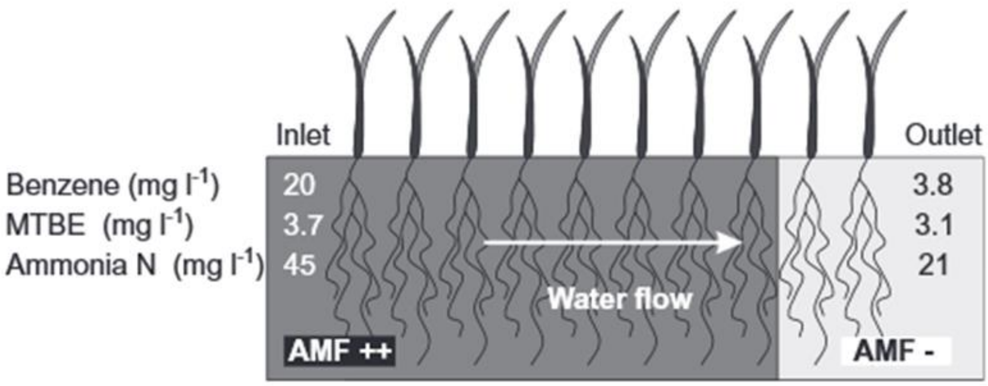


Figure 6 – Design parameters of CWs by Fester 2013 assessing AMF colonization under contaminated conditions

The growth of AMF and eventual treatment of water can be further supplemented using well thought-out CW design which includes the aeration of roots, intermittent influx of water using batch operation, and sewage backflow which increases oxygen availability (Li et al. 2011). For example, an experiment studying the development of *Glomus mosseae*, a species of AMF using various plant species demonstrated that a high colonization rate was observed using alternating aeration of two hours, four times a day (Hawkins and George 1997). Another study by Miller 2000 verified a higher rate of AMF colonization in CWs using alternating batch operation, and flooding conditions than that of those with continuous flooding in the root systems of wetland plant species *Panicum hemitomon* and *Leersia hexandra*. Although an appropriate level of water depth and mode of operation seems to improve AMF growth in CWs (Xu et al. 2016), establishment of AMF propagules was also witnesssed in substrates immediately after an extended flooding period as demonstrated by (Wolfe et al. 2007). Besides choice of substrate and mode of operation, the level of abiotic factors such as nitrogen (N), phosphorus (P) in soil also seems to have a major role in AMF colonization and expansion in wetland plants (Wang et al. 2011).

For example, it has been suggested that in nitrogen rich environments, plants do not require nitrogen allocation from fungi resulting in a disproportionate relationship where the fungi request more energy sources, in the form of carbon (C), than the nutrients they provide for the plant (Blanke et al. 2005). Ultimately, the plants decreased the amount of donated carbon to fungi which resulted in decreased growth and colonization (Blanke et al. 2005). To further illustrate, a study by Confer and Niering 1992 that compared colonization of AMF in the roots of *Phragmites australis* and *Typha angustifolia* between natural wetlands and artificially constructed wetlands found that the development of fungi was greater in artificial CWs than natural wetlands due to decreased organic matter availability at the artificial sites.

Consequently, influent wastewater quality and amount of organic matter has a direct influence on AMF growth rate in wetland plant roots (Confer and Niering 1992). It is therefore important to investigate the role of abiotic factors in soil substrate to understand their role in the establishment of AMF-plant relationships in order to enhance CWs remediation capabilities (Xu et al. 2016). Based on these examples, arbuscular mycorrhizal fungi may serve as model co-inhabitants of technical installations such as constructed wetlands due to their potential to accelerate bioremediation of water contaminated by pollutants including PPCPs in disturbed areas (Hildebrandt et al. 2007; Meier et al. 2011; Fester et al. 2014). AMF have been found to be crucial for the health of plants in distressed areas. Nevertheless, the specific role of AMF in bioremediation is understudied as constructed wetlands and other such installations are frequently fashioned without factoring for AMF, or even without a substantial amount of its propagules (Fester 2013). Also, the stressful conditions within the potentially contaminated substrates of constructed wetlands may hamper the development of AMF (Debiane et al. 2009; Fester 2013). However future studies must address the specific roles of AMF as studies regarding dissipation of pollutants rarely go beyond experimental installations “leaving the question of who is doing what largely unresolved” (Fester et al. 2014). Furthermore, remediation capacity of CWs enriched with AMF must be analyzed for potentially increasing the removal of emerging contaminants and to better optimize the CW ecosystem. (Xu et al. 2016).

Overall, bioremediation may well be regarded as the preferred method for the removal of pollutants including PPCPs. However, as evidenced in studies and literature, most approaches focus on single organisms or methods that do not take into account the vast amount of interplays that exist within the soil microenvironment. In the future, the effectiveness of bioremediation will depend on our understanding of the complete ecosystems found in natural and artificial constructed wetlands, with plant-microbial interactions at the forefront being of vital importance for CWs design (Fester et al. 2014)

3. Objectives

As mentioned above, constructed wetlands provide a natural, reliable, and low-cost alternative in removing ever present contaminants discharged by humans into the surrounding environment. However, the abovementioned numerous processes and mechanisms involved in the removal of PPCPs in constructed wetlands are understudied, and knowledge gaps concerning key elements of CWs design remain. These gaps must be addressed to enhance the relevance of CWs and their success in achieving maximum contaminant removal. The main objectives of this study are to compare the effects of substrates in the removal of ibuprofen in combination with the occurrence of arbuscular mycorrhiza. The study aims to demonstrate that choice substrates and AMF prevalence positively impact not only the removal of emerging contaminants but also positively benefit the established plants used in such designations. The experiment intends to provide the groundwork for further studies of design parameters and demonstrate the significance of constructed wetlands application in waste-water treatments across the globe.

4. Methods and Materials

Small-scale laboratory sub-surface vertical constructed wetlands were developed on the grounds of the Czech Agricultural University in Prague. The experimental setup consisted of 24 reactors grouped into 4 categories (A, B, C, D) characterized by the presence of plants (*Glyceria maxima*) from the Czech Agricultural University, and the inclusion of arbuscular mycorrhiza obtained from the Institute of Botany in Czech Republic. Group A reactors contained gravel and sand based substrate while B, C, D reactors had a gravel/sand and either a perlite, vermiculite or biochar substrate respectively. **Figure 7** details the specifications of the reactors.

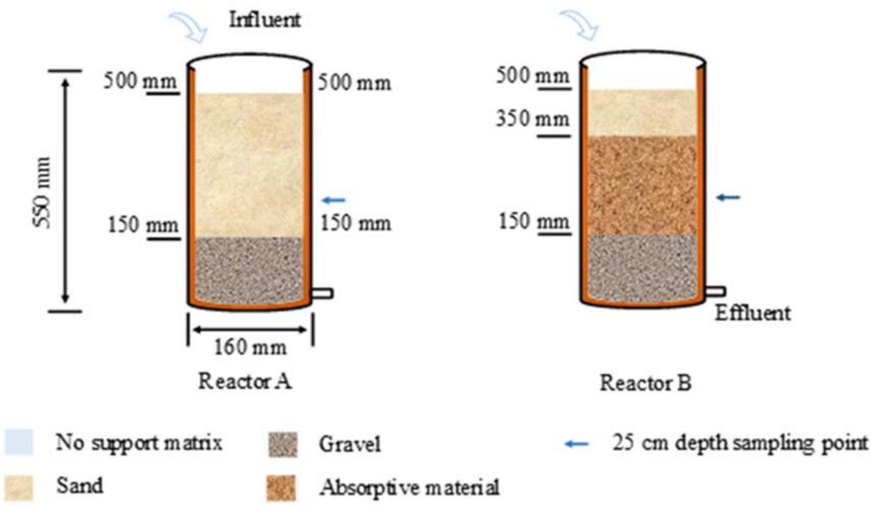


Figure 7 – Schematic diagram of reactors in CWs

Two types of reactors were developed with a total height of 550 mm and a width of 160 mm. Each reactor consisted of a plastic bottom and column which was made watertight. The reactors had an exposed top for the entry of influent which required the construction of a shelter which protected the reactors from external environmental factors such as incursion of rain water. Furthermore, all of the reactors had a sampling point at 25 cm above the plastic bottom which consisted of a hose for the effluent. The reactors further differed in the number of layers, with group A reactors having two layers with a 400 mm sand substrate matrix and a 150 mm gravel-based matrix. All reactors from the groups B, C, D had a three-layer matrix which consisted of 50 mm sand layer, a second 350 mm layer consisting of absorptive material that differed among the three groups, and a third 150 mm gravel matrix layer. The second layer in groups B, C, D differed in the type of substrate with reactors in group B having expanded perlite, Group C consisting of expanded vermiculite and reactors from group D with biochar.

The breakdown of reactor parameters is further specified by each group having 2 variations in triplicates containing Glyceria maxima (Gly+) and the other *Glyceria maxima* and arbuscular mycorrhiza (Gly+ AMF+) and triplicate of reactors having both AMF and plants. A schematic diagram (**Figure 8**) detailing specification of the constructed wetlands can be viewed below.

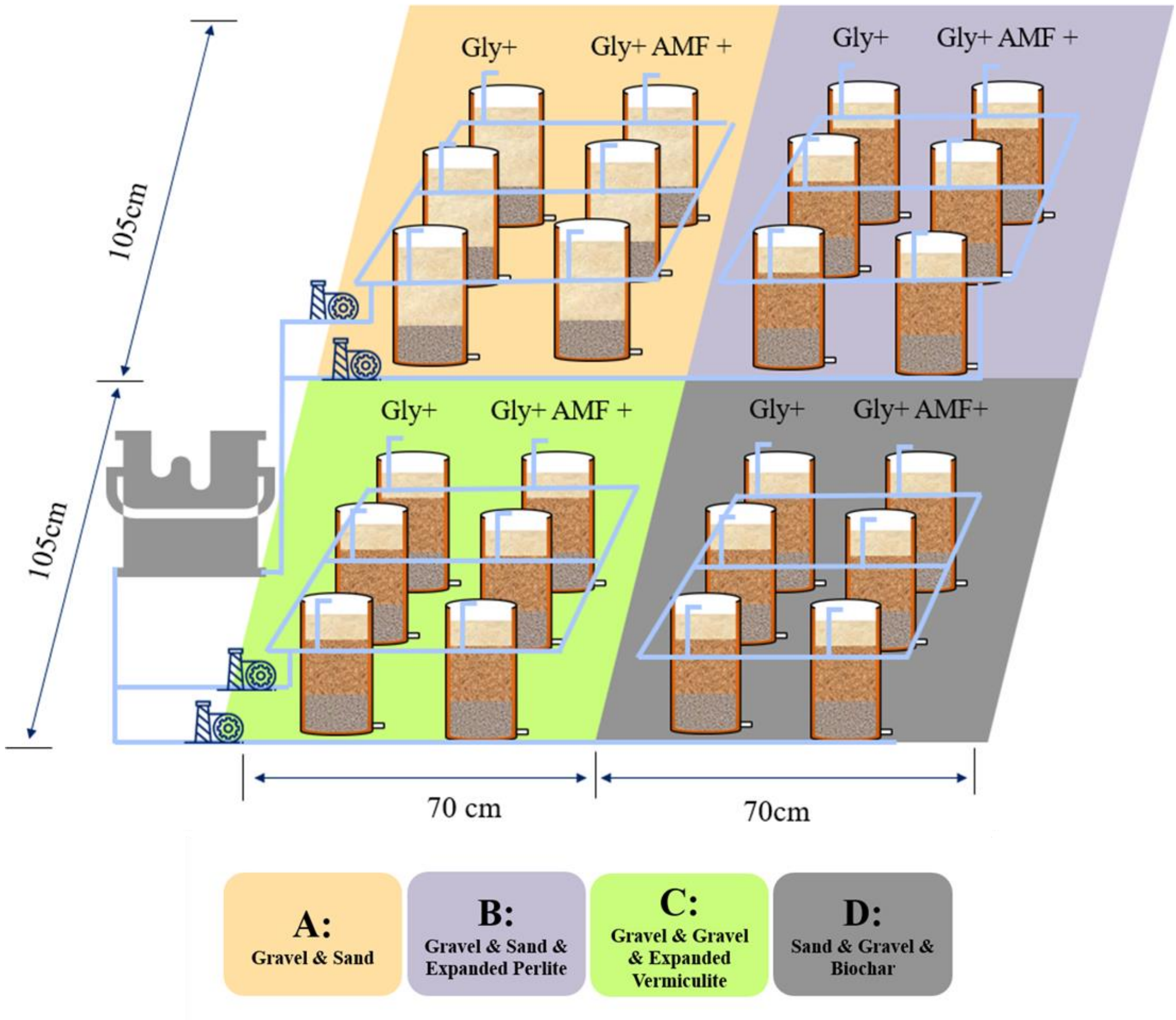


Figure 8 - Schematic diagram of laboratory scale CWs

The first phase of the experimental setup lasted four weeks, and was designed so we could see whether plants could successfully adapt to their new environment as well as for the establishment of symbiotic system between plants and the AMF. During this time, plants were fed a low concentrated nutrient solution intermittingly within a four-day cycle. The composition of the nutrient solution is illustrated by **Table 1**. The cycle comprised of 24 hours in which the reactors were kept water free, followed by a 72-hour cycle during which plants were watered every two days with 1 L of the influent, and water was allowed to directly flow out. After this stage of the experiment, a simulated sewage commissioning phase followed which lasted for two weeks. During this phase, a four-day cycle was kept with 2 L of the influent every two days which was allowed to directly flow out. At this stage measurements and data began to be collected for the following water quality indicators: total carbon (TC), total organic carbon (TOC), inorganic carbon (IC), total nitrogen (TN), as well as ammonium nitrogen (NH_4^+ N), Chloride, Nitrate (NO_3^-N), Nitrite (NO_2^-N), Phosphate and Sulphate. Furthermore, data was also collected for the measurements of dissolved oxygen (DO), electrical conductivity (EC), oxidation/reduction potential (ORP) and pH.

Reagent	Concentration	Microelements	Concentration
Urea	104 mg/L	$CuSO_4{\cdot}5H2O$	0.01 mg/L
NH_4Cl	16 mg/L	$FeSO_4{\cdot}7H_2O$	0.45 mg/L
$CH_3COONa{\cdot}3H_2O$	255 mg/L	$MnSO4{\cdot}H_2O$	0.02 mg/L
Peptone	20 mg/L	$Pb(NO_3)_2$	0.02 mg/L
KH_2PO_4	41 mg/L	H_3BO_3	0.04 mg/L
Yeast extract	132 mg/L	$Na_2MoO_4{\cdot}2H_2O$	0.02 mg/L
Skim milk	59 mg/L	$KCr(SO_4)_2{\cdot}12H_2O$	0.02 mg/L
$NaHCO_3$	25 mg/L		
$MgSO_4{\cdot}7H_2O$	41 mg/L		
$CaCl_2{\cdot}6H_2O$	28 mg/L		

Table 1 - Concentration (mg/L) of nutrients in wastewater solution

Sampling of water quality consisted of collection at the 25 cm depth sampling point at each of the 48 reactors. For each sample a 0,5 L plastic water container was marked and used for each of the reactors. Each sampling consisted of collection from the effluent. For each sample, the water containers were filled with 0,5 L which was then directly emptied, and once again filled to the brim. All 24 containers comprising effluent samples were then transported to be measured in laboratories. As mentioned above, a number of measurements were collected starting with the dissolved oxygen (DO). Measurements were taken using the Hach HQ30D flexi portable multimeter used for the assessment of water quality application. Connected to the instrument was a probe which upon installation was automatically calibrated and measurements of each water sample could start immediately. The probe connected to the multimeter was inserted into each of the 48 water containers and following every measurement, the probe was then cleaned using deionized water in order to lower risk of data discrepancy and misinterpretation. Data was measured and recorded in mg/L. Assessments of electrical conductivity (EC), oxidation/reduction potential (ORP) and pH were carried out using the HI 98121 digital combo pH & EC meter, and the HI 98129 pH & ORP probe manufactured by Hanna Instruments. As with the measurement of dissolved oxygen, each electrode was automatically calibrated following insertion of the electrode into each of the 48 samples. Following each measurement, the probes were rinsed using deionized water.

For the measurement of ammonia ions, an indophenol method using Agilent Technologies Cary 60 UV-Vis spectrophotometer was carried out. In order for each sample to be measured, an alkaline and a coloring agent solution had to be prepared. For the preparation of the alkaline solution 16 g of sodium hydroxide (NaOH) was dissolved in 250 mL of deionized water and 1g of sodium dichloroisocyanurate dihydrate ($C_3N_3O_3CL_2Na2H_2O$) was added following a period of incubation until the solution reached room temperature. After dissolving, the solution was kept in a dark container and stored in the refrigerator. For the preparation of the dyeing solution, 32.5 g of sodium salicylate ($C_7H_5O_3Na$) and 32.5 g of sodium citrate dihydrate ($Na_3C_6H_5O_7\ 2H_2O$) was added to 250 ml deionized water. After dissolution, 0.238 g of sodium nitroprusside dihydrate ($Na_2[Fe\ (CN)_5\ NO)].2H_2O$) was added to the solution and allowed to dissolve. Same as the alkaline solution, the coloring agent was kept in a dark container and stored in the refrigerator. Following the preparation of the reagent solutions, each sample (700 µl) along with an alkaline solution and a dyeing agent was pipetted into a reaction tube and allowed to stand for 60 minutes. Furthermore, the same amount of the influent was also prepared using the same reaction agents as a blank. Following the allotted time, each sample was measured using the aforementioned spectrophotometer at the pre-determined wavelength of 655 nm using a 1 cm cuvette.

The automatic SKALAR Formacs TOC/TN analyzer supplied an advantageous method for the measurement of total organic carbon (TOC), inorganic carbon (IC) as well as nitrogen (TN). Each sample is fitted into a rotating carousel which works as an autosampler following placement into a high temperature reactor (750-950 °C). Once in the reactor, all organic and inorganic carbon (TC) is oxidized into gaseous carbon dioxide (CO_2) while all bonded nitrogen is converted into Nitric oxide (NO). Air in the analyzer carries these converted products onto detectors where their concentration is measured. First, Carbon dioxide is detected using an infrared detector, while nitric oxide is measured using a chemiluminescence detector. Inorganic carbon is measured in an IC detector chamber where it is converted into gaseous CO_2. The total amount of organic carbon is then measured from the equation TOC = TC – IC.

The third official operational phase of the experiment began on the 8th of September which also marked the first time the emerging pollutants were added to wastewater. Among the ECs that were added and analyzed was ibuprofen, the subject of this study as well as organic compounds carbon, nitrogen, phosphate and sulphate. The third phase lasted for three months and was comprised of a four-day cycle in which 2 L of the influent containing EC's was fed to the reactors in a semi-saturated setting. Following the four-day cycle, water samples containing emerging contaminants were analyzed by the aforementioned methods stated above. Sample collection and analysis was consistent with that of the second phase. Each of the 24 samples were collected in plastic water containers and the same series of analysis steps were taken. Following measurements, each sample was stored for further analysis. After the end of the experiment which lasted until the end of November 2019, all of the 24 reactors were disassembled and samples of substrates, the plant itself as well as roots and shoots were measured, weighed and stored for future analysis.

5. Results and Discussion

The results of our experiment over the course of several months beginning in late August and concluding in November have been compiled and assessed using the aforementioned measurement techniques described in the methodology section. **Table 2** below exemplifies the types and acronyms used for each of our CWs reactor variations, as well as the number and dates of measurements taken for easier understanding. Data representation has been apportioned based on the subject of interests which was primarily the differences of ibuprofen and organic compound concentrations between substrates, but also the variable represented by the presence of arbuscular mycorrhiza. Furthermore, the average removal rate in each type of substrate has been calculated for ibuprofen and organic compounds to further assess the efficacy of each substrate.

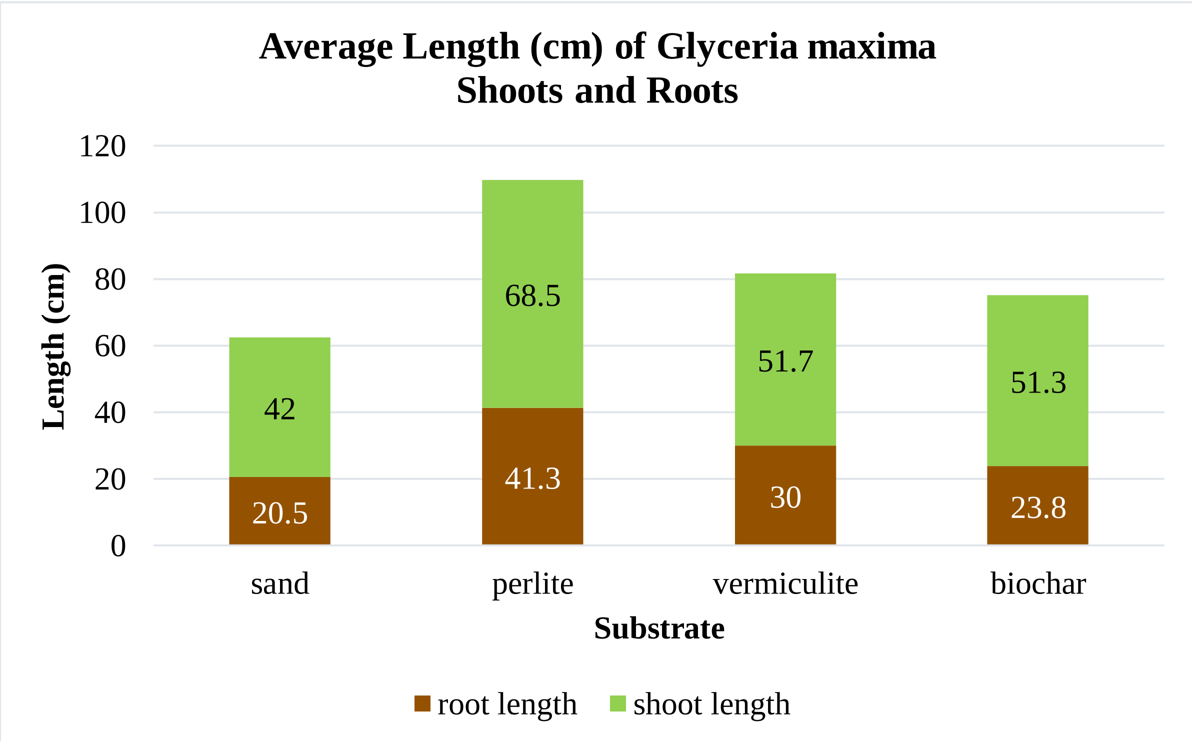
Plant + Sand	PS
Plant + Sand + AMF	PSA
Plant + Expanded Perlite	PP
Plant + Expanded Perlite + AMF	PPA
Plant + Expanded Vermiculite	PV
Plant + Expanded Vermiculite + AMF	PVA
Plant + Biochar	PB
Plant + Biochar + AMF	PBA

NO. of measurement	Date
1	23/8/2019
2	31/8/2019
3	8/9/2019
4	16/9/2019
5	24/9/2019
6	2/10/2019
7	10/10/2019
8	18/10/2019
9	26/10/2019
10	30/10/2019
11	03/11/2019

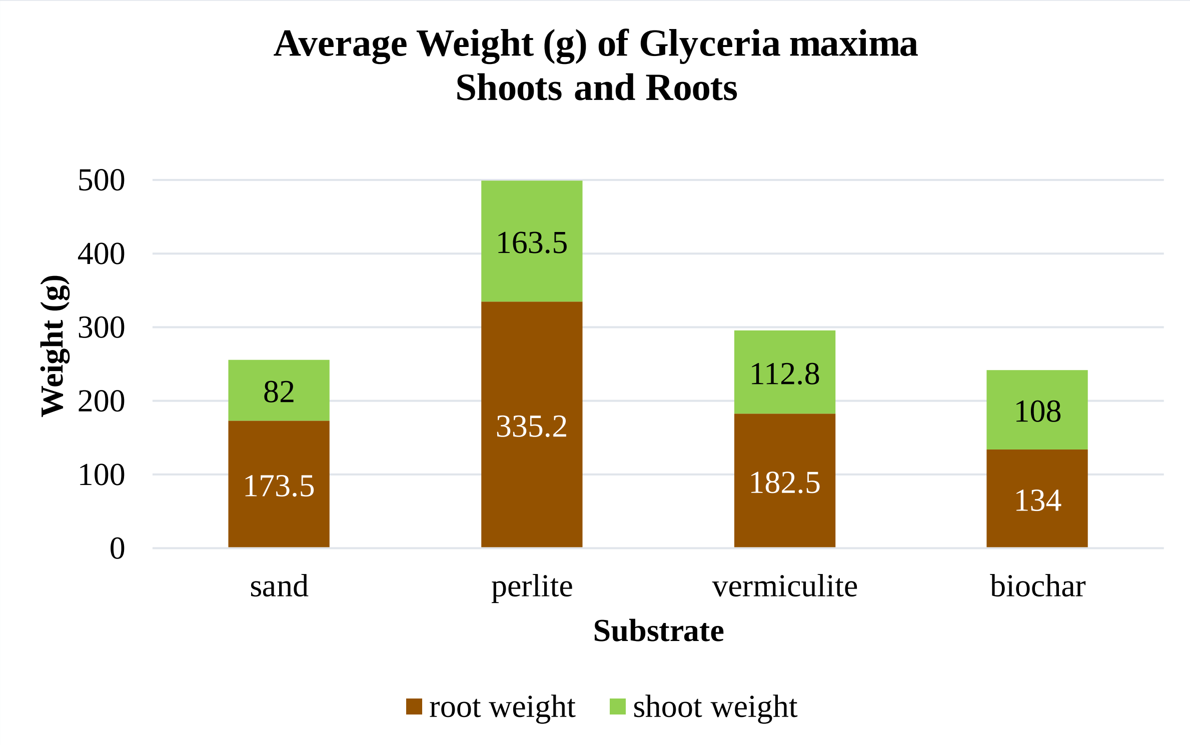
Table 2 – Reactor acronyms/Dates of measurements

5.1 Plant Biomass

The biomass of planted *Glyceria maxima* including the length (cm) of shoots and roots (**Graph 1**) and as well as shoot/root weight (g) (**Graph 2**) may have significant consequences for the uptake of nutrients as well as pollutant and subsequent removal and microbial processes within the soil. As demonstrated by our results, the choice of substrates can have great influence on the biomass of plants'. Our data shows that the use of perlite as a substrate has the most significant effect on average plant biomass with a root length of 41,3 cm and shoots with 68,5 cm for a total of 109,8 cm. The average weight of plants in perlite substrate was also much higher with 335,2 g root weight and shoots of 163,5 g. Second largest length and weight were plants in vermiculite with 30 cm long roots weighing 182,5 g, and shoots with a length of 51,7 cm weighing at 182,5 g. Plants having biochar as a substrate had larger lengths than sand based plants with total length of 75,1 cm compared to 62,5 cm respectively, though the weight of sand based plants was higher with 255,5 g compared to 242 g biochar plants. These differences may be explained by the properties of the substrates. Vermiculite being less porous than perlite for example, has the ability to absorb and retain water and nutrients more efficiently which may result in better conditions for pollutant removal and also increased plant growth (Kang et al. 2004). On the other hand, the more aerated perlite, though it doesn't retain water as efficiently, allows for better oxygen diffusion (Jackson 1974; Kang et al. 2004) may provide better nitrifying conditions in the rhizosphere. Biochar has the advantage of providing a rich carbon source necessary for the denitrification process, is valued for its soil amendment properties, encourages growth of symbiotic microorganisms and have the ability to remove pollutants while also being highly porous (Gupta et al. 2016). These results indicate that use of different substrates causes significant changes not only to plant biomass, but most likely to mechanical and biological processes within the rhizosphere and the complex network of nutrient and pollutant removal pathways.

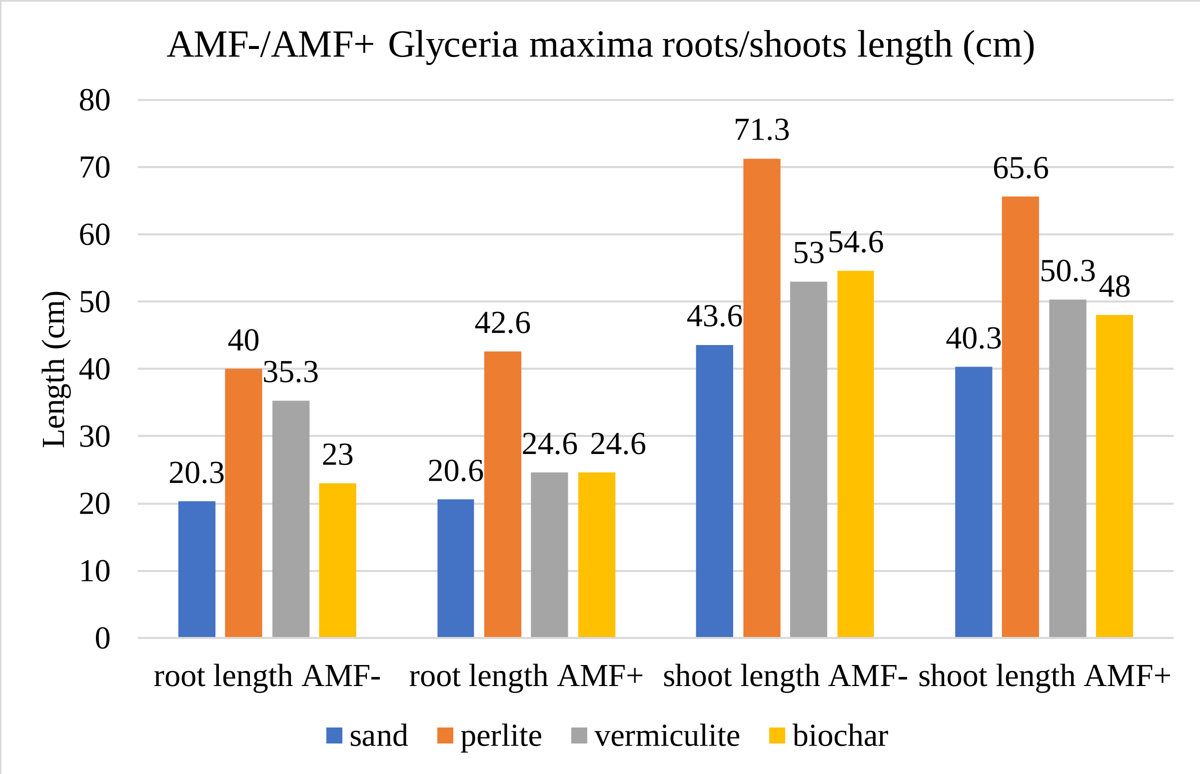


Graph 1 – Root/Shoot length (cm) of *Glyceria maxima*

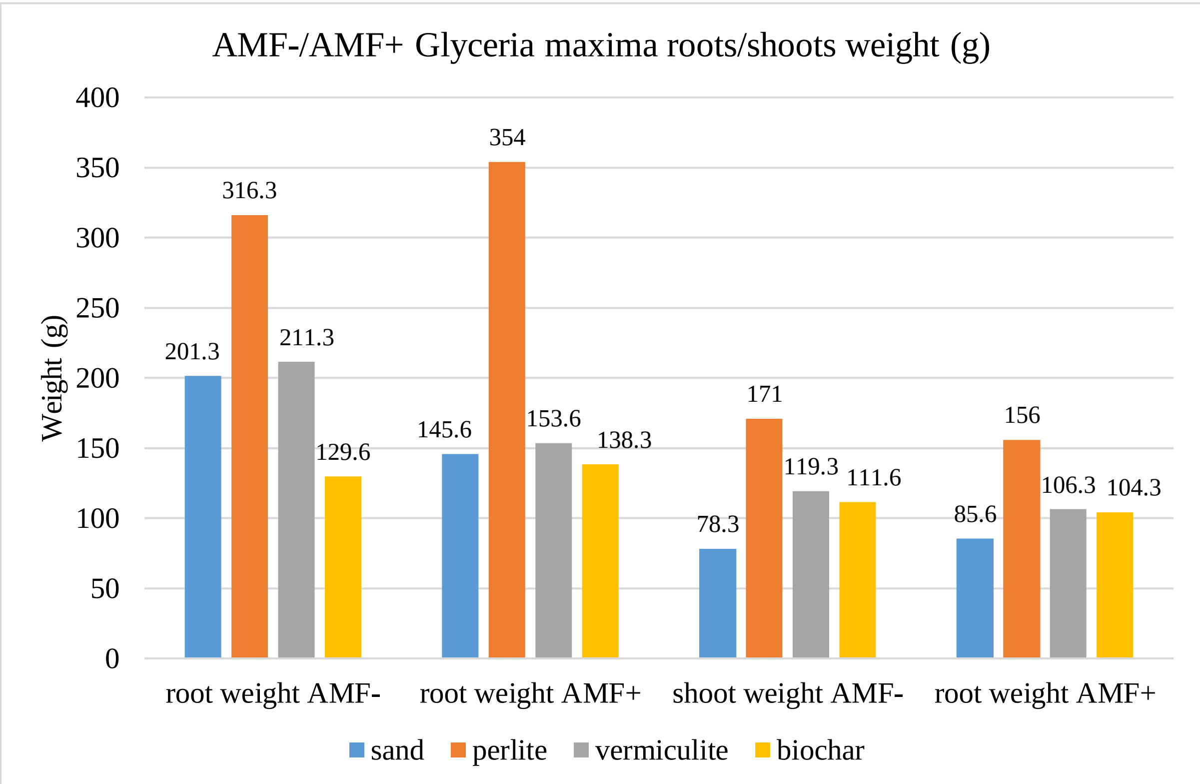


Graph 2 – Biomass of *Glyceria maxima*; root and shoot weight (g)

The impact of AMF on the biomass of plants is shown by the **Graphs 3** and **4** which show the differences between AMF inoculated and non-inoculated plant length and weight. According to previous studies, it has been found that AMF may cause physiological changes resulting in increased plant growth and biomass along with improved nutrient uptake (Miransari 2011; Xu et al. 2016; Hu et al. 2020). On the contrary, other studies found that mycorrhizal inoculation negatively affected plant growth and biomass (Barbera et al. 2020) Our results support the latter as we have a general decrease in the length and weight of AMF inoculated *Glyceria maxima* with some exceptions. In sand AMF+ reactors, we report a +1,56% root length, -7,57% shoot length, -27,67% root weight and +8,53% shoot weight. For perlite AMF+ in the aforementioned order, +6,11% root length, -8,00% shoots length, +10,65 root weight and -8,78% shoot weight. Vermiculite AMF+ plants exhibited -30,32% root length, -5,1% shoot length, -27,31% root weight and -10,9% shoot weight. Finally, biochar AMF+ *Glyceria maxima* had +6,51% root length, -12,09% shoot length, +6,30% root weight and -6,55% shoot weight. It must be noted that the results are not only affected by the inclusion and abundance of AMF, but also the use of different substrates, which poses another variable which affects the rhizosphere environment, microorganisms within it, nutrient uptake by plant and consequently the physiological differences among plant biomass. Therefore, more research should be performed on substrate induced rhizosphere changes.



Graph 3 – Influence of AMF on *Glyceria maxima* length (cm)



Graph 4 – Influence of AMF on *Glyceria maxima* weight (g)

5.2 AMF Colonization

	F%	M%	A%
SAP	61.34±10.48	11.24±2.47	0.54±0.07
VAP	92.27±3.57	58.17±0.56	1.32±0.02
PAP	83.54±5.63	26.23±1.34	0.79±0.04
BAP	78.36±6.34	24.43±1.62	0.67±0.05

Table 3 – The differences of AMF colonization among different substrates. (F%) mycorrhiza occurrence in the rhizosphere, (M%) intensity of AMF colonization and (A%) arbuscule abundance.

AMF communities were successfully established in CWs of all types of substrates as illustrated by **Table 3** above. The AMF colonization may be visually seen by **photos 1-4** below showing AMF arbuscules stained by Trypan blue. Differing colonization percentages can be explained by physiological changes among the substrates It has been reported that nutrient accessibility and retention of carbon, nitrogen and phosphorus, along with pH and oxygen availability were important defining factors of AMF colonization (Xu et al. 2016; Hu et al. 2020). Vermiculite had the highest colonization intensity (58,17%) possibly due to the substrates ability to retain nutrients more effectively (Kang et al. 2004). 26,23% and 24,43% colonization intensity was found for perlite and biochar respectively, again possibly due to the substrates porous structure allowing for better oxygen diffusion while sand had the lowest intensity (11,24%).

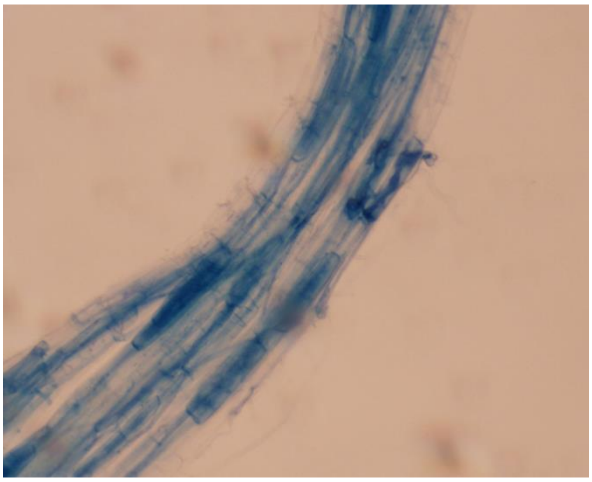


Photo 1 – Sand AMF+

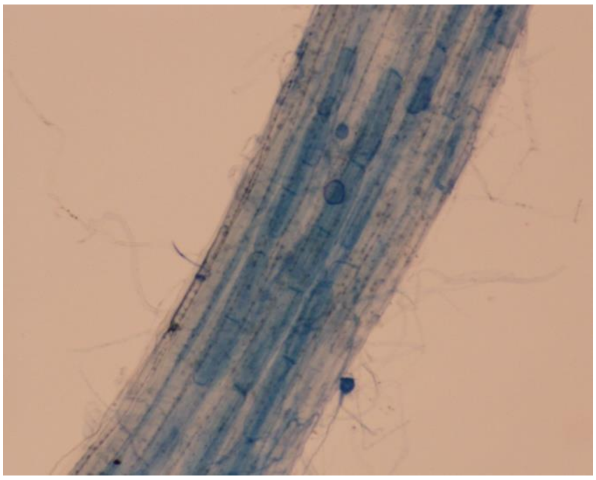
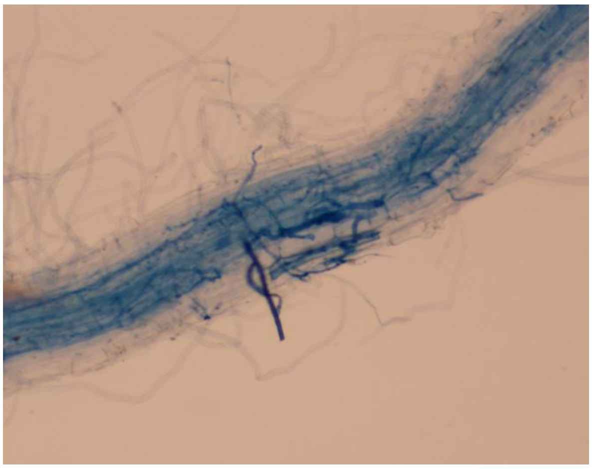


Photo 2 – Perlite AMF+



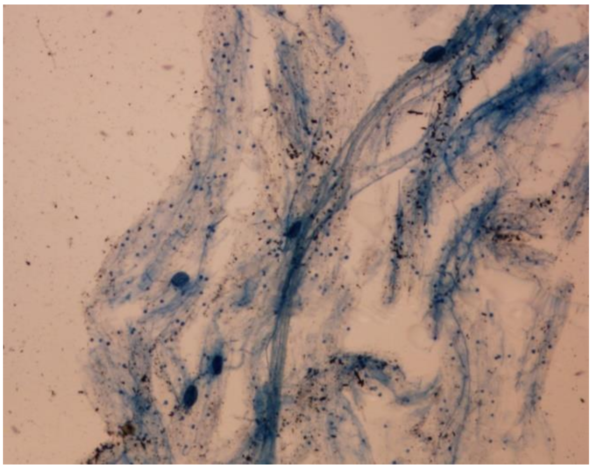
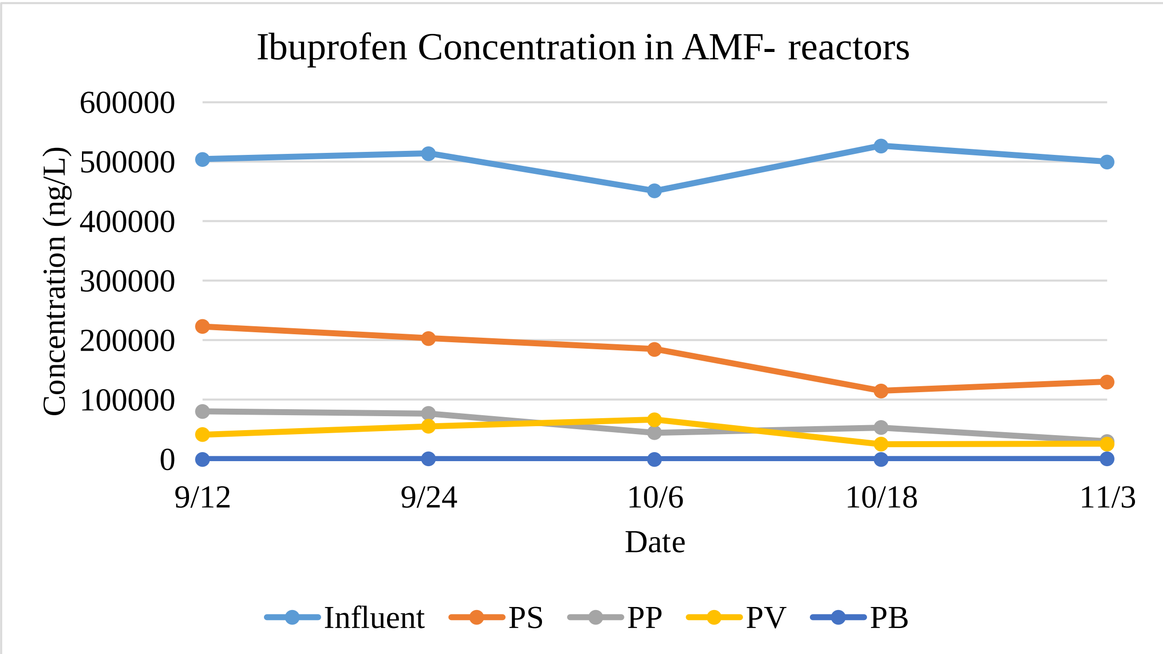


Photo 3 – Vermiculite AMF+

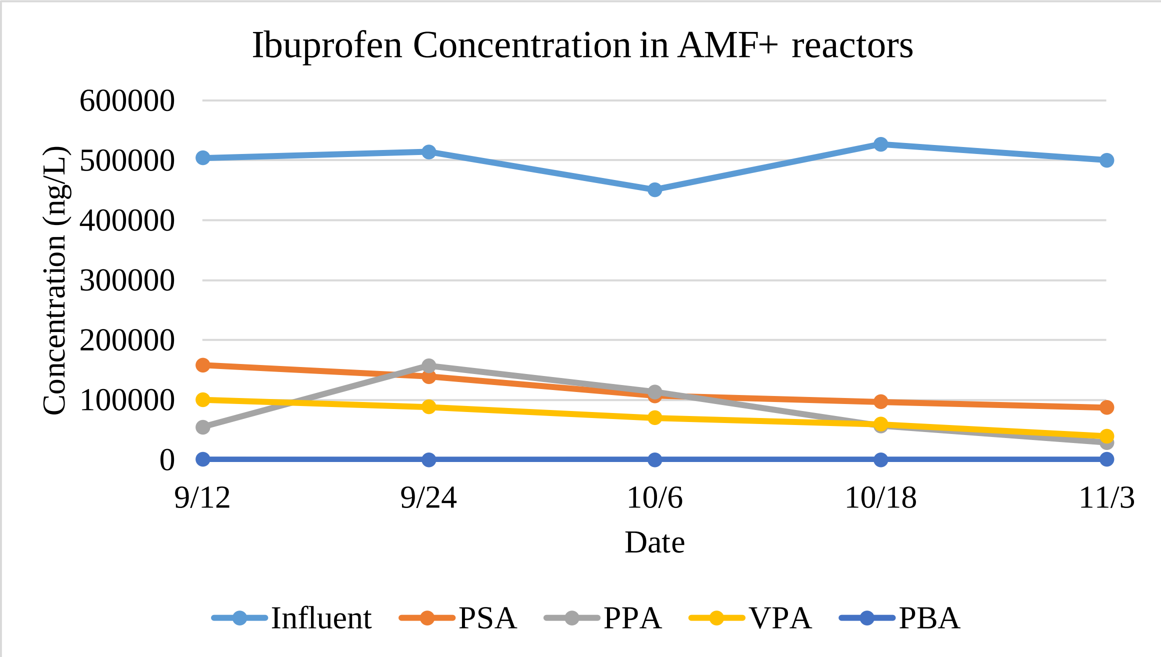
Photo 4 – Biochar AMF+

5.3 Ibuprofen Removal

The removal efficiency of ibuprofen and other PPCPs is governed by numerous biotic and abiotic factors including and not limited to adsorption, sedimentation, plant uptake and assimilation, biodegradation by means of microbial and fungi interactions (Matamoros et al. 2008; Hijosa-Valsero et al. 2010; Gupta et al. 2016). Our experiment sought to determine the influence of distinctive substrates on the removal efficiency of ibuprofen. Experimental results demonstrate that substrates have a noteworthy influence on ibuprofen removal as illustrated by **Graphs 5** (AMF-) and **Graph 6** (AMF+).



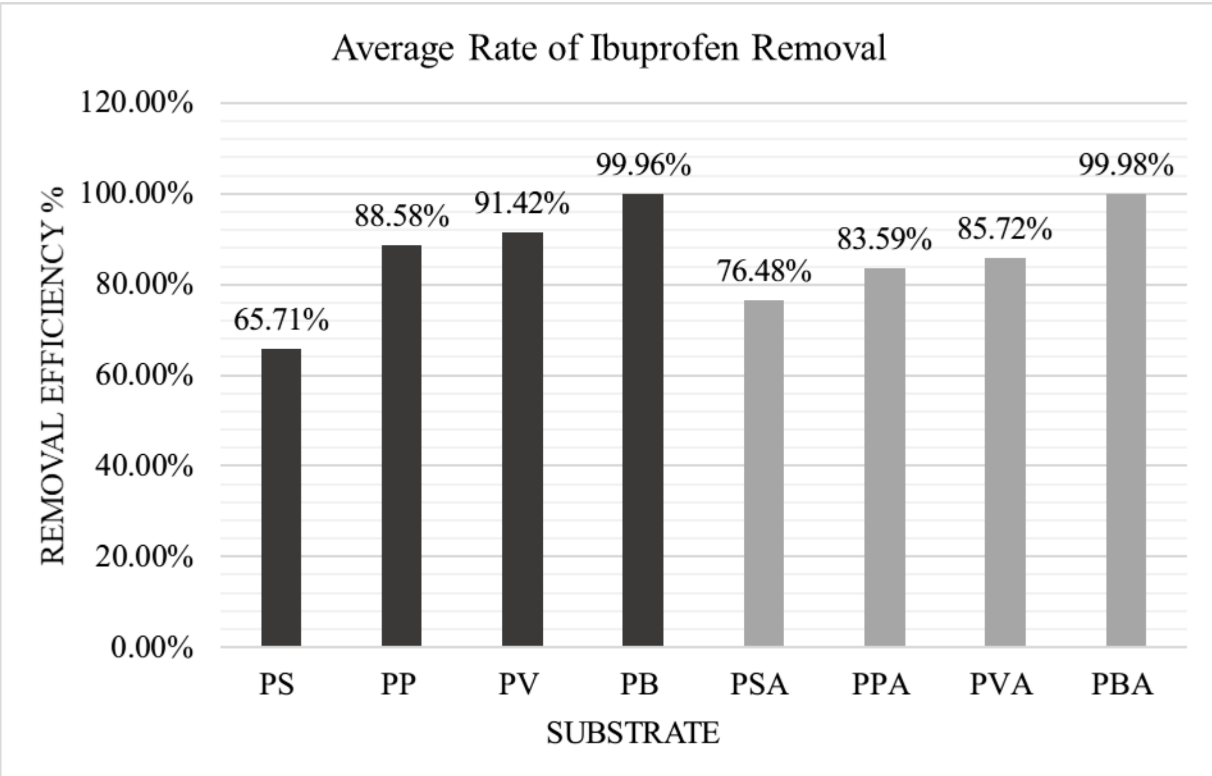
Graph 5 – Ibuprofen concentration in AMF- reactors



Graph 6 – Ibuprofen concentration in AMF+ reactors

In general, ibuprofen was significantly removed in reactors of all substrates at varying degrees. A 65,71 - 76,48% removal efficiency was observed in sand-based reactors, 83,59 - 88,55% removal efficiency in perlite CWs, 85,72 - 91,42% in vermiculite CWs and a noteworthy 99,6 - 99,98% removal efficiency in biochar containing CWs as illustrated by **Graph 7**. The differences between the substrates were most probably due to different sorption capacities and substrate induced changed on the rhizosphere environment affecting microbial activity (Gupta et al. 2016). The striking removal efficiency of biochar (but also vermiculite, perlite and sand respectively) is possibly due to its high surface area and porous structure, potential to ameliorate and purify soil and water, enduring sorption capacity with high redox potential which was found to be positively correlated with ibuprofen removal (Marschner et al. 2013; Zhang et al. 2017; Gupta et al. 2016). AMF contributions to removal rates were mixed with +10,77% increase in AMF+ sand reactors, and +0,02% in AMF+ biochar while a -4,99% and -5,70% decrease has been reported in AMF+ perlite and vermiculite reactors respectively. These mixed results are possibly a result of changes to the rhizosphere environment caused by the presence of AMF. Overall, the influence of AMF on ibuprofen removal is debatable and needs to be researched more thoroughly to assess its impact.

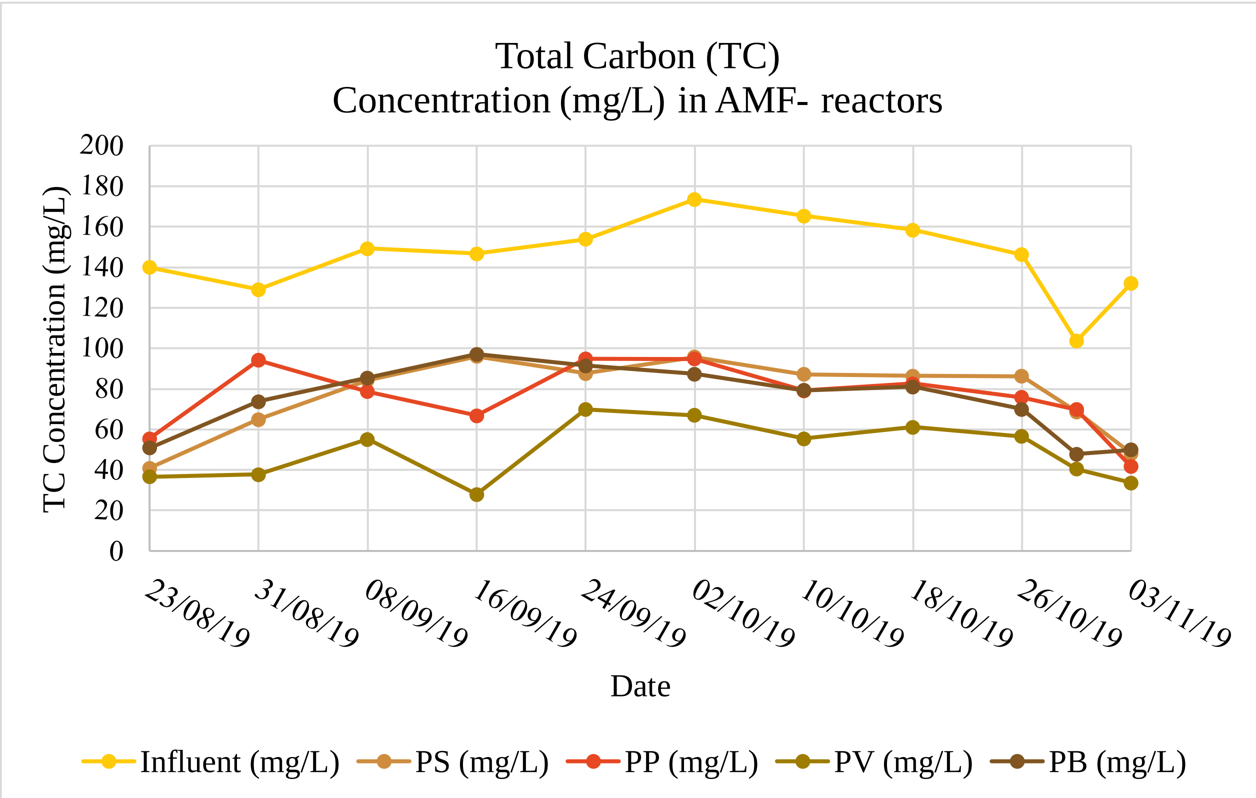
Literature describes that substrate adsorption is an important element that contributes to the reduction and removal of PPCPs reaching over 90% removal efficiency consistent with our findings (Oliveira et al. 2019). Furthermore, the use of a sand substrate along with a lower gravel layer in our CWs may have also contributed to ibuprofen removal as the substrates' adsorption capacities were reported to increase PPCP removal rates (Matamoros et. al 2005; Matamoros and Bayona 2006). In addition Zhang et al. 2017 reported that microbial degradation pathways in CWs along with pollutant assimilation by plants also contributes to total pollutant removal. It must be mentioned that other studies reported that variables such as nitrogen, organic carbon, and pH did not affect removal efficiency of ibuprofen while temperature, redox potential, and the mechanisms of phytodegradation, sorption onto organic matter and microbial activity played key roles in PPCP removal (Hijosa-Valsero et al. 2010; Park et al. 2018; Lancheros et al. 2019). It was also described that phytodegradation accelerated ibuprofen removal as significant removal differences were found in planted vs unplanted CWs (Hijosa-Valsero et al. 2010; Zhang et al. 2016; Zhang et al. 2017). Therefore, the presence of *Glyceria maxima* in our experiment may have contributed to ibuprofen removal. However, due to the complex interactions within the aerobic as well as anaerobic microbial communicates of the rhizosphere, it is difficult to determine specific phytodegradation pathways of PPCPs and should be further studied to elucidate key networks for ibuprofen removal. (Lancheros et al. 2019).



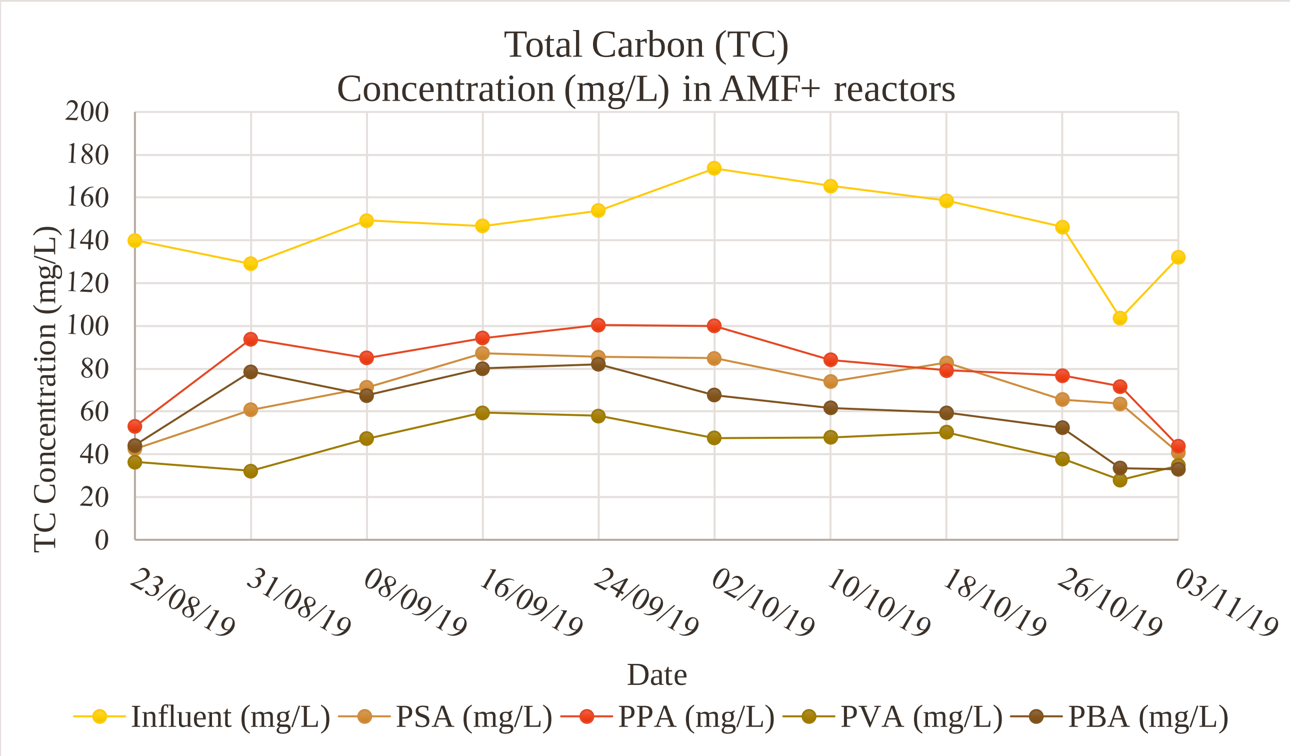
Graph 7 – Average rate of removal for ibuprofen in AMF- and AMF+ reactors

5.4 Total Carbon Removal

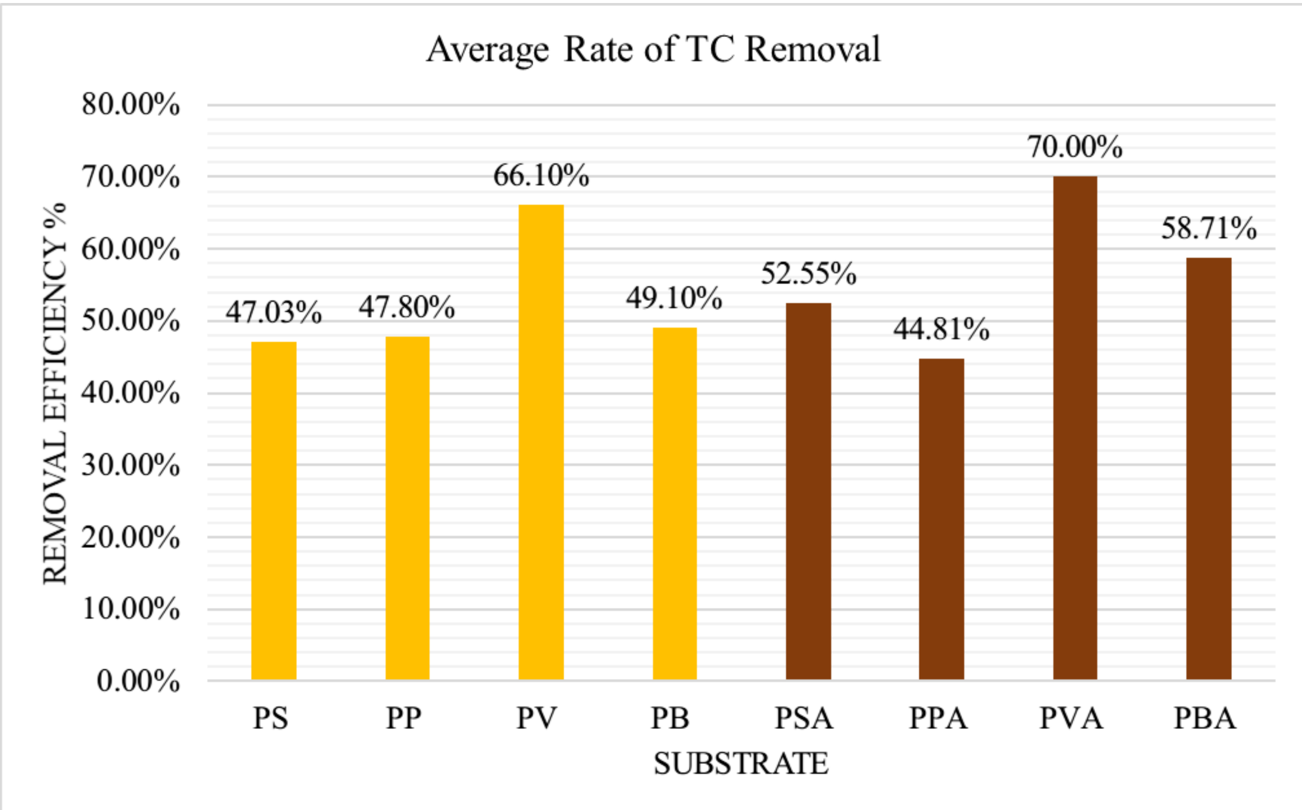
The interaction that comes into being between roots and the substrate matrix is central to the effectiveness of various complex biotic and abiotic processes occurring in rhizosphere. Therefore, the type of substrate used can have great effect on the removal of organic compounds as well as PPCPs and other pollutant (Stottmeister et al. 2003). The transformation and removal of carbon in the soil is mostly carried out by microorganisms found in the rhizosphere as it was found that higher removal efficiencies for carbon were in unplanted CWs compared to planted mesocosms (Baptista et al. 2003). However, it must also be mentioned that plants provide microorganisms a favorable growing environment as well as nutrients in the form of root exudates (Stottmeister et al. 2003). Therefore, the role of plants for direct carbon removal may be minimal, however, due to symbiotic relationships with bacteria and fungi, plants have a substantial indirect influence. Different substrate matrixes cause changes to the composition of the rhizosphere environment along with changes to the rates of carbon cycling and removal. Our results support these observations as different concentrations of total carbon (TC) were found in different substrates illustrated by **Graph 8** for TC concentration in AMF- reactors and **Graph 9** for AMF+ reactors.



Graph 8 – Total carbon concentration (mg/L) in AMF- reactors

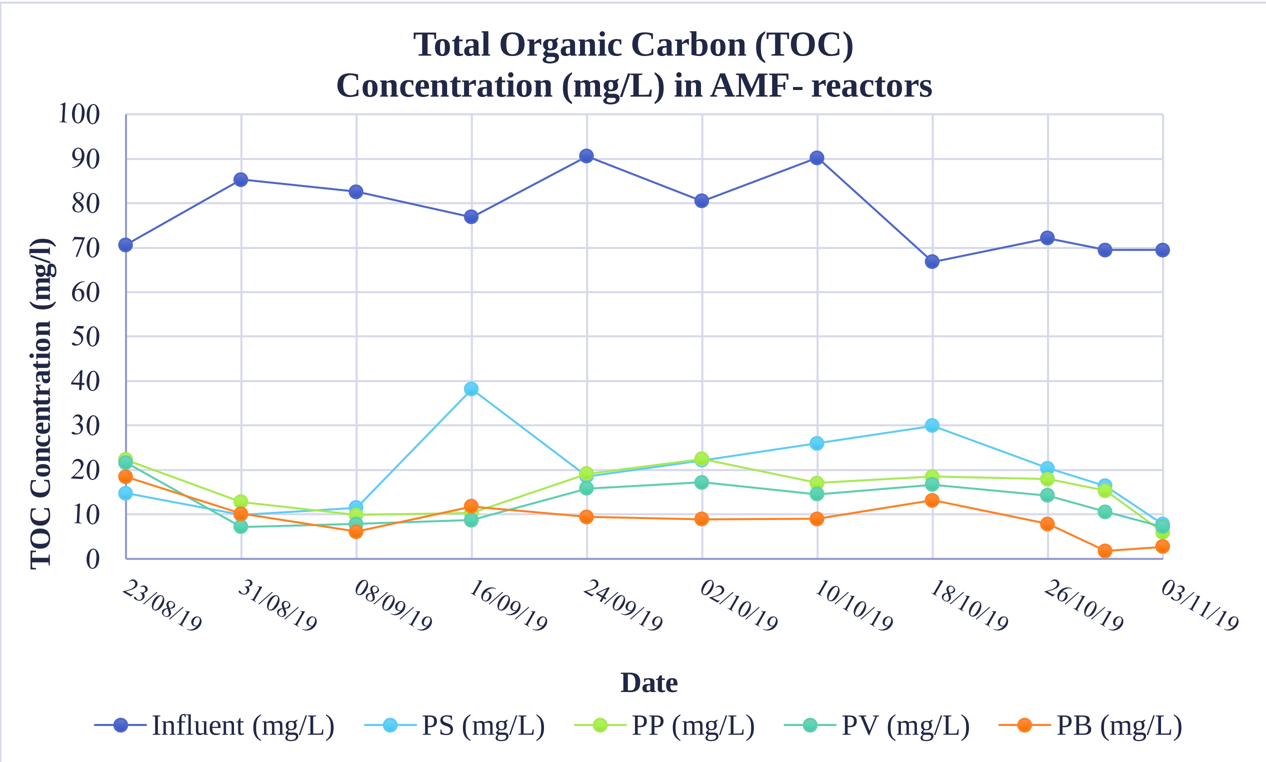


Graph 9 – Total carbon concentration (mg/L) in AMF+ reactors

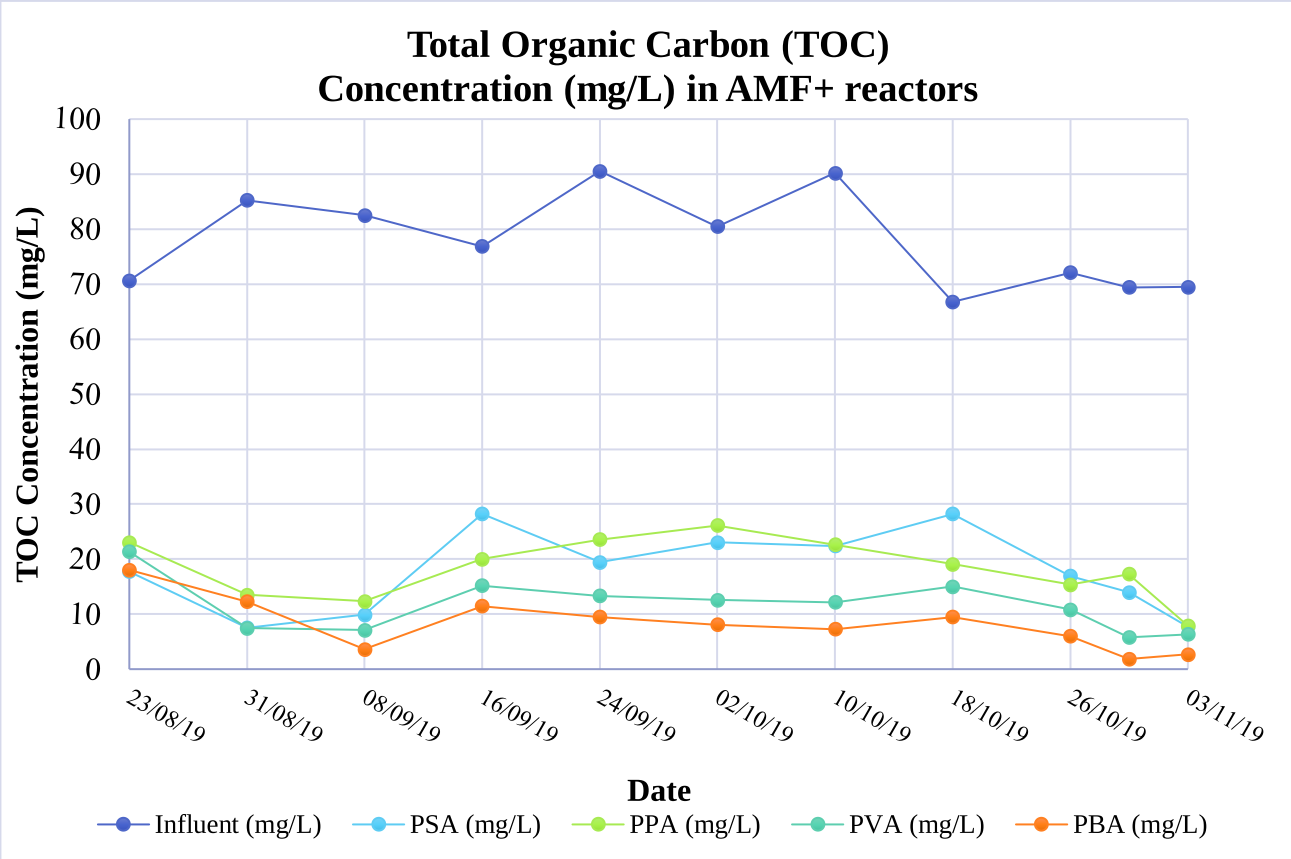


Graph 10 - Average rate of removal for Total Carbon (TC) in AMF- and AMF+ reactors

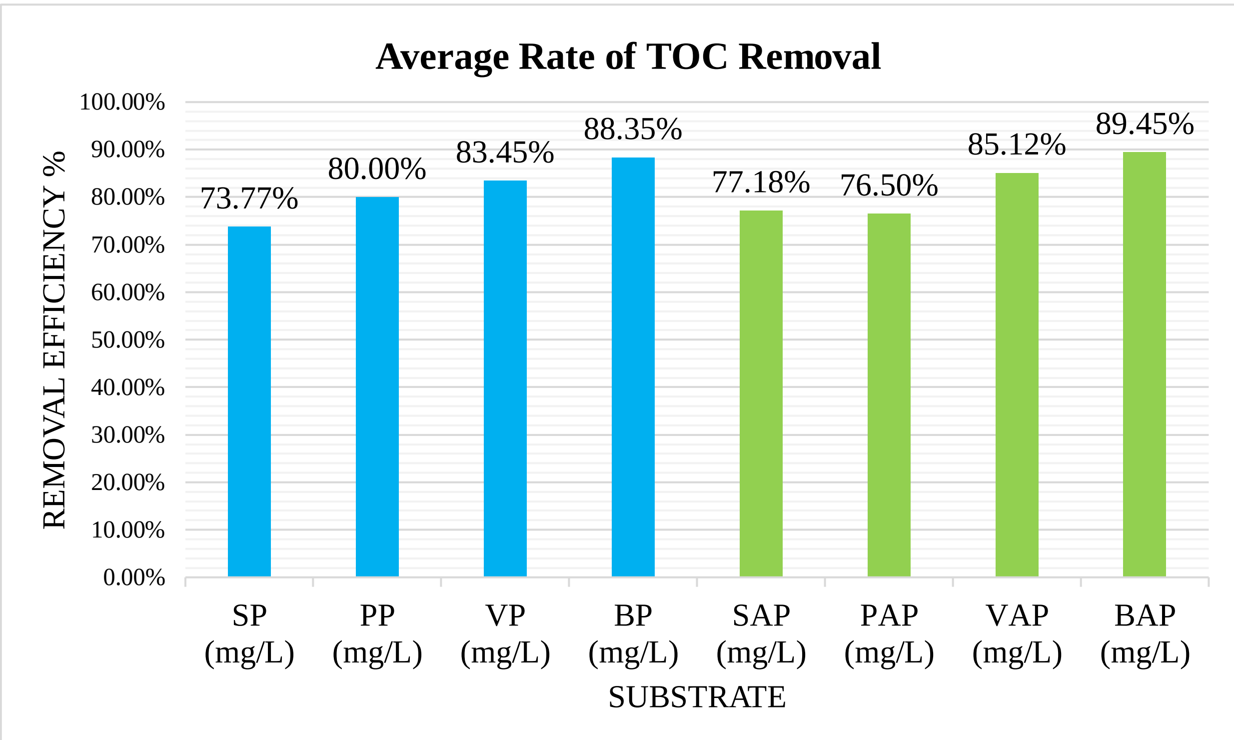
Removal efficacy (TC) illustrated by **Graph 10** was found to be 47,03 - 52,55% for sand, 44,81 - 47,80% for perlite, 66,10 - 70,00% for vermiculite, and 49,10 - 58,71% for biochar substrates. The highest removal rate was found in vermiculite reactors, which could be attributed to the substrate's properties of improved water and nutrient retention which are needed for microbial metabolism processes. The influence of AMF on TC concentration is also non-negligible as AMF increased TC removal rate by +5,52% in sand, +3,9% in vermiculite, +9,61% in biochar. However, a -2,99% decrease was reported in perlite reactors. These changes may have occurred as a result of a change in the microenvironment due to AMF presence. TC concentrations may have also been affected by root exudate leakage in the effluent, and the total removal efficiency affected by the release of CO2 as a result of respiration from plants as well as aerobic organisms in the soil (Wiessner et al. 2005). Large gaps between influent and effluent concentrations have also been found for total organic carbon (TOC) shown by **Graph 11** (AMF-) and **Graph 12** (AMF+). All substrates have shown to be valuable for TOC removal with a removal rate ranging from 73,77 – 89,45% (**Graph 13**). Biochar was found to have the highest removal efficiency with 88,35 -89,45% indicating that the substrates properties are suitable for expansion of microbial communities that utilize carbon for metabolic activity and thus are able to transform and remove other nutrients such as nitrogen (N) or pollutants including ibuprofen.



Graph 11 – Total organic carbon concentration (mg/L) in AMF- reactors

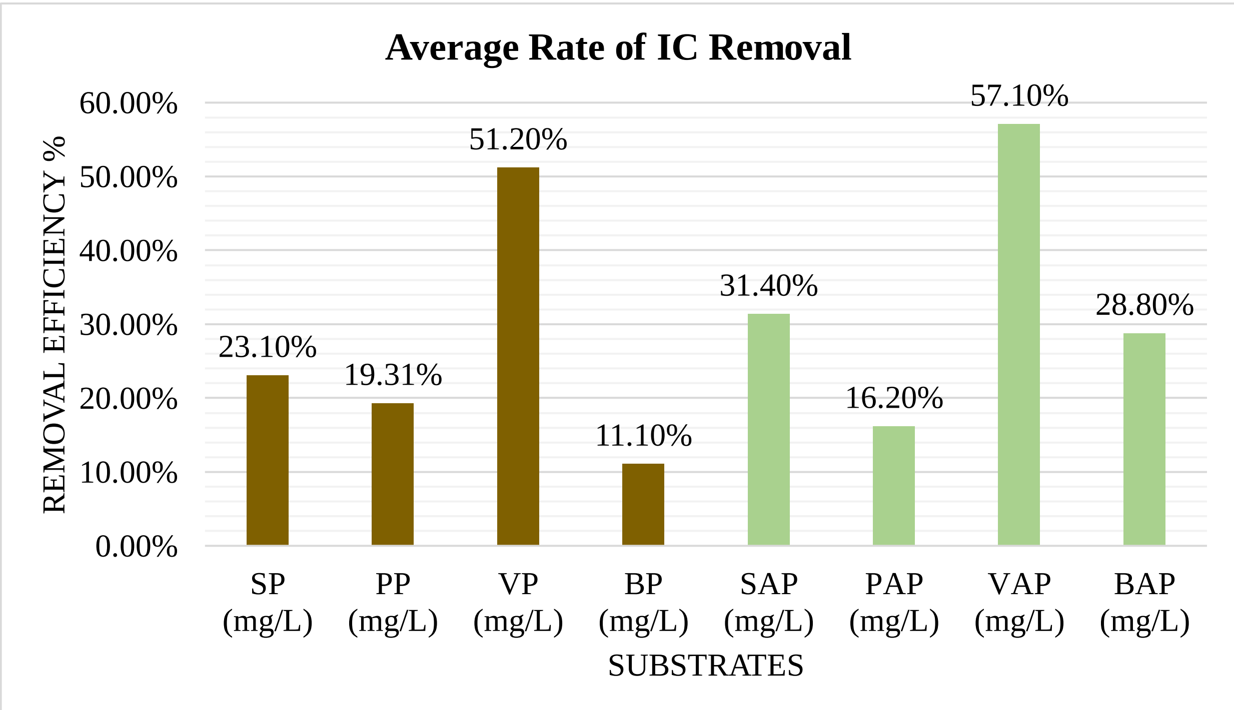


Graph 12 – Total organic carbon concentration (mg/L) in AMF+ reactors



Graph 13 - Average rate of Total organic carbon (TOC) removal in AMF- and AMF+ reactors

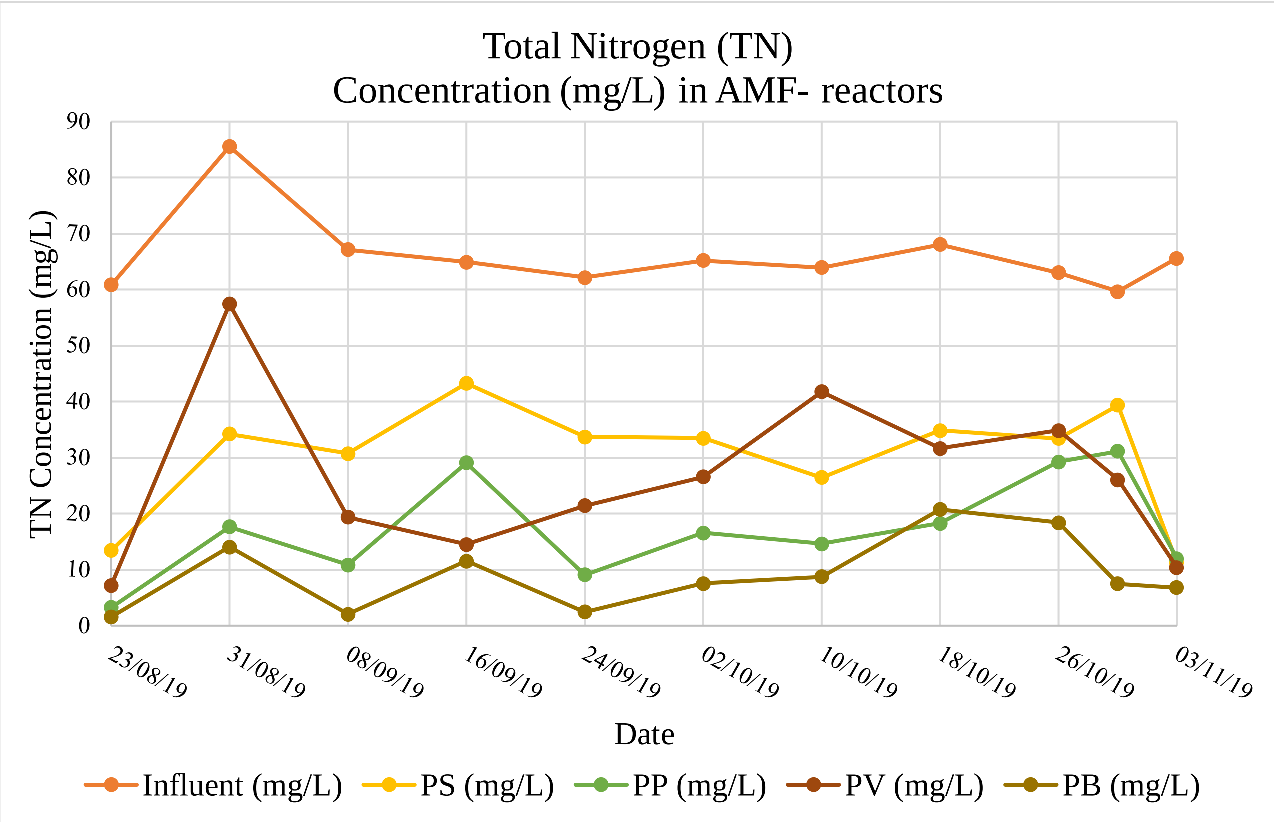
On the contrary, inorganic carbon (IC) removal rate was found in biochar substrates with 11,10 - 28,80% efficiency in AMF-/AMF+ shown by **Graph 14**. As the substrate is very rich in carbon, the soil matrix could have been fully saturated by carbon, unable to remove it as efficiently. Vermiculite was found to be the most efficient in eliminating IC with 51,20 - 57,10% removal rate. TOC and IC removal rate disparities between substrates can be explained by differences in soil temperature, water and nutrient retention, pH, redox potential, adsorption and sedimentation, and more importantly metabolic interactions among soil microbes. AMF have shown to have a very slight influence on the increase of removal rates of carbon except in perlite reactors where both TOC and IC rates decreased by -3,5% and -3,11 % respectively. It has been reported by Wang et al. 2016 that AMF contributed to carbon partitioning, the mechanisms by which this occurs is however still poorly understood. To sum up, substrates were found to have an effect on carbon cycling and transformation of organic matter in CWs. Vermiculite was found to the have the greatest removal efficiency for TC and IC while biochar demonstrated highest efficiency for total organic carbon removal. Nonetheless, the mechanisms by which substrates as well as AMF affect carbon transformation are still poorly understood, and although these changes are most likely due to structure and composition changes of the microbial environment, further research should be done to clarify carbon cycling in CWs to better optimize design parameters to improve CW function.



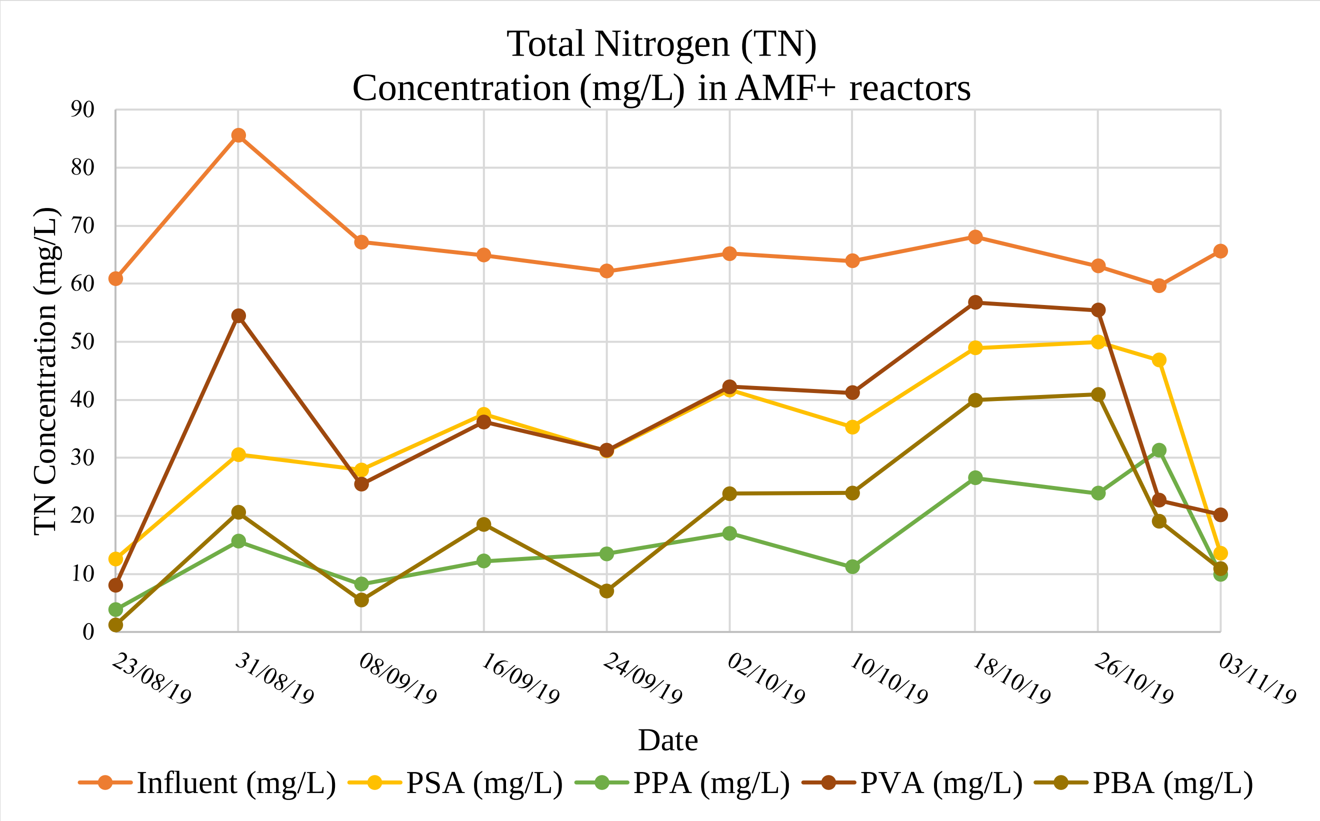
Graph 14 - Average rate of removal for inorganic carbon (IC) in AMF- and AMF+ reactors

5.5 Nitrogen Removal

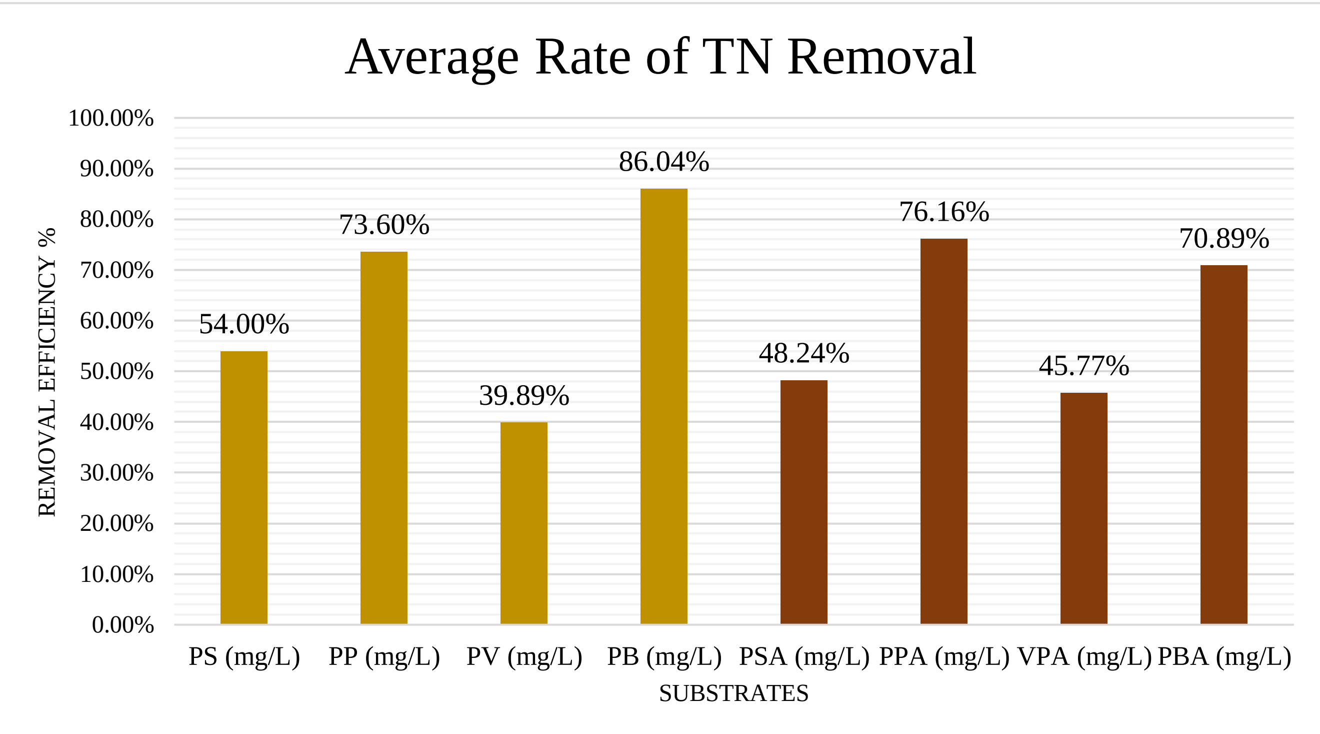
As with removal of other pollutants and organic compounds, the expulsion of nitrogen from constructed wetlands is governed by numerous complex biological and mechanical pathways that can be enhanced by factors such as temperature, pH, carbon availability and operational factors of CWs. Nitrogen removal is deemed especially important as increased release into the surrounding environment causes eutrophication of waters consequently leading to depleted oxygen levels and the death of numerous aquatic species (Grinberga and Lagzdins 2017). Although processes such as adsorption, plant uptake and assimilation play a role in nitrogen transformation, the predominant pathways by which nitrogen is transformed and subsequently removed are ammonification, nitrification, and denitrification carried out by microbiological organisms (Saeed and Sun 2012). Our experimental results (TN concentrations of influent vs effluent in AMF- and AMF+ reactors illustrated by **Graphs 15** and **16** respectively) exemplify that substrates do in fact contribute to the effectiveness of total nitrogen (TN) removal with 39,89 - 86,04% efficiency in AMF- reactors and 45,77 - 76,16% removal efficiency in AMF+ reactors as can be viewed in **Graph 17**.



Graph 15 – Total nitrogen concentration (mg/L) in AMF- reactors

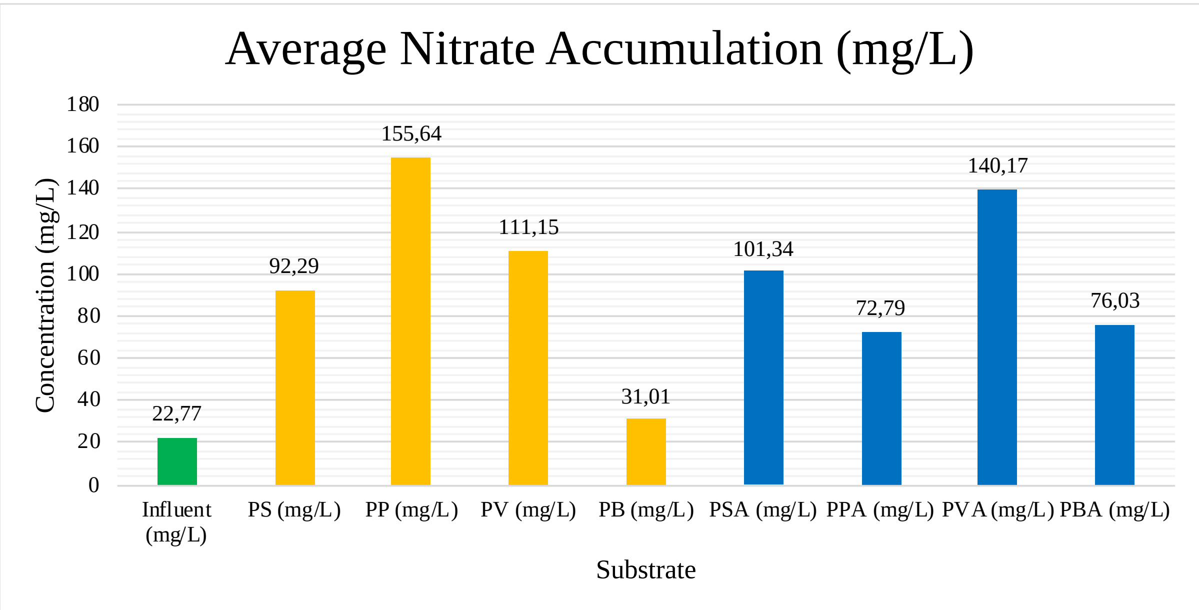


Graph 16 – Total nitrogen concentration (mg/L) in AMF+ reactors



Graph 17 - Average rate of removal for total nitrogen (TN) in AMF- and AMF+ reactors

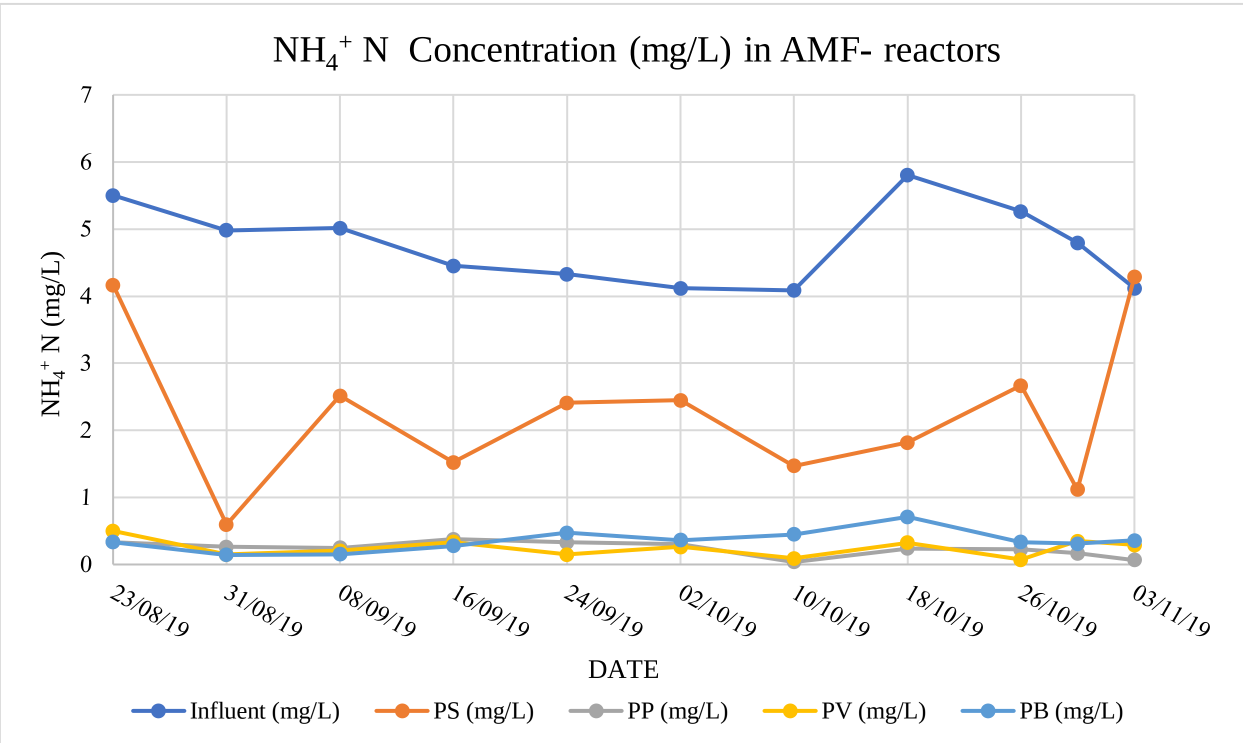
The highest removal rate (86,04 %) was found to be in reactors containing biochar. This can be explained by the fact that many microbiological processes that support nitrogen removal such as denitrification and nitrification require carbon which biochar, acting as a rich carbon sink readily provides, and thus, facilities the enhancement of these processes as shown by studies performed by Laber et al. 1997; Rustige and Nolde 2007; Lu et al. 2009. Furthermore, the importance of carbon as demonstrated by the studies above is further exemplified by nitrate concentrations due to denitrification, an observation which was also detected in our experiment shown by **Graph 18**. Biochar enhanced reactors had lowest concentrations of nitrate (31,01 – 76,03 mg/L) which could be accredited to higher denitrification rates in those reactors. TN removal rate was also high in perlite (73,60 - 76,16%) and sand (48,24 – 54%). This could be explained by the porous structure of the substrates providing more aeriated conditions for the growth of nitrifying bacteria, and the lower gravel layer having anoxic conditions which simultaneously enhances nitrification and denitrification (Saeed and Sun 2012). A study conducted by Białowiec et al. 2011 used porous light-weight aggregates in the upper level and gravel in the lower level of their wetland system and achieved 60% removal of TN. The lower removal efficiency of TN in vermiculite reactors, a light-weight aggregate substrate could possibly be explained by the length of *Glyceria maxima* (**Graph 1 and Graph 2**) planted in these reactors as the smaller length of roots compared to perlite provides less surface area for microbial organisms governing nitrification-denitrification processes resulting in decreased TN removal.



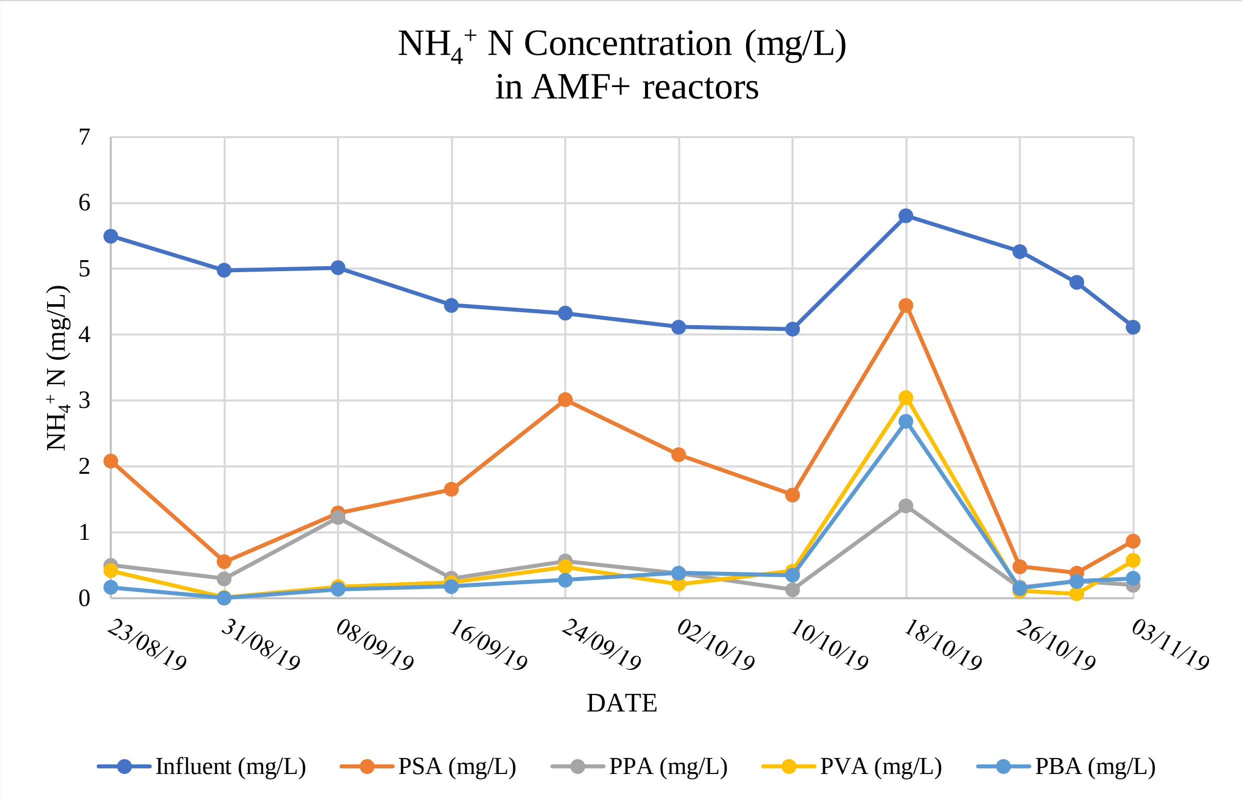
Graph 18 – Average nitrate accumulation in AMF- and AMF+ reactors

The influence of AMF generally decreased TN removal, only in the substrates perlite and vermiculite did we see an increase of TN removal with only 2,56% and 5,88% respectively (**Graph 17**). This small increase could be explained by fungi hyphae which increase total surface area providing more oxygen to microorganisms in the rhizosphere and thus, enhancing nitrification (Saeed and Sun 2012). Generally, however, the decrease in nitrogen removal can be explained by a change in the rhizosphere environment as the presence of AMF can have an impact on the metabolism of nitrogen fixing microorganisms.

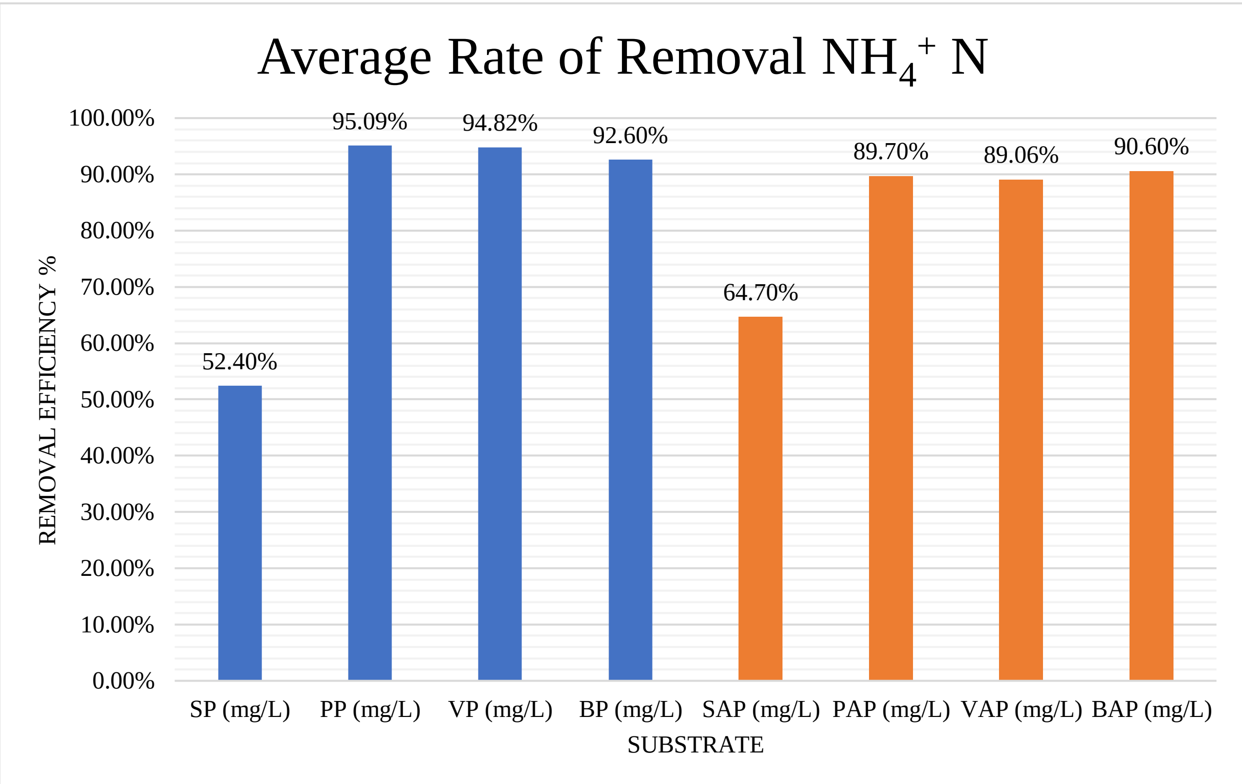
The results of our experiment show that concentration of ammonia (NH_4^+ N) as can be seen in **Graph 19** (AMF- reactors) and **Graph 20** (AMF+ reactors) differs depending on the type of substrates employed. Removal efficiency (**Graph 21**) ranged from 52,40 - 95,09% in AMF- and 64,70 - 90,60% for AMF+ reactors. Perlite, vermiculite and biochar proved to be more beneficial for ammonia removal compared to sand in both AMF inoculated and non-inoculated reactors due to more favorable conditions that promote oxygen diffusion in more porous perlite and vermiculite increasing nitrification (Noorvee et al. 2007), and the carbon rich biochar allowing for faster metabolism of nitrifying bacteria which convert NH_4^+ N to nitrite (NO_2-N) and then to nitrate (NO_3-N).



Graph 19 – Ammonia Concentration (mg/L) in AMF- reactors

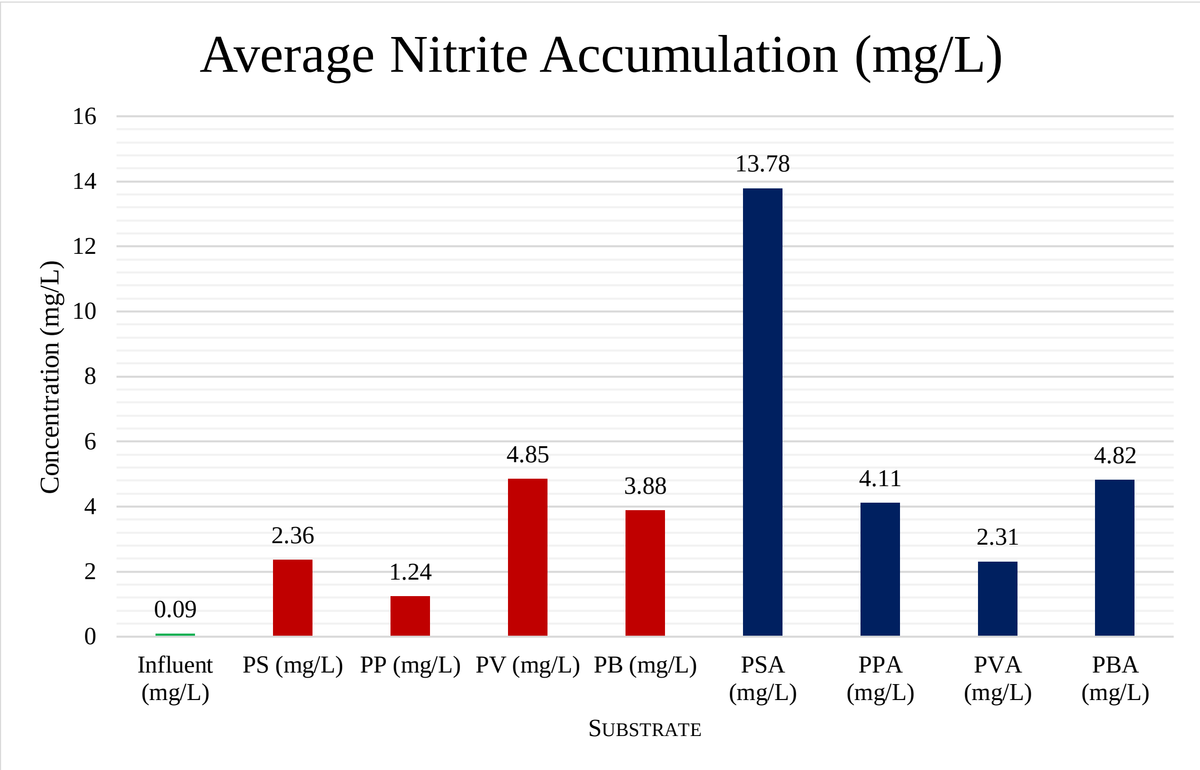


Graph 20 – Ammonia Concentration (mg/L) in AMF+ reactors



Graph 21 – Average rate of removal for ammonia in AMF- and AMF+ reactors

This increase of nitrification may be observed in concentration increases of nitrates and nitrites illustrated by **Graphs 18 and 22**. However, it must be noted that the significant spike in nitrite removal may be attributed to mistakes during the measurement. Overall, although the influence of AMF on nitrogen metabolism did not prove to be significant according to our results, the potential of using different substrates proved to be effective in increasing metabolic microbial processes such as denitrification and nitrification that determine total nitrogen removal. For future studies, more research should be done in order to identify optimal conditions of CW parameters that limit nitrogen removal such as temperature, pH, carbon and oxygen concentrations to further enhance pollutant removal in specific substrates.

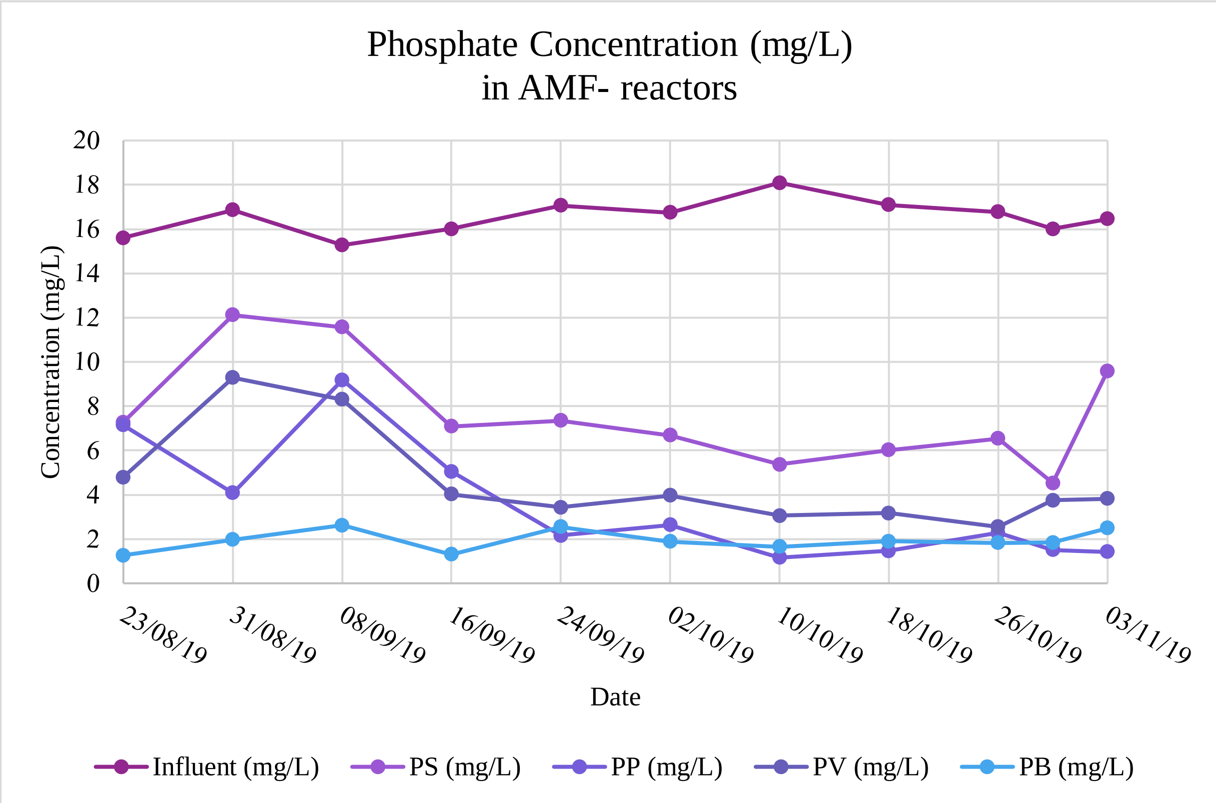


Graph 22 – Average nitrite accumulation in AMF- and AMF+ reactors

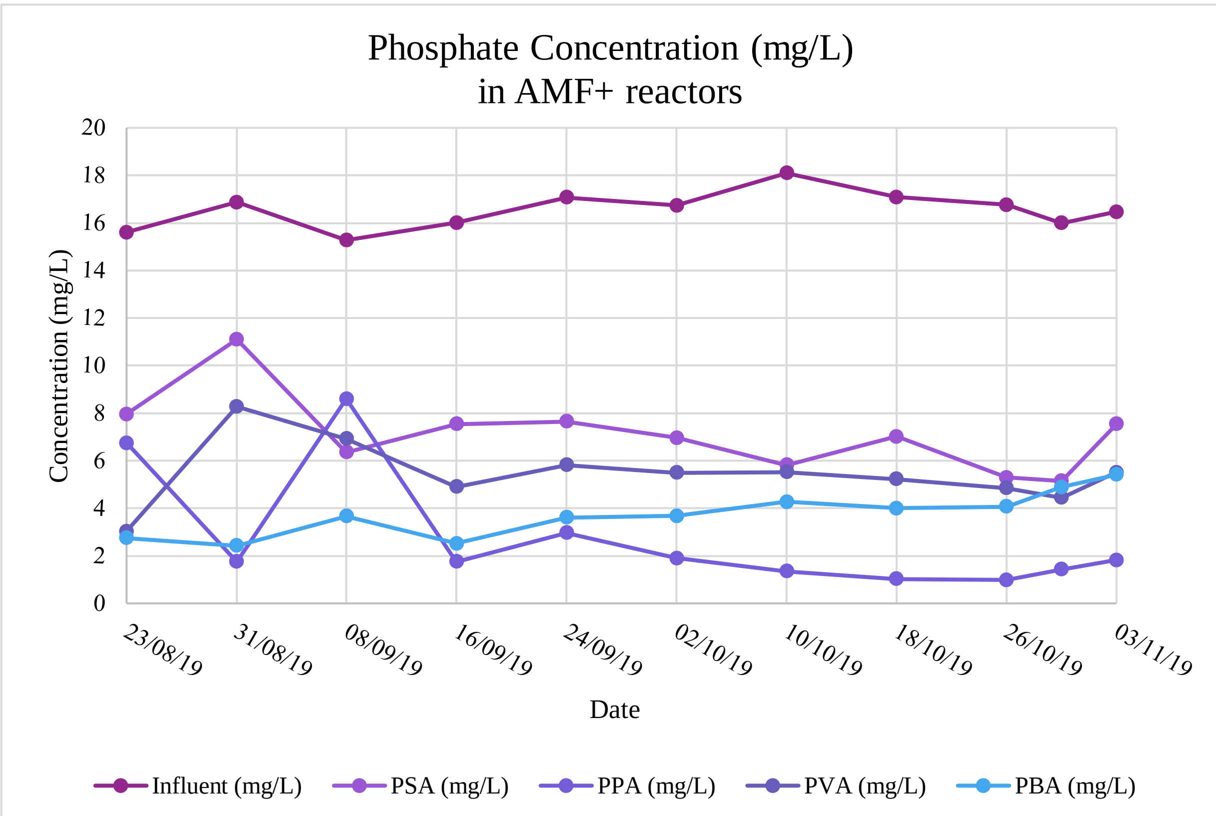
5.6 Phosphate Removal

Phosphorus is one of the key compounds which cause eutrophication of surface waters and its removal from wastewater using CWs has been of great interest (Prochaska and Zouboulis 2006). However, this is a problematic issue that has yet to be adequately resolved. The combination of several processes such as uptake by plants, adsorption, and precipitation within substrates play a major role in phosphate removal (Wang et al. 2013). The initial potential of each constructed wetland system to remove phosphorus is limited and dependent on types of materials used such as choice of substrates, plants as well as symbiotic microbes (Arias and Brix 2005). Plant uptake is generally seen as one of the main mechanisms by which phosphates are removed, however, this represents only a fraction of the total amount of the compound that is usually present in wastewater and is only a temporary mechanism by which phosphates are stored (Brix et al. 2001; Gupta et al. 2016). Also, microbial uptake of phosphates is not considered substantial and only temporary as phosphates are released back into the environment following decay of organisms (Vymazal 2007). One of the ways by which phosphate removal may be enhanced is by utilizing substrates which are able to bind phosphorus more efficiently and have high sorption capacities. However, this comes with its own drawbacks as the removal of the compound is specifically tied to the mineral makeup of the substrate and does not solve long-term removal due to finite sorption capacity of substrates (Vymazal 2007; Brix et al. 2001).

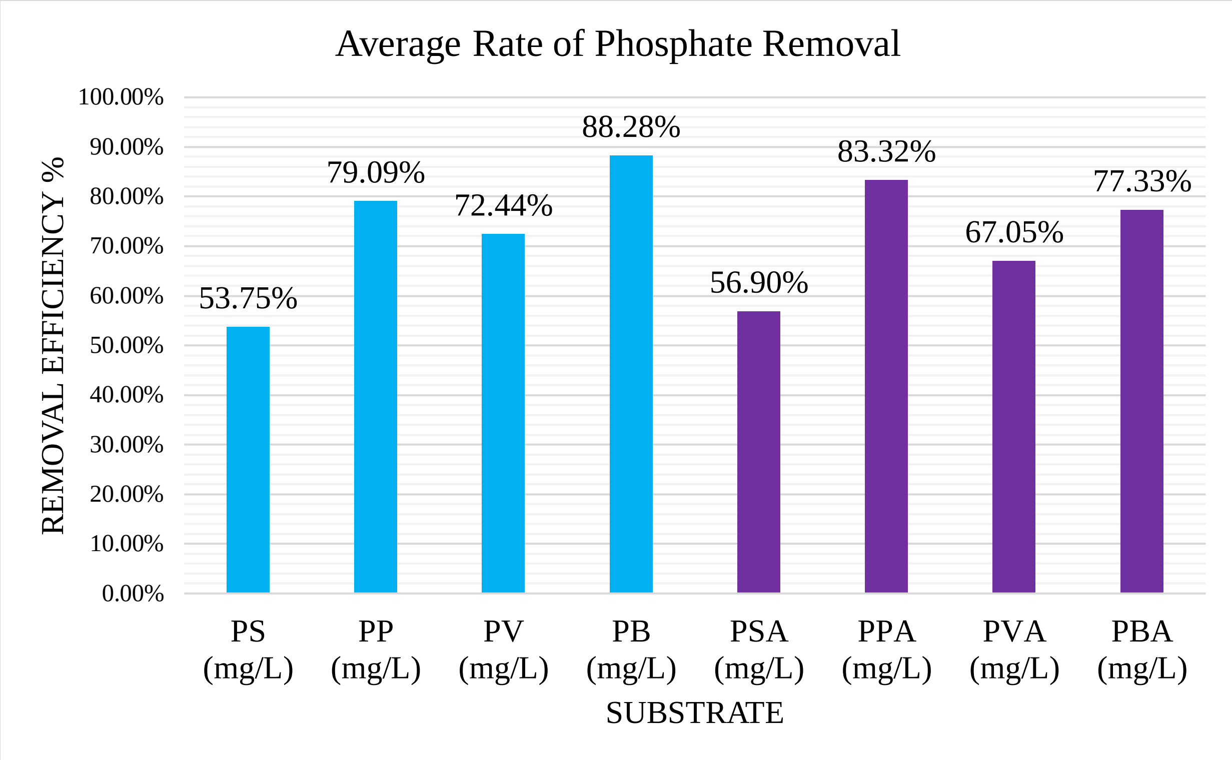
The results of our experiment illustrated by **Graphs 23** for AMF- reactors and **Graph 24** for AMF+ show that the use of constructed wetlands and specific substrates can successfully remove phosphates with varying degrees of success based on a number of variables. Average rate of removal (**Graph 25**) was found to range from 53,75 – 88, 28% efficiency in sand-based, perlite, vermiculite as well as biochar media possibly due to the substrates high surface area, ability to attract and successfully bind organic compounds, as well as sorption onto the lower layer of gravel media. In our experiment, biochar (73,33 – 88,28%) and perlite (79,09 – 83,32%) have been shown to have the greatest removal of phosphates. Furthermore, the inclusion of AMF may have increased the removal of phosphorus resulting from increased uptake by plants and microbes. An observed exception was AMF+ biochar reactors which showed a decreased removal, perhaps due to lower total surface area of roots because plants in biochar contained reactors exhibited lower total length and weight of roots which could negatively affect temporary phosphate uptake. In the latter stages of the experiment, phosphate concentration began to increase, which could indicate maximal saturation of phosphate compounds in the substrates. For future study recommendations concerning phosphorus and phosphate removal, it is necessary to more closely examine selected substrates and their mineral makeup, choose materials that have high phosphorus adsorption and retention capacities with determining factors that include grain size distribution and total surface area helpful for sorption and precipitation processes (Ballantine and Tanner 2010).



Graph 23 - Phosphate concentration (mg/L) in AMF- reactors



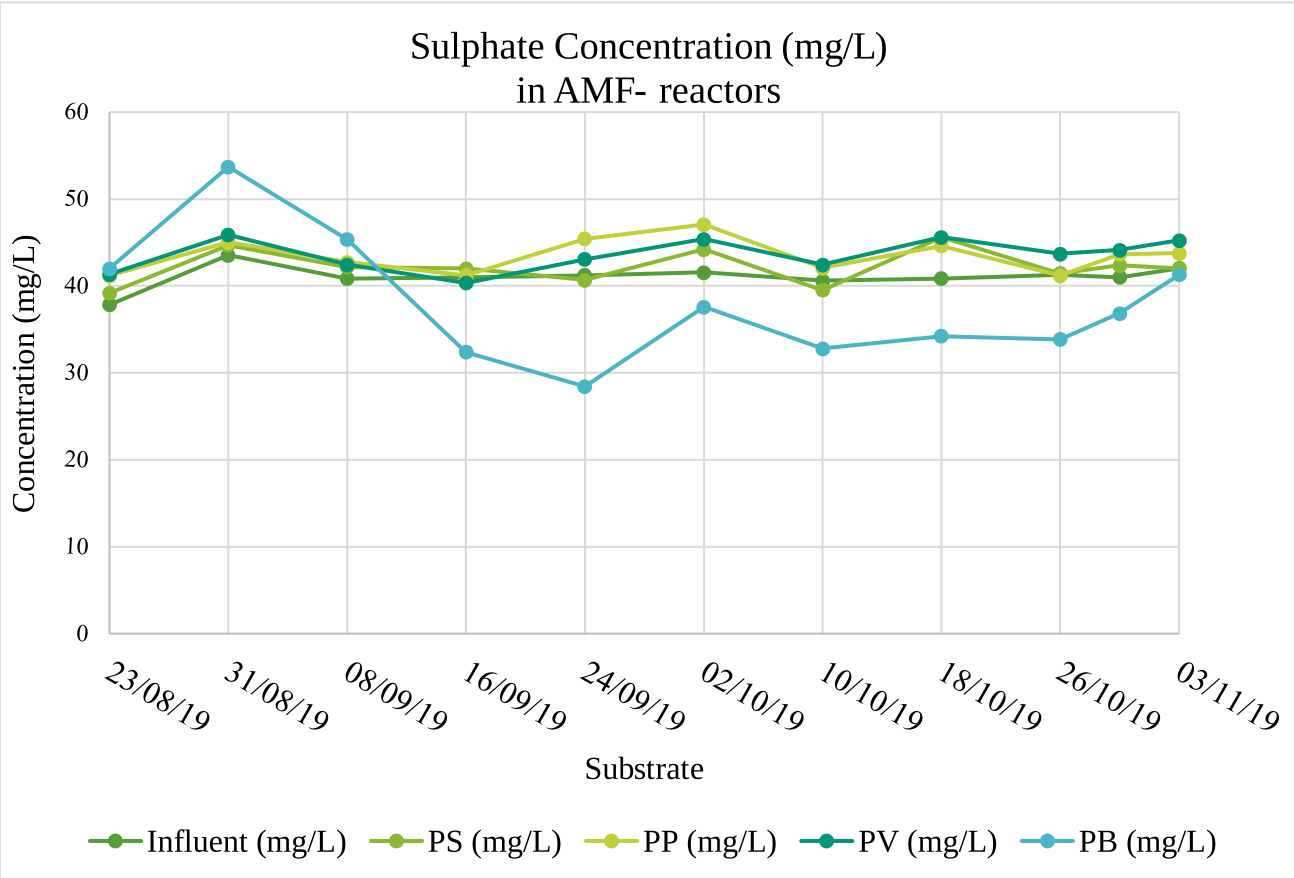
Graph 24 - Phosphate concentration (mg/L) in AMF+ reactors



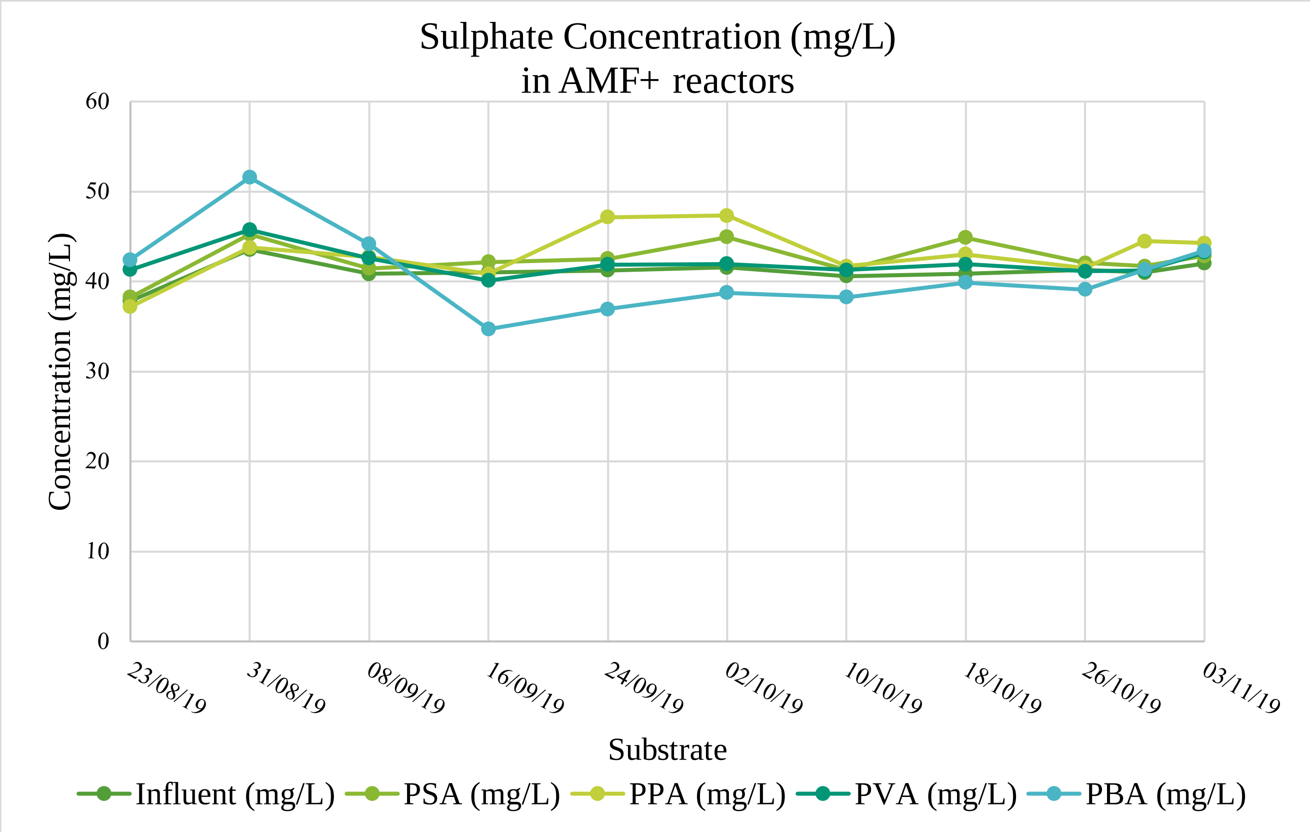
Graph 25 – Average rate of phosphate removal in AMF- and AMF+ reactors

5.7 Sulphate Removal

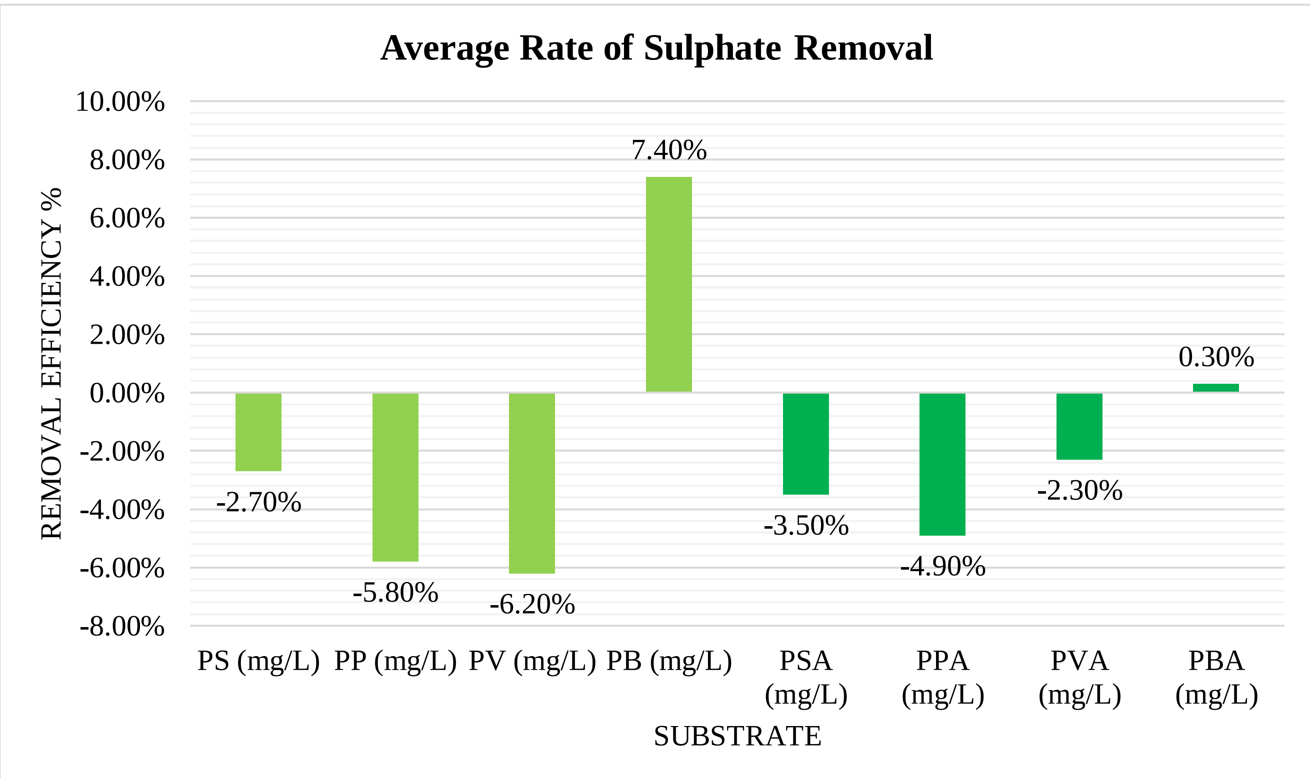
Sulphate is a common wastewater pollutant, and although it does not usually pose health risks, reduced sulphate compounds may inhibit plant growth and negatively affect microbial communities (Wiessner et al. 2005; Chen et al. 2016). Furthermore, the transformation of sulphate compounds to hydrogen sulfide (H_2S) for example can have detrimental effects on waterpipe corrosion, which in turn may cause phytotoxicity (Chen et al. 2016). The removal of sulphate in CWs is closely associated with the cycling of carbon and nitrogen, however, these relationships are not well understood (Wiessner et al. 2005). Our experimental results, illustrated by sulphate concentrations in **Graph 26** (AMF-) and **Graph 27** (AMF+) indicate that removal of sulphates was not substantial, but suggested that's substrates do have an effect on its concentration. **Graph 28** details removal efficiency of sulphate for all substrates as well as the influence of AMF. The use of biochar as a substrate was shown in the experiment to be a promising agent enhancing sulphate removal with a 7,38% efficiency possibly due biochar's properties as a rich carbon sink, as the rhizosphere by itself does not contain enough internal carbon to successfully facilitate sulphate removal by its reduction (Stein et al. 2007). Other substrate types were not efficient in sulphate removal nor did AMF colonization have a substantial effect and conversely, decreased removal rate of sulphate in biochar (AMF+) reactors by -7,08%. This could signify that AMF induce changes to sulphate reducing bacteria community structure. The efficiency of biochar is consistent with a study by Wiessner et al. 2005 which found that sulphate reduction intensity improved with an increased carbon load. Over time however, the removal of carbon in biochar enriched reactors decreased, possibly due to the reduction of carbon supply provided by the biochar substrate. This would also be in accordance with a study by Chen et al. 2016 that utilized plant litter, a rich carbon sink. In this study, sulphate removal decreased in the later phases of the experiment due to a decreased carbon sources (Chen et al. 2016). Our experiment suggests that the presence of a rich carbon source, such as biochar may positively affect the removal of sulphate at a time-dependent rate decreasing over time, a factor which should be more closely considered in future studies. Therefore, CWs can be considered a feasible option as long as carbon is continuously provided to maintain sulfate reduction (Gruyer et al. 2013).



Graph 26 – Sulphate concentration (mg/L) in AMF- reactors



Graph 27 – Sulphate concentration (mg/L) in AMF+ reactors



Graph 28 – Average rate of removal for sulphate in AMF- and AMF+ reactors

To recapitulate, the objectives of this work were to examine whether and how substrates might affect ibuprofen removal as representative of PPCPs, and other emerging contaminants in constructed wetlands. Our results suggest that substrates are a key operational factor that must be thoroughly assessed when designing such a system. Nonetheless, the number and types of substrates which were analyzed remain a limiting factor that needs to be examined more closely, and on a wider scale to gain further insight into the role that substrates play in PPCP removal, and to determine if and which substrate is most efficient in wastewater remediation. Furthermore, while substrate choice is a crucial factor of CWs design configuration, it is only one of the many operational factors that influence the efficacy of the total system.

Although our experiment has been successful at removing ibuprofen and organic compounds, the total removal efficiency of the system was impeded by a lack of assessment of other mechanical, chemical and biological factors that determine CWs. The relative short duration of the experiment meant that factors such as seasonal variations of temperature and light, central for photodegradation pathways for example have not been thoroughly inspected. Most importantly, a key limiting factor was a lack of microbial community evaluation as biodegradation processes are deemed as the major removal mechanisms by which contaminants are removed. However, although some of these processes such as denitrification and nitrification have been observed, other biodegradation pathways occurring within the microenvironment of the rhizosphere remain poorly understood. The success of future CWs installments will depend on the understanding of the complex relationships within the rhizosphere network and its inhabitants and should be a principal priority for upcoming CWs research.

6. Conclusion

Our experiment has demonstrated that substrates have a considerable role in determining the removal efficiency of not only ibuprofen, but other contaminant compounds usually present in wastewater. Our results, however, exemplify that substrates are only one piece of a far more complex puzzle consisting of a multitude of biotic and abiotic factors. Differences which have been observed in the removal rates of ibuprofen and organic compounds between AMF+ and AMF- reactors suggest that AMF induced changes to the rhizosphere environment. Nevertheless, the multitude of factors which influence CWs efficacy is highlighted by our experiment’s shortcomings. A relatively short duration of the experiment forgoing seasonal variations in light and temperature, lack of substrates with more diverse characteristics, and an absence of a microbial community assessment may hinder our results, and must be addressed in future CWs designs. Therefore, future studies on the removal efficacy of constructed wetlands in relation to the effects of substrates should focus on the elucidation of microbial interactions and how substrates’ properties might enhance them in order to augment the remediation capacity of the whole CW system. The objective to establish an ideal design configuration of CWs to remove pollutants may be hindered by the multitude of interactions occurring at the same time. Our results imply that a single, most efficient design configuration that optimally removes all pollutants present in wastewater may not exist, because removal pathways for a certain type of pollutant may adversely impact the removal of other contaminant compounds. Therefore, the composition of CWs including substrates should be designed around the removal pathways specific for the type of pollutant in question. This study was able to identify that a combination of different substrates in CWs allows for simultaneous aerobic and anerobic degradation pathways resulting in improved contaminant removal. While the inclusion of perlite and vermiculite has shown increases to the removal rates of organic compounds with vermiculite being most efficient in TC and IC removal, biochar has been shown to be most efficient in the removal of over 98% ibuprofen, OC, TN, phosphate, and sulphate. This indicates that biochar is a suitable candidate for PPCP remediation from soil and could be a useful constituent of future CWs designed for the removal of PPCPs and other emerging contaminants.

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