CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Environmental Sciences
Department of Applied Ecology

Effect of arbuscular mycorrhizal fungi on treatment of heavy metal under different N:P ratios in constructed wetlands

Diploma Thesis

Carlos René Cárdenas Muñoz

Director: doc. Dr.-Ing. Zhongbing Chen

2021

CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Environmental Sciences

DIPLOMA THESIS ASSIGNMENT

B.Sc. Carlos René Cárdenas Muñoz

Environmental Geosciences

Thesis title

Effect of arbuscular mycorrhizal fungi on treatment of heavy metal under different N:P ratios in constructed wetlands

---

Objectives of thesis

This thesis aims to investigate the AMF colonization with plant roots in constructed wetlands under different N:P ratios, as well as the effects of AMF colonization on the removal of heavy metal.

Methodology

This study will be conducted using 12 vertical subsurface flow CWs at the campus of the Czech University of Life Sciences Prague. The experimental device consisted of the innovative KG-System (PVC) pipes, substrate, and water outlet. The 12 PVC pipes will be established to simulate the subsurface flow CWs with the dimensions of each system is 15× 55 cm (diameter ×Height). Each CW will be filled with 10 cm gravel (4-5 cm) and 35 cm mixture of sand will be used as substrates. Glyceria maxima will be selected as a wetland plant. AMF inoculum will be Rhizophagus irregularis. The influencing factors of this study are AMF (with AMF, without AMF), heavy metals, N:P ratio. The experiment will last 5 months.

The proposed extent of the thesis

50

Keywords

Arbuscular mycorrhizal fungi, heavy metal, N:P ratio, constructed wetlands

Recommended information sources

Abdelhameed RE, Metwally RA (2019) Alleviation of cadmium stress by arbuscular mycorrhizal symbiosis. Int J Phytoremediation 21:663–671.

Akbar Karimi (2011) Arbuscular mycorrhizal fungi and heavy metal contaminated soils. African J Microbiol Res 5

Akhtar O, Mishra R, Kehri HK (2019) Arbuscular Mycorrhizal Association Contributes to Cr Accumulation and Tolerance in Plants Growing on Cr Contaminated Soils. Proc Natl Acad Sci India Sect B – Biol Sci 89:63–70.

Bao X, Wang Y, Olsson PA (2019) Arbuscular mycorrhiza under water — Carbon–phosphorus exchange between rice and arbuscular mycorrhizal fungi under different flooding regimes. Soil Biol Biochem 129:169–177.

Fusconi A, Mucciarelli M (2018) How important is arbuscular mycorrhizal colonization in wetland and aquatic habitats? Environ Exp Bot 155:128–141.

He Y mei, Yang R, Lei G, et al (2020) Arbuscular mycorrhizal fungi reduce cadmium leaching from polluted soils under simulated heavy rainfall. Environ Pollut 263:

Huang M, Ai H, Xu X, et al (2018) Nitric oxide alleviates toxicity of hexavalent chromium on tall fescue and improves performance of photosystem II. Ecotoxicol Environ Saf 164:32–40.

Huang X, Ho SH, Zhu S, et al (2017) Adaptive response of arbuscular mycorrhizal symbiosis to accumulation of elements and translocation in Phragmites australis affected by cadmium stress. J. Environ. Manage.

Huang X, Wang L, Zhu S, et al (2018b) Unraveling the effects of arbuscular mycorrhizal fungus on uptake, translocation, and distribution of cadmium in Phragmites australis (Cav.) Trin. ex Steud. Ecotoxicol. Environ. Saf. 149:43–50

Li H, Huang WX, Gao MY, et al (2020a) AM fungi increase uptake of Cd and BDE-209 and activities of dismutase and catalase in amaranth (Amaranthus hypochondriacus L.) in two contaminants spiked soil. Ecotoxicol Environ Saf 195

Expected date of thesis defense

2020/21 SS – FES

The Diploma Thesis Supervisor

doc. Zhongbing Chen

Supervising department

Department of Applied Ecology

Advisor of thesis

Shanshan Hu

Electronic approval: 2. 2. 2021

prof. Ing. Jan Vymazal, CSc.

Head of department

Electronic approval: 10. 2. 2021

prof. RNDr. Vladimír Bejček, CSc.

Dean

Prague on 26. 03. 2021

Statement

I hereby declare that I have independently elaborated the diploma/final thesis with the topic of: Effect of arbuscular mycorrhizal fungi on treatment of heavy metal under different N:P ratios in constructed wetlands, and that I have cited all the information sources that I used in the thesis and that are also listed at the end of the thesis in the list of used information sources.

I am aware that my diploma/final thesis is subject to Act No. 121/2000 Coll., on copyright, on rights related to copyright and on amendment of some acts, as amended by later regulations, particularly the provisions of Section 35(3) of the act on the use of the thesis.

I am aware that by submitting the diploma/final thesis I agree with its publication under Act No. 111/1998 Coll., on universities and on the change and amendments of some acts, as amended, regardless of the result of its defense.

With my own signature, I also declare that the electronic version is identical to the printed version and the data stated in the thesis has been processed in relation to the GDPR.

In Prague, Czech Republic 26.03.2021
Carlos René Cárdenas Muñoz

Acknowledgements

First of all, I would like to thank doc. Dr.-Ing. Zhongbing Chen and his PhD student Shanshan Hu, for the opportunity and trust they put into me for the writing of this diploma thesis and conduction of the research. Also, for all the consultations, patience, time and effort invested in the review of this document and all the support provided during the development of this project. Finally, I want to thank my family and friends as well for the encouragement and support.

Thank you very much,

Carlos René Cárdenas Muñoz

Abstract

The bioremediation of heavy metal pollution assisted by arbuscular mycorrhizal fungi (AMF) is one of the new natural approaches to enhance water treatment, moreover, the effect of the AMF in the removal of metals from wastewater has been widely studied. However, the influence of different ratios of N:P on the effectiveness of this removal has not been much evaluated even knowing that agricultural or industrial usage of compounds of nitrogen and phosphorus is growing with the increase of economic activities.

The purpose of this thesis is to assess the effect of AMF on the treatment of heavy metal under different N:P ratios in constructed wetlands (CWs). The experimental setup consisted of 12 different CWs made out of PVC pipes that worked as subsurface vertical flow CW, vegetated with *Iris Pseudacorus* and the AMF was *Rhizophagus irregularis.* From these experimental systems, there were treatments with and without the presence of AMF, with and without heavy metal pollution and three different N:P ratios (1:1, 1:5 and 1:10).

Water samples were taken for further analysis of pH, Rh, NH_4^+, Total Organic Carbon (TOC), Total Nitrogen (TN), PO_4^3, NO_2^-, NO_3^-, copper (Cu) and lead (Pb) every five days starting from September 16th to November 15th, 2020 Furthermore, samples from plant roots, shoots and stems were taken to analyze AMF colonization and heavy metal distribution in the systems.

The results showed that the highest intensity of AMF colonization with 26.8% is from the system M_P1_HM corresponding to the system with mycorrhiza presence under a N:P ratio of 1:1 and treated with heavy metal polluted water, the intensity was decreasing when the N:P ratio was changing to 1:5 and 1:10. Regarding the metal distribution in the experimental CW, the concentrations were analyzed in the laboratory for each part (substrate, plant roots, shoots and water). The highest concentration was found in the roots for both Cu and Pb, and the concentrations were decreasing with the increase of N:P ratio.

The heavy metal removal efficiency was also assessed and the results were that the impressive efficiencies of more than 95% in all the systems were possible to the complex reactions between different aspects of the SSVF CW such as soil, plants and microbiota. The presence of the AMF had a positive influence on the difference of these efficiencies and it was related also to the different N:P ratios and their effect on AMF colonization.

Key words: *Constructed wetlands, AMF, N:P ratios, heavy metals, water pollution, IRIS.*

Table of Contents

Introduction 10
1. Objectives 10
2. Literature review 11
2.1. Metal pollution in wastewater 11
2.2. Constructed wetlands 12
    2.2.1. Types of constructed wetlands 13
    2.2.2. Free Water Surface Constructed Wetlands 13
    2.2.3. Horizontal subsurface flow constructed wetlands 14
    2.2.4. Vertical subsurface flow constructed wetlands 15
  2.3. N and P removal in CW 16
  2.4. Cu and Pb removal in CW 17
2.5. Arbuscular Mycorrhizal Fungi (AMF) 17
  2.5.1. Factors affecting the application of AMF in CW 18
2.6. Influence of N and P in wetlands vegetation 19
  2.6.1. Nitrogen in wetland vegetation 19
  2.6.2. Phosphorus in wetland vegetation 19
2.7. Influence of AMF in metal removal from wastewater 20
3. Methodology 21
3.1. Experimental setup 21
3.2. Sample analysis 23
  3.2.1. AMF Colonization Assessment 23
  3.2.2. Water, soil and plant sample analysis 24
4. Results 25
  4.1. AMF Colonization 25
  4.2. Metal concentration distribution in SSVF CW 28
  4.3. Removal of Cu and Pb from wastewater 31
    4.3.1. Cu particles removal in SSVF CW 31
    4.3.2. Pb particles removal in SSVF CW 33
  4.4. Water parameters analysis 36

4.4.1. pH 36
4.4.2. Oxidation-Reduction Potential 37
4.4.3. Ammonium- N (NH_4^+) Removal 38
4.4.4. Total Nitrogen 40
4.4.5. Total Carbon 42
4.4.6. Total Organic Carbon 43
4.4.7. Phosphate 44
5. Discussion 46
5.1. Effect of different N:P ratios in AMF colonization 46
5.2. Effect of different N:P ratios in the distribution of metals in CW 47
5.3. Influence of AMF under different N:P ratios in the removal of Ammonium (NH_4^+-N) and Phosphorus (PO_4 -P) 48
5.4. Influence of AMF at different N:P ratios in the removal of heavy metals from wastewater 49
6. Conclusions 50
Reference 51

List of Tables

Table 1: Composition of simulated sewage water 22
Table 2: AMF Colonization intensity in root system, mean values with standard deviation 26
Table 3: Arbuscule abundance in the root system........ 26
Table 4: Concentrations of Cu and Pb in the plant’s roots........ 28
Table 5: Concentration of Cu and Pb in plant shoots 29
Table 6: Cu and Pb concentration in substrate........ 30
Table 7: Mean concentration of Cu in the systems and in inflow wastewater 32
Table 8: Mean Cu removal efficiency (%) in each system 33
Table 9: Pb concentration in each system and in inflow wastewater 33
Table 10: Pb removal efficiency mean values and standard deviation 35
Table 11: Pb removal efficiency in % 35
Table 12: pH in outflow water from each experimental system........ 36
Table 13: ORP mean values from all experimental systems 37
Table 14: NH4 mean concentration values (mg/L) 38

Table of Plots

Plot 1: M(%) Intensity of AMF colonization........ 26
Plot 2: Cu and Pb concentration in roots........ 28
Plot 3: Cu and Pb concentration in plant shoots........ 29
Plot 4: Cu and Pb concentrations in substrate 30
Plot 5: Cu concentration in experimental systems........ 31
Plot 6: Cu mass removal efficiency (%)........ 32
Plot 7: Pb concentration in experimental SSVF CW in mg/kg........ 34
Plot 8: pH from outflow water 37
Plot 9: ORP mean values for each experimental system........ 38
Plot 10: Ammonium concentrations (mg/L) 39
Plot 11: Total Nitrogen concentration in mg/L 40
Plot 12: Total Carbon concentration (mg/L)........ 42
Plot 13: Total organic carbon concentration (mg/L)........ 43
Plot 14: P concentration (mg/L)........ 44

Introduction

Modern life and its economic activities produced various byproducts or residues that are affecting the environment. Sectors such as industry and mining have dangerous wastes containing metals that are toxic or carcinogenic, also these pollutant particles are being transported to water sources and soils creating toxic environments full of acidity.

From soil and water, these pollutant particles are introduced into the food chain where animals and humans are vulnerable to develop carcinogenic diseases or organ malfunctions due to the presence of these particles in the body.

As an environmentally efficient way to reduce this risk and to be able to treat wastewater from these industries, the constructed wetlands have grown as an option that through phytoremediation or phytostabilization could remove the pollutant particles from the water due to chemical and microbiological processes that are carried out in these artificial environments (Najeeb et al., 2017). These constructed wetlands are special flooded systems where vegetation, soil and microorganism associations aid the treatment of waters, replicating the natural processes but in controlled conditions to focus on specific components extraction (Vymazal, 2014).

Arbuscular mycorrhizal fungi (AMF) is a fungus that creates a symbiotic relationship with the plants helping them to uptake nutrients in a much more efficient way to enhance the uptake of these pollutants. AMF can improve the resistance of the plants and help them to keep these metals in their biomass (XU et al., 2016). Controlling the conditions and with proper design and operational techniques, it is possible to decrease the harmfulness of polluted waters and places in order to recover them for future use of these resources.

In this study, the idea is to analyze the influence of arbuscular mycorrhizal fungi (AMF) in the extraction of metals such as lead and copper from wastewater under different ratios of N:P and its effect. This research was developed to explore its possibilities in the treatment of industrial or mining wastewaters.

1. Objectives

- To assess the effect of arbuscular mycorrhizal fungi on the treatment of heavy metals under different N:P ratios in constructed wetlands
- To evaluate the effect of AMF in the treatment of heavy metal in constructed wetlands
- To analyze the influence of different N:P ratios in the treatment of heavy metal in constructed wetlands

2. Literature review

2.1. Metal pollution in wastewater

Metal pollution in water is one of the biggest concerns because they can enter the food chain of aquatic ecosystems or get deposited in soil (Censi et al., 2006). The presence of Cu and Pb metals affects aquatic habitats greatly because they have severe effects of toxicity on living organisms. There are two main ways of how metals reach the water or pollute water sources: first the natural processes of chemical and physical weathering of rocks or several plant processes (Gacia et al., 2020), the second consist of anthropogenic activities such as the burning of fossil fuels, batteries production, smelting, mining and more (Ahmed et al., 2021).

The metals usually get deposited heavily in sediments through adsorption and other chemical reactions such as precipitation, diffusion or biochemical processes, and depending on the properties of the sediments, these pollutant particles could be present in the water column (Pandiyan et al., 2020). The heavy metal concentrations can fluctuate depending on the source of the wastewaters, for copper (Cu), the daily average concentrations from industrial wastewater is 400-1500 µg/L and mine drainage concentrations of Cu can range from 10-50 µg/L as average monthly. The concentrations of lead (Pb) from industrial wastewater can reach 1560 µg/L and it can range from 2 to 6 µg/L for mining drainage (Galletti et al., 2010).

Cu is a very common metal that is present in most living beings as a primary constituent of cytochrome oxidase, which is a respiratory enzyme. In humans, traces of Cu are present in the bones, liver, and muscles (Al-Fartusie & Mohssan, 2017). Wastewater polluted with Cu can be the result of the production of metal, electrical appliances, fungicides, pesticides, and other products that are often transported to bigger water sources. The consequence of this Cu pollution in water is that animals can absorb Cu when feeding or drinking from contaminated water and soil and this leads to poor health due to damaged kidneys, liver, and affections to the nervous system (Kondzior & Butarewicz, 2018).

Pb is one of the most abundant metals on the planet, in water under usual conditions it doesn’t react however when it has contact with air the reactivity of Pb increases because a small layer of lead oxide (PbO) is formed in the surface of this metal (Habte et al., 2020). The presence of Pb in water is caused by anthropogenic activities such as the burning of fossil fuels where it binds to different oxides in the air and eventually, these fall in the form of precipitation into the water and soil, also the mining activities, accidental leaks, and spills, old water piping lines, improper disposal of batteries and smelting can be sources of this metal in water (Tiwari & Tripathi, 2012).

Pb often binds to sulphide (S^{2-}), or phosphate (PO_4^{3-}). In these forms, Pb is insoluble and is present as immobile compounds in the environment. Pb compounds are generally soluble in soft and slightly acidic water (Wilson et al., 2015). The environmental consequences or effects of lead are an important issue due to the toxicity of its compounds. Pb compounds such as lead carbonate, lead nitrate, and lead oxide can limit plant chlorophyll synthesis even in plants with resistance to lead, also, high concentrations can impact negatively on plant growth (Victoria et al., 2008). Due to the uptake of Pb from plants this compound can enter into the food chain carrying dangers to animals and humans.

For humans, the effects of lead poisoning are teratogenic or neurological because it causes necrosis of neurons, it has also a carcinogenic effect, and the interaction of lead and free sulfhydryl groups of proteins generates the deactivations of some enzymes (Farooq et al., 2008).

There are several available methods to remove these pollutant and harmful particles such as: complex fibers as a filtrating matrix like polyacrylic acid materials (Esfahani et al., 2020), the use of hydrochar is also another possible option obtained from biomass as rice straw carbonized (Nadarajah et al., 2021), also other from the non-traditional techniques is the application of porous aerogels as adsorbent (Hasanpour & Hatami, 2020).

These methods are highly efficient but they are difficult to implement as a proper system of extraction of metals from water due to their price and availability of materials, that is why systems of constructed wetlands are considered a reliable option because they are a low price treatment system that can be highly efficient if designed, constructed and operated properly, also, it can have recreational attributes or landscape properties, moreover these constructed wetlands perform a sustainable treatment method that reduces the impact in the downstream water sources (Parde et al., 2020).

2.2. Constructed wetlands

Constructed wetlands (CW) are wetlands created after a process of design and construction to control and optimize natural processes in order to develop water treatments specialized to treat different types of wastewater (Wang et al., 2020). These systems have proven to be efficient not only in the treatment but in the economical and sustainability aspects and they can differ depending on their hydrology and flow path and being classified on subsurface flow wetlands, surface wetlands and hybrid constructed wetlands (Fan et al., 2021).

All wetlands, natural or constructed with freshwater or salt have one characteristic in common that is the presence of a surface or near-surface water, at least intermittently. In the majority of wetlands the hydrological conditions allow the substrate to be saturated long enough during the growing season to create oxygen-poor conditions in the substrate (DuPoldt et al., 1996). The lack of oxygen creates a reduction (oxygen-poor) conditions within the substrate and limits the vegetation to those species that are adapted to low-oxygen environments (DuPoldt et al., 1996).

A constructed wetland (CW) comprises a correctly designed basin that contains water, substrate, and most commonly, vascular plants (Mihelcic, 2018), these components can be manipulated in the design and construction of a wetland, other important components of wetlands, such as the communities of microbes and aquatic invertebrates can develop in these ecosystems (Hoffmann et al., 2011).

CWs are used for primary and secondary treatment to treat wastewater of domestic origin, agricultural wastewater, coal drainage wastewater, petroleum refineries wastewater, landfill leachates, compost, and many other polluted water (Parde et al., 2020).

2.2.1. Types of constructed wetlands

CWs used as wastewater treatment could be classified according to the life form of the macrophyte that dominates the systems, or into systems with floating-leaved, free-floating, rooted emergent, and submerged macrophytes. The further classification could be made according to the wetland water movement, subsurface systems or free water surface. Subsurface flow CWs could be classified according to the direction in which water flows (horizontal and vertical) (Vymazal, 2010).

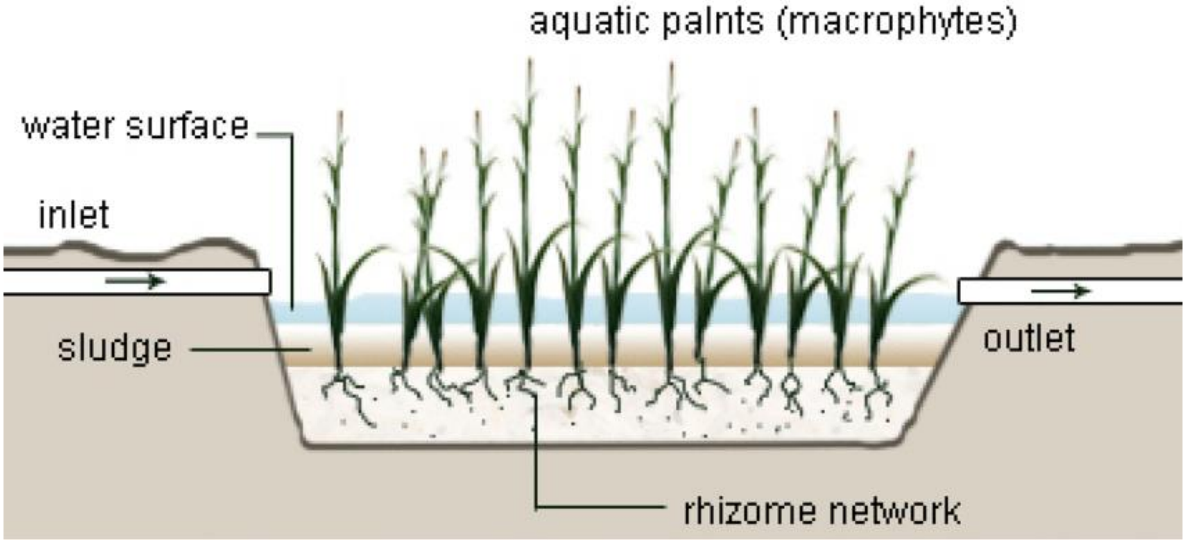
Different types of CW may be combined to achieve a higher treatment effect, especially for nitrogen. Hybrid CW systems include most frequently vertical flow and horizontal flow systems arranged in sections or stages but, in general, all types of constructed wetlands could be adapted and combined to focus on specific parameters achieving more complex treatment processes with high efficiency (Vymazal, 2005).

2.2.2. Free Water Surface Constructed Wetlands

Free water surface constructed wetland is a natural water remediation system that is commonly used in non-point source control of pollution in which wastewater flows over the surface. This type of CW are useful for flood prevention and also help to control shoreline erosion along with the improvement of wastewater quality (Farooqi et al., 2008). A wide variety of vegetation can be applied in the free water surface CW as emergent plants (*Typha, Scirpus, Phragmites*), submerged plants (*Elodea, Potamogeon*), and floating plants (*Eichornia, Lemna*) (Parde et al, 2020).

Plants in these systems are not harvested frequently, hence the litter supplies organic carbon used for denitrification which may happen in anaerobic parts within the litter layer (Vymazal, 2010).

Figure 1: Free water surface constructed wetland



Source: Technical Guide to EcoSan Promotion

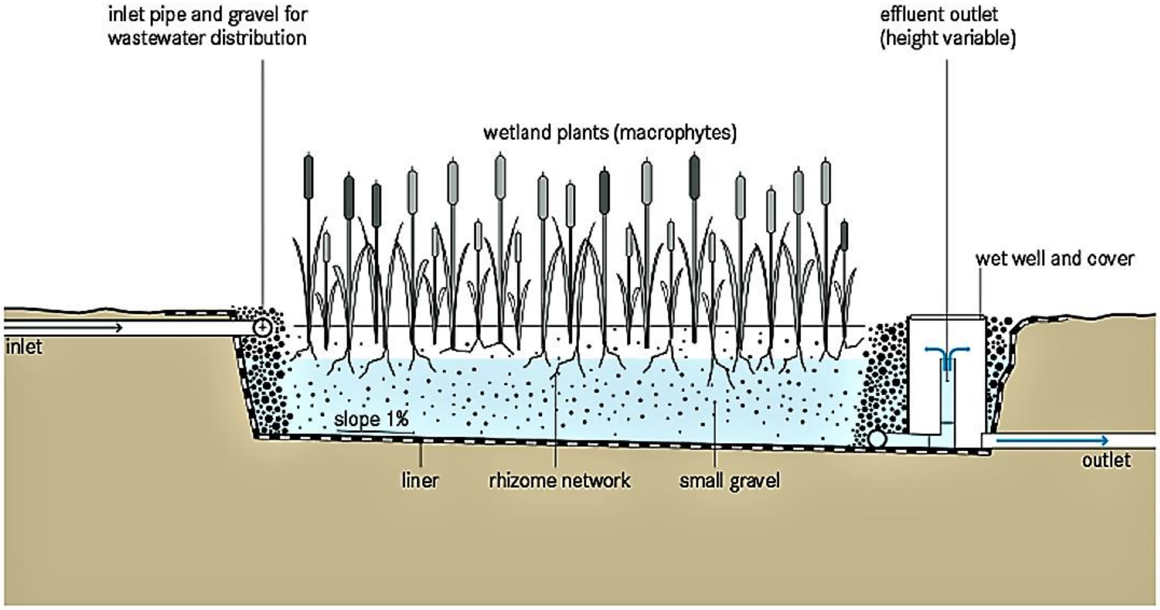
2.2.3. Horizontal subsurface flow constructed wetlands

In this type of wetland the wastewater flows horizontally in the bed of the CW, this wastewater goes under aerobic and anaerobic conditions being the aerobic conditions produced in the root zone and the anaerobic conditions degrade the organic matter (Boyd, 2006). Aerobic and anaerobic processes performed by bacteria attached to plant underground organs (i.e. roots and rhizomes) and media surface degrade the organic compounds, the removal of organic compounds is generally very high in Horizontal subsurface flow CWs (Vymazal, 2005).

This type of constructed wetland is recommended for warm climates, but it can be designed to stand some colder environments and periods of low microbiological activity. However, if the outflow water is going to be reused, the system can face losses due to high evapotranspiration rates depending on the climate (Khan et al., 2020).

In a study on the removal of metals conducted by Vymazal (2006) was found that this type of system proved to be able to remove different elements with efficiencies such as Al (>98.9%), Zn (94.1%), and Cr (>92.8%), Cu (>75%), Pb (>73%), Ni (55.7%).

Figure 2: Horizontal subsurface flow constructed wetland



Source: Compendium of Sanitation Systems and Technologies. 2nd Revised Edition

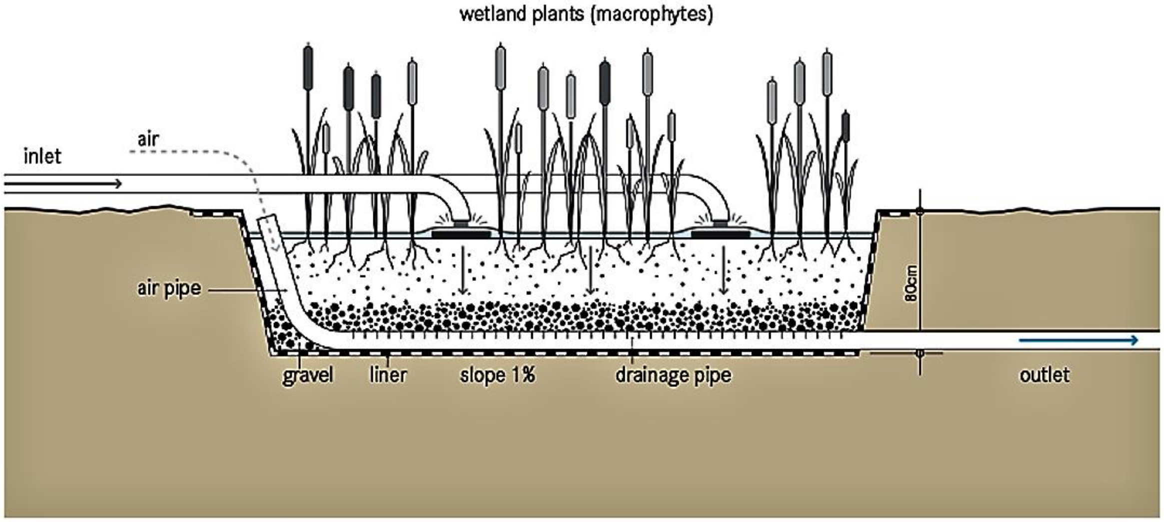
2.2.4. Vertical subsurface flow constructed wetlands

The constructed wetlands with a vertical subsurface flow are characterized because they have the capability to nitrify and they have a good oxygen transmission because the water flows vertically being filtrated between sand or gravel (Vymazal, 2014).

VF CWs are fed in an intermittent way with a large charge thus flooding the surface. The wastewater then percolates gradually down through the substrate and is collected by a drainage network at the bottom. The bottom or the bed drains completely giving space and allowing air to refill the bed. This kind of dosing leads to good oxygen transfer and hence the ability to nitrify (Jain et al., 2020).

VF CWs could remove heavy metals in wastewater. For example, Mustapha et al (2017) found *Cyperus alternifolius*, *Typha latifolia*, and *Cynodon dactylon* all can remove Cu, Zn, Pb, Fe, Cd, and Cr from the refinery wastewater in VF CWs, but the one planted with *T. latifolia* had the best heavy metal removing results. In other experiments carried out by Lee & Scholz (2006) showed that metals such as Cu and Ni were significantly removed from the water showing the efficiency of the vertical subsurface flow constructed wetlands.

Figure 3: Vertical subsurface flow constructed wetland



Source: Compendium of Sanitation Systems and Technologies. 2nd Revised Edition

2.3. N and P removal in CW

Studies such as the one performed by Zeng et al. (2020) provide an understanding of the importance of different factors that affect the effectiveness of the constructed wetland on the removal of specific compounds. The difference in the construction of wetlands leads to different physicochemical conditions that will change the metabolic pathways of microbes and will also change the intensity of the reactions (Bernardes et al., 2019).

The interaction between microorganisms and their cooperation or competition in the environment leads to the degradation and conversion of nutrients involving fungi and bacteria creating a complex interaction network (Yang et al., 2020). This bacteria-algae-fungus interaction can achieve the removal of different compounds such as NH_4, TN and PO_4^{3-} (Sun et al., 2020). The heterotrophic bacteria as a source of carbon, use organics for the removal of N and P because Nitrite nitrogen (NO2) is used as an electron acceptor by denitrifying phosphorus accumulating organisms (DPAO) (Zhimiao et al., 2016). The interaction of different types of bacteria such as the nitrite-oxidizing bacteria and the ammonia-oxidizing bacteria cooperates to remove Nitrogen in CW (Hu et al., 2019).

One of the main problems or issues in wastewater treatment is the avoidance of the consideration on the relationship between P removal and N. Some systems that involve organics degradation, denitrification, nitrification and other microbial activities take into account the interaction of biochemical reactions and how they affect the distribution of P (Chang et al., 2012). Studies such as the one performed by Zhimiao et al. (2016) aim to improve phosphorus removal under different N:P ratios and different compounds as PO_4, PO_3 and P_2O_7 , based on the performance of nitrogen removal.

2.4. Cu and Pb removal in CW

Plant and animals require micronutrients that help with their growth but these nutrients are present in very small amounts. Some of these nutrients are copper, zinc, selenium and others but in higher concentrations they can be toxic. And other metals such as cadmium, lead and mercury are toxic even in small concentrations and they are usually found in industrial wastewater.

There are different methods of removal of metals in constructed wetlands and they are plant uptake, soil adsorption and precipitation. The rate of contaminants uptaken by plants depends on the type of plant and metal of interest because some plant species are capable of storing large amounts of metals in their roots and biomass. Duckweed is a great example of a plant that can store large amounts of metals such as copper and cadmium. When the metals pass close to the root structure they tend to accumulate there instead of being absorbed by the plant (Norton, 2003).

The soils of wetlands also can retain or trap pollutant particles including, lead, cadmium, copper, nickel and more. These metals can form insoluble compounds after interacting with sulfides in anaerobic environments in the soils of the constructed wetlands minimizing the resolubilization of these metals in also anaerobic conditions (Huang et al., 2017).

Metals such as copper, lead, chromium, and zinc form strong chemical complexes with organic material present in soil and water through a process called chemisorption, and copper can be bound to clays and oxides that will settle out (Yeh, 2008).

2.5. Arbuscular Mycorrhizal Fungi (AMF)

An arbuscular mycorrhiza is a close mutualistic relationship between diverse plant species and a small group of soil fungi that develops in root systems. The association permits the exchange of mineral nutrients provided by the fungi and carbohydrates provided by the plant, increasing the host plant's tolerance to different stress factors of biotic and abiotic sources (Riaz et al., 2021).

Mycorrhizas are usually classified around the world into arbuscular mycorrhizas, ectomycorrhizal, ericoid mycorrhizas , and orchid mycorrhiza. Arbuscular mycorrhizal fungi (AMF) form symbiotic relationships with more than 80% of vascular plants cultivated (French, 2017). This mycorrhizal association with plants helps to perform a major role in natural ecosystems by colonizing plant roots intracellularly (Zhan et al., 2019) and depend on the host throughout their life cycle for food.

As one of the most ancient and important groups of symbionts with plants , AMF provides phosphorus (P) and other nutrients of mineral origin, , to plants in exchange for photosynthetically fixed carbon (C) (Bao et al., 2019). Plants transfer 10–30% of their photosynthetic products, about 5 billion tons of C per year, to fungal symbiont (Bago et al., 2000), and receive up to 90% of the mineral nutrients (especially P) from AMF (Smith &

Smith, 2011). Therefore, global C and P cycling get significantly benefited by AM symbiosis contribution.

The AMF and plant roots association denotes one of the most common and significant symbiotic relationships in nature. In broad terms, the relationship is usually mutualistic and involves bidirectional transfers of fixed organic carbon from the plant and soil-derived nutrients from the fungi (Jones & Smith, 2004).

Extraradical mycelium extends outside the plant, for meters and have important roles in nutrient (nitrogen, zinc , and phosphorous in particular) uptake and translocation to the intraradical structures. Extraradical mycelia also play a role in foraging for new carbon sources and hence may associate with plants from the same or different species, forming an underground highway (Amalero et al., 2003).

Arbuscular mycorrhizal fungi has an impact in the transport and distribution of organic pollutants within plants (Ruytinx et al., 2020), seemingly reducing these concentrations in shoots of colonized plants, while increasing their concentrations in root system, especially in the rhizodermis (Huang et al., 2007).

AMF may have a lot of positive effects on host plants, for example, it may improve plant growth by increasing nutrients such as nitrogen and phosphorus (Zhang et al., 2020). AMF might improve the photosynthesis process (Zhang et al. 2020). It may improve the heavy metals, salinity, drought tolerance of plants (Wężowicz et al., 2015). AMF may decrease the damage caused by environmental stress through decreasing lipid peroxidation and increasing antioxidant enzymes (Zhang et al., 2010). It could affect the transportation and distribution of pollutants in plants (Schneider et al., 2013). In addition, it may improve the microbial activity of the host rhizosphere, the stability and diversity of the plant community (XU et al., 2016).

2.5.1. Factors affecting the application of AMF in CW

The prominent effect of AMF on plant development under various stress conditions, including salinity and drought in terrestrial environments, and their capacity to enhance biodegradation of organic pollutants, also, the phytoremediation of inorganic ones, is well recognized. However, in aquatic and wetland plants the diversity of AMF is still poorly studied and recognized (Calheiros et al., 2019).

Both microorganisms, bacteria and fungi, play important roles in the assimilation, transformation, and recycling of chemical constituents present in various wastewaters. In addition, mycorrhizae increase the rates and efficiency of the host plant for the absorption of nutrients from the water, soil , and air (Kadlec & Wallace, 2009).

There is a hypothesis that reduced environments with low oxygen levels might limit the survival of AMF in the roots of plants (Cooke et al, 2018). Nonetheless, Fester (2013) showed the ability of AMF to colonize roots of *Phragmites Australis* inhabiting a CW, implemented for the phytoremediation of groundwater contaminated with benzene, methyl tert-butyl ether, and ammonia.

The alternation of drying-rewetting or intermittent operation can bring oxygen into wetlands, which improved the growth, abundance, and diversity of AMF in wetland ecosystems shown in studies performed by Li et al. (2011) and Shi et al. (2015). The growth of wetland plants can be enhanced and previous studies showed that AMF colonization in continuously flooding conditions was significantly lower than in intermittent flood conditions, such as drying-rewetting cycles or fluctuating water depth (Hu et al., 2020).

2.6. Influence of N and P in wetlands vegetation

2.6.1. Nitrogen in wetland vegetation

Wetlands perform many important biogeochemical functions in watersheds. Among these are transformation of inorganic nutrients to organic forms; nutrient storage, release and removal, and sediment trapping . The N cycle in wetlands plays an important role in the transport, storage, and biological availability of N in the surrounding watershed.

Inorganic forms of N (NH_4^+ and NO_3^-) are taken up by plants rooted in the soil or floating in the water (including algae). Dead plant matter as leaves or stems fall down from the living plants and collects at the surface of the soil to form a litter layer, also known as detritus (Debusk, 1999).

Denitrification, is a process performed by microorganisms in which nitrate (NO_3) is constantly removed from ecosystems to the atmosphere as inert dinitrogen gas (N_2), can help to reduce and mitigate the effects of mineralized-nitrogen pollution. Wetland beds or sediments are predominantly important places where denitrification is performed because their anaerobic conditions favors the complete reduction of mineralized nitrogen to N_2 gas while decreasing the release of the intermediate product, NO_2, a powerful greenhouse gas (Alldred & Baines, 2016).

In quite a few cases has been demonstrated that plants are able control denitrification dynamics in sediment bed by competing for nitrate thus providing organic carbon and introducing oxygen by diffusion from roots. Plant communities can have a major effect on sediment microbial processes including denitrification. Plants vary in unique and functional features that might influence denitrification producing changes in the composition of plant communities, altering denitrification (Alldred & Baines, 2016).

2.6.2. Phosphorus in wetland vegetation

Phosphorus (P) is one of the major nutrient-limiting in many freshwater ecosystems. Wetlands are not only capable of accumulate nutrients but also alter them from biologically available forms into non-available forms and vice versa (Jakubaszek, 2020).

Retention can be considered as the ability of wetlands and streams to remove P from the water column through chemical, physical, and biological processes in order to retain it in a form that is not ready to be released under normal conditions. Retention of nutrients at wetland interfaces decreases the load to downstream aquatic systems. Dissolved inorganic P is considered bioavailable, whereas organic and particulate P forms generally must transform inorganic forms before being considered bioavailable (Reddy et al., 1999).

In wetlands, vegetation has an important role in P assimilation and storage and it depends on the vegetative type and growth characteristics. Mostly floating and emergent macrophyte roots are not in direct contact with the P transported in the stream but the submerged vegetation having a limited storage capacity can alter the physicochemical properties in the water resulting in the precipitation of Phosphorus (Reddy et al., 1999).

Emergent plants are rooted in soil, and the majority of their P requirements are usually met from soil porewater P. For example, Walton et al., (2020) found surface roots of *Typha* to be responsible for removal of added P, but there was no evidence of direct absorption from the water column.

2.7. Influence of AMF in metal removal from wastewater

The presence of AMF in constructed wetlands depends on many factors such as N and P amounts, organics, dissolved oxygen and more (Calheiros et al., 2019). The assumption that in low oxygen environments the AMF colonization survival is threatened by these conditions, but Fester (2013) found and demonstrated the ability of AMF to colonize the root parts of vegetation such as *Phragmites Australis*, present in a constructed wetland created for the phytoremediation of groundwater contaminated with organic compounds. AMF colonization was highly dependent on the solid substrate due to the lack of AMF in free-floating plants (Calheiros et al., 2019). The AMF colonization plus bacteria play an important role in the removal of metals from water but there is no much information about the mechanisms used by the AMF to improve metal removal in constructed wetlands (Xu et al., 2018). However, an important study performed by Hu et al. (2020) demonstrated the benefits and methods that AMF colonization uses to enhance the removal of metals from wastewater such as enhancing host plants resistance to different stresses provided by abiotic parameters, increases the activity of antioxidant enzymes as Catalase, Peroxidase, superoxide dismutase, also, AMF enhances photosynthesis and works for better uptake of nutrients, also, decrease the accumulation ox reactive oxygen species (ROS).

3. Methodology

3.1. Experimental setup

This study was performed using 12 lab-scale vertical subsurface flow CWs. The experimental set-up consists of PVC pipes, substrate, and a water outlet. These 12 vertical subsurface flow CW (SSVF CW) were established with the dimensions of each system is 15x 55 cm (diameter x height). Each CW was filled with 15 cm gravel (4-5 cm) and 30 cm of sand as substrate inoculated with mycorrhiza fungi. In terms of vegetation for the experiment, *Iris Pseudacorus* was selected as the wetland plant and the AMF inoculum was *Rhizophagus irregularis*.

The influencing factors of this study are: 1) presence of AMF (with AMF, without AMF), 2) heavy metal presence (with, without), 3) N:P ratios (1:1, 1:5, 1:10). Hence 12 different treatments will be set. Inlet water of CWs will simulate municipal sewage fed into each CW and the hydraulic retention time was 5 days.

These lab-scale SSVF CW in the bottom have a watertight cap, the top is exposed to allow plant growth and it is the way to introduce the simulated sewage water into the systems. Due to this opening in the top, the experimental set-up had to be covered by a roof to protect it from rainwater or any other external influencing parameters.

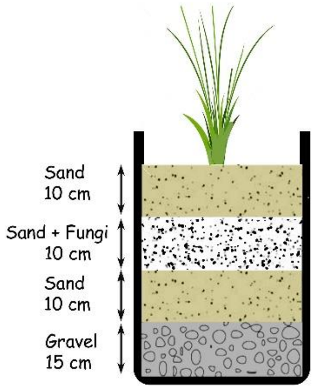
The beginning of the experiment consisted of the set-up of these experimental reactors and then waiting for the plants (*I. Pseudacorus*) to grow or show signs of proper adaptation to this environment plus the development of the symbiotic relationship with the AMF. Then the plants were watered with a nutrient solution that represents sewage water. The amount of wastewater introduced to the system was 1.5 L in each system. Then the data recollection started and it consisted of getting different volumes available inside each system in plastic bottles labeled for each one of the experimental systems. To recollect this outflow water first each plastic container was washed with the water inside of each reactor to avoid the possibility of contaminating the sample, then the samples were transported to laboratory for further analysis.

Table 1: Composition of simulated sewage water

Reagent	Concentration (mg/L)	Microelements	Concentration (mg/L)
Urea	104	$CuSO_4 \cdot 5H_2O$ Copper sulfate pentahydrate	0.01
NH_4Cl Ammonium Chloride	16	$FeSO_4 \cdot 7H_2O$ Iron(II) Sulfate Heptahydrate	0.45
$CH_3COONa \cdot 3H_2O$ Sodium Acetate	255	$MnSO_4 \cdot H_2O$ Manganese sulfate monohydrate	0.02
Peptone	20	$Pb(NO_3)_2$ Lead(II) nitrate	0.02
KH_2PO_4 Potassium dihydrogen phosphate	41	H_3BO_3 Boric acid	0.04
Yeast extract	132	$Na_2MoO_4 \cdot 2H_2O$ Sodium Molybdate Dihydrate	0.02
Skim milk	59	$KCr(SO_4)_2 \cdot 12H_2O$ Chromium Potassium Sulfate Dodecahydrate	0.02
$NaHCO_3$ Sodium bicarbonate	25		
$MgSO_4 \cdot 7H_2O$ Magnesium Sulfate Heptahydrate	41		
$CaCl_2 \cdot 6H_2O$ Calcium Chloride Hexahydrate	28		

Source: Author

Figure 4: Small scale vertical subsurface flow constructed wetland experiment



Source: Author

3.2. Sample analysis

3.2.1. AMF Colonization Assessment

This process starts with the root preparation where the roots were washed to remove any soil particle and then they are cut into 1cm long segments.

The second step is the Trypan blue staining of total mycelium according to the Phillips and Hayman, 1970 process. This step starts with clear roots in 10% (w/v) KOH for 1h 90°C in a water bath or oven, another option is to put the samples for 15 min at 120° C in a pressure cooker.

After this step the roots are rinsed with water three times on a fine sieve or using a mesh and forceps, then the roots are covered with 2% (v/v) HCl for 5 minutes and after this time passes the HCl is discarded and the roots are covered with 0.05% trypan blue in lactoglycerol that consists in one part lactic acid, one part of glycerol and one part of water, for 30 minutes at 90°C in an oven or even a water bath. The roots are then placed into a Petri dish with the lactoglycerol mix for further analysis under the stereomicroscope.

For the estimation of Arbuscular Mycorrhizal Fungi, the test was based on the method of Trouvelot et al, 1986. The root fragments, if possible 15 of them, are mounted into one slide. The ideal procedure recommends having two slides with 30 root fragments in total.

The samples are then analyzed under the microscope and rated according to the range of classes and the AM form. This range of classes provides a quick estimation of the level of colonization of mycorrhiza on each root fragment and also provides information on the abundance of arbuscular.

With this information, the usage of the software “Mycocale” allows the calculation of the parameters **%F** frequency of mycorrhization that is calculated as the number of mycorrhiza fragments divided by the total number of fragments observed, **%M** is the intensity of mycorrhization and this value shows the best grade of mycorrhization, **%m** represents the Intensity of mycorrhization of mycorrhizae fragments, **%a** is the shrub intensity of the mycorrhizae part and lastly the **%A** that is the shrub intensity in the root system.

3.2.2. Water, soil and plant sample analysis

For the water samples analysis, there are different parameters of interest such as pH, Rh, NH_4^+, Total Organic Carbon (TOC), Total Nitrogen (TN), PO_4^{3-}, NO_2^-, NO_3^-, copper (Cu) and lead (Pb).

In terms of pH and ORP (oxidation/reduction potential) analysis was made in the laboratory with the device Multi 3430 (WTW). After calibrated, this device was connected to a probe that was introduced into each sample to get the results. After each sample was analyzed the probe was cleaned with deionized water to eradicate any contamination of the sample and to reduce the risk of misinterpretation or error.

For the Ammonia (NH_4^+) ions the methodology of Indophenol was followed and consists of the preparation of two solutions, one alkaline solution and one dyeing solution. The alkaline solution is made of 16g of NaOH (sodium hydroxide) that is dissolved in 250 mL of deionized water plus 1g of sodium dichloroisocyanurate dihydrate ($C_3N_3O_3CL_2Na_2H_2O$) that was added after the solution reached room temperature following a period of incubation. After this dissolution, the solution is stored in a dark glass container inside the fridge to preserve its characteristics.

In the case of the dyeing solution preparation, the procedure is to dissolve 65 g of sodium salicylate and 65 g of trisodic citrate dihydrate ($Na_3C_6H_5O_7\ 2H_2O$) in a 500 ml graduated flask then add 0.475 g of sodium nitroprusside. After complete dissolution, complete up to a volume of 500 ml. Keep in a dark bottle in the fridge to preserve its characteristics, same as the alkaline solution.

Having these two solutions and the samples the procedure continues by using 10 mL of the water sample filtered with a 45 µm filter + 2 mL of the dyeing solution and 2 mL of the alkaline solution. To this, some deionized water was added to complete 25 mL of the prepared sample for further analysis in the Agilent Technologies Cary 60 UV-Vis spectrophotometer with a wavelength of 655 nm. The blank samples were just tapped water as comparison samples.

Total organic carbon (TOC) and total nitrogen (TN) were measured by the Primacs SERIES TOC analyzer (Skalar, Dutch). This device provides accurate analysis because it contains a large autosampler that can analyze large batches of samples. These samples are introduced vertically into the analyzer and then exposed to high temperatures ranging from 750-950°C in the reactor where all the bonded nitrogen is converted into NO (Nitric Oxide) and the total inorganic Carbon is oxidized and converted to CO_2. The machine then catches these particles and they are directed into a detector that uses infrared light to measure. The total organic carbon is measured by subtracting the inorganic carbon from the total carbon.

The PO_4^{3-}, NO_2^-, NO_3^- analysis was conducted using the 883 Basic IC plus (Metrohm, Switzerland) that is used to identify ions by separating them based on their charge, with this technique is possible to measure the concentration of a specific ion in water samples. The device works first pumping a small volume of the sample, through a column of packed particles and the retention time is recorded as the time it takes for the ion to pass through this column.

The metals of interest for this study, Cu and Pb in water, roots, shoots, and substrate were analyzed by the ICP-MS method (Inductively Coupled Plasma Mass Spectrometry).

In the case of the plant samples and substrate analysis, the process starts with the digestion of the samples in HNO3/HCl (1/3 v/v) at 210°C that takes place on a heating plate for 12 h. The samples were diluted to 50 mL with deionized water and then filtered through a 0.22 µm filter. When this process of digestion is finished the samples are placed in centrifuge tubes and adjusted to 45 mL with deionized water from the Milli-Q® system.

For the analysis of Total Carbon (TC) and Total Nitrogen (TN) in plants, the dry plant samples were analyzed by the Primacs^{SN} analyzer (Skalar, Dutch).

4. Results

4.1. AMF Colonization

The analysis of the AMF colonization provides two results, the first one was the M(%) or the intensity of the mycorrhiza colonization in the root system. These results show that the highest M(%) was found in the M_P1 with a value of 43.89%, this resulting from the first set of experimental systems that were not treated with metals but there is a difference in the values from the first and the third set of experimental systems M_P3, this could be caused by differences in the conditions of that exact environment or during the inoculation probably there was a slight variance in the amount of mycorrhiza or it could be caused by a high concentration of P in this CW. Comparing the first two sets of systems the values were relatively similar providing a base for the understanding and comparison with the systems that were treated with metal-polluted waters during their cycle.

Taking into account these values and differences the results clearly show a reduction of 38.82% in the M(%) for the first set of systems treated with wastewater polluted with metals and other compounds previously mentioned above in Table 1, for the second set of systems the difference in the M(%) was 39.99% and for the third set of systems the reduction was 42.31% the most considerable one. Knowing these results is possible to say that the presence of different pollutant compounds in the waters affects the intensity of the mycorrhiza colonization in the root system.

Table 2: AMF Colonization intensity in root system, mean values with standard deviation

M (%) = Intensity of the Mycorrhiza colonization in root system					
M_P1	**M_P2**	**M_P3**	**M_P1_HM**	**M_P2_HM**	**M_P3_HM**
43.89±2.04	41.9±1.91	32.1±3.51	26.85±1.22	25.14±2.08	18.51±0.76

Source: Author

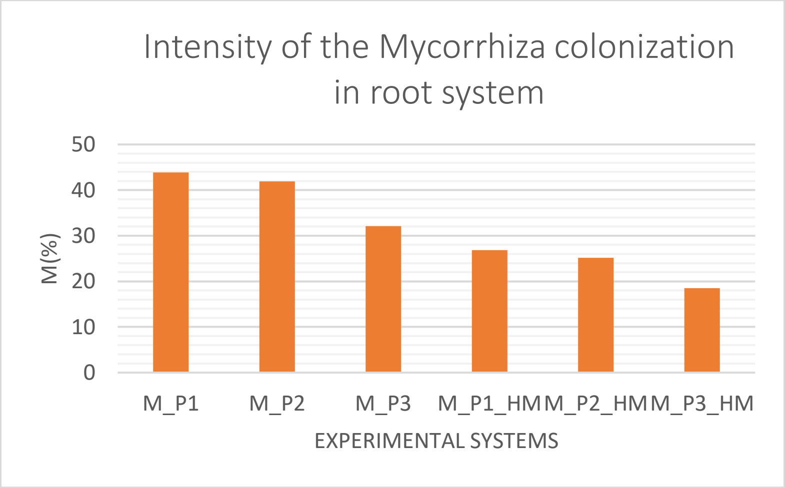
The A(%) or Arbuscule abundance in the root system showed a behavior similar to the M(%) where the first two sets of experimental systems had a higher percentage value compared to the third one. In this case, the A(%) from the first set M_P1 of experimental SSVF CW presented a higher value than any other system with 10.36%, the second with 6.3% and the third system registered 3.78%. The differences between these values were repeated in the results from the systems that were treated with the simulated sewage water. The presence of pollutant particles of Cu, Pb and others, affected the % of arbuscule abundance in the root system. The difference in the A(%) from the non-treated system 1 to the treated system M_P1_HM was 59.12%, M_P2 to M_P2_HM was 46.22% and 54.64% for the last set of treated systems.

Table 3: Arbuscule abundance in the root system

A (%)= Arbuscule abundance in the root system					
M_P1	**M_P2**	**M_P3**	**M_P1_HM**	**M_P2_HM**	**M_P3_HM**
10.36±1.90	6.3±1.67	3.78±0.40	4.24±0.87	3.39±1.19	1.71±0.93

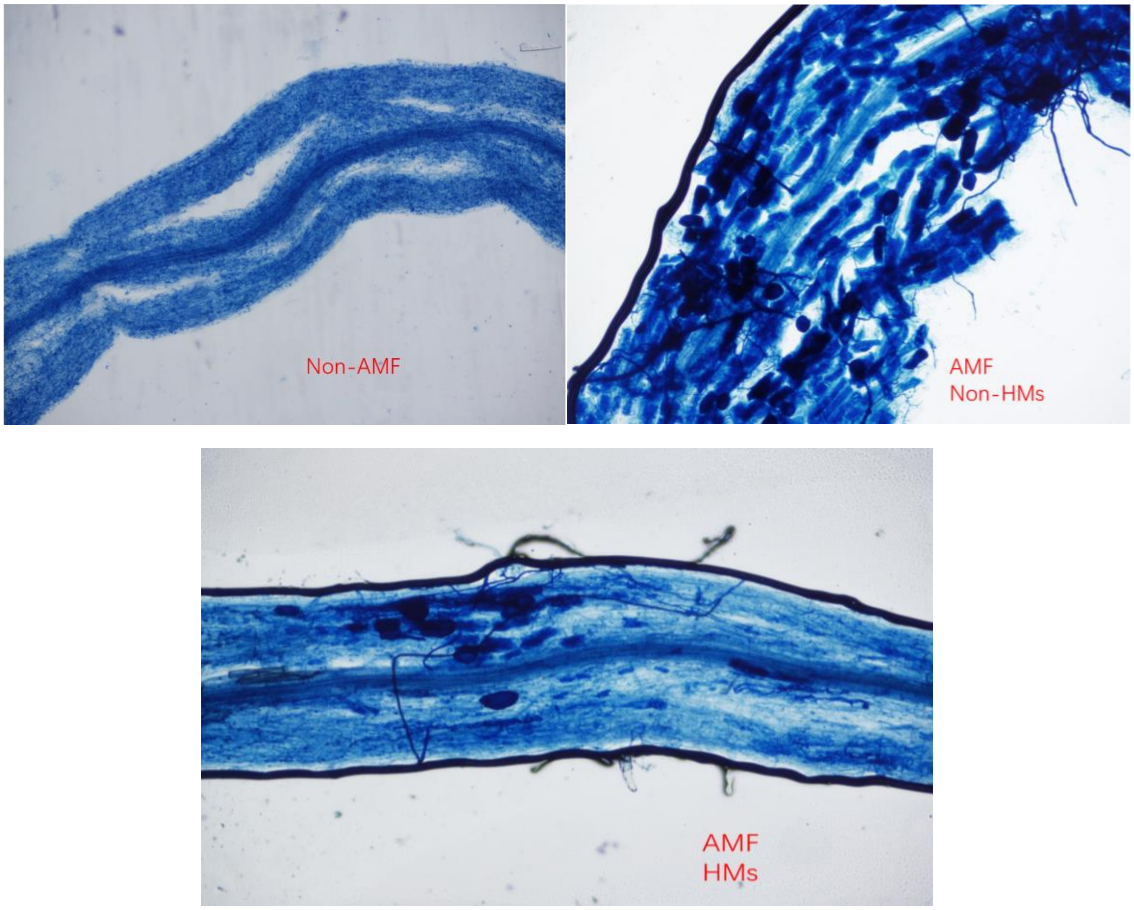
Source: Author

Plot 1: M(%) Intensity of AMF colonization



Source: Author

Figure 5: AMF Colonization



Source: Author

The above presented images are the final result of the dyeing process to analyze the AMF colonization. The first image presents the root segment without any inoculation of AMF, the second image shows the root segment with the AMF inoculated represented by this conglomeration of oval-shaped spots, this fungus is attached to the roots in a symbiotic relationship. The third image in the bottom part shows the presence of the AMF attached to the root segment but in a less amount, this due to the addition of water mixed with different compounds that represent sewage water where there is a concentration of Cu and Pb particles that changes the properties of the environment reducing the number of mycorrhizas.

4.2. Metal concentration distribution in SSVF CW

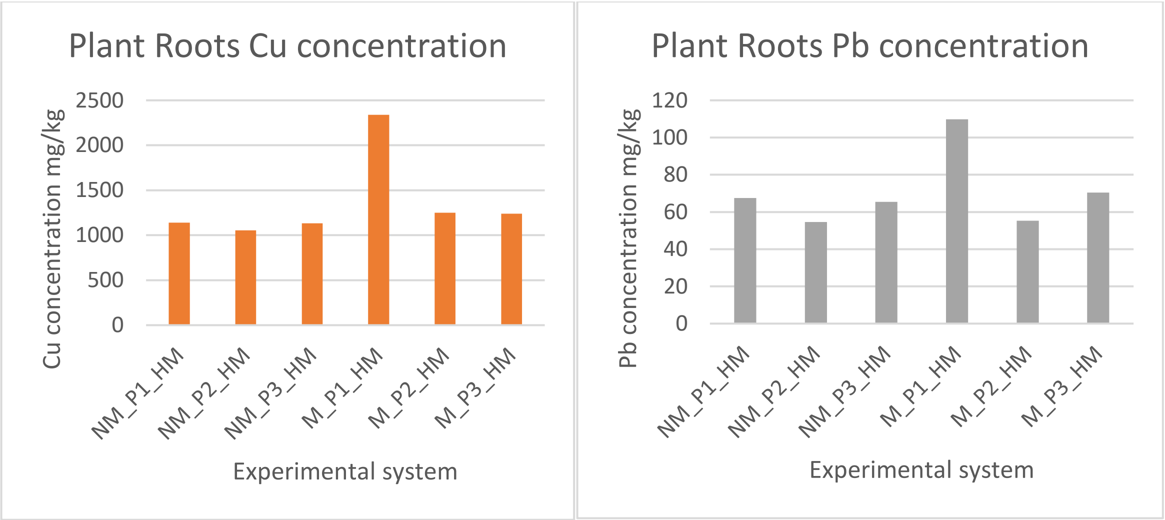
This analysis was made by the assessment of the concentration of metals in different parts of the experimental SSVF CW, the substrate, plant roots and shoots in order to understand how these pollutant particles are distributed in the systems.

Table 4: Concentrations of Cu and Pb in the plant's roots

	Experimental System	Cu (mg/kg)	Pb (mg/kg)
Plant root	NM_P1_HM	1140.72	67.43
	NM_P2_HM	1054.89	54.63
	NM_P3_HM	1131.95	65.48
	M_P1_HM	2339.53	109.78
	M_P2_HM	1249.60	55.21
	M_P3_HM	1238.45	70.46

Source: Author

Plot 2: Cu and Pb concentration in roots



Source: Author

The results shown in Table 4 where NM_P1_HM means No Micorrhiza_P1_Heavy Metal treated water and M_P1_HM means Mycorrhiza inoculated system with heavy metal polluted water, so, thanks to this information is possible to understand that in the systems with the presence of the mycorrhiza there is a slight increase in the concentration of both metals of interest Cu and Pb. This can be considered as a way to enhance the plant uptake of compounds from water and soil demonstrating that the AMF inoculated plants have a better performance in terms of uptake rates than the plants without this symbiotic relationship.

The difference in the concentration of Cu from the plant root in the first system without mycorrhiza and with mycorrhiza was 1198.82 mg/kg that represents an increase in the concentration of Cu in the root with mycorrhiza of 51.24% that is a considerable amount compared to the other two systems, that also presented an increase in the concentration of Cu due to the presence of mycorrhiza with 194.71 mg/kg and 106.50 mg/kg or an increase of 15.50% and 8.60% respectively.

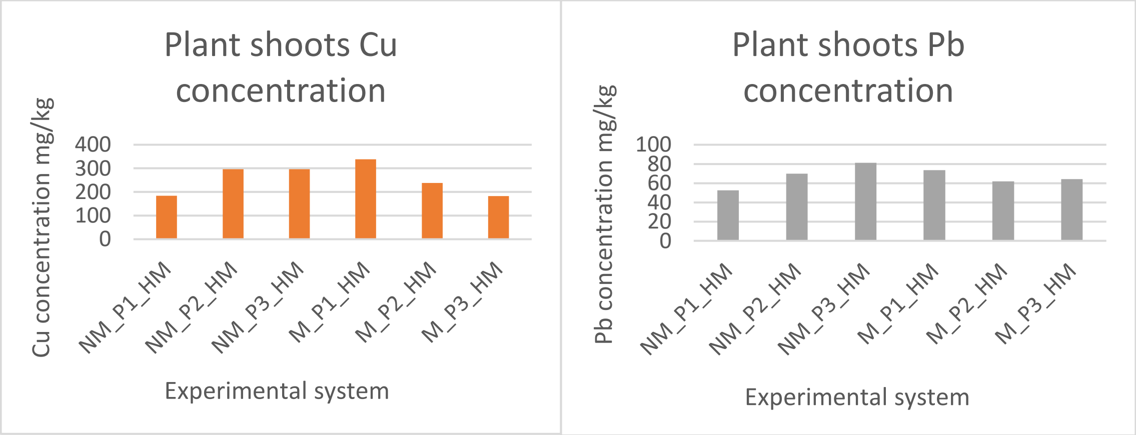
The Pb differences have a similar behavior where the first system with the presence of mycorrhizas had an increase of 42.38 mg/kg or 38.57% compared to the one without this fungus. The same happened with the other systems but the difference was not as high showing an increase of 0.57 mg/kg and 4.98 mg/kg or in terms of percentage this means a 1.04% and a 7.07% of increase respectively for systems P2 and P3.

Table 5: Concentration of Cu and Pb in plant shoots

	Experimental System	**Cu**	**Pb**
Plant shoot (mg/kg)	NM_P1_HM	183.48	52.48
	NM_P2_HM	296.69	69.83
	NM_P3_HM	296.56	81.06
	M_P1_HM	337.93	73.42
	M_P2_HM	238.78	62.03
	M_P3_HM	182.06	64.06

Source: Author

Plot 3: Cu and Pb concentration in plant shoots



Source: Author

The behavior of the concentrations of Cu and Pb in the plant shoots was similar just for the first system where there was a considerable difference in the concentrations due to the presence of mycorrhizas resulting in a difference of 154.45 mg/kg for Cu and 20.94 mg/kg for Pb or what it represents a difference of 45.7% and 28.52% respectively for Cu and Pb.

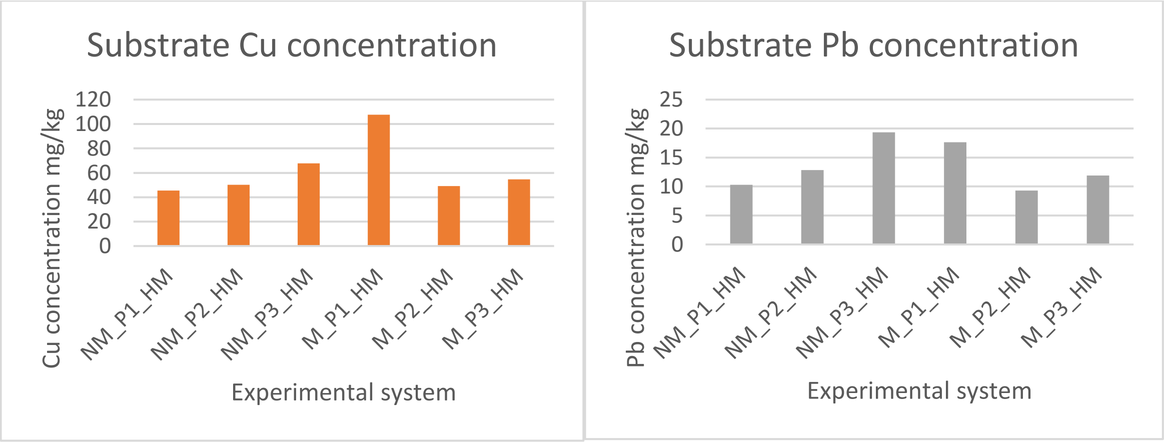
For the second and third experimental systems the behavior was completely different, instead of experiencing an increase in the concentration of Cu and Pb in the plant shoots there was a decrease in the systems with mycorrhiza. For Cu in the M_P2_HM there is 57.90 mg/kg or 19.51% less concentration than in the system without mycorrhiza, same talking about Pb where there was a decrease of 7.8 mg/kg or 11.17%. The third system M_P3_HM presented similar results with a decrease in the concentration of Cu and Pb in plant shoots of 114.49 mg/kg or 38.60% and 16.99 mg/kg or 20.90% for Cu and Pb respectively. This behavior shows that the metals in the plant travel more freely when they are not inoculated with mycorrhizas and that this fungus retains somehow a part of these pollutant particles.

Table 6: Cu and Pb concentration in substrate

	Experimental System	**Cu**	**Pb**
Substrate (mg/kg)	NM_P1_HM	45.51	10.27
	NM_P2_HM	50.18	12.85
	NM_P3_HM	67.70	19.34
	M_P1_HM	107.73	17.61
	M_P2_HM	49.02	9.28
	M_P3_HM	54.68	11.90

Source: Author

Plot 4: Cu and Pb concentrations in substrate



Source: Author

About the analysis of the concentrations of Cu and Pb in the substrate, the behavior of these pollutant particles was similar than to the shoots analysis where the M_P1_HM system presented a higher concentration of both Cu and Pb but for M_P2_HM and M_P3_HM the concentrations were lower than in the no mycorrhiza presence that can be considered as the difference of the pollutant particles that were retained by the plant-AMF association.

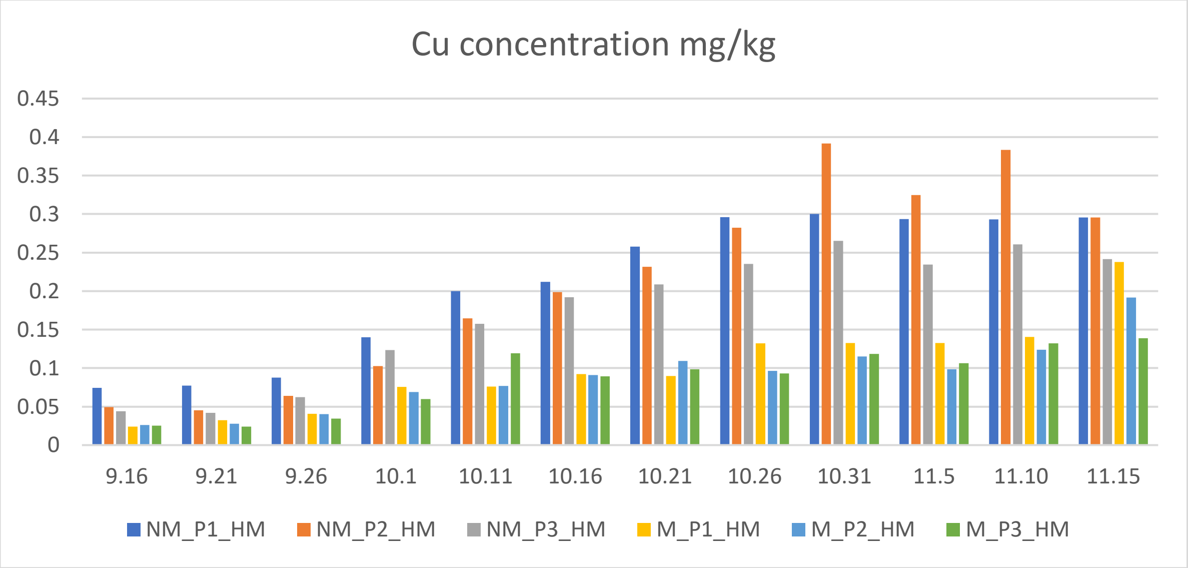
4.3. Removal of Cu and Pb from wastewater

This analysis started on September 16th and went until November 15th and it consisted of the addition of wastewater simulating sewage wastewater and through this method, it was possible to monitor the inlet concentration of the pollutant particles and the outflow concentration of metals and other compounds, so, every 5 days the outflow water was taken from the systems and then analyzed.

It is worth mentioning that every day that the systems were emptied from the water they have then filled again with the same 1.5 L described in the methodology section, this wastewater that was introduced into the systems was also monitored to know the initial concentration of Cu and Pb to compare these concentrations from inflow to outflow water through the systems.

4.3.1. Cu particles removal in SSVF CW

Plot 5: Cu concentration in experimental systems



Source: Author

Table 7: Mean concentration of Cu in the systems and in inflow wastewater

Mean Cu concentration (mg/L)						
Inflow	**NM_P1_HM**	**NM_P2_HM**	**NM_P3_HM**	**M_P1_HM**	**M_P2_HM**	**M_P3_HM**
25.35±1.545	0.21±0.093	0.21±0.127	0.17±0.085	0.10±0.060	0.09±0.046	0.09±0.041

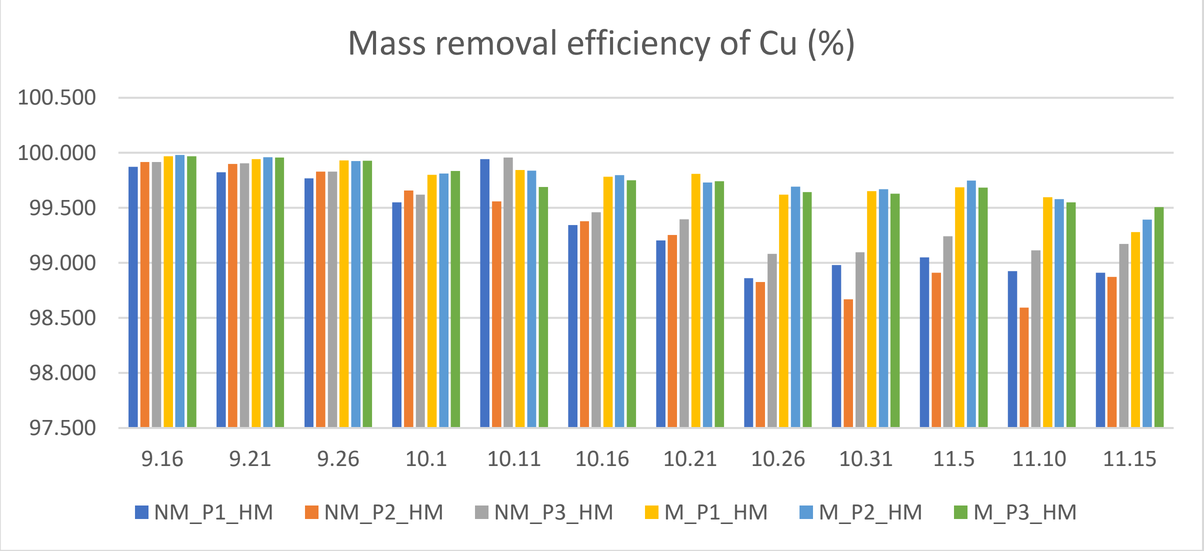
Source: Author

The results from the Cu concentration in the systems showed that the highest concentrations were registered in the systems that did not have any presence of AMF also from these NM_Px_HM systems at the beginning of the experiment until October, NM_P1_HM presented the highest concentration of Cu but from the samples taken at the end of October until the end of the experiment or November 15 the NM_P2_HM presented the highest values.

The systems that had the AMF and plant symbiotic relationship presented a similar behavior between each other through the whole experiment except for the last sample taken on November 15 where the values had a considerable difference. The samples were registering higher values and this behavior can be influenced by the temperatures or other external parameters.

Due to the different volumes obtained as outflow in each sampling day, it was necessary to calculate the mass removal efficiency by multiplying the inflow concentration by the volume introduced to the system in this case 1.5L then from this value the multiplication of the concentration in each system timed the outflow volume, was subtracted. This value is multiplicated by 100 and then divided by the inflow concentration times the inflow volume (1.5 L).

Plot 6: Cu mass removal efficiency (%)



Source: Author

Table 8: Mean Cu removal efficiency (%) in each system

Mean Cu removal (%)					
NM_P1_HM	**NM_P2_HM**	**NM_P3_HM**	**M_P1_HM**	**M_P2_HM**	**M_P3_HM**
99.87±0.07	99.90±0.05	99.89±0.05	99.93±0.03	99.92±0.04	99.91±0.06

Source: Author

Having the mass removal efficiency calculated is possible to assess the efficiency of Cu removal from wastewater because from the initial concentration of the inflowing wastewater these particles were included in the uptake by the plants. In this research, the efficiency results were high ranging from 98.7% to 99.97% showing favorable results.

In terms of efficiency in most of the cases, the systems that presented mycorrhizas were more efficient compared to the ones without it, this behavior was constant during all the sample analysis except for the one made on October 11th because it shows that the systems NM_P1_HM and NM_P3_HM had a higher removal efficiency than any other, even more than the systems with mycorrhizas. From the samples taken on October 16th until the end of the experiment the difference in the efficiency of NM_Px_HM and M_Px_HM is more noticeable but in general terms with such high efficiency of removal this difference of 0.5% is not considerable, only if the comparison is made between no mycorrhiza and the systems with the presence of this fungus.

4.3.2. Pb particles removal in SSVF CW

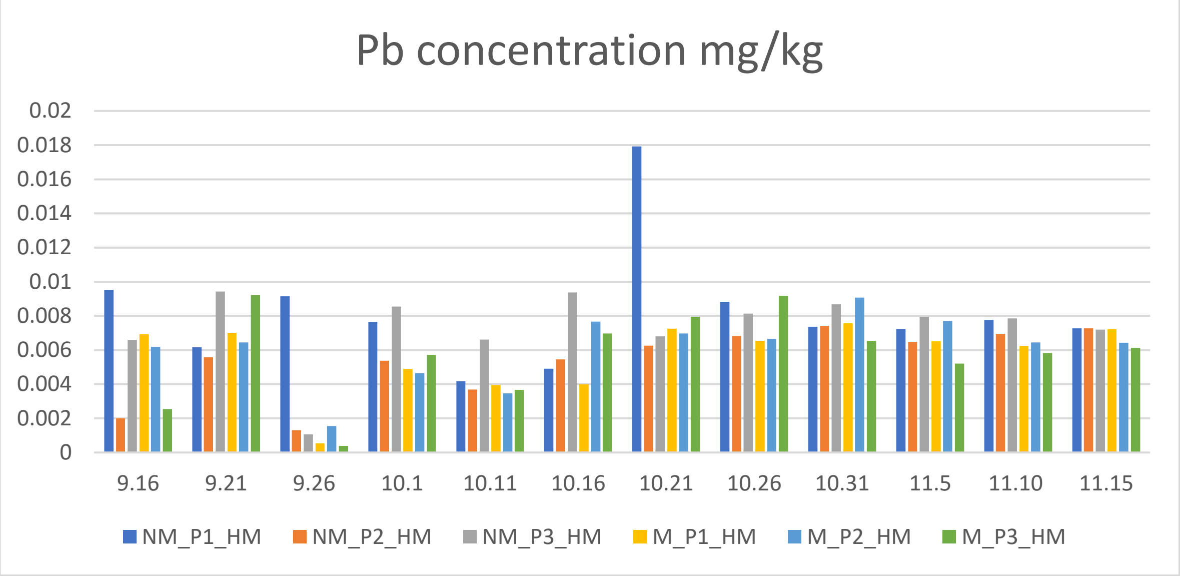
Table 9: Pb concentration in each system and in inflow wastewater

Pb concentration (mg/L)						
Inflow	**NM_P1_HM**	**NM_P2_HM**	**NM_P3_HM**	**M_P1_HM**	**M_P2_HM**	**M_P3_HM**
4.85±0.59	0.0082±0.003	0.0054±0.002	0.0074±0.002	0.0057±0.002	0.0061±0.002	0.0058±0.002

Source: Author

In Table 9 mean values of inflow wastewater and mean values of Pb concentration in each experimental SSVF CW are expressed with each respective standard deviation of the data and a considerable difference is shown between the concentration of Pb in the inflow water and the concentration of Pb in the outflow water from each system.

Plot 7: Pb concentration in experimental SSVF CW in mg/kg



Source: Author

In this analysis of the concentration of Pb in the outflow water on each system, the results are more varied compared to the ones of the Cu concentration, in this case, there are different interesting data such as the first half of the samples, the ones that were taken from September 16th to October 16th had a strange behavior showing in some cases higher concentrations of Pb in outflow water in the systems which had mycorrhizas for example in the sample taken in September 21st where the M_P1_HM and M_P2_HM presented higher concentration than their counterpart systems without mycorrhiza presence. Something similar occurred in samples taken on September 26th and on October 16th.

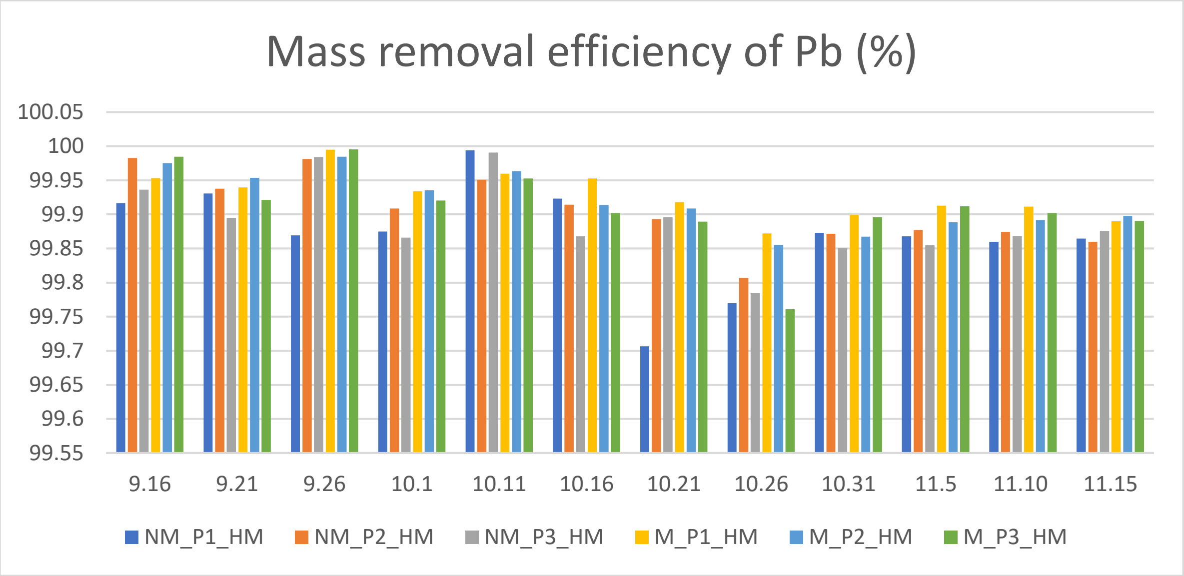
From the samples taken on October 21st the sample corresponding to NM_P1_HM had an odd behavior with the most concentration of Pb in the whole experiment and also the samples of outflow wastewater presented the behavior in which the systems treated with mycorrhizas had a higher concentration than the ones without it. This behavior was repeated throughout the second half of the samples taken and analyzed. This performance of the systems comparing it to the Cu ones had a different distribution which allows us to infer that both pollutant metals behave differently in these environments and is influenced by the conditions in each experimental system.

Table 10: Pb removal efficiency mean values and standard deviation

Pb removal efficiency (%)					
NM_P1_HM	**NM_P2_HM**	**NM_P3_HM**	**M_P1_HM**	**M_P2_HM**	**M_P3_HM**
99.87±0.075	99.90±0.052	99.89±0.058	99.92±0.034	99.92±0.042	99.91±0.059

Source: Author

Table 11: Pb removal efficiency in %



Source: Author

Regarding the efficiency data of Pb mass removal, the lowest value was 99.7% and the highest was 99.99% showing that de difference is small and that there is a positive result in the treatment of wastewater polluted with metals in this case Cu and Pb. The distribution of this data had a bigger variance but it is not too considerable because it is just a difference of 0.2% and the efficiency of the removal was more than 99%.

In most of the samples taken the highest efficiency can be found in the systems with mycorrhizas but there are different examples where the result is different such as the NM_P1_HM and NM_P3_HM samples taken on October 10th and showing a similar performance, the system of NM_P2_HM from the analysis of the sample taken on September 16th had a higher efficiency that its counterpart even without the presence of AMF.

The efficiency of mass removal of pollutant metals from wastewater using SSVF CW was considerably high in the ranges higher than 95%, indeed they were more in the 99% range, this could also be thanks to the AMF symbiotic relationship and this though is based in the higher efficiency of removal showed by these systems even considering the values that were not following this behavior.

In both analysis of Cu and Pb mass removal, it was found that the systems with AMF colonization had a better performance compared to the experimental systems without any fungus presence, these results are more consistent in the Cu analysis because from all the samples taken throughout the experiment there was just only one that presented a different behavior.

In terms of Pb removal analysis the majority of the sample analysis the AMF inoculated systems had a higher efficiency in the removal, however, more samples expressed different information showing that the systems without AMF had a higher removal efficiency of Pb compared to the ones with mycorrhizas.

Taking into account the small variances, this information in terms of removal of metals from wastewater using subsurface vertical flow constructed wetlands is not considerable due to the positive results showing that the AMF influences the efficiency of the treatment systems.

4.4. Water parameters analysis

Besides the previous analysis of each sample, other parameters were also measured in order to control or have a record of all the characteristics of the water both in inlet and outflow wastewater, parameters such as pH, ORP, Ammonium-N, TN, TOC, Nitrate-N, TC and Phosphate.

4.4.1. pH

The data related to the pH shows that all the water samples taken registered pH values ranging from 7.05 to 7.32 with the minimum being the sample corresponding to the system M_P2 with mycorrhizas but without heavy metals in its inlet water and the maximum pH value registered were the sample corresponding to NM_P2_HM that means the system without mycorrhizas and with inlet water polluted with Cu and Pb.

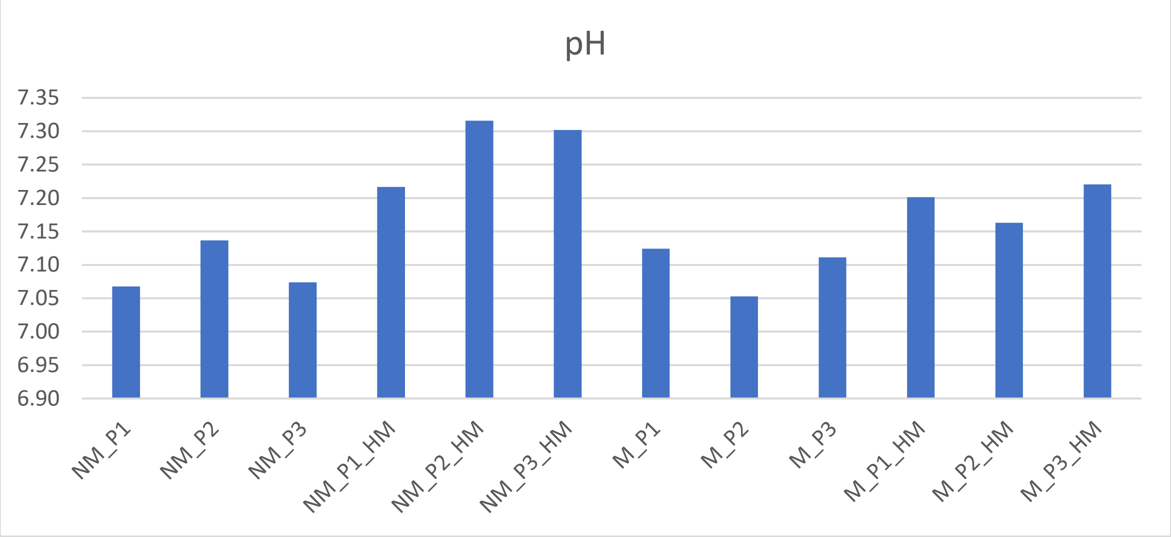
Even with all the compounds described in Table 1 the pH in all systems was somehow constant just showing a variance of 0.27 in the pH levels being a parameter that didn’t influence considerably the reactions inside each system allowing the normal behavior of particles and microorganisms.

Table 12: pH in outflow water from each experimental system

pH					
NM_P1	**NM_P2**	**NM_P3**	**NM_P1_HM**	**NM_P2_HM**	**NM_P3_HM**
7.07±0.18	7.14±0.03	7.07±0.11	7.22±0.10	7.32±0.14	7.30±0.08
M_P1	**M_P2**	**M_P3**	**M_P1_HM**	**M_P2_HM**	**M_P3_HM**
7.12±0.07	7.05±0.11	7.11±0.09	7.20±0.05	7.16±0.06	7.22±0.07

Source: Author

Plot 8: pH from outflow water



Source: Author

4.4.2. Oxidation-Reduction Potential

Table 13: ORP mean values from all experimental systems

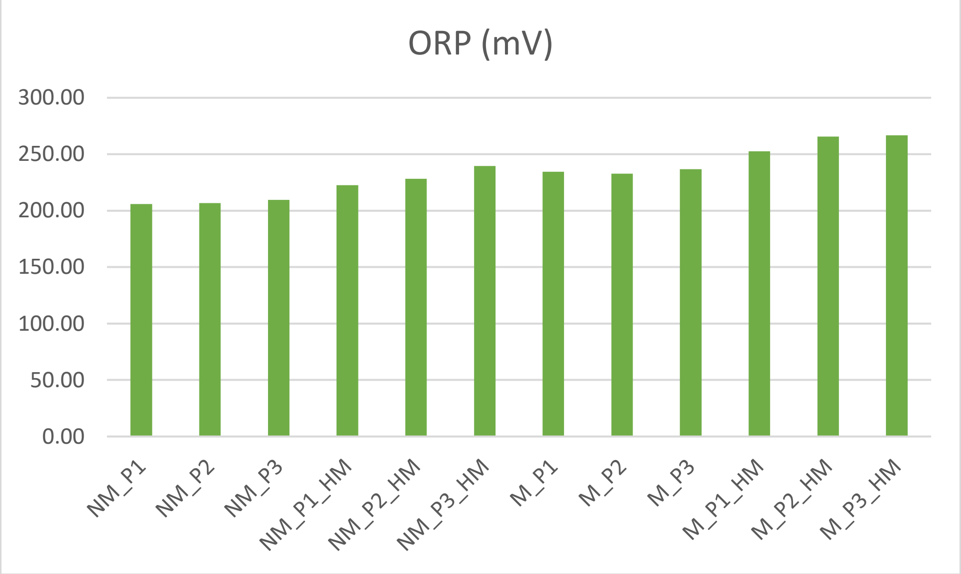
Oxidation-Reduction Potential (mV)					
NM_P1	**NM_P2**	**NM_P3**	**NM_P1_HM**	**NM_P2_HM**	**NM_P3_HM**
205.77±33.5	206.82±7.41	209.51±22.59	222.51±21.08	228.18+24.27	239.64±28.22
M_P1	**M_P2**	**M_P3**	**M_P1_HM**	**M_P2_HM**	**M_P3_HM**
234.52±28.49	232.85±26.52	236.66±37.40	252.56±36.30	265.59±37.85	266.8±42.31

Source: Author

The ORP represents the capacity of a solution for electron transfer this means that there are oxidation or reduction chemical reactions and one cannot occur without the other one. It is measured in millivolts when an inactive chemically electrode made out of metal is introduced into a solution where a redox reaction is happening, an electric measurement appears in the electrode and this is called ORP or redox potential (Banhidi, 2001).

This measurement could take up to several minutes if not hours because the equilibrium needs to be reached and there could be different types of reactions at different rates and this behavior is influenced by the metal surface condition and it can differ from electrode to electrode (Zhang & Lin, 2020).

Plot 9: ORP mean values for each experimental system



Source: Author

In this research, the ORP or Redox potential or just Eh was measured in all the samples taken from September 8th to November 15th obtaining the results shown in table 13 where the highest value of 266.80 mV was registered in the samples taken from the system with the presence of mycorrhizas and treated with the wastewater with heavy metals (M_P3_HM) and the lowest value of 205.77 mV registered in the mean of the set of samples from the experimental system without any AMF colonization and heavy metals in its water outflow NM_P1. The results of the ORP measurement were consistent only fluctuating for 61.03 mV but considering the activity of the pollutant particles, the highest values registered were from the systems with mycorrhizas and treated with heavy metal polluted wastewater, this is the proof of the activity of all the chemical reactions inside these systems.

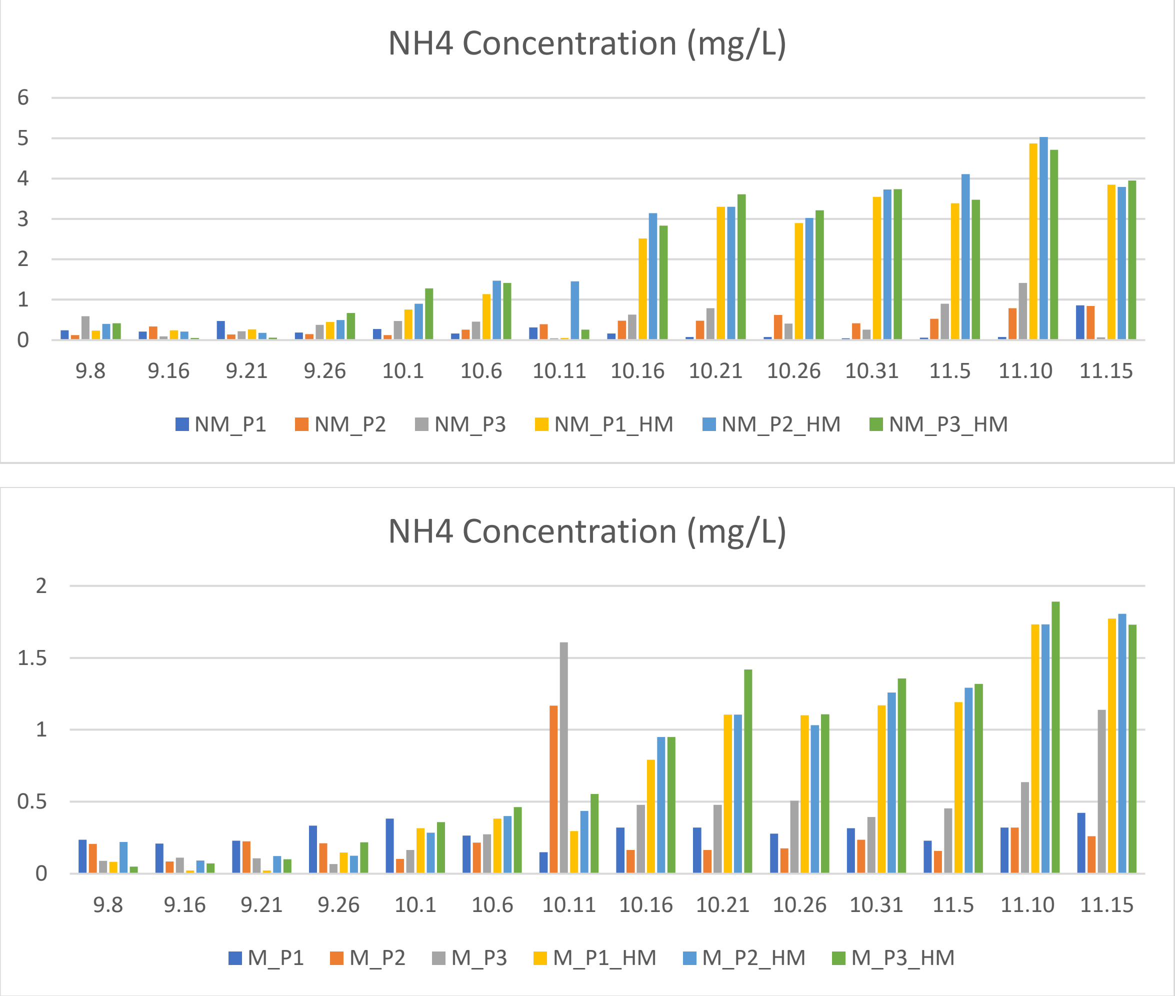
4.4.3. Ammonium- N (NH_4^+) Removal

Table 14: NH4 mean concentration values (mg/L)

NH4 mean concentrations					
NM_P1	**NM_P2**	**NM_P3**	**NM_P1_HM**	**NM_P2_HM**	**NM_P3_HM**
0.23±0.22	0.41±0.24	0.48±0.38	1.97±1.67	2.23±1.67	2.12±1.68
M_P1	**M_P2**	**M_P3**	**M_P1_HM**	**M_P2_HM**	**M_P3_HM**
0.29±0.07	0.26±0.27	0.46±0.44	0.72±0.62	0.78±0.61	0.83±0.65

Source: Author

Plot 10: Ammonium concentrations (mg/L)



Source: Author

From the results shown above in Plot 10 is possible to infer that the samples that presented a higher amount of ammonium were the ones taken from October 16th to November 15th, also, from these results the samples with a higher concentration of ammonia were the ones without mycorrhizas compared to the experimental systems with mycorrhizas. The behavior of these data in this comparison between systems with AMF and without it demonstrates that the addition of the simulated sewage wastewater had an important impact in the concentrations of NH_4^+ where the values were at least twice as high as the results of the systems without wastewater inflow.

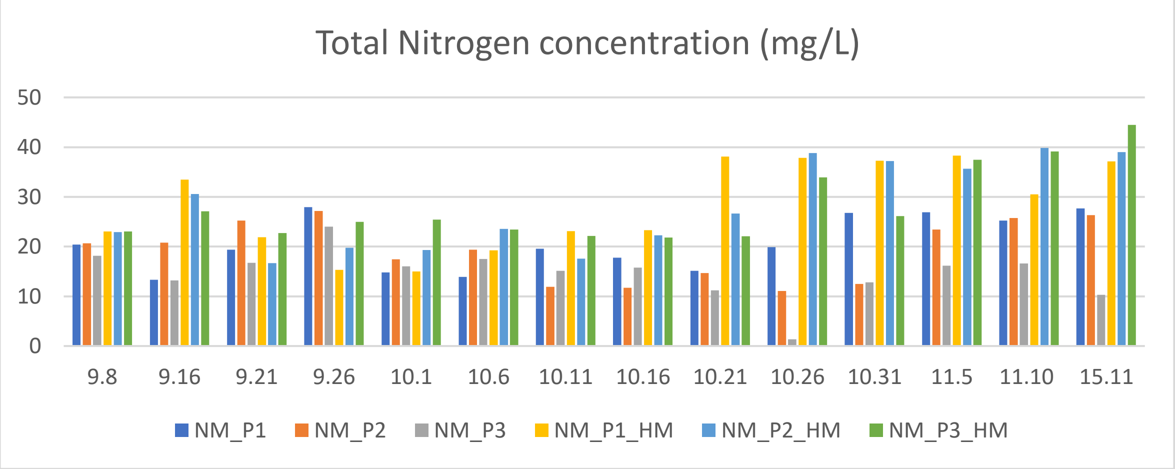
The mycorrhiza presence reduced the concentration of ammonium where the highest concentration was 1.89 mg/L related to the M_P3_HM system this means the system with mycorrhizas, the third treatment of phosphorous or with a proportion of 1:10 in N:P rates and treated with wastewater that simulates sewage water. The highest value from the experimental systems without mycorrhizas registered a value of 5.03 mg/L corresponding to NM_P2_HM or the systems that didn't have mycorrhiza presence, treated with the second phosphorus treatment (1:5 in terms of N:P ratio) and with polluted water in it. The difference was 3.14 mg/L that is a considerable fluctuation in the efficiency of the systems.

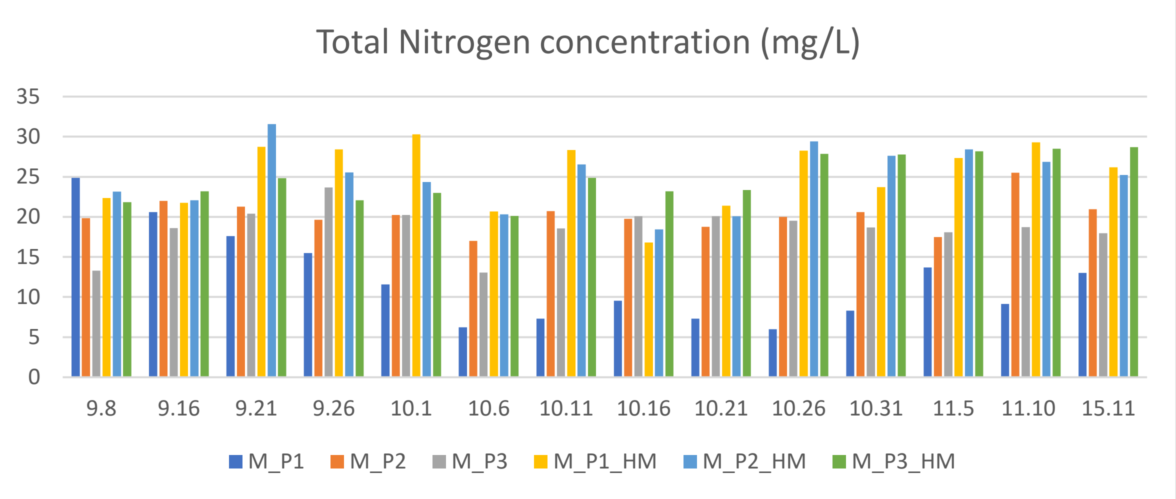
The values that behaved differently such as the M_P3 and M_P2 had an unexpected value even being systems that were not treated with the polluted wastewater, this can be caused by different factors in the sampling process or in the laboratory analysis. The removal efficiency was above 90% in most of the cases but there were measurements of low efficiencies under 20%.

4.4.4. Total Nitrogen

The total Nitrogen was measured in each sample during the whole experiment and the results were the following

Plot 11: Total Nitrogen concentration in mg/L



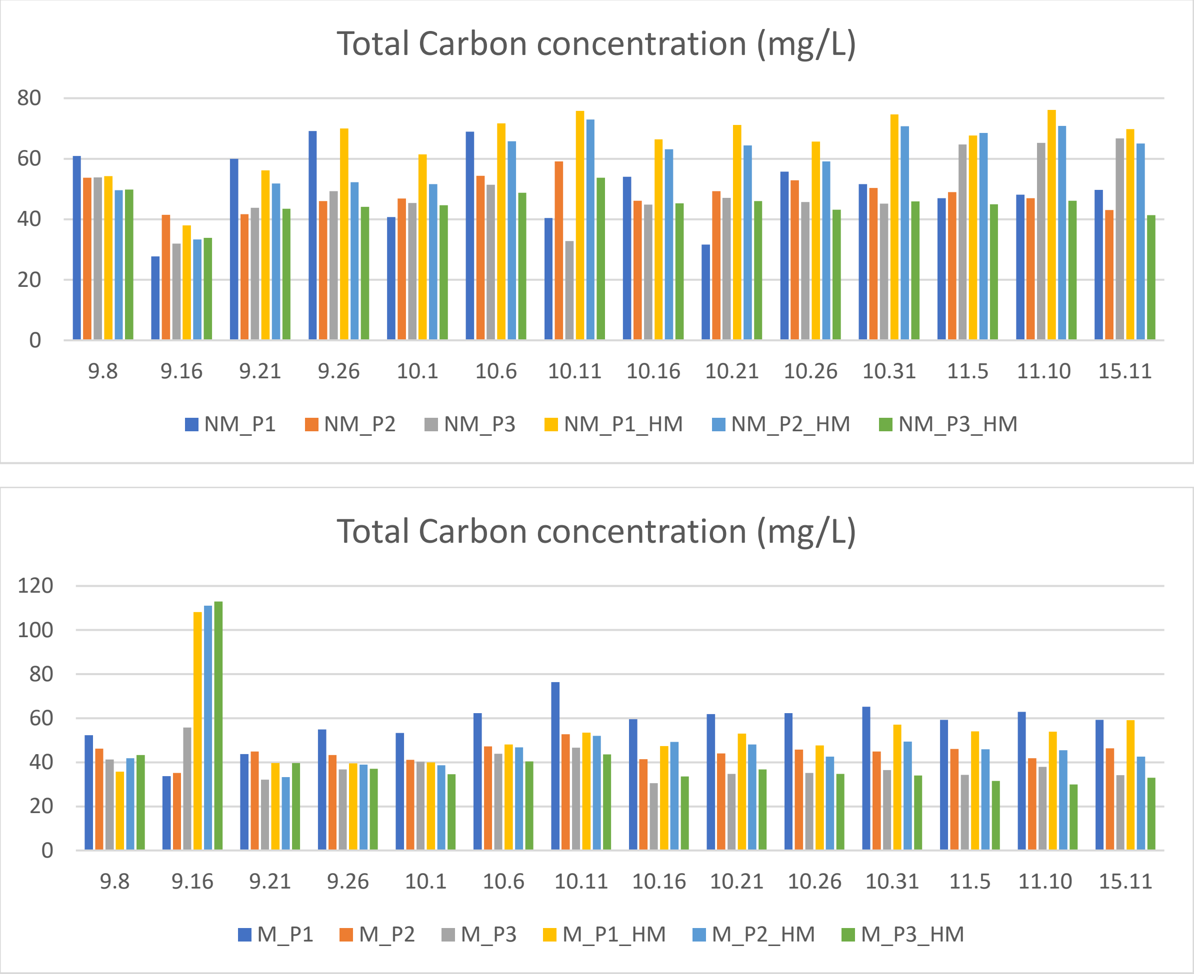


Source: Author

From the results showed above in plot 10 where the total nitrogen or TN was measured in all the systems, the ones without any mycorrhiza presence showed higher concentrations presenting values of up to 44.45 mg/l in the experimental system NM_P3_HM, this behavior is probably influenced by the addition of wastewater. For the results of the measurements of TN in the systems with mycorrhizas, the highest concentration value was 31.58 mg/L that belongs to the M_P2_HM. The data follows a logical behavior where the systems treated with wastewater presented a higher concentration of TN compared to the systems that didn't have wastewater as inlet water, furthermore the difference in the concentrations between the presence of AMF and no AMF shows that there is an influence of the AMF in the concentration of TN in the outflow water. The same behavior was present in the removal efficiency of these systems, the highest removal efficiency was 86.47% and the system responsible had mycorrhizas but it was not treated with wastewater, from the systems treated with wastewater the highest removal efficiency was 70.86% and it had AMF colonization, the difference between the efficiencies of the systems treated with wastewater and presence of mycorrhiza and their counterparts without AMF colonization was 11.84% being this an indicator on the influence of AMF in the TN removal from wastewater.

4.4.5. Total Carbon

Plot 12: Total Carbon concentration (mg/L)

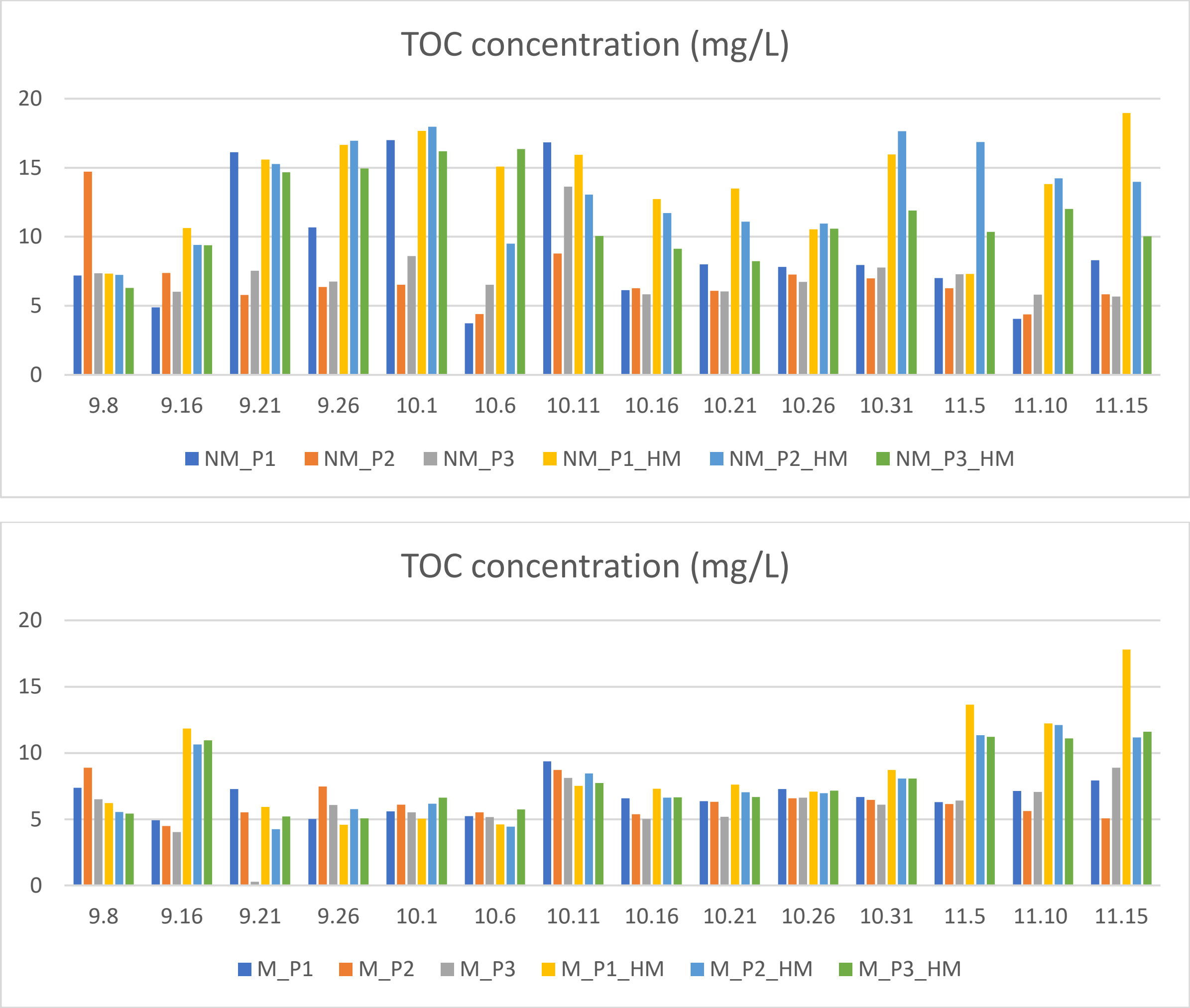


Source: Author

The measurement of total carbon concentration values shows that the systems with no presence of mycorrhizas had a slightly higher concentration compared to the systems with AMF colonization in general but the measurement of M_P1_HM, M_P2_HM, M_P3_HM presented the highest concentration of total carbon with 112.04 mg/L even with mycorrhizas in their systems. The behavior of the rest of the SSVF CW with mycorrhizas was more regular or similar between each other but there is an interesting performance in the M_P1 in all the samples because in most of the cases its concentration of TC is the highest even greater than the CW treated with polluted wastewater. Analyzing the mean values from all the sample sets (days of sampling) the difference between no mycorrhizas CW and the ones with AMF colonization is 5.81 mg/L that means that the AMF has not a distinct influence in the removal of TC from wastewater. The mean efficiency of the experimental CW in the removal of TC from wastewater is 64.80% in the systems without the presence of mycorrhizas and 73.92% for the systems that had AMF activity, this means a difference of 9.12% in the performance of these experimental systems in the removal of TC.

4.4.6. Total Organic Carbon

Plot 13: Total organic carbon concentration (mg/L)



Source: Author

Results from TOC concentration measurements showed that the experimental CW with no mycorrhizas and treated with polluted wastewater had the highest values this one being 18.95 mg/L belonging to NM_P1_HM experimental system, there are some irregular behaviors in the CW that didn't have mycorrhizas such as the NM_P1 from the sample taken in September 21st that presented higher concentrations of TOC than the systems which were treated with wastewater and similar results were obtained in the samples taken on September 8th and October 11th. Regarding the results obtained from the measurement of

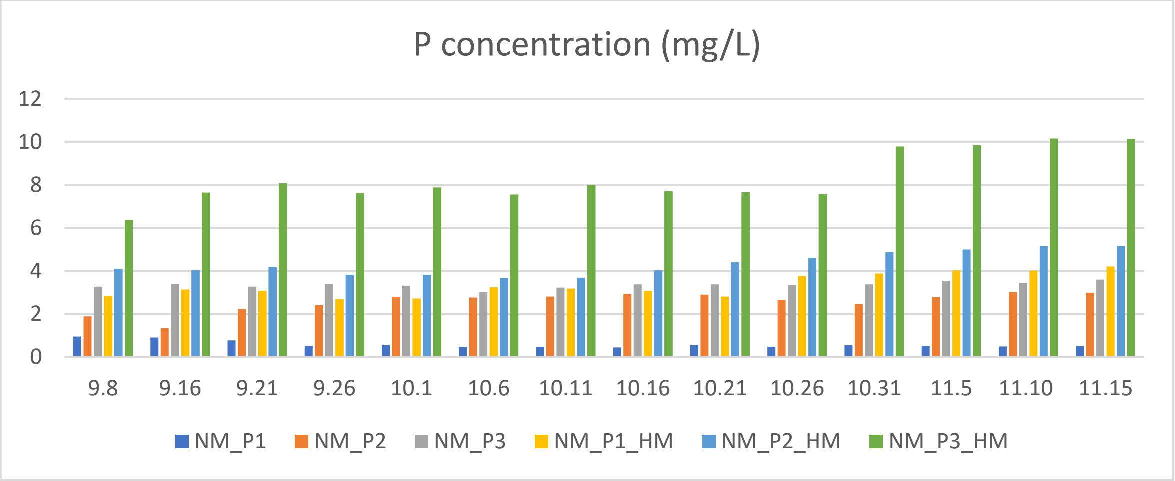
TOC from the CW that had mycorrhiza presence, the behavior of the data was more regular but it also showed some results of interest because some systems that were not treated with wastewater presented higher concentrations of TOC than the ones with polluted wastewater such as the samples taken on September 8th and on October 11th but in the majority of the cases the CW with mycorrhizas and treated with wastewater had a higher TOC concentration.

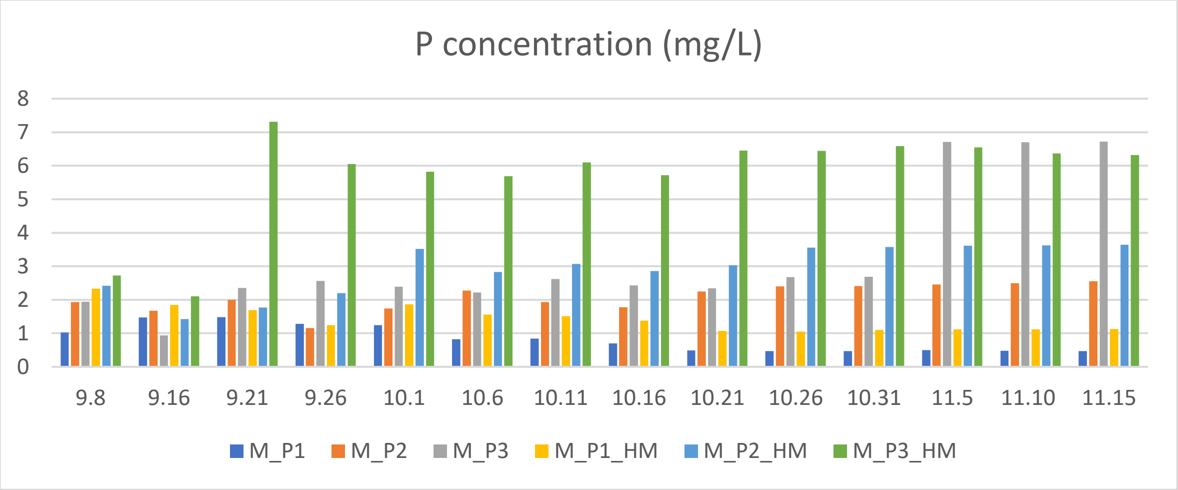
The average TOC concentrations of the CW with no mycorrhiza presence was 10.26 mg/L and the mean of the concentrations of TOC corresponding to the systems with AMF in them was 7.14% so the difference in the concentrations was 3.12 mg/L, this just considering the performance of the mycorrhizas in the removal of TOC from polluted wastewater. The highest efficiency in the removal of TOC from the wastewater was 92.89% corresponding to the CW with mycorrhiza and a ratio of N:P of 1:5, the highest removal efficiency from the systems without mycorrhizas and treated with wastewater was 87.97% that belongs to the CW with a ratio of M:P of 1:10, and the difference between this two was 4.92%.

4.4.7. Phosphate

In this experiment, three different inflow water was added with three different concentrations of P to assess the influence of P and its different rates in the removal of heavy metals. The first P treatment had average concentrations of 5.03 mg/L, the second P inflow had average concentrations of 11.03 mg/L and the last P inflow recorded average concentrations of 24.95 mg/L. This is the reason why in the results there are three different experimental systems for no mycorrhiza, no mycorrhiza, and wastewater addition, mycorrhiza, mycorrhiza and wastewater inlet.

Plot 14: P concentration (mg/L)





Source: Author

The highest concentrations were found in the CW without the presence of mycorrhizas and had wastewater added and the greater value from these was 10.14 mg/L corresponding to the NM_P3_HM sample from November 11th, the behavior of the data was consistent in terms of the highest concentration showed always by the CW with no mycorrhiza and with the highest pollution of P added. Regarding the values presented by the CW with mycorrhizas, the behavior was similar where M_P3_HM had the highest value of 7.31 mg/L but there were some unexpected results as the ones from November 5th, 11th and 15th where the CW M_P3 showed more concentration of P than the systems with the wastewater addition where the expected behavior was that the concentration of this system should be less than the one with wastewater in it.

Comparing the results between the CW with mycorrhizas and the ones without them, the second ones had lower concentrations in general where the highest value registered was 5.73 mg/L coming from the M_P3_HM, the highest concentration found in the systems without AMF colonization was 8.28 mg/L. This result shows the influence of the AMF in the removal of P from the wastewater with an efficiency of 83.5% for the first P concentration, 82.78% for the second P concentration and 83.76% for the highest P concentration. The results of the efficiency from the CW that didn’t have mycorrhiza and were treated with the different P concentrations in the inflow wastewater, were 50.73%, 68.59%, 76.10% for the corresponding P concentrations of inlet water. These last three efficiencies reflect the efficiency of the SSVF CW systems in the removal of P particles from wastewater without the influence of AMF colonization.

5. Discussion

This research focused on the evaluation of the influence of AMF in the removal of heavy metals and other parameters such as Ammonium (NH_4^+) or phosphorus in addition to the influence in the metal’s distribution in the plant roots, shoots and the substrate. Furthermore, the analysis of the effect of different N:P ratios in the colonization of AMF in SSVF CW and the efficiency on the removal of the metals Cu and Pb from polluted wastewater.

5.1. Effect of different N:P ratios in AMF colonization

Understanding the differences of the intensity of mycorrhiza colonization and arbuscule abundance in the root system at different N:P ratios allows to assess the effect of these different proportions of N:P in the development of AMF association, therefore the procedure was to treat the CW with different ratios of the abovementioned parameters to compare the behavior of the occurrence of AMF in systems with and without wastewater.

The results expressed that the CW with mycorrhiza and with a ratio of 1:1 of N and P had the highest intensity of mycorrhiza colonization or M(%) of 43.89% but the experimental system treated with the ratio of 1:10 of N and P got a M(%) of 32.08%, same as the systems where the wastewater was added showing that the CW with a N:P ratio of 1:1 had the highest M(%) with 26.85% than the other two CW´s with a different proportion of Nitrogen to Phosphorus of 1:5 and 1:10 that got 25.14% and 18.51% M(%) respectively. The results of the A(%) or arbuscule abundance in the root system showed the same nature as the ones of M(%) where the CW without the treatment of wastewater and a N to P ratio of 1:1 presented the highest arbuscule abundance with 10.36% and it got lesser and lesser with the addition of P to the systems being 6.3% and 3.78% respectively in systems with N:P of 1:5 and 1:10, a behavior similar to the one registered by Merlin et al. (2020) where the researchers found that the addition of P reduces the AMF colonization in plant roots demonstrating that the increase in the nutrient availability and uptake of P especially, replaces sometimes the AMF effect or impact in the plant development (Smith & Smith, 2012). The P availability in high concentrations frequently reduces the spore formation hence the root colonization by AMF is also decreased impacting the plant growth even knowing that the P is an important element in plant development (Lermen et al., 2017).

The AMF symbiosis functions are determined by the N and P availability and their stoichiometric ratios, this, according to Johnson et al. (2015) who found that the mutual paybacks were mostly predictable when the contents and availability of N were high and the ones of P were low in the environment. Yu et al., (2020) found that AMF had an important influence in mediating the plant community diversity in environments with lower P concentrations, moreover, the limitation on the availability of N causes a higher rate of colonization of AMF promoting a higher uptake of N and plant growth, an inverse effect than the one of P where the mycorrhizal growth or population response was due to increased P allocation in the aboveground biomass meaning that there is less P in the substrate or water enhancing the production of spores (De Vries et al., 2011).

5.2. Effect of different N:P ratios in the distribution of metals in CW

From the results of the analysis of metal concentration distribution in SSVF CW the behavior of metals in the wetland distribution was that most of the concentration of metals was found in the roots of the plants. The different N:P ratios showed that the systems had a different behavior regarding the Cu and Pb concentrations in the experimental systems for example, the M_P1_HM presented the highest concentration of Cu with 2339.54 (mg/kg) compared to the other two systems that were treated with a higher concentration of P thus an increased P availability. A similar nature in the results from the analysis of Pb particles in the roots of the plants of *I. Pseudacorus* where the highest concentration (109.78 mg/kg) was present in the M_P1_HM experimental system.

The tendency of the results in showing that the experimental systems with the less N:P ratios difference presented the highest concentration of Cu and Pb not only in the roots but also in the plant shoots and in the substrate, this demonstrates that the P availability affects the uptake of nutrients and other particles like, in this case, the pollutant particles of metals Cu and Pb where the CW with higher ratios of N:P reduces the rates of particle uptake from plants due to the lesser presence of AMF and its symbiotic relationship with the plants (Bostic & White, 2007; Reddy et al., 1999).

In the experimental systems where there is no mycorrhiza presence and they were treated with polluted wastewater and different N:P ratios, the concentrations of Cu were mostly found in the roots of the plants, then, in the plant shoots and finally with the less concentration found in the CW substrates. The fluctuation between the different N:P ratios and the concentrations in each part of the experimental systems and plants was significant for example the maximum difference in the Cu concentrations in the roots was 7.54%, in the shoots was more considerable with 38% and in the substrate was 32.83%. The highest concentration found in the plant roots was with the N:P of 1:1, in the shoots the highest ratios of 1:5 and 1:10 got the same concentration of 296 mg/kg and in the substrate the highest concentration of Cu was found in the system with a 1:10 ratio of N to P. The highest concentration of Pb in the experimental systems without mycorrhizas was found in the plant shoots in the system with the highest N:P ratio of 1:10 followed by the plant roots where the concentration of Pb was similar in all three different N:P ratios. The results were similar to the ones found by Vymazal & Březinová (2015) where the highest metal concentrations in plants from wetlands, in that case *P. Australis,* were registered in the roots of the plants and also Vymazal et al. (2009) explained that the standing stock or total storage of a substance in a particular compartment, is higher in the belowground biomass (roots) comparted to the aboveground biomass but due to the poor development of roots in constructed wetlands that are heavily loaded with pollutant particles, the higher concentrations of metals in roots perhaps could not result in an increased standing stock comparing it with the aboveground biomass (Bonanno & Lo Giudice, 2010).

5.3. Influence of AMF under different N:P ratios in the removal of Ammonium (NH_4^+-N) and Phosphorus (PO_4 -P)

From the results of the experiment related with the systems that presented mycorrhizal associations, in the removal of ammonium and phosphorus (P) compared to the experimental systems without mycorrhiza and under different N:P ratios, the efficiencies of removal increased with the mycorrhizal activity but the impact of the different N:P ratios resulted in a decrease of the efficiencies of the experimental systems.

In wetlands there are different mechanisms for the removal of N from wastewater such as nitrification, denitrification, volatilization and adsorption or plant uptake; these mechanisms depend on different parameters to work properly for example: nitrification requires an aerobic environment to be performed by bacteria, denitrification takes place in anoxic conditions, volatilization happens when the ammonia in the water is volatilized to gas at the air/water interface and then it is released into the air but it needs high pH levels of 9.0 and above (Gu et al., 2021; Z. Hu et al., 2019; Lu et al., 2020).

The ammonium removal results showed that in general the presence of the AMF enhanced the removal of this nutrient from the wastewater presenting an efficiency higher than 60% in the comparison of the systems treated with wastewater and the differences were the presence of AMF and the N:P ratios that were changing (P1=1:1, P2=1:5, P3=1:10). The experimental CW with the first ratio of N:P presented an efficiency of the removal of 63.45%, the CW with the second N:P ratio showed an efficiency in the removal of 65.02% and lastly the CW that had the third N:P ratio resulted in an efficiency of 60.84% being the least effective one in the removal of this nutrient. These efficiencies were similar to the ones experienced by Thalla et al. (2019) in their research where their CW obtained a minimum of 67.19% and is also one proof that the SSVF CW is more efficient than any other constructed wetland system in the removal of NH_4^+ but this also depends on other factors such as the plant species because they differ in their preferred nitrogen forms to be absorbed and also depends on the forms available N in the water and soil (Kadlec & Wallace, 2009).

Phosphorus removal in subsurface flow wetlands can be performed by different mechanisms but it is mostly influenced by physicochemical characteristics of the used substrate (Brix & Arias, 2005) but the soluble part of the P will be transported by the water flow and the P attached to particulate matter will be removed in a filtration and interception process in the wetland bed (Kadlec & Wallace, 2009). In this research the efficiencies of P removal in the form of PO_4-P were higher in the experimental systems which had AMF colonization in them compared to the systems without it, showing that the first system which had AMF presence in it and treated with a N:P of 1:1 was 83.5% and for the CW with ratios of 1:5 and 1:10 the efficiencies were 82.78% and 81.76% respectively. The difference in the efficiencies of these systems let us infer that the influence of AMF in the removal of P was higher by 32.76, 14.18 and 5.65% compared to the systems without AMF colonization, but the efficiency decreases when the ratio of N:P changes from 1:1 to 1:5 and finally 1:10. In previous researches performed by Hu et al. (2020) was demonstrated that AMF increases the plant ability to uptake P, N and other micronutrients because in the inoculated plants there are more pathways for the transport of nutrients to the plant such as the normal or direct plant uptake and the mycorrhizal uptake pathway which is missing in the plants that were not inoculated by AMF lacking from this ability (Bücking & Kafle, 2015).

5.4. Influence of AMF at different N:P ratios in the removal of heavy metals from wastewater

As it is known the removal of heavy metals from wastewater through the application of constructed wetlands is complex due to all the processes that are involved in it including abiotic and biotic reactions or plant uptake (Batool & Saleh, 2020). There are different mechanisms in the removal of heavy metals from wastewater in constructed wetlands such as the adsorption to fine-textured sediments and organic matter, precipitation as insoluble salts, absorption and induced changes in the biogeochemical cycles by pants and microorganisms and the deposition of suspended particles as a result of low flow rates (Xu et al., 2018). Through all these processes there is an accumulation of heavy metals in the wetland systems thus removing these particles from the inlet water, furthermore, from the mechanisms mentioned the microorganisms such as plant-growth-promoting rhizobacteria and AMF play an important role in the HM removal using different metabolic properties and complementing them with their host metabolism processes (Siani et al., 2017).

From this experiment, the results shown in the efficiencies of the removal of Cu and Pb from wastewater, the systems which presented AMF colonization had higher efficiency compared to the ones that did not count with this AMF symbiotic association but the difference is not considered being the greatest of just 0.05%. The highest efficiency from the systems with AMF inoculation was 99.92% corresponding to the system classified as M_P1_HM or the experimental CW with treated with a ratio of N:P of 1:1 and the least efficient was the M_P3_HM related to the experimental system with a N:P ratio of 1:10 with a mass removal efficiency of 99.91%; the difference was just 0.017% being this a really small difference in the Cu removal. In the Pb removal efficiency from these systems the values were similar to the Cu removal showing exceptional results of 99.9% efficiency and the fluctuation of the results between the different N:P ratios are just 0.01%. These results are similar to the researches of (Huang et al., 2017),(Hussain et al., 2018), (Chen et al., 2009) where the efficiencies of removal of Cu and Pb using constructed wetlands were between 95 and 99% taking into account that the processes of the behavior of heavy metals in wetlands are complex and depend on different parameters, the presence of AMF is considered to influence the removal of HM from wastewater due to its properties of improving the morphological and physiological status plus they promote growth and stress tolerance protecting plant tissue (Hu et al., 2020; Xu et al., 2018).

AMF can improve the efficiency of the removal of heavy metals from water controlling the transformation of heavy metals in roots and shoots and also AMF reduces stress caused by heavy metals by immobilizing this particles in the roots and the rhizosphere of the plants avoiding the accumulation of them in the aboveground biomass (Meier et al., 2012).

6. Conclusions

This experiment showed that the different N:P ratios have a negative influence on the AMF colonization intensity where the experimental wetlands with the highest ratio of N:P (1:10) had the least mycorrhizal presence in their systems, moreover, the addition of the simulated sewage water reduced the amount of AMF colonization in the systems by 17.04%.

Regarding the destiny of the heavy metals in the system, most of the concentration of Cu and Pb was present in the roots of the *I. Pseudacorus* plants, this happened due to the presence of arbuscular mycorrhizal fungi in the systems preventing the absorption to other parts of the plant where these particles will be more harmful such as the leaves and shoots.

As researches support and this thesis demonstrate, the AMF affects the bioavailability in the plants that act as hosts, promoting the retention of heavy metals in the roots improving shoot biomass by the restriction of the translocation to the aboveground parts.

After testing the effect of AMF in the treatment of wastewater to remove heavy metals (Cu and Pb), the systems showed an exceptional ability to deal with this job of the retention of these metals improving the quality of the outlet water with efficiencies of above 95% and the AMF had an important role in this matter due to the symbiotic relationship with the plants, enhancing the complex processes that take place into wetlands where soil, microbiota and plants perform their daily life cycles.

The efficiencies were decreasing when the ratios of N:P were higher due to the limitation on the AMF colonization caused by phosphorus thus reducing the AMF population and, in that way, reducing the tolerance of the plants to heavy metals present in their roots. Further analysis in more adverse conditions and testing different types of metals or even different species of AMF and plants could be done to enhance or improve these results, creating a deeper understanding and knowledge to design an optimized treatment system.

This experiment was done under controlled conditions to be able to understand the effect of AMF in the removal of heavy metals under different ratios of N:P in constructed wetlands but in real life, these conditions can vary or can be affected by external factors changing the properties of the constructed wetlands changing their processes and reducing the efficiencies, so, the real application of these systems for the treatment of wastewater need to be calibrated in situ to adapt the conditions of the constructed wetlands to the new external factors.

Reference

Al-fartusie, F. S., & Mohssan, S. N. (2017). Indian Journal of Advances in Chemical Science Essential Trace Elements and Their Vital Roles in Human Body. *Indian Journal of Advances in Chemical Science*, *5*, 127–136. https://doi.org/10.22607/IJACS.2017.503003

Alldred, M., & Baines, S. B. (2016). *Effects of wetland plants on denitrification rates : a meta-analysis Author (s): Mary Alldred and Stephen B . Baines Published by : Wiley on behalf of the Ecological Society of America Stable URL : https://www.jstor.org/stable/24701977 Effects of wetlan*. *26*(3), 676–685.

Amalero, E. G., Ingua, G. L., Erta, G. B., & Emanceau, P. L. (2003). Review article Methods for studying root colonization by introduced. *Agronomie*, *23*, 407–418. https://doi.org/10.1051/agro

Bago, B., Pfeffer, P., & Shachar-hill, Y. (2000). Update on Symbiosis Arbuscular Mycorrhizas. *Plant Physiology*, *124*(November), 949–957.

Banhidi, M. (2001). pH and ORP. *Metal Finishing*, *99*(March), 593–599. https://doi.org/10.1016/s0026-0576(01)85317-4

Bao, X., Wang, Y., & Olsson, P. A. (2019). Arbuscular mycorrhiza under water — Carbon–phosphorus exchange between rice and arbuscular mycorrhizal fungi under different flooding regimes. In *Soil Biology and Biochemistry* (Vol. 129, pp. 169–177). https://doi.org/10.1016/j.soilbio.2018.11.020

Batool, A., & Saleh, T. A. (2020). Removal of toxic metals from wastewater in constructed wetlands as a green technology; catalyst role of substrates and chelators. *Ecotoxicology and Environmental Safety*, *189*(November 2019). https://doi.org/10.1016/j.ecoenv.2019.109924

Bernardes, F. S., Herrera, P. G., Chiquito, G. M., Morales, M. F., Castro, A. P., & Paulo, P. L. (2019). Relationship between microbial community and environmental conditions in a constructed wetland system treating greywater. *Ecological Engineering*, *139*(August), 105581. https://doi.org/10.1016/j.ecoleng.2019.105581

Bonanno, G., & Lo Giudice, R. (2010). Heavy metal bioaccumulation by the organs of Phragmites australis (common reed) and their potential use as contamination indicators. *Ecological Indicators*, *10*(3), 639–645. https://doi.org/10.1016/j.ecolind.2009.11.002

Bostic, E. M., & White, J. R. (2007). Soil Phosphorus and Vegetation Influence on Wetland Phosphorus Release after Simulated Drought. *Soil Science Society of America Journal*, *71*(1), 238–244. https://doi.org/10.2136/sssaj2006.0137

Boyd, C. H. (2006). Horizontal Subsurface Flow Constructed Wetlands for on-Site Wastewater Treatment. In *WRIGHT STATE UNIVERSITY*.

Brix, H., & Arias, C. A. (2005). The use of vertical flow constructed wetlands for on-site treatment of domestic wastewater: New Danish guidelines. *Ecological Engineering*, *25*(5), 491–500. https://doi.org/10.1016/j.ecoleng.2005.07.009

Bücking, H., & Kafle, A. (2015). Role of arbuscular mycorrhizal fungi in the nitrogen uptake of plants: Current knowledge and research gaps. *Agronomy*, *5*(4), 587–612. https://doi.org/10.3390/agronomy5040587

Calheiros, C. S. C., Pereira, S. I. A., Franco, A. R., & Castro, P. M. L. (2019). Diverse arbuscular mycorrhizal fungi (AMF) communities colonize plants inhabiting a constructedwetland for wastewater treatment. *Water (Switzerland)*, *11*(8). https://doi.org/10.3390/w11081535

Censi, P., Spoto, S. E., Saiano, F., Sprovieri, M., Mazzola, S., Nardone, G., Di Geronimo, S. I., Punturo, R., & Ottonello, D. (2006). Heavy metals in coastal water systems. A case study from the northwestern Gulf of Thailand. *Chemosphere*, *64*(7), 1167–1176. https://doi.org/10.1016/j.chemosphere.2005.11.008

Chang, J. jun, Wu, S. qing, Dai, Y. ran, Liang, W., & Wu, Z. bin. (2012). Treatment performance of integrated vertical-flow constructed wetland plots for domestic wastewater. *Ecological Engineering*, *44*, 152–159. https://doi.org/10.1016/j.ecoleng.2012.03.019

CHEN, M., TANG, Y., LI, X., & YU, Z. (2009). Study on the Heavy Metals Removal Efficiencies of Constructed Wetlands with Different Substrates. *Journal of Water Resource and Protection*, *01*(01), 22–28. https://doi.org/10.4236/jwarp.2009.11004

De Vries, F. T., van Groenigen, J. W., Hoffland, E., & Bloem, J. (2011). Nitrogen losses from two grassland soils with different fungal biomass. *Soil Biology and Biochemistry*, *43*(5), 997–1005. https://doi.org/10.1016/j.soilbio.2011.01.016

Debusk, W. F. (1999). Nitrogen Cycling in Wetlands. *University of Florida*, 2–4.

DuPoldt, C., Edwards, R., Garber, L., Isaacs, B., & Lapp, J. (1996). A Handbook of Constructed Wetlands: General Considerations. *Ecological Engineering*, *1*(1996), 53. http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.169.7471&rep=rep1&type=pdf%0Awww.unhabitat.org

Esfahani, A. R., Zhang, Z., Sip, Y. Y. L., Zhai, L., & Sadmani, A. H. M. A. (2020). Removal of heavy metals from water using electrospun polyelectrolyte complex fiber mats. *Journal of Water Process Engineering*, *37*(June), 101438. https://doi.org/10.1016/j.jwpe.2020.101438

Fan, Y., Wu, X., Shao, L., Han, M., Chen, B., Meng, J., Wang, P., & Chen, G. (2021). Can constructed wetlands be more land efficient than centralized wastewater treatment systems? A case study based on direct and indirect land use. *Science of the Total Environment*, *770*, 144841. https://doi.org/10.1016/j.scitotenv.2020.144841

Farooq, Y., Hussain, M. M., Aleem, S. Bin, & Farooq, M. A. (2008). Lead Intoxication: the Extent of Problem and Its Management. *Pak J Physiol*, *4*(2), 36–41. http://www.pps.org.pk/PJP/4

Farooqi, I. H., Basheer, F., & Chaudhari, R. J. (2008). Constructed Wetland System (CWS) for Wastewater Treatment. *Proceedings of Taal 2007: The World Lake Conference*, *May 2014*, 1004–1009.

Fester, T. (2013). Arbuscular mycorrhizal fungi in a wetland constructed for benzene-, methyl tert-butyl ether- and ammonia-contaminated groundwater bioremediation. *Microbial Biotechnology*, *6*(1), 80–84. https://doi.org/10.1111/j.1751-7915.2012.00357.x

Gacia, E., Soto, D. X., Roig, R., & Catalan, J. (2020). Phragmites australis as a dual indicator (air and sediment) of trace metal pollution in wetlands – the key case of Flix reservoir (Ebro River). *Science of the Total Environment*, *xxxx*, 142789. https://doi.org/10.1016/j.scitotenv.2020.142789

Galletti, A., Verlicchi, P., & Ranieri, E. (2010). Removal and accumulation of Cu, Ni and Zn in horizontal subsurface flow constructed wetlands: Contribution of vegetation and filling medium. *Science of the Total Environment*, *408*(21), 5097–5105. https://doi.org/10.1016/j.scitotenv.2010.07.045

Ghasemi Siani, N., Fallah, S., Pokhrel, L. R., & Rostamnejadi, A. (2017). Natural amelioration of Zinc oxide nanoparticle toxicity in fenugreek (Trigonella foenum-gracum) by arbuscular mycorrhizal (Glomus intraradices) secretion of glomalin. *Plant Physiology and Biochemistry*, *112*, 227–238. https://doi.org/10.1016/j.plaphy.2017.01.001

Gu, X., Chen, D., Wu, F., He, S., & Huang, J. (2021). Recycled utilization of Iris pseudacorus in constructed wetlands: Litters self-consumption and nitrogen removal improvement. *Chemosphere*, *262*, 127863. https://doi.org/10.1016/j.chemosphere.2020.127863

Habte, L., Shiferaw, N., Thriveni, T., Mulatu, D., Lee, M. hye, Jung, S. ho, & Ahn, J. W. (2020). Removal of Cd(II) and Pb(II) from wastewater via carbonation of aqueous Ca(OH)2 derived from eggshell. *Process Safety and Environmental Protection*, *141*, 278–287. https://doi.org/10.1016/j.psep.2020.05.036

Hasanpour, M., & Hatami, M. (2020). Application of three dimensional porous aerogels as adsorbent for removal of heavy metal ions from water/wastewater: A review study. *Advances in Colloid and Interface Science*, *284*, 102247. https://doi.org/10.1016/j.cis.2020.102247

Hoffmann, H., Platzer, C., Winker, M., & Von Muench, E. (2011). *Technology review of constructed wetlands Subsurface flow constructed wetlands for greywater and*.

Hu, B., Hu, S., Chen, Z., & Vymazal, J. (2020). Employ of arbuscular mycorrhizal fungi for pharmaceuticals ibuprofen and diclofenac removal in mesocosm-scale constructed wetlands. *Journal of Hazardous Materials*, *409*(November 2020). https://doi.org/10.1016/j.jhazmat.2020.124524

Hu, S., Chen, Z., Vosátka, M., & Vymazal, J. (2020). Arbuscular mycorrhizal fungi colonization and physiological functions toward wetland plants under different water regimes. *Science of the Total Environment*, *716*, 137040. https://doi.org/10.1016/j.scitotenv.2020.137040

Hu, S., Hu, B., Chen, Z., Vosátka, M., & Vymazal, J. (2020). Antioxidant response in arbuscular mycorrhizal fungi inoculated wetland plant under Cr stress. *Environmental Research*, *191*(August). https://doi.org/10.1016/j.envres.2020.110203

Hu, Z., Liu, J., Zheng, W., Li, D., Liu, Y., & Yao, H. (2019). Highly-efficient nitrogen removal from domestic wastewater based on enriched aerobic/anoxic biological filters and functional microbial community characteristics. *Journal of Cleaner Production*, *238*, 117867. https://doi.org/10.1016/j.jclepro.2019.117867

Huang, H., Zhang, S., Shan, X. quan, Chen, B. D., Zhu, Y. G., & Bell, J. N. B. (2007). Effect of arbuscular mycorrhizal fungus (Glomus caledonium) on the accumulation and metabolism of atrazine in maize (Zea mays L.) and atrazine dissipation in soil. *Environmental Pollution*, *146*(2), 452–457. https://doi.org/10.1016/j.envpol.2006.07.001

Huang, X., Zhao, F., Yu, G., Song, C., Geng, Z., & Zhuang, P. (2017). Removal of Cu, Zn, Pb, and Cr from Yangtze Estuary Using the Phragmites australis Artificial Floating Wetlands. *BioMed Research International*, *2017*. https://doi.org/10.1155/2017/6201048

Hussain, F., Mustufa, G., Zia, R., Faiq, A., Matloob, M., Rehman Shah, H. ur, Rafique Ali, W., & Irfan, J. A. (2018). Constructed Wetlands and their Role in Remediation of

Industrial Effluents via Plant-Microbe Interaction – A Mini Review. *Journal of Bioremediation & Biodegradation*, *09*(04). https://doi.org/10.4172/2155-6199.1000447

Jain, M., Majumder, A., Ghosal, P. S., & Gupta, A. K. (2020). A review on treatment of petroleum refinery and petrochemical plant wastewater: A special emphasis on constructed wetlands. *Journal of Environmental Management*, *272*(July), 111057. https://doi.org/10.1016/j.jenvman.2020.111057

Jakubaszek, A. (2020). Nitrogen and Phosphorus Content in Constructed Wetlands. *Civil and Environmental Engineering Reports*, *30*(2), 247–257. https://doi.org/10.2478/ceer-2020-0030

Johnson, N. C., Wilson, G. W. T., Wilson, J. A., Miller, R. M., & Bowker, M. A. (2015). Mycorrhizal phenotypes and the Law of the Minimum. *New Phytologist*, *205*(4), 1473–1484. https://doi.org/10.1111/nph.13172

Jones, M. D., & Smith, S. E. (2004). Exploring functional definitions of mycorrhizas: Are mycorrhizas always mutualisms? *Canadian Journal of Botany*, *82*(8), 1089–1109. https://doi.org/10.1139/B04-110

Kadlec, R. H., & Wallace, S. D. (2009). Treatment Wetlands, Second Edition TOC and References. In *Treatment Wetlands, Second Edition*.

Khan, N. A., El Morabet, R., Khan, R. A., Ahmed, S., Dhingra, A., Alsubih, M., & Khan, A. R. (2020). Horizontal sub surface flow Constructed Wetlands coupled with tubesettler for hospital wastewater treatment. *Journal of Environmental Management*, *267*(February), 110627. https://doi.org/10.1016/j.jenvman.2020.110627

Kondzior, P., & Butarewicz, A. (2018). Effect of heavy metals (Cu and Zn) on the content of photosynthetic pigments in the cells of algae Chlorella vulgaris. *Journal of Ecological Engineering*, *19*(3), 18–28. https://doi.org/10.12911/22998993/85375

Lee, B. H., & Scholz, M. (2006). Application of the self-organizing map (SOM) to assess the heavy metal removal performance in experimental constructed wetlands. *Water Research*, *40*(18), 3367–3374. https://doi.org/10.1016/j.watres.2006.07.027

Lermen, C., da Cruz, R. M. S., de Souza, J. S., de Almeida Marchi, B., & Alberton, O. (2017). Growth of Lippia alba (Mill.) N. E. Brown inoculated with arbuscular mycorrhizal fungi with different levels of humic substances and phosphorus in the soil. *Journal of Applied Research on Medicinal and Aromatic Plants*, *7*(July), 48–53. https://doi.org/10.1016/j.jarmap.2017.05.002

Li, Y., Li, H., Sun, T., & Wang, X. (2011). Study on nitrogen removal enhanced by shunt distributing wastewater in a constructed subsurface infiltration system under intermittent operation mode. *Journal of Hazardous Materials*, *189*(1–2), 336–341. https://doi.org/10.1016/j.jhazmat.2011.02.039

Lu, J., Guo, Z., Kang, Y., Fan, J., & Zhang, J. (2020). Recent advances in the enhanced nitrogen removal by oxygen-increasing technology in constructed wetlands. *Ecotoxicology and Environmental Safety*, *205*(September), 111330. https://doi.org/10.1016/j.ecoenv.2020.111330

Meier, S., Borie, F., Bolan, N., & Cornejo, P. (2012). Phytoremediation of metal-polluted soils by arbuscular mycorrhizal fungi. *Critical Reviews in Environmental Science and Technology*, *42*(7), 741–775. https://doi.org/10.1080/10643389.2010.528518

Merlin, E., Melato, E., Lourenço, E. L. B., Jacomassi, E., Junior, A. G., da Cruz, R. M. S., Otênio, J. K., da Silva, C., & Alberton, O. (2020). Inoculation of arbuscular mycorrhizal fungi and phosphorus addition increase coarse mint (Plectranthus amboinicus Lour.) plant growth and essential oil content. *Rhizosphere*, *15*(March), 100217. https://doi.org/10.1016/j.rhisph.2020.100217

Mihelcic, J. (2018). *CONSTRUCTED WETLANDS: MANAGEMENT OF RISK FROM EXCRETA AND WASTEWATER SLUDGE MANAGEMENT*.

Nadarajah, K., Bandala, E. R., Zhang, Z., Mundree, S., & Goonetilleke, A. (2021). Removal of heavy metals from water using engineered hydrochar: Kinetics and mechanistic approach. *Journal of Water Process Engineering*, *40*(January), 101929. https://doi.org/10.1016/j.jwpe.2021.101929

Najeeb, U., Ahmad, W., Zia, M. H., Zaffar, M., & Zhou, W. (2017). Enhancing the lead phytostabilization in wetland plant Juncus effusus L. through somaclonal manipulation and EDTA enrichment. *Arabian Journal of Chemistry*, *10*, S3310–S3317. https://doi.org/10.1016/j.arabjc.2014.01.009

Norton, S. (2003). Removal Mechanisms in Constructed Wastewater Wetlands Stephen Norton. *Removal Mechanisms in Constructed Wastewater Wetlands*.

Pandiyan, J., Mahboob, S., Govindarajan, M., Al-Ghanim, K. A., Ahmed, Z., Al-Mulhm, N., Jagadheesan, R., & Krishnappa, K. (2020). An assessment of level of heavy metals pollution in the water, sediment and aquatic organisms: A perspective of tackling environmental threats for food security. *Saudi Journal of Biological Sciences*, *xxxx*. https://doi.org/10.1016/j.sjbs.2020.11.072

Parde, D., Patwa, A., Shukla, A., D, R. V. P., D, D. J. K. P., & D, R. K. P. (2020). Environmental Technology & Innovation A review of constructed wetland on type , treatment and. *Environmental Technology & Innovation*. https://doi.org/10.1016/j.eti.2020.101261

Parde, D., Patwa, A., Shukla, A., Vijay, R., Killedar, D. J., & Kumar, R. (2020). A review of constructed wetland on type, treatment and technology of wastewater. *Environmental Technology and Innovation*, *21*, 101261. https://doi.org/10.1016/j.eti.2020.101261

Reddy, K. R., Kadlec, R. H., Flaig, E., & Gale, P. M. (1999). Streams and Wetlands: A Review. *Critical Reviews in Environmental Science and Technology*, *29*(1), 83–146.

Riaz, M., Kamran, M., Fang, Y., Wang, Q., Cao, H., Yang, G., Deng, L., Wang, Y., Zhou, Y., Anastopoulos, I., & Wang, X. (2021). Arbuscular mycorrhizal fungi-induced mitigation of heavy metal phytotoxicity in metal contaminated soils: A critical review. *Journal of Hazardous Materials*, *402*(September 2020), 123919. https://doi.org/10.1016/j.jhazmat.2020.123919

Ruytinx, J., Kafle, A., Usman, M., Coninx, L., Zimmermann, S. D., & Garcia, K. (2020). Micronutrient transport in mycorrhizal symbiosis; zinc steals the show. *Fungal Biology Reviews*, *34*(1), 1–9. https://doi.org/10.1016/j.fbr.2019.09.001

Schneider, J., Labory, C. R. G., Rangel, W. M., Alves, E., & Guilherme, L. R. G. (2013). Anatomy and ultrastructure alterations of Leucaena leucocephala (Lam.) Inoculated with mycorrhizal fungi in response to arsenic-contaminated soil. *Journal of Hazardous Materials*, *262*, 1245–1258. https://doi.org/10.1016/j.jhazmat.2012.05.091

Shafiuddin Ahmed, A. S., Hossain, M. B., Babu, S. M. O. F., Rahman, M. M., & Sarker, M. S. I. (2021). Human health risk assessment of heavy metals in water from the subtropical river, Gomti, Bangladesh. *Environmental Nanotechnology, Monitoring and Management*, *15*(July 2020), 100416. https://doi.org/10.1016/j.enmm.2020.100416

Shi, A., Yan, N., & Marschner, P. (2015). Cumulative respiration in two drying and rewetting cycles depends on the number and distribution of moist days. *Geoderma*, *243–244*, 168–174. https://doi.org/10.1016/j.geoderma.2014.12.019

Smith, S. E., & Smith, F. A. (2012). Fresh perspectives on the roles of arbuscular mycorrhizal fungi in plant nutrition and growth. *Mycologia*, *104*(1), 1–13. https://doi.org/10.3852/11-229

Sun, L., Ma, J., Li, L., Tian, Y., Zhang, Z., Liao, H., Li, J., Tang, W., & He, D. (2020). Exploring the essential factors of performance improvement in sludge membrane bioreactor technology coupled with symbiotic algae. *Water Research*, *181*. https://doi.org/10.1016/j.watres.2020.115843

Thalla, A. K., Devatha, C. P., Anagh, K., & Sony, E. (2019). Performance evaluation of horizontal and vertical flow constructed wetlands as tertiary treatment option for secondary effluents. *Applied Water Science*, *9*(6), 1–9. https://doi.org/10.1007/s13201-019-1014-9

Tiwari, S., & Tripathi, I. P. (2012). Lead Pollution -An Overview. *International Research Journal of Environment Sciences*, *1*(4), 84–86.

Victoria, L., Makokha, A., Anselimo Makokha, K. O., Mghweno, L. R., Magoha, H. S., Nakajugo, A., & Wekesa, J. M. (2008). Environmental lead pollution and contamination in food aroud Environmental lead pollution and contamination in food around Lake Victoria, Kisumu, Kenya. *African Journal of Environmental Science and Technology*, *2*(10), 349–353. http://www.academicjournals.org/AJest

Vymazal, J., & Březinová, T. (2015). Heavy metals in plants in constructed and natural wetlands: Concentration, accumulation and seasonality. *Water Science and Technology*, *71*(2), 268–276. https://doi.org/10.2166/wst.2014.507

Vymazal, Jan. (2005). Horizontal sub-surface flow and hybrid constructed wetlands systems for wastewater treatment. *Ecological Engineering*, *25*(5), 478–490. https://doi.org/10.1016/j.ecoleng.2005.07.010

Vymazal, Jan. (2006). Removal of heavy metals. *Journal of Environmental Science and Health*, *40*, 1369–1379. https://doi.org/10.1016/s0026-0576(01)81526-9

Vymazal, Jan. (2010). Constructed wetlands for wastewater treatment. *Water (Switzerland)*, *2*(3), 530–549. https://doi.org/10.3390/w2030530

Vymazal, Jan. (2014). Constructed wetlands for treatment of industrial wastewaters: A review. *Ecological Engineering*, *73*, 724–751. https://doi.org/10.1016/j.ecoleng.2014.09.034

Vymazal, Jan, Kröpfelová, L., Švehla, J., Chrastný, V., & Štíchová, J. (2009). Trace elements in Phragmites australis growing in constructed wetlands for treatment of municipal wastewater. *Ecological Engineering*, *35*(2), 303–309. https://doi.org/10.1016/j.ecoleng.2008.04.007

Walton, C. R., Zak, D., Audet, J., Petersen, R. J., Lange, J., Oehmke, C., Wichtmann, W., Kreyling, J., Grygoruk, M., Jabłońska, E., Kotowski, W., Wiśniewska, M. M., Ziegler, R., & Hoffmann, C. C. (2020). Wetland buffer zones for nitrogen and phosphorus retention: Impacts of soil type, hydrology and vegetation. *Science of the Total Environment*, *727*. https://doi.org/10.1016/j.scitotenv.2020.138709

Wang, Y., Cai, Z., Sheng, S., Pan, F., Chen, F., & Fu, J. (2020). Comprehensive evaluation of substrate materials for contaminants removal in constructed wetlands. *Science of the Total Environment*, *701*, 134736. https://doi.org/10.1016/j.scitotenv.2019.134736

Wilson, V., Hou, A., & Meng, G. N. (2015). Source of lead pollution, its influence on public health and the countermeasures. *International Journal of Health*, *2*(1), 1–1. https://doi.org/10.13130/2283-3927/4785

XU, Z., BAN, Y., JIANG, Y., ZHANG, X., & LIU, X. (2016). Arbuscular Mycorrhizal Fungi in

Wetland Habitats and Their Application in Constructed Wetland: A Review. *Pedosphere*, *26*(5), 592–617. https://doi.org/10.1016/S1002-0160(15)60067-4

Xu, Z., Wu, Y., Jiang, Y., Zhang, X., Li, J., & Ban, Y. (2018). Arbuscular mycorrhizal fungi in two vertical-flow wetlands constructed for heavy metal-contaminated wastewater bioremediation. *Environmental Science and Pollution Research*, *25*(13), 12830–12840. https://doi.org/10.1007/s11356-018-1527-z

Yang, Y., Pan, J., Zhou, Z., Wu, J., Liu, Y., Lin, J. G., Hong, Y., Li, X., Li, M., & Gu, J. D. (2020). Complex microbial nitrogen-cycling networks in three distinct anammox-inoculated wastewater treatment systems. *Water Research*, *168*, 115142. https://doi.org/10.1016/j.watres.2019.115142

Yeh, T. Y. (2008). Removal of metals in constructed wetlands: Review. *Practice Periodical of Hazardous, Toxic, and Radioactive Waste Management*, *12*(2), 96–101. https://doi.org/10.1061/(ASCE)1090-025X(2008)12:2(96)

Yu, M., Wang, Q., Tao, W., Liu, G., Liu, W., Wang, L., & Ma, L. (2020). Interactions between arbuscular mycorrhizal fungi and soil properties jointly influence plant C, N, and P stoichiometry in West Lake, Hangzhou. *RSC Advances*, *10*(65), 39943–39953. https://doi.org/10.1039/d0ra08185j

Zeng, L., Tao, R., Tam, N. F. yee, Huang, W., Zhang, L., Man, Y., Xu, X., Dai, Y., & Yang, Y. (2020). Differences in bacterial N, P, and COD removal in pilot-scale constructed wetlands with varying flow types. *Bioresource Technology*, *318*(July), 124061. https://doi.org/10.1016/j.biortech.2020.124061

Zhang, H. H., Tang, M., Chen, H., Zheng, C. L., & Niu, Z. C. (2010). Effect of inoculation with AM fungi on lead uptake, translocation and stress alleviation of Zea mays L. seedlings planting in soil with increasing lead concentrations. *European Journal of Soil Biology*, *46*(5), 306–311. https://doi.org/10.1016/j.ejsobi.2010.05.006

Zhang, J., Qian, Y., Chen, Z., Amee, M., Niu, H., Du, D., Yao, J., Chen, K., Chen, L., & Sun, J. (2020). Lead-induced oxidative stress triggers root cell wall remodeling and increases lead absorption through esterification of cell wall polysaccharide. *Journal of Hazardous Materials*, *385*(July 2019), 121524. https://doi.org/10.1016/j.jhazmat.2019.121524

Zhang, W. J., & Lin, M. F. (2020). Influence of redox potential on leaching behavior of a solidified chromium contaminated soil. *Science of the Total Environment*, *733*. https://doi.org/10.1016/j.scitotenv.2020.139410

Zhimiao, Z., Xinshan, S., Yanping, X., Yufeng, Z., Zhijie, G., Fanda, L., Yi, D., Wei, W., & Tianling, Q. (2016). Influences of seasons, N/P ratios and chemical compounds on phosphorus removal performance in algal pond combined with constructed wetlands. *Science of the Total Environment*, *573*, 906–914. https://doi.org/10.1016/j.scitotenv.2016.08.148

Wężowicz, K., Turnau, K., Anielska, T., Zhebrak, I., Gołuszka, K., Błaszkowski, J., & Rozpądek, P. (2015). Metal toxicity differently affects the Iris pseudacorus-arbuscular mycorrhiza fungi symbiosis in terrestrial and semi-aquatic habitats. Environmental Science and Pollution Research International, 1940-1947.

Cooke, J. C., Butler, R. H., & Madole, G. (2018). Some observations on the vertical distribution of vesicular arbuscular mycorrhizae in roots of salt marsh grasses growing in saturated soils. Ecology and Biogeography, 547-550.

Fester, T. (2013). Arbuscular mycorrhizal fungi in a wetland constructed for benzene-, methyl tert-butyl ether- and ammonia-contaminated groundwater bioremediation.

Microbial Technology, 80-84.

French, K. E. (2017). Engineering Mycorrhizal Symbioses to Alter Plant Metabolism and Improve Crop Health. Frontiers in Microbiology.

Kadlec, R. H., & Wallace, S. (2009). Treatment Wetlands 2nd Edition. CRC Press.

Keddy, P. A. (2010). Wetland Ecology: Principles and Conservation. Cambridge: Cambridge University Press.

Mustapha, H. I., Van Bruggen, J. A., & Lens, P. L. (2017). Fate of heavy metals in vertical subsurface flow constructed wetlands treating secondary treated petroleum refinery wastewater in Kaduna, Nigeria. International Journal of Phytoremediation.

Smith, S. E., & Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. Annual Review of Plant Biology, 227-250.

Zhan, F., Bo, L., Ming, J., Tianguo , L., Yongmei, H., Yuan , L., & Youshan, W. (2019). Effects of arbuscular mycorrhizal fungi on the growth and heavy metal accumulation of bermudagrass [Cynodon dactylon (L.) Pers.] grown in a lead–zinc mine wasteland. International Jurnal of Phytoremediation, 849-856.