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HEAVY METALS REMOVAL IN ARBUSCULAR MYCORRHIZAL FUNGI ASSISTANT CONSTRUCTED WETLANDS

DIPLOMA THESIS

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DIPLOMA THESIS ASSIGNMENT

B.Sc. Sodiq Ajo, BSc

Engineering Ecology
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Thesis title

Heavy metals removal in arbuscular mycorrhizal fungi assistant constructed wetlands

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Objectives of thesis

This study aims to evaluate the capacity of arbuscular mycorrhizal fungi (AMF) for the improvement ofCWs' treatment performance regarding the single and complex heavy metals removal from wastewater.

Methodology

Serval vertical subsurface flow constructed wetlands (CWs) will be established at the campus of the CzechUniversity of Life Sciences Prague. The experimental device consisted of the innovative KG-System (PVC) pipes, substrate, and water outlet. The dimensions of each system are 15× 55 cm (diameter ×Height). Each CW will be filled with 10 cm gravel (4-5cm) and 35 cm mixture of sand will be used as substrates. *Iris pseudacorus* will be selected as a experimental plant. AMF inoculum will be *Rhizophagus irregularis*. Single andcomplex heavy metals will be added into the artificial wastewater to be treated in those CWs.

The proposed extent of the thesis

50

Keywords

Arbuscular mycorrhizal fungi, heavy metal, constructed wetlands

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Recommended information sources

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Prague on 30. 03. 2021

DECLARATION

I, AJO SODIQ OLUSEGUN, hereby declare that except for reference to other work from different authors which I have dully cited and acknowledged, this action research is the result of my effort and that it has neither in whole nor in part been presented elsewhere.

SIGNATURE: ……………… DATE:………………….

(AJO SODIQ OLUSEGUN)

SUPERVISOR'S DECLARATION

I hereby certify that the preparation and presentation of this thesis was supervised following the guidelines binding the supervision of Diploma thesis laid down by the Czech University of Life Sciences Prague.

SIGNATURE:…………………. DATE:…………………

(doc. ZHONGBING CHEN)

DEDICATION

I dedicate this project first and foremost to Almighty God who has been there right from the beginning to this very point. Special dedication to my late father, whom I promised to dedicate this work to before he left this world, without him, I would never have been able to achieve this.

AJO SODIQ OLUSEGUN

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ABSTRAKT

Umělé mokřady (CW) zažívají celosvětový růst popularity jako přijatelný způsob řešení environmentálního znečištění díky biologické schopnosti odstraňovat odpadní materiály a znečišťující látky obsahující těžké kovy. Bylo popsáno, že arbuskulární mykorhizní houby (AMF) jsou schopny vychytávat a translokovat určité těžké kovy. Tento výzkum se proto zabýval zejména schopností AMF účinně odstraňovat zinek, mangan a kadmium z uměle vybudovaných mokřadů. Tato studie byla realizována na České zemědělské univerzitě v Praze s využitím 10 vertikálně podpovrchově protékaných umělých mokřadů a modelové mokřadní rostliny *Iris pseudacorus*, která se s oblibou používá jako běžná rostlina umělých mokřadů.

Nálezy z této studie ukazují, že ošetření jedním těžkým kovem vede k významně vyšší kolonizaci AM hub než přidání kombinace tří těžkých kovů. Nejvyšší intenzita mykorhizní kolonizace (M %) a abundance arbuskulů (A %) byla zjištěna při ošetření samotnými AMF (41,3 %, resp. 13,7 %). AMF významně zvýšily hmotnost sušiny kořenů o 7 – 49 %, hmotnost sušiny nadzemní části o 2 – 31 %, koncentraci P (mg/kg) v kořeni rostliny o 13 – 40 % a koncentraci K v kořeni rostliny o 20 – 98 %. AMF také zvýšily koncentraci K (mg/kg) v nadzemní části o 72 – 152 % a koncentraci P v nadzemní části o 10 – 72 %. Tato studie dále prokázala, že AMF snižují koncentraci Zn (10 – 13 %), Mn (35 – 55 %) a hmotnostní obsah v kořeni rostliny indukovaný HM a AMF v porovnání s indukcí bez použití AMF. AMF však zvýšily koncentraci Cd o 38 – 278 % a hmotnostní obsah o 47 – 283 %. Dále bylo zjištěno, že účinnost odstraňování těžkých kovů (%) se pohybuje v rozmezí 99,91 – 99,99 % pro Cd, 98,64 – 99,99 % pro Zn a 96,23 – 99,92 % pro Mn. AMF zvýšily účinnost odstraňování Cd o 0,07 – 0,32 %, účinnost odstraňování Zn o 0,14 – 0,33 % a účinnost odstraňování Mn o 0,12 – 1,44 %. AMF navíc snížily koncentraci NH_4^+- N o 20 – 67 % a celkový dusík (N) o 9 – 36 %. Vlivem AMF se však zvýšila účinnost odstraňování NH_4^+- N o 0,3 – 13 % a účinnost odstraňování celkového dusíku (N) o 1 – 40 %. Tato studie dále prokázala, že AMF snižuje celkovou koncentraci C o 3 – 33 % a TOC o 36 – 49 %, zatímco průměrná účinnost odstraňování celkového C se zvýšila o 6 – 17 % a TOC o 2 – 7 %. AMF také snížily koncentraci P v inokulovaných umělých mokřadech a zvýšily účinnost odstraňování hmoty. Tato studie obecně poskytuje povzbudivé důkazy o tom, že zavedení AMF do CW může zlepšit odstraňování těžkých kovů a vývoj sazenic po výsadbě.

Klíčová slova: Arbuskulární mykorhizní houby (AMF), umělé mokřady (CW), těžké kovy, účinnost odstraňování.

ABSTRACT

Constructed wetlands (CWs) are becoming globally popular as an acceptable way of dealing with environmental pollution due to their biological ability of removing waste matter and heavy metal pollutants. Arbuscular mycorrhizal fungi (AMF) have been reported to be capable of depleting certain heavy metals through their uptake and translocation. This research therefore focused primarily on the capacity of AMF to effectively remove Zinc, Manganese and Cadmium from constructed wetlands. This study was conducted using 10 vertical subsurface flow CWs in the Czech University of Life Sciences Prague, using *Iris pseudacorus* as a wetland plant due to its popularity as a commonly used plant in CWs.

The findings of this study revealed that treatments with a single heavy metal had significantly higher AMF colonization than the combination of the three HMs. The highest intensity of Mycorrhiza colonization (M%) and Arbuscule abundance (A%) was found in AMF only treatment (41.3% and 13.7% respectively). AMF significantly increased root dry weight by 7% - 49%, shoot dry weight by 2% - 31%, P concentration (mg/kg) in the plant root by 13% - 40% and K by 20% - 98%. AMF also increased K concentration (mg/kg) in the shoot by 72% - 152% and P concentration by 10%-72%. This study also showed that AMF reduced Zn (10% - 13%), Mn (35% - 55%) concentration and mass content in the root of plants induced with HM and AMF than without AMF. However, AMF increased Cd concentration by 38% - 278% and mass content by 47% - 283%. It was also revealed that heavy metals removal efficiency (%) ranged between 99.91% - 99.99% for Cd, 98.64% - 99.99% for Zn and 96.23% - 99.92% for Mn. AMF increased Cd removal efficiency by 0.07% - 0.32%, Zn removal efficiency by 0.14% - 0.33% and Mn removal efficiency by 0.12% - 1.44%. Furthermore, AMF reduced NH_4^+ - N concentration between 20%-67% and total N between 9%-36%. However, NH_4^+ - N removal efficiency was increased by AMF by 0.3% - 13% and total N by 1%-40%. This study also showed that AMF reduced total C concentration by 3% - 33% and TOC by 36% - 49%, while the average removal efficiency of total C was increased by 6%- 17% and TOC by 2% - 7%. AMF also decreased P concentration in constructed wetlands inoculated while increasing mass removal efficiency. Generally, this study provides encouraging evidence that the introduction of AMF into CWs can enhance the removal of heavy metals and improve plant performance.

Keywords: Arbuscular mycorrhiza fungi (AMF), constructed wetlands (CWs), heavy metals, removal efficiency.

Contents

1.0 INTRODUCTION

1.1 Background

Heavy metals are referred to as metallic elements that have a relatively high density, and are toxic at low concentrations (Fergusson, 1990). Heavy metals are also considered trace elements because of their availability in very low concentrations (Kabala-Pendia, 2001). The drastic industrialization of almost every part of the world is the major reason for the uncontrolled proliferation of heavy metals into our ecosystem (He *et al*, 2005; Bradl, 2002). Chief amongst these heavy metal pollutants are Mercury (Hg), Lead (Pb), and Cadmium (Cd). Heavy metals pose a lot of health risks and environmental hazards because they tend to bio-accumulate. Bio-accumulation or ecotoxicity is the rise in the level of chemical components in biological organisms with time in relationship with the chemical components in their environment (Chojnacka & Mikulewicz, 2014). Heavy metals may leach into constructed wetlands by industrial and consumer wastes, or even from acidic rain which is capable of breaking down soils and releasing these metals into streams, rivers, groundwater, and even wetlands.

Arbuscular mycorrhizal fungi (AMF) are fungi present in the soil and are capable of improving plant nutrients uptake and resistance to several non-living stress factors, including the risk of toxic heavy metals (Sun *et al*, 2018). AMF is touted to be very invaluable for sustainable crop improvement (Gianinazzi *et al*, 2010) because they play a vital symbiotic relationship by effectively affecting plant productivity and the active performance of the ecosystem (Smith & Read, 2008; Jung *et al*, 2012). Several researchers have tried to explain the symbiosis of AMF and improved resistance of plants to varieties of stress to include drought, salinity, temperature, and most importantly for this research work, the presence of toxic heavy metals (Rodriguez *et al*, 2008; Ahanger *et al*, 2014; Salam *et al*, 2017). However, the use of an appropriate plant that can absorb a high amount of heavy metals with the help of AMF can be a viable option in the removal of heavy metals from constructed wetlands (Chen, 2020), the exact role of AMF in achieving this onerous task of getting rid of heavy metals still needs to be studied.

A wetland is a unique ecosystem between land and water which is capable of providing ecological functions (Chen, 2020). A Constructed Wetland (CW) is however termed as a man-made wetland technologically designed to treat municipal or industrial wastewater. A CW often acts as a bio-filter commonly used to remove a wide range of pollutants (including heavy metals and other water quality constituents only) (Zheng *et al*, 2015). Since AMF and wetland plants form a symbiotic relationship that makes it possible for these plants to be able to remove heavy metals as they can uptake and absorb them, there is justification to study how effective this process can be in order to be adopted as a method of sanitizing wetlands of heavy metals.

1.2 AIM

The aim of this thesis is to evaluate the capacity of arbuscular mycorrhizal fungi (AMF) in the removal of heavy metals from AMF assisted constructed wetlands (CWs).

1.3 Research Justification

The massive global industrialization and human activities have led to an increased pollution level. The whole world is very concerned about the effects of pollution in our ecosystem and how it is decreasing our quality of life. Several attempts including the use of technology have been deployed to decrease or ameliorate the effects of pollution in our society entirely. One of the effective technological means of achieving this is the construction of artificial wetlands.

Constructed wetlands are fast becoming globally popular as an acceptable way of dealing with environmental waste pollution because of their bio-filtration properties of removing waste matter and heavy metal pollutants. Arbuscular mycorrhizal fungi are micro-organisms that are believed to be capable of depleting certain heavy metals through their uptake and translocation.

This study is necessary at this point because no research has been able to prove or evaluate the effectiveness of the AMF in removing heavy metals pollution in CWs. This research will focus primarily on the capacity of AMF to effectively remove Zinc, Manganese, and Cadmium from wetlands.

2.0 LITERATURE REVIEW

2.1 Heavy Metals Pollution

Pollution can be defined as the introduction of hazardous substances into the environment. Pollutants can be natural such as volcanic ash and can at the same time be created by human activities solid waste disposal, effluent discharges from factories. Pollutants spoil the condition of air, water, and land.

According to (Muralikrishna *et al*, 2017), Environmental pollution is the contamination of the earth or atmosphere's physical and biological components affecting the normal environmental processes. Environmental pollution could also be the adverse change of our surroundings fully or substantially as a by-product of man's actions through the direct or indirect effect of the changes in radiation levels, energy pattern, abundance of organisms, and chemical and physical compositions (Prabhat, 2016). Environmental pollution has been adjudged a global challenge that is common to both developed and developing countries, which often attracts the attention of relevant stakeholders for its severe long-term consequences. The decrease in environmental quality as a result of pollution is displayed by the loss of biological diversity, vegetation, excessive amounts of harmful chemicals in the ambient atmosphere and food grains, and growing risks of environmental accidents and threats to the life support system (Prabhat,2016).

For over a decade, industrialization has been rapid globally leading to an increase in demand for the exploitation of the Earth's natural resources without control, therefore, contributing to the world's problem of environmental pollution (Gautam *et al*, 2016). The environment has been severely polluted by numerous pollutants, chief amongst them are heavy metals. Heavy metals are being described as metallic chemical elements and metalloids which are toxic to the environment and humans, while some heavy metals are typically not toxic (Tchounwou *et al*, 2012). A list of heavy metals according to their density of being greater than $5 g/cm^3$, and which are more common in our present environment are Zinc (Zn), Cadmium (Cd), Manganese (Mn), Iron (Fe), Cobalt (Co), Mercury (Hg), Lead (Pb), Gold (Au), Arsenic (Ar), Titanium (Ti), Chromium (Cr), Selenium (Se), Silver (Ag), Copper (Cu) (Wang, 2009; Kushwaha *et al*, 2018).

2.2 Sources of Heavy Metal Pollution

Heavy metals are naturally found on the Earth's crust and have been since the Earth's formation. The overwhelming increase of the use of heavy metals for various human endeavours has resulted in an imminent surge of metallic substances in both terrestrial and aquatic environments (Gautam *et al*, 2016). The most important cause of heavy metal pollution is incessant anthropogenic activities (Masindi and Muedi, 2018. This is majorly as a result of mining the metal, smelting foundries, and all other industries that use metals and leaching of metals from various sources such as waste dumps, landfills, excretion, industrial effluent runoff, automobiles (Jessica *et al*, 2020). The secondary source of heavy metals pollution is from the application of agricultural chemicals such as pesticides, fertilizers, insecticides, herbicides on agricultural farmlands (Tchounwou *et al*, 2012). Natural causes can also add to heavy metal pollution such as volcanic activity, metal corrosion, metal evaporation from soil, water and sediment re-suspension, soil erosion, geological weathering (Herawati *et al*, 2000; He *et al*, 2005).

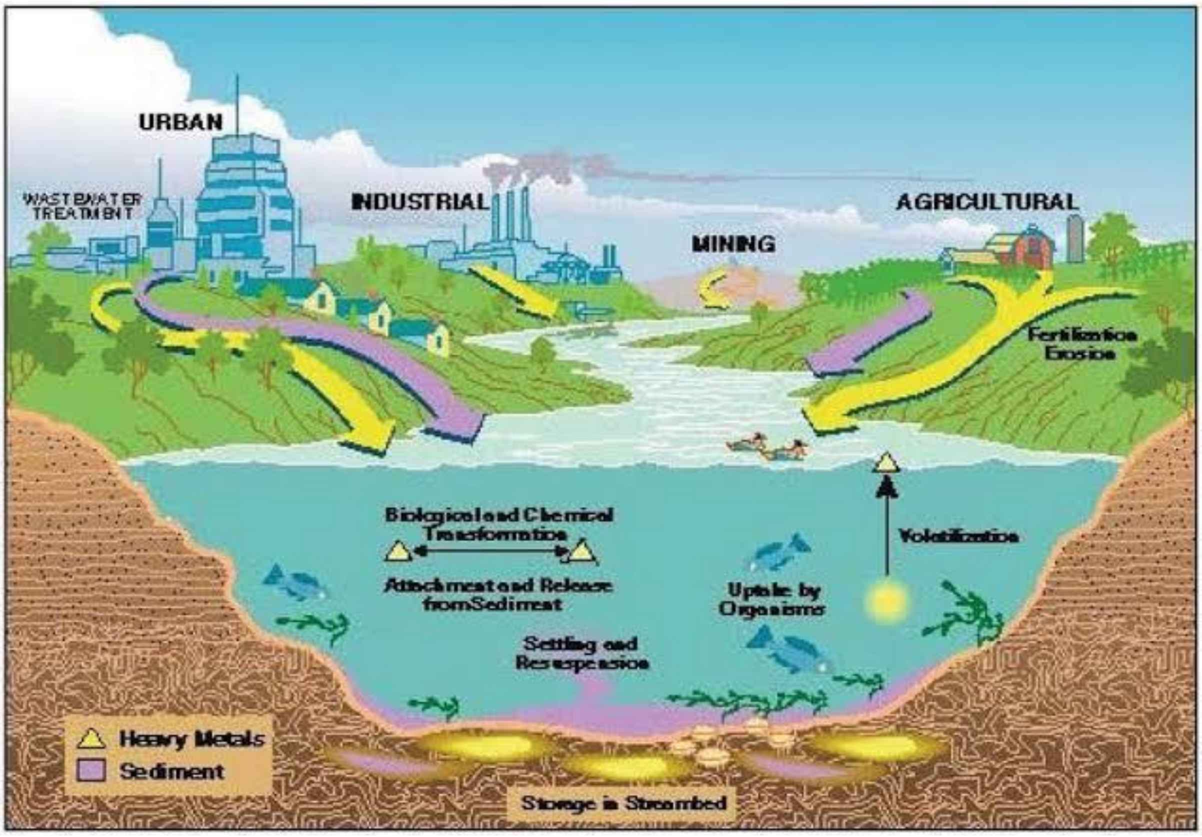

Figure 1: Sources of Heavy Metal Pollution

(https://www.intechopen.com/books/heavy-metals/environmental-contamination-by-heavy-metals)

2.3 Properties of Heavy Metals (HM)

Metals cannot be broken down and are non-biodegradable (Masindi and Muedi, 2018). Organisms may detoxify metal ions by hiding the active elements within a protein (Walker *et al*, 2012). When heavy metals are swallowed or inhaled by humans, they bio-accumulate in the body. Bio-accumulation is the increase in the concentration of a chemical in the biological organism over time compared to the chemical's concentration in the environment (Chojnacka & Mikulewicz, 2014) and hence are termed dangerous. However, some heavy metals are needed for life and are called essential elements which are required for a spread of biochemical and physiological body functions (Duffus, 2002), although, they too can be toxic when present in large amounts (Wang, 2009).

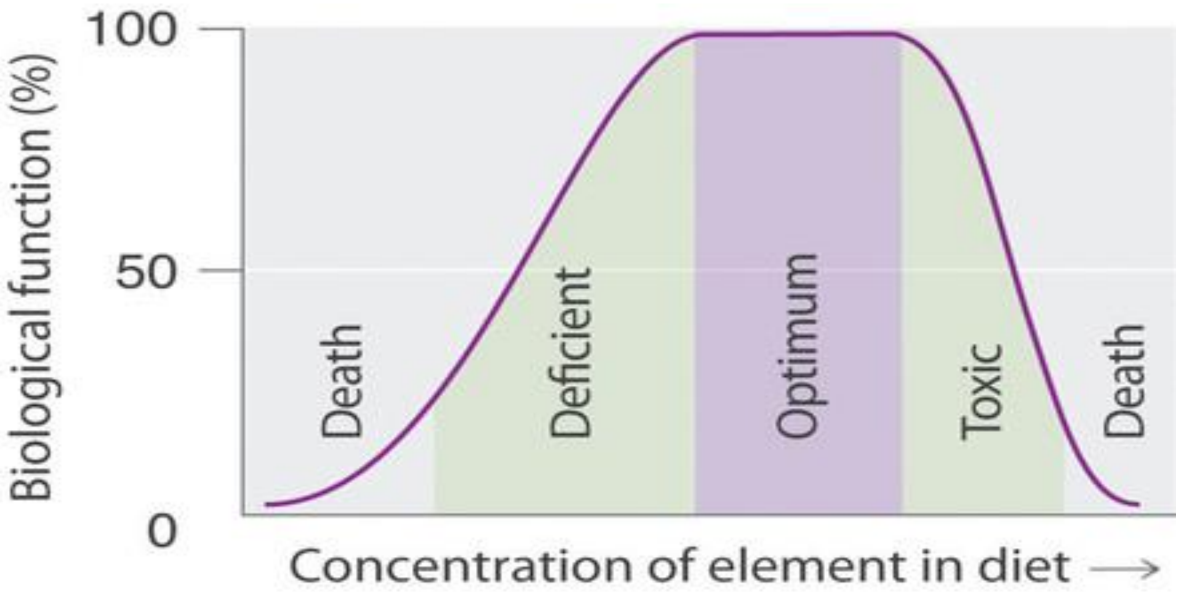

Figure 2: The Relationship Between human performances concerning the concentration of the essential element in the diet

(https://chem.libretexts.org/Bookshelves/General_Chemistry/Book%3A_Chemistry_(Averill_and_Eldredge)/01%3A_Introduction_to_Chemistry/1.8%3A_Essential_Elements_for_Life)

Such heavy metals have been widely used in agriculture, medicine, and other industries to the effect that they dispersed into the environment including our atmosphere, water, and soil.

2.4 Zinc as a Heavy Metal

Zinc (Zn) is an essential heavy metal with atomic number '30' that is commonly used and can enter the environment as a result of numerous industrial processes (Nemery, 1990; Murgia *et al*, 2006). Zinc is an element frequently found within the Earth's crust. It is released to the environment through both natural and anthropogenic sources; however, releases from anthropogenic sources are larger than those from natural sources. The primary anthropogenic sources of zinc in the environment (air, water, soil) and are related to mining and metallurgic operations involving zinc and the use of commercial products accommodating zinc (N. Roney,2005). Zinc is able to form complexes with different organic and inorganic groups (ligands). Biological activity can affect the movement of zinc within the aquatic environment, while the biota contains relatively little zinc compared to the sediments. Zinc bio-accumulates reasonably in aquatic organisms (N. Roney, 2005).

Figure 3: The Aftermath of Zinc Pollution in Aquashicola, Pennsylvania, USA.

(https://www.greenspec.co.uk/building-design/zinc-production-environmental-impact/)

Although, Zinc contributes to human health, however, very large concentrations may cause health problems (Murgia *et al*, 2006). In the environment, Zinc contagion can lead to flu-like conditions known as 'metal fever'. This condition is caused by over-sensitivity to Zinc. Zinc can also cause problems to unborn and new-born children when their mothers have taken in large concentrations of Zinc and the children may be exposed to it through milk and blood from their mothers (Tony Milkins, 2013).

2.5 Manganese as a Heavy Metal

Manganese, an important element of the human diet, is a naturally exisitng component of the earth's crust. After iron, Mn is the second most abundant heavy metal. Unlike Pb^2+, which has no known physiological role, Mn has several favorable roles in human physiology (Aschner and Aschner, 2005). Elevated Mn levels can cause human neurotoxicity. Notably, workers exposed to high levels of Mn are at elevated risk of developing a Parkinson's disease (PD)-like neurological disorder known as manganism (Cersosimo and Koller, 2006), and recently adverse effects of exposure to elevated Mn in drinking water have been observed in children (Khan *et al*, 2011).

While Mn can exist in 11 different oxidation states, Mn(II) and Mn(III) are the most biologically relevant (Yokel, 2009; Michalke *et al*, 2007). In particular, miners, welders, smelters, workers of ferroalloy plants, and dry cell battery workers are more prone to Mn-related toxicity (O'Neal *et al.* 2015).

Manganese exists in the Earth's crust at an average concentration of 950 mg/kg, principally in ores: pyrolusite (Mn4+O2), rhodochrosite ($MnCO_3$), manganite (Mn3+O(OH)), hausmannite ($Mn^{2+}Mn^{3+}{}_2O_4$), biotite mica (K(Mg,Fe)3(AlSi3O10)(OH)2), and amphibole (Fe,Mg)7Si8O22(OH)2). Manganese is an important chemical element required by both plants and animals. In some waters it can either limit the growth of algae; directly or indirectly; it is also an essential component of several enzyme systems in animals (Moore J.W. 1991). Although manganese is of little toxicologic significance, it may control the concentration of other elements, including toxic heavy metals, in surface waters (Moore J.W. 1991).

2.6 Cadmium as a Heavy Metal

Cadmium (Cd) is a naturally occurring heavy metal of considerable toxicity with a destructive impact on the environment and particularly on human health. Cadmium is situated on the Periodic Table of Elements between Zinc (Zn) and Mercury (Hg), with chemical behaviour similar to Zinc. Cadmium exists in the earth's crust at about 0.1 part per million (Wedepohl, 1995). Cadmium chiefly occurs as an impurity in Zinc or Lead deposits, and hence, being produced primarily as a by-product of Zinc and Lead smelting.

Human exposure to Cadmium occurs primarily through inhalation and ingestion. About 5 – 10% of ingested Cadmium is taken in, with intestinal absorption greater in persons with Iron, Calcium or Zinc deficiency (Nordberg *et al*, 2007). Cadmium poisoning has been reported from several parts of the world. Long-term exposure to Cadmium through the air, water, soil, and food may lead to cancer and organ system toxicity. Cadmium noticeably exists in the environment due to human activities such as the use of fossil fuels, metal ore combustion and waste burning. Leaking sewage sludge to agricultural soil may cause the transfer of Cadmium compounds absorbed by plants that may play a significant role in the food chain, and also bio-accumulate in various human organs.

2.7 Constructed Wetlands (CW)

A Constructed Wetland is an engineered system designed for wastewater treatment with moderated soil, plants, microbial as natural wetlands (Vymazal, 2005). CWs are also artificial wetlands to treat municipal or industrial wastewater, greywater, or storm-water run-off. It may also be designed for land reclamation after mining, or as a way of reducing natural areas lost to land development. Based on the geographic and topographic location of wetland, the wetland may have different functions on the ecosystem, such as water storage (flood control), groundwater refilling, reservoirs of biodiversity, climate change modification, trap sediments and heavy metals (United Nations Millennium Ecosystem Assessment and Ramsar Convention).

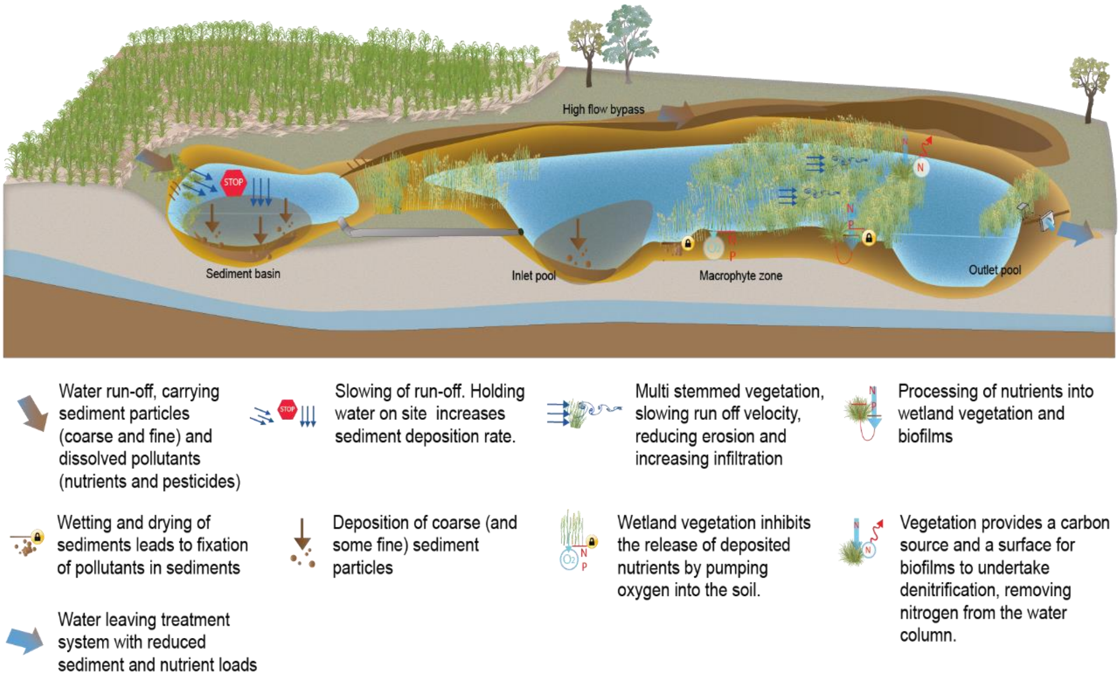

Figure 4: Schematic diagram of constructed wetland (https://wetlandinfo.des.qld.gov.au/wetlands/management/treatment-systems/for-agriculture/treatment-sys-nav-page/constructed-wetlands/)

Like natural wetlands, constructed wetlands also act as a bio-filter which can help in the extraction of pollutants such as organic matter, nutrients, heavy metals, and so on from the water (Maiga *et al*, 2017). There are two main types of Constructed Wetlands: Sub-surface Flow and Surface Flow Constructed Wetlands (Vymazal, 2005; 2008).

In Sub-surface Flow Constructed Wetlands, wastewater flows between the roots of the plants and there is no water surfacing (goes through the gravel and sand bed). This system has been proven to be more efficient because of certain characteristics such as its lesser area needed for construction, unattractiveness to mosquitoes, lesser odour, and sensitivity to environmental conditions (Tilley *et al*, 2014).

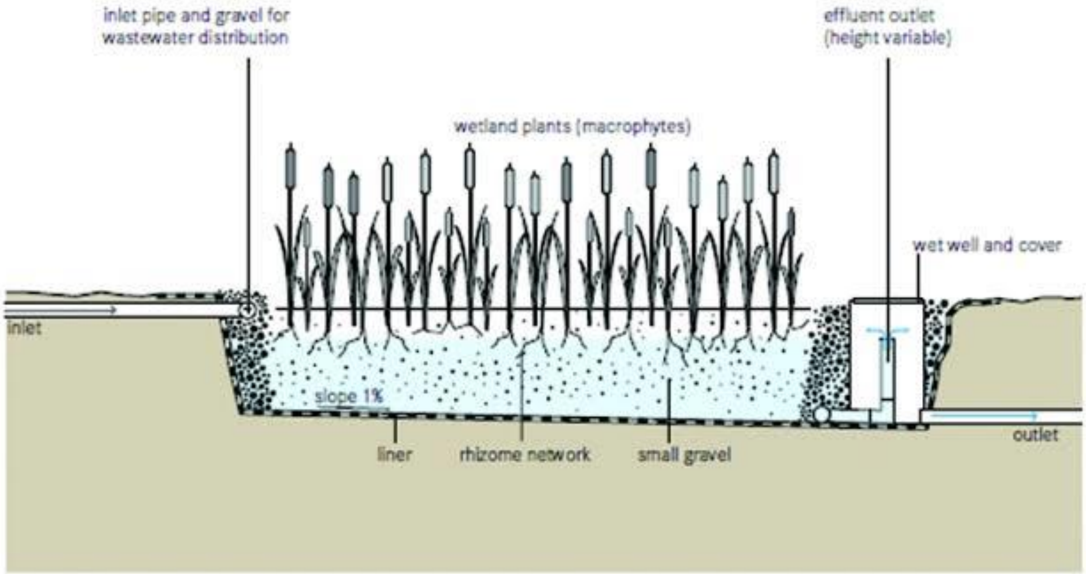

Figure 5: Horizontal subsurface flow constructed wetland

(https://www.waterpathogens.org/book/constructed-wetlands)

A sub-surface Flow Constructed Wetlands require the following maintenance tasks, such as regular checking of;

1. The pre-treatment processes
2. Pumps when they are used
3. Influent loads
4. Distribution of the filter beds

It should be noted strongly that Constructed Sun-surface Flow wetlands are meant as secondary treatment systems. This means that the effluent needs to first pass through a primary treatment that will effectively remove solids (such as sand and grits, grease trap, etc). An important disadvantage of the Sub-surface Flow Constructed Wetlands are the intakes which can clog or bio-clog easily.

A Sub-Surface Flow can be further classified as Horizontal or Vertical flows. In the vertical flow constructed wetlands, the effluent (outflow) moves vertically from the planted layer down through the substrate and out (needing air pumps to aerate the bed), (Alexandros *et al*, 2014).

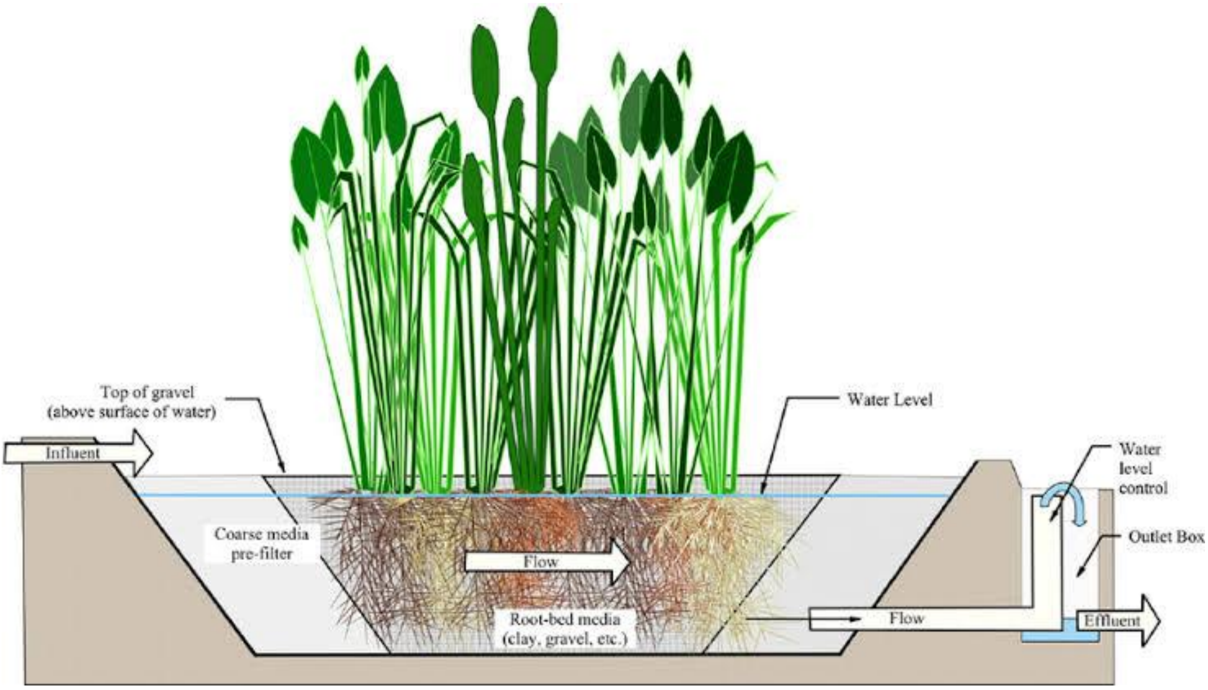

Figure 6: Flow of water through a subsurface flow constructed wetland

(https://www.researchgate.net/figure/Water-flow-path-through-a-subsurface-flow-constructed-wetland_fig2_258697505)

Surface flow constructed wetlands is composed of basins (or channels) with soil (or other substrates) to assist the plants and water flow through CW with shallow depth (Vymazal, 2014). Surface flow constructed wetlands with emergent macrophytes can be used as a biological treatment system. Surface Flow Constructed Wetlands could also help to remove organic compounds, nitrogen, and phosphorus. It can also remove suspended solids by filtrating, precipitation, aggregation, and surface adhesion. Wetland vegetation can help the precipitation process by decreasing water mixture and re-suspension of particles on the surface of the precipitation (Vymazal, 2014).

Surface flow constructed wetlands can remove heavy metals. Heavy metals can be absorbed in plants and soils in wetlands. The study has proved surface flow constructed wetlands could remove Cadmium, Nickel, Lead, Copper, Zinc, and Iron that were mostly accumulated in roots compared with shoots. And there is no obvious heavy metals accumulation in the vertical soil profile (Lavrnic *et al*. 2018). In addition, surface flow constructed wetlands are suitable to remove Iron, Manganese, Mercury, and Silver from wastewater (Vymazal, 2005; Ghermandietal, 2007).

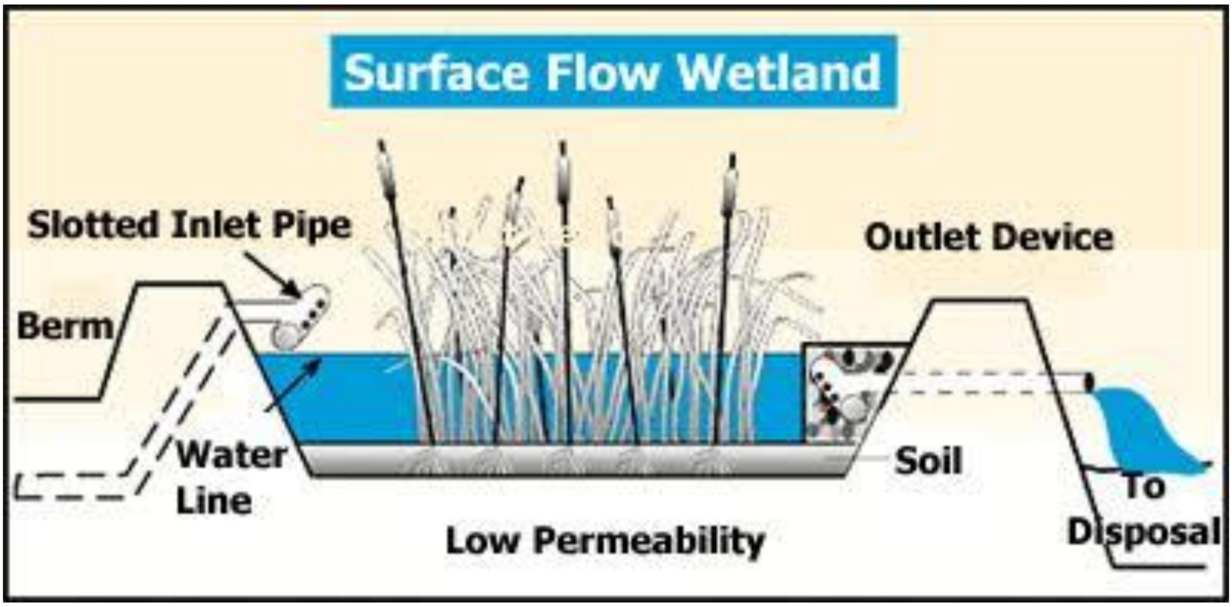

Figure 7: Surface flow constructed wetland

(https://www.wateronline.com/doc/surface-flow-systems-work-like-natural-wetlan-0001)

2.8 Arbuscular Mycorrhizal Fungi (AMF)

Arbuscular mycorrhizal fungi (AMF) are a ubiquitous soil micro-organism, which can form a symbiotic association with most terrestrial plants. These beneficial microbes have been proven to offer an array of benefits to host plants (Bonfante & Genre, 2010). During mycorrhization, besides significant improvement of plant nutritional status, AMF can enhance plant performance and tolerance against several stresses including heavy metals pollution (Balestrini & Lumini, 2018). The exploitation of AMF is considered as one of the most efficient practices to increase plant's tolerance to environmental stresses (Birhane *et al*, 2012).

2.9 Factors Affecting the Application of AMF in Constructed Wetlands

A lot of factors affect the application of AMF in Constructed Wetlands. For example, flooding (hydrologic condition), phosphorus, salinity, plant species and aerenchyma, CW types, the quality of wastewater and so on (Xuetal, 2016).

1. Flooding (Hydrologic Condition)

There are divergent views about the flooding effect on AMF colonization. There is a decreasing AMF colonization with flooding, (Miller, 2000; Wang *et al*, 2010; Xu *et al*, 2016). Wirsel (2004) even found continuing flooding could cause zero colonization. Colonization decreased because flooding conditions might affect root morphology and physiology (Xu *et al*. 2016). Therefore, flooding condition is variable in different situations, but it is essential for AMF colonization. It is important to take into account the flooding conditions in Constructed Wetlands regarding the AMF colonization.

2. Phosphorus (P)

Phosphorus level in the rhizosphere is a major abiotic factor that influences the AMF colonization in roots (Xu *et al*. 2016). But AMF colonization and phosphorus level of the environment have a complicated relationship. *Typha angustifolia* is colonized in low-phosphorus treatment but does not exist in high-phosphorus treatment (Xu *et al*, 2016; Tang *et al*. 2001). However, *Carex Lasiocarpa* and *Typha latifolia* have no mycorrhizal in a low Phosphorus condition (Xu *et al*, 2016; Cornwell *et al*, 2001). So, Wang *et al* (2010) and (Xu *et al*, 2016) both suggested that there is a "bell-shaped" relationship between AMF colonization and soil Phosphorus in wetland ecosystems. That is, AMF colonization is inhibited at high or low Phosphorus levels. Therefore, attention should be paid to the content of phosphorus in constructed wetland to ensure AMF colonization.

3. Operation Modes of Constructed Wetland

Intermittent operation of water flow, variation of wet and dry, and aeration would supply oxygen to Constructed Wetlands, which brings benefit to the growth, richness, and variety of AMF (Xu *et al*, 2016). For example, Miller (2000) and (Xu *et al*, 2016) found the AMF colonization is higher in intermittent flood conditions than continuous flood conditions. In addition, a study found adjustment of operation modes in surface flow constructed wetlands could improve the oxygen transfer capacity to provide enough oxygen for microorganisms (including bacteria and AMF) to eliminate pollutants. These operations include frequent fluctuations in water levels (tidal flow), passive air pumps (vertical flow), or direct aeration of water in gravel-bed (horizontal flow) (Xu *et al*, 2016). Therefore, the means of operation of constructed wetlands and the right water depth are essential for AMF colonization.

4. Abiotic Environmental Factors

A study was conducted on the effects of temperature on fungal growth and tested if there are differences in fungal growth and if they were linked to the effect's temperature had on how carbon moved to, or within, the fungus. The transfer-translocation measurements of Growth curves and C uptake were derived for three arbuscular mycorrhizal fungi (AMF) isolates cultured within a 6–30°C temperature range. Some experiments with a model fungal isolate, *Glomus intraradices*, was used to examine how temperature affects lipid body and P movement, and to know the role of acclimation and incubation time. Despite clear independent root and AMF growth responses, temperature effects on AMF growth were both direct and indirect. Translocation of C in the fungus, were also lessened by low temperatures (< 18°C). Uptake and translocation of P by fungal hyphae were, by contrast, similar between 10 and 25°C. It was deduced that temperature between 6 and 18°C, reduces the growth of AMF, and that movement of C to the fungus is involved in this response.

Arbuscular mycorrhizal fungi (AMF) are associated with the roots of over 80% of terrestrial plant species. Mycorrhizal fungi are critical and essential microbes for plant growth and survival. It is mostly accepted that environmental conditions that support host plant growth tend to increase mycorrhizal infection and sporulation. Mycorrhizal colonization is known to prompt different physiological, morphological, and biochemical changes in host plants. Environmental factors and soil conditions affects the mycorrhizal associations in ecosystems, but to study the impacts of these factors on mycorrhizal fungi is not an easy task because they hardly occur in nature without a host (Monther and Kamaruzaman, 2012).

Among the biofertilizers, mycorrhizal fungi form the most important group of soil microorganisms. The review showed the main abiotic conditions that interacted with mycorrhizal fungi and they are; soil temperature, crop rotation, soil acidity, fertilizer and organic matter, drought stress and soil moisture, pesticides, heavy metals, and salt stress (Monther and Kamaruzaman, 2012).

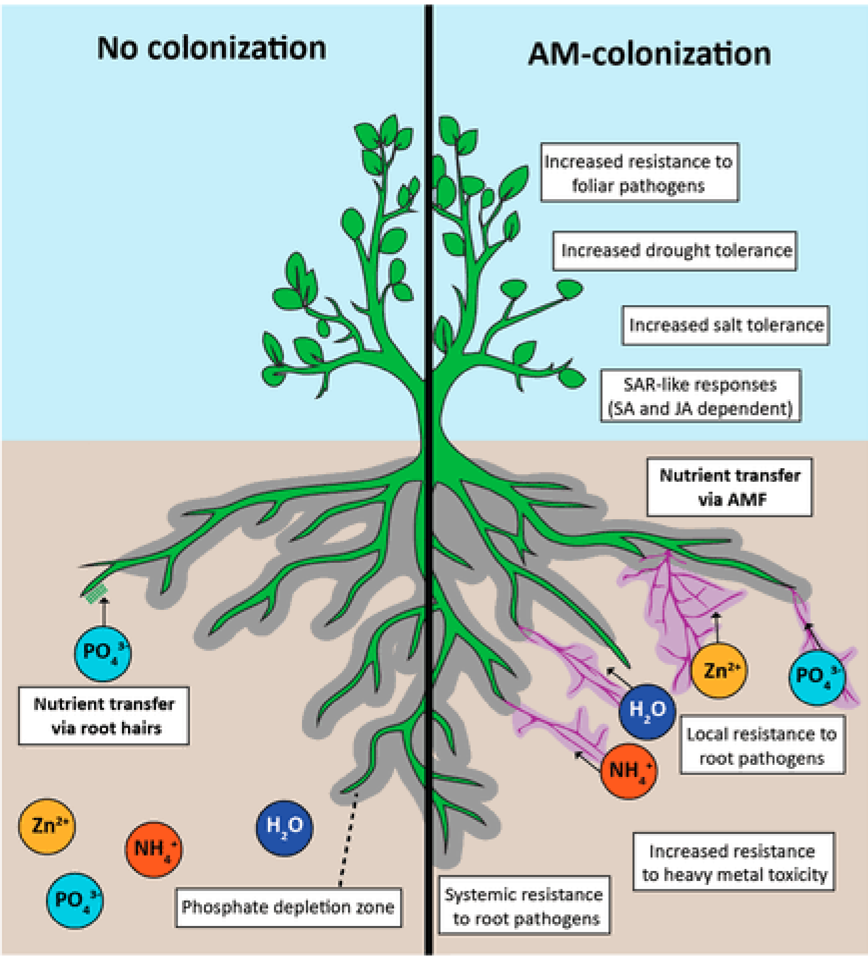

Figure 8: Arbuscular Mycorrhizal Fungi

(https://en.wikipedia.org/wiki/Arbuscular_mycorrhiza)

3.0 MATERIALS AND METHODOLOGY

3.1 EXPERIMENTAL SETUP

Iris pseudacorus was selected as a wetland plant in this study because it is a commonly used plant in CWs. The seedlings of *I. pseudacorus* were collected from natural ponds on the campus of the Czech University of Life Sciences Prague. The roots of each *I. pseudacorus* were surface sterilized with 75% ethyl alcohol for 10s and 1% sodium hypochlorite (NaClO) for 15 min, washed carefully with sterile distilled water five times before transplanted into the sterilized pots. AMF inoculum (*Rhizophagus irregularis* BEG140) was purchased from Symbiom Ltd., Lanˇskroun, Czech Republic.

This study was conducted using 10 vertical subsurface flow CWs in the Czech University of Life Sciences Prague. The experimental device consisted of the innovative KG-System (PVC) pipes, substrate, and water outlet. The 10 PVC pipes were established to simulate the subsurface flow CWs with the dimensions of each system is 15× 55 cm (diameter × height). Each CW was filled with 15cm gravel (4 - 5 cm) and 30cm sand was used as substrates. Factors that influenced this study were AMF, and Heavy Metals (without Zn, Mn & Cd, and with Zn, Mn & Cd). Pollutants included heavy metals. The concentrations of Zn, Mn and Cd were 5mg/L, 5mg/L, and 0.2mg/L, respectively. Inlet water of CWs was simulated municipal sewage (Table 1). Considering the AMF colonization in the roots of *I. pseudacorus,* simulated municipal sewage without heavy metals was fed into each CW for 2 months, and hydraulic retention time is 5 days. The successful AMF colonization successfully necessitated the simulated municipal sewage with different heavy metals which were fed into CWs afterward. The duration of the experiment was 16 weeks. The CWs were protected from rain throughout the experiment.

Figure 9: Experimental Pots

3.2 SAMPLE ANALYSIS

Outflow samples in the 10 CWs were taken every 5 days. pH, oxidation-reduction potential (ORP), total nitrogen (TN), total organic carbon (TOC), ammonium (NH_4^+), nitrite (NO_3^-), nitrite (NO_2^-), phosphate (PO_4^{3-}). Heavy metal concentrations in water were analyzed every 10 days. Wetland plant samples and substrates in CWs were analyzed after the experiment. Plant shoots and roots were harvested individually, the height and fresh weight were measured afterwards, then washed carefully with deionized water for more analysis. Samples of about 5g fresh weight of plants were used to determine chlorophyll and MDA concentrations. Dry weights of shoots and roots were obtained after oven drying at 70 °C for 48 h. The dried samples were used to measure the heavy metal concentrations, biomass, total phosphorus (TP), TN, total carbon (TC) and mycorrhizal dependency. Subsamples of about 2g fresh weight of roots were collected for the determination of AMF colonization. Heavy metal concentrations were analyzed in substrates, pH and ORP in outflow were monitored using HQD Field Case (HACH), TOC and TN in outflow will be monitored by Formacs^{SERIES}. Total Organic Carbon (TOC)/Total Nitrogen analyzers, NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-} concentrations were determined by 883 Basic IC plus. Heavy metal in the outflow, substrate and plants was extracted in the microwave with HNO_3 and H_2O_2 presence, and then analysed by ICP-OES. TP contents in plants were also analysed by ICP-OES. Chlorophyll content was determined by spectrophotometry with acetone extraction (Palta, 1990). Biomass in shoots and roots was measured by the gravimetric method. MDA content in shoots and roots was determined according to (Chen *et al*., 2013). TC and TN contents in the substrates and plants were directly determined by a Skalar Primacs SNC analyzer (Breda, the Netherlands), NIST 1547 Peach Leaves was used as the standard (National Institute of Standards and Technology, Gaithersburg, MD, USA). AMF colonization was analysed by (Phillips and Hayman, 1970).

Reagent	**Concentration (mg/L)**	**Microelements**	**Concentration (mg/L)**	**Heavy metals**	**Concentration (mg/L)**
Urea	104	$CuSO_4.5H_2O$	0.01	$ZnSO_4.7H_2O$	135
NH_4Cl	16	$FESO_4.7H_2O$	0.45	$MnSO_4.H_2O$	95
$CH_3COONa.3H_2O$	255	H_3B0_3	0.04	$CdSO_4.8H_2O$	3.5
Peptone	20	$Na_2MoO_4.2H_2O$	0.02		
KH_2PO_4	41	$KCr(SO_4)_2.12H_2O$	0.02		
Yeast extract	132				
Skim milk	59				
$NaHCO_3$	25				
$MgCl_2.6H_2O$	34				
$CaCl.6H_2O$	28				

Table 1: Characteristics of simulated municipal sewage (Sample analysis)

3.3 AMF colonization

AMF colonization was accessed according to the description of Phillips and Hayman (1970). Firstly, 0.5-1g root samples were washed, cut it to be around 1cm afterward. Then root was heated at 90℃ in 10% KOH for one hour. Rinsed with 2% HCl around 5 minutes. Stained for 5 minutes in 0.05% trypan blue with lactophenol then heated at 90 ℃ for 30 minutes. Discoloration in a petri dish with lactic acid glycerol. Later taken 30 root sections for slice preparation and observed with a 100×400 microscope. The mycorrhizal colonization (M%), and the arbuscular abundance (A%) were calculated with MYCOCALC software.

3.4 AMMONIA IONS

Ammonium ion (measured as N-NH4) was determined by N-NH4 by the indophenol method. Determination of CSN EN ISO 7150-1, without determination of concentration up to 1.2 mg/l.

Instrument equipment: Spectrophotometer (setting wavelengths to 655nm) + 1cm cuvette, or 5 m cuvette (for low concentrations)

Chemicals: Sodium salicylate ($C_7H_5NaO_3$), Sodium citrate ($Na_3C_6H_5O_7$), Sodium nitroprusside ($C_5FeN_6Na_2O$), Sodium dichloroisocyanurate ($C_3Cl_2N_3NaO_3$), Sodium hydroxide (NaOH), Ammonium chloride, (NH4Cl) Ethanol (95%), Distilled water.

Preparation of coloring agent: 65g of sodium salicylate and 65g of trisodium citrate dihydrate were dissolved in a 500ml volumetric flask. then 0.475g of sodium nitroprusside is added. After complete dissolution, it was made up to a volume of 500ml and stored in a dark bottle in the refrigerator.

Preparation of alkaline solution: 16g of NaOH was dissolved in 250ml of deionized water, cooled to room temperature, then added 1g of sodium dichloroisocyanurate dihydrate. After dissolution, it was transferred to a 500ml volumetric flask and made up to 500 ml. It was stored in a dark bottle in the refrigerator.

Preparation of the standard - stock solution: 3,819g of NH4Cl (dried for 2 hours at room temperature) was dissolved in a 1000ml volumetric flask with 500 billion water, (105 ° C), and made up to the mark, i.e., concentration 1000 mg/l.

Working solution 1: The diluted SOx standard stock solution (2ml to 00ml), i.e., 20mg/l

Working solution 2: 4x credit working solution 1 (25ml to 100ml), i.e., 5mg/I

Method: Into a 40ml volumetric flask, 4 coloring agents were added, mixed, and also 4 ml of alkaline solution, mixed, and made up to the mark with 50ml of distilled water and left to stand for at least 60 minutes. Then it was changed at a wavelength of 655nm in a 1cm cuvette (green color).

3.5 Cd, Zn, Mn, K, and P Concentration in Plants

Cd, Zn, Mn, K and P contents in plants (roots and shoots) were determined with the pseudo-total digestion method according to US EPA Method 3051A with some modifications. 0.2 g grind sample was added 2 ml H2O2 and 8 ml HNO3 in this order then digested with an electric heating plate at 150℃ overnight. Later diluted at 25ml with distilled water and filtered and then passed the sample for ICP-OES.

3.6 The Ph, Cd, Zn, and Mn Concentration in Substrate

pH

The pH values were determined with a pH meter according to Hanlon, E.A. CIR1081. The dried substrate was sieved with a 0.710 mm sieve. Added 25 ml deionized water in a beaker. Measured the pH after 30 minutes of standing with a pH meter.

Cd, Zn, and Mn Concentration

Cd, Zn, and Mn concentrations in substrates were determined by the pseudo-total digestion method (US EPA Method 3051A) as analyzed in plants. 500mg sand materials were added to 2.5ml hydrochloric acid (HCl) and 7.5ml nitric acid (HNO3). Then heated and filtered samples for ICP-OES.

3.7 STATISTICAL ANALYSIS

The collected data were subjected to Analysis of Variance (ANOVA) and the means were separated using DMRT and standard error. All statistical computations were performed with SAS statistical software.

4.0 RESULTS

4.1 Intensity of mycorrhiza colonization and arbuscule abundance in the root system

The intensity of mycorrhiza colonization (M%) was found to be significantly different among the treatments ($p=0.0018$). The highest intensity of Mycorrhiza colonization (M%) was found in AMF treatment (41.3%) followed by AMF + Zn treatment (40.2%), AMF + Cd treatment (40.1%) and AMF + Mn treatment (39.99%), while the least was found in AMF + Zn+Mn+Cd treatment (28.0%). However, the intensity of Mycorrhiza colonization observed in AMF treatment was not significantly different from AMF + Zn, AMF + Cd and AMF + Mn but was significantly higher than the intensity of Mycorrhiza colonization observed in AMF + Zn+Mn+Cd treatment from which the lowest value was recorded (Table 2).

As shown in Table 2, the Arbuscule abundance in the root system (A%) was discovered to be significantly different among the treatments ($p<0.0001$). The highest Arbuscule abundance (A%) was recorded in AMF treatment (13.7%), followed by AMF + Zn+Mn+Cd treatment (6.8%), AMF + Mn treatment (6.8%) and AMF + Cd treatment (6.7%), while the least Arbuscule abundance (A%) was observed in AMF + Zn treatment (6.2%). The highest value of Arbuscule abundance (A%) which was observed in AMF treatment was significantly higher than other treatments. However, the values of Arbuscule abundance (A%) observed in AMF + Zn+Mn+Cd, AMF + Zn, AMF + Mn and AMF + Cd treatments were not significantly different (Table 2).

Table 2: Intensity of Mycorrhiza colonization (M%) and Arbuscule abundance in the root system (A%)

Treatment	Intensity of Mycorrhiza colonization in root system M (%) ± SE	Arbuscule abundance in the root system A (%) ± SE
AMF	41.26 ± 1.32^{a}	13.73 ± 1.05^{a}
AMF + Zn+Mn+Cd	28.08 ± 1.06^{b}	6.81 ± 0.61^{b}
AMF + Zn	40.18 ± 2.66^{a}	6.23 ± 0.57^{b}
AMF + Mn	39.99 ± 1.34^{a}	6.75 ± 0.73^{b}
AMF + Cd	40.12 ± 2.00^{a}	6.65 ± 0.41^{b}
p-value	0.0018	<0.0001

Note: Means with the same alphabet along the column are not significantly different

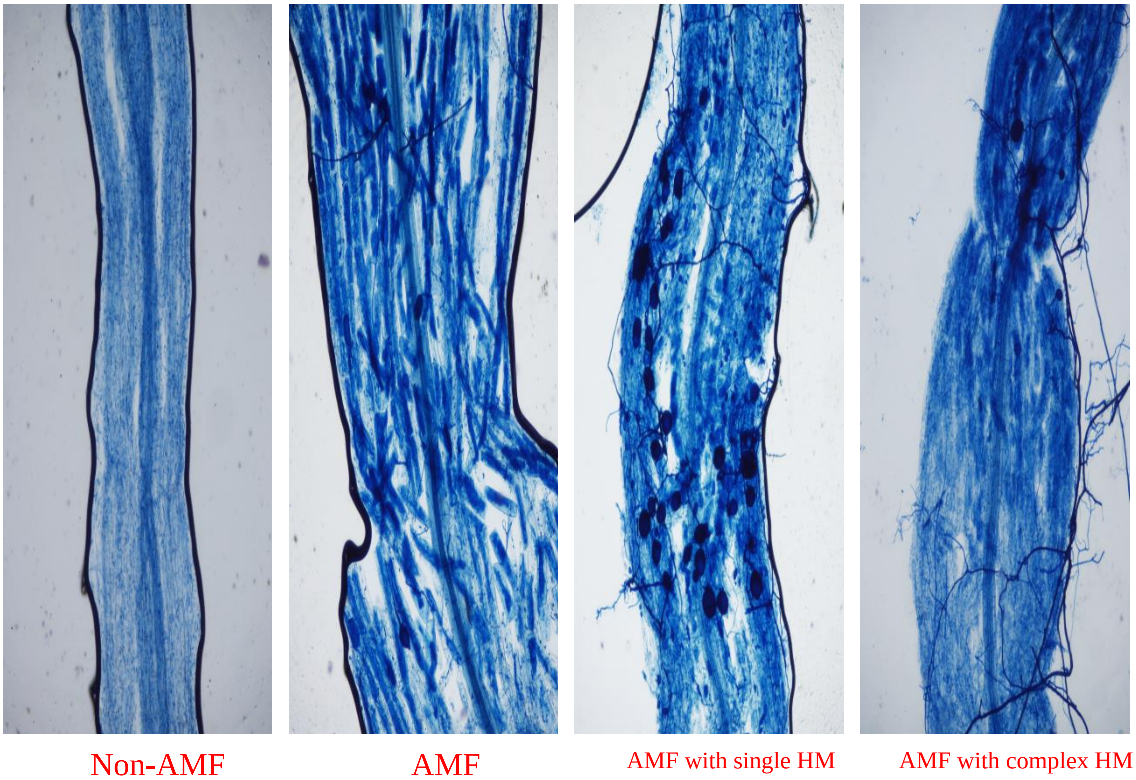

Plate 1: AMF colonization in different treatments

4.2 Biomass content of experimental plant

Plant response in terms of root dry weight (g) to HM and AMF revealed a significant difference ($p<0.0001$) between the various treatments. The highest root dry weight was found in AMF treatment (18.76g) which was significantly higher than all other treatments, followed by Control treatment (12.07g), AMF + Zn (9.48g), AMF + Mn (6.93g), AMF + Cd (6.88g), Cd (6.79g), Zn (6.37g), and AMF +Zn+Mn+Cd (5.93g), while the lowest root dry weight was found in Zn+Mn+Cd treatment (5.55g). The root dry weight value observed in the control treatment was significantly higher than other treatments with HM. Also, a significant difference ($p=0.0052$) was observed among the treatments in terms of shoot dry weight (g). The highest shoot dry weight was found in AMF treatment (7.41g), followed by Control treatment (7.39g), AMF + Mn (6.39g), AMF +Zn (5.95g), Zn (5.85g), Mn (5.80g), Cd (5.32g) and the least value was recorded in Zn+Mn+Cd (3.70g). However, AMF increased root dry weight by 7% with Zn+Mn+Cd, by 49% with Zn, by 12% with Mn and by 1% with Cd. AMF also increased shoot dry weight by 31% with Zn+Mn+Cd, by 10% with Mn and by 2% with Zn. On the other hand, AMF decreased shoot dry weight by 18% with Cd (Table 3).

Table 3: Biomass content of experimental plant

Treatment	Root dry weight (g) ± SE	Shoot dry weight (g) ±SE
Control	12.07 ± 0.23^{b}	$7.39\pm0.0.80^{a}$
Zn+Mn+Cd	5.55 ± 0.24^{e}	3.70 ± 0.10^{d}
AMF	18.76 ± 0.45^{a}	7.41 ± 0.82^{a}
AMF + Zn+Mn+Cd	5.93 ± 0.29^{de}	4.86 ± 0.57^{bcd}
AMF + Zn	9.48 ± 0.79^{c}	5.95 ± 0.50^{abc}
AMF + Mn	6.93 ± 0.36^{d}	6.39 ± 0.72^{ab}
AMF + Cd	6.88 ± 0.50^{d}	4.35 ± 0.28^{cd}
Zn	6.37 ± 0.15^{de}	5.85 ± 0.66^{abc}
Mn	6.18 ± 0.15^{de}	5.80 ± 0.22^{abc}
Cd	6.79 ± 0.10^{d}	5.32 ± 0.84^{abc}
p-value	<0.0001	0.0052

Note: Means with the same alphabet along the column are not significantly different

4.3 Nutrient concentration in plant

Plant response to AMF and HM in terms of nutrient concentration (mg/kg) in the root and shoot varied significantly ($p<0.0001$) among the treatments. The highest K concentration (mg/kg) in the plant root was observed in AMF+Zn+Mn+Cd (28317.80mg/kg), while the lowest was observed in Zn (12668.87mg/kg). AMF significantly increased K concentration (mg/kg) in the root by 54% without HM, by 98% with Zn+Mn+Cd, by 77% with Zn, by 20% with Mn and by 79% with Cd. The highest P concentration (mg/kg) in the plant root was also observed in AMF+Zn+Mn+Cd (6465.13mg/kg), while the lowest was recorded in Mn (3581.97mg/kg). AMF significantly increased P concentration (mg/kg) in the root by 13% without HM, by 33% with Zn+Mn+Cd, by 8% with Zn, by 40% with Mn and by 16% with Cd.

A significant difference was also observed in nutrient concentration (mg/kg) of the plant shoot. The highest P concentration (mg/kg) in the plant shoot was observed in AMF+Zn+Mn+Cd (37484.42mg/kg), while the least was observed in Cd (11049.84mg/kg). AMF significantly increased K concentration (mg/kg) in the shoot by 74% without HM, by 127% with Zn+Mn+Cd, by 124% with Zn, by 72% with Mn and by 152% with Cd. The highest P concentration (mg/kg) in the plant shoot was also observed in AMF+Zn+Mn+Cd (5987.82mg/kg), while the lowest was also recorded in Cd (3294.78mg/kg). AMF significantly increased P concentration (mg/kg) in the shoot by 72% with Zn+Mn+Cd and by 10% with Cd. On the other hand, AMF significantly decreased P concentration (mg/kg) by 1% without HM, by 6% with Zn, and by 3% with Mn.

Table 4: Nutrient concentration in experimental plant (mg/kg)

Treatment	Root		Shoot	
	K (mg/kg) ± SEM	P (mg/kg) ± SEM	K(mg/kg) ± SEM	P(mg/kg) ± SEM
Control	14573.49±6.53[f]	4185.68±2.21[g]	16103.48±7.95[g]	4580.25±15.46[b]
Zn+Mn+Cd	14315.58±11.32[g]	4846.70±21.28[d]	16505.02±13.19[f]	3484.29±18.42[e]
AMF	22405.95±14.91[c]	4707.06±21.31[e]	28059.62±10.65[b]	4521.68±20.98[c]
AMF + Zn+Mn+Cd	28317.80±12.04[a]	6465.13±33.89[a]	37484.42±13.68[a]	5987.82±15.18[a]
AMF + Zn	22358.78±19.80[d]	4025.51±22.54[h]	26840.75±15.13[d]	3858.28±39.25[e]
AMF + Mn	17171.99±18.38[e]	5008.81±9.90[c]	23908.11±12.24[e]	3453.49±13.08[h]
AMF + Cd	26071.62±7.99[b]	5384.64±13.39[b]	27840.68±15.63[c]	3623.19±11.08[f]
Zn	12668.87±8.02[h]	3719.18±11.88[i]	11981.01±15.40[i]	4114.30±8.70[d]
Mn	14284.39±10.37[g]	3581.97±13.46[j]	13940.72±17.44[h]	3567.51±29.06[g]
Cd	14541.96±13.36[f]	4627.97±7.94[f]	11049.84±9.48[j]	3294.78±7.31[i]
p-value	<0.0001	<0.0001	<0.0001	<0.0001

4.4 Heavy metal content in plant

Heavy metals concentration in plant root and shoot varied significantly among the treatments. The highest concentration (mg/kg) of Cd in plant root was observed in AMF + Cd treatment (136.48mg/kg) while the least Cd concentration (mg/kg) was found in Cd only treatment (36.12mg/kg). AMF increased Cd concentration (mg/kg) in the plant root by 38% with Zn+Mn+Cd and by 278% with Cd only. The highest Mn concentration (mg/kg) in the root was found in Mn only treatment (1907.69mg/kg) while the least Mn concentration (mg/kg) was found in AMF+Zn+Mn+Cd treatment (547.88mg/kg). AMF reduced Mn concentration in the plant root by 35% with Zn+Mn+Cd and by 55% with Mn only. The highest concentration of Zn (mg/kg) was found in AMF+Zn treatment (998.71mg/kg) while the lowest was recorded in AMF+Zn+Mn+Cd treatment (784.10mg/kg). AMF reduced Zn concentration in the plant root by 10% in Zn+Mn+Cd with AMF than without AMF and increased Zn concentration by 13% in Zn with AMF than without AMF (Figure 10).

The mass content of HM in the plant root also varied among the treatments. The highest mass content (mg) of Cd in plant root was observed in AMF + Cd treatment (0.94mg) while the least Cd mass content (mg) was found in Cd only treatment (0.24mg). AMF increased Cd mass content (mg) in the plant root by 47% with Zn+Mn+Cd and by 283% with Cd only. The highest Mn mass content (mg) in the root was found in Mn only treatment (11.79mg) while the least Mn mass content (mg) was found in AMF+Zn+Mn+Cd treatment (3.25mg). AMF reduced Mn mass content (mg) in the plant root by 31% in Zn+Mn+Cd with AMF than without AMF and by 50% in Mn with AMF than without AMF. The highest mass content (mg) of Zn was found in AMF+Zn treatment (9.47mg) while the lowest was recorded in AMF+Zn+Mn+Cd treatment (74.65mg). AMF reduced Zn mass content (mg) in the plant root by 31% in Zn+Mn+Cd with AMF than without AMF and increased Zn mass content (mg) by 68% in Zn with AMF than without AMF (Figure 11).

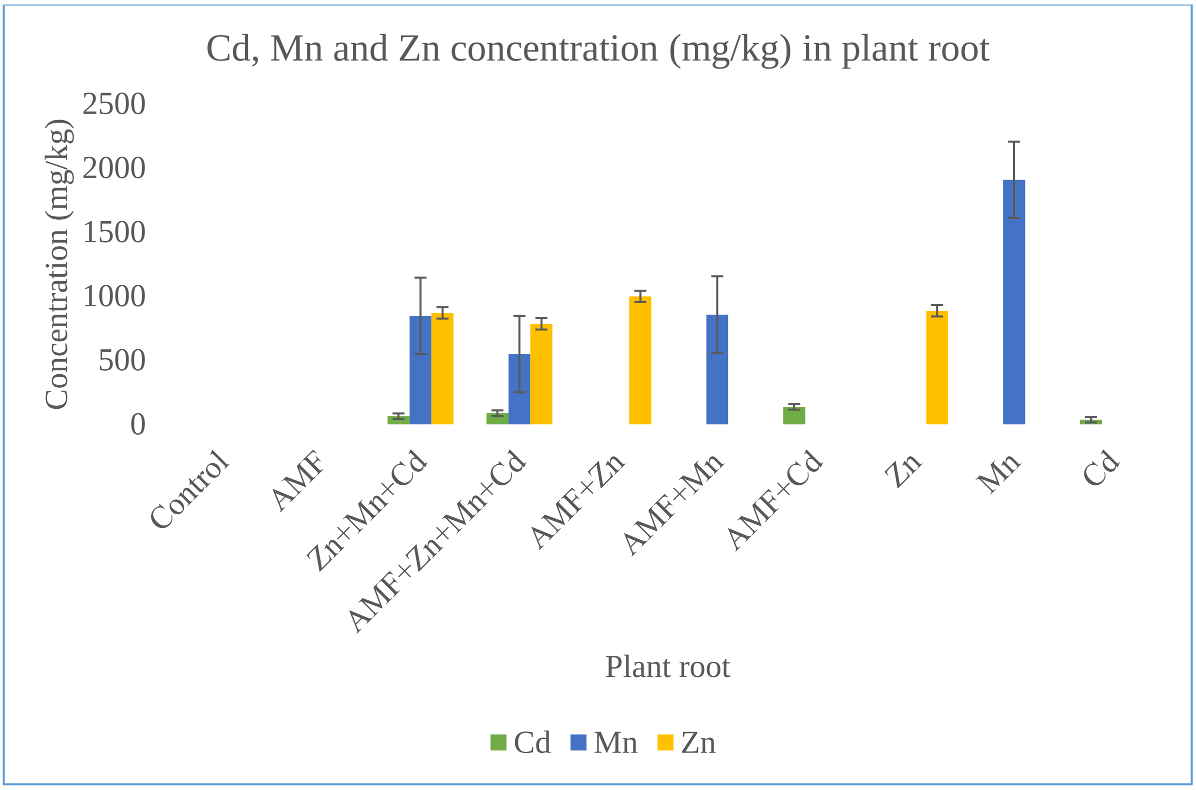

Figure 10: HM concentration (mg/kg) in plant root

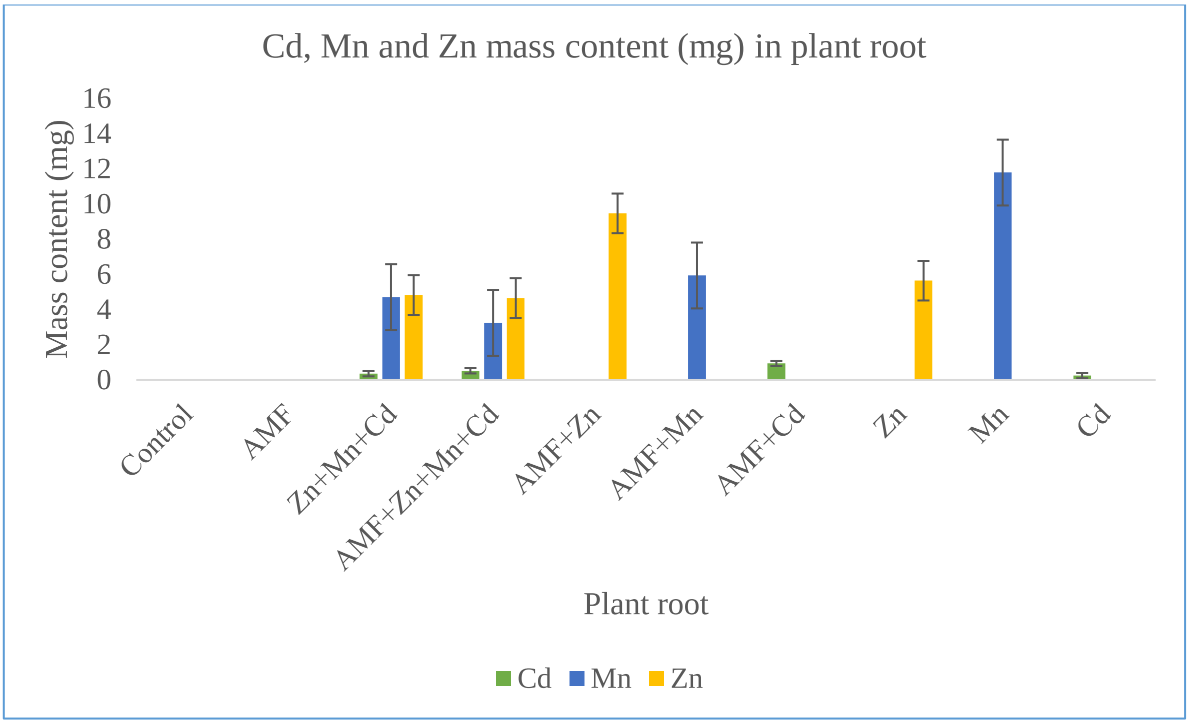

Figure 11: HM mass content (g) in plant root

The highest concentration (mg/kg) of Cd in plant shoot was observed in Zn+Mn+Cd treatment (16.11mg/kg) while the least Cd concentration (mg/kg) was found in Cd only treatment (8.72mg/kg). AMF decreased Cd concentration (mg/kg) in the plant shoot by 11% with Zn+Mn+Cd while it increased Cd concentration by 64% with Cd only. The highest Mn concentration (mg/kg) in the shoot was found in Zn+Mn+Cd treatment (388.27mg/kg) while the least Mn concentration (mg/kg) was found in Mn treatment (172.50mg/kg). AMF reduced Mn concentration in the plant shoot by 34% in Zn+Mn+Cd with AMF than without AMF and by 37% in Mn with AMF than without AMF. The highest concentration of Zn (mg/kg) was found in AMF+Zn+Mn+Cd treatment (252.36mg/kg) while the lowest was recorded in Zn only treatment (191.08mg/kg). AMF reduced Zn concentration in the plant shoot by 24% in Zn+Mn+Cd with AMF than without AMF and increased Zn concentration by 26% in Zn with AMF than without AMF (Figure 12).

The mass content of HM in the plant shoots also varied among the treatments. The highest mass content (mg) of Cd in plant shoot was observed in AMF+Zn+Mn+Cd treatment (0.70mg) while the least Cd mass content (mg) was found in Cd only treatment (0.05mg). AMF increased Cd mass content (mg) in the plant shoot by 17% in Zn+Mn+Cd with AMF than without AMF and by 34% in Cd with AMF than without AMF. The highest Mn mass content (mg) in the shoot was found in Mn only treatment (1.59mg) while the least Mn mass content (mg) was found in AMF+Mn treatment (1.10mg). AMF reduced Mn mass content (mg) in the plant shoot by 13% with Zn+Mn+Cd and by 31% with Mn only. The highest mass content (mg) of Zn was found in Zn only treatment (1.12mg) while the lowest was recorded in AMF+Zn+Mn+Cd treatment (0.93mg). AMF reduced Zn mass content (mg) in the plant shoot by 0.2% in Zn+Mn+Cd with AMF than without AMF and increased Zn mass content (mg) by 29% in Zn with AMF than without AMF (Figure 13).

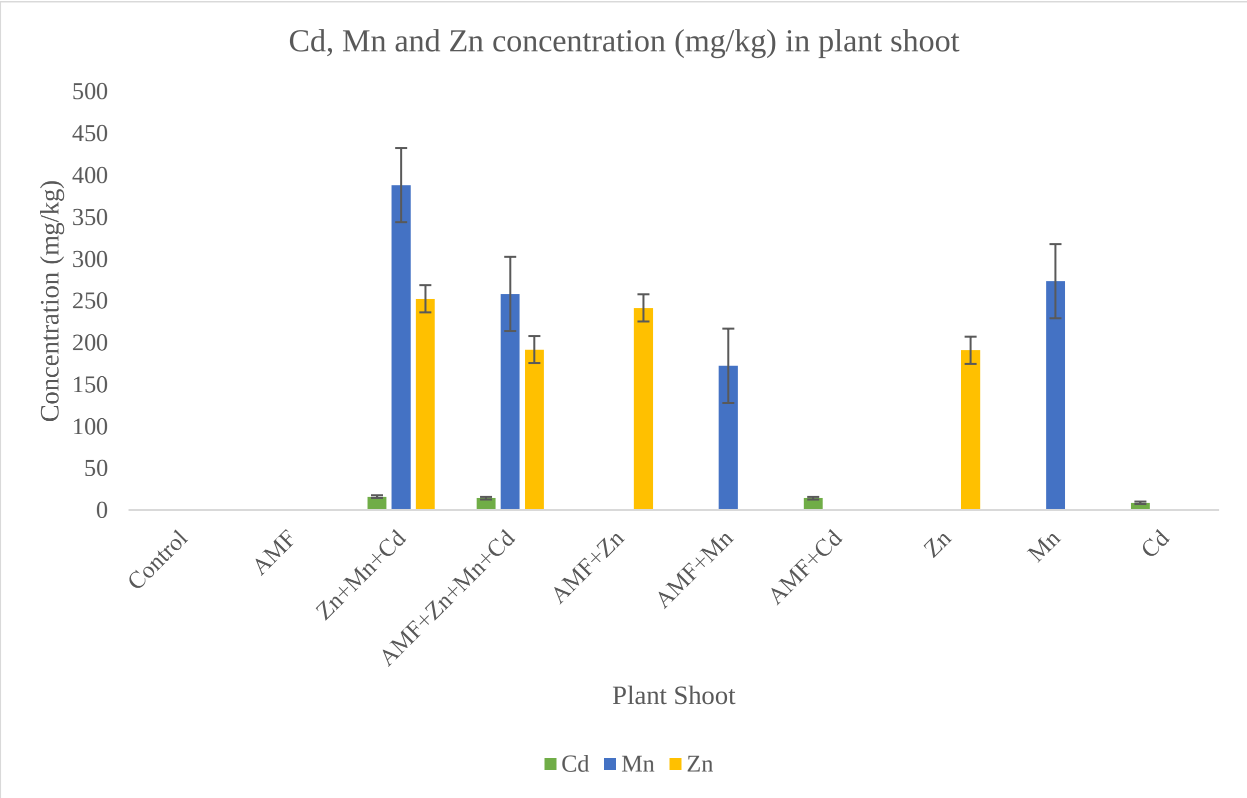

Figure 12: HM concentration (mg/kg) in plant shoot

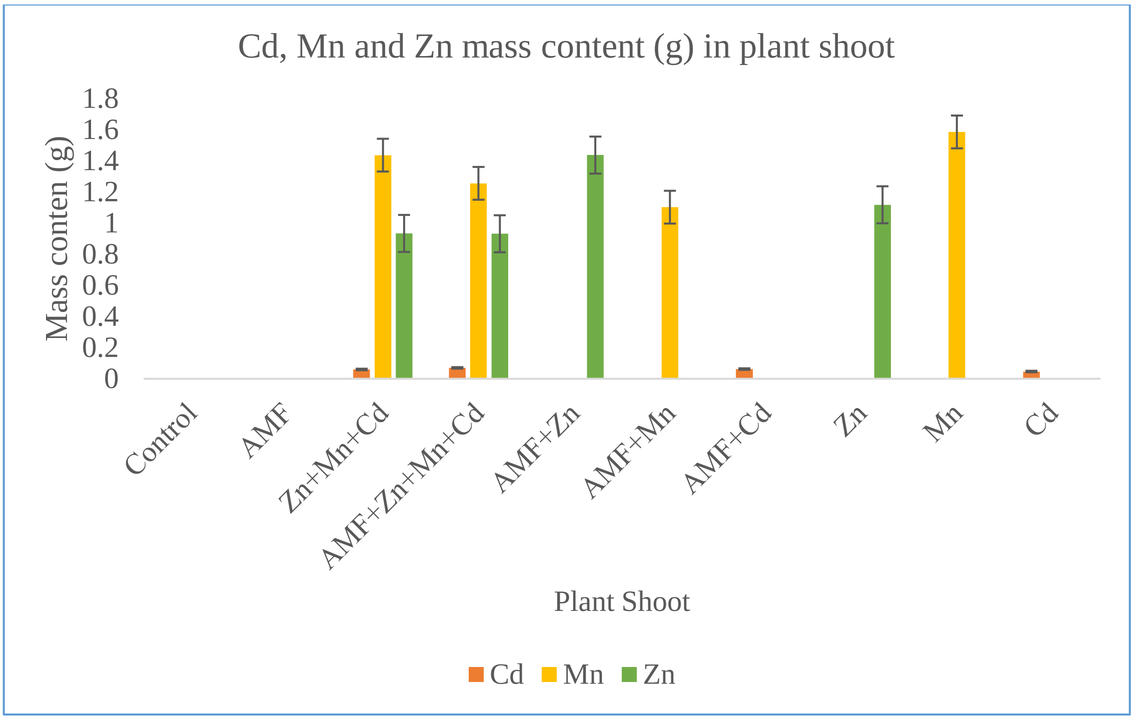

Figure 13: HM mass content (g) in plant shoot

The highest concentration (mg/kg) of Cd in plant substrate was observed in AMF+Cd treatment (1.15 mg/kg) while the least Cd concentration (mg/kg) was found in Cd only treatment (0.64mg/kg). AMF decreased Cd concentration (mg/kg) in the plant substrate by 27% in Zn+Mn+Cd with AMF than without AMF while it increased Cd concentration by 80% in Cd with AMF than without AMF. The highest Mn concentration (mg/kg) in the substrate was found in AMF+Mn treatment (14.98mg/kg) while the least Mn concentration (mg/kg) was found in Mn only treatment (5.04mg/kg). AMF increased Mn concentration in the plant substrate by 312% with Zn+Mn+Cd and by 197% with Mn only. The highest concentration of Zn (mg/kg) was found in AMF+Zn treatment (23.71mg/kg) while the lowest was recorded in Zn only treatment (6.09mg/kg). AMF increased Zn concentration in the plant substrate by 34% with Zn+Mn+Cd in AMF than without AMF and by 290% in Zn with AMF than without AMF (Figure 14).

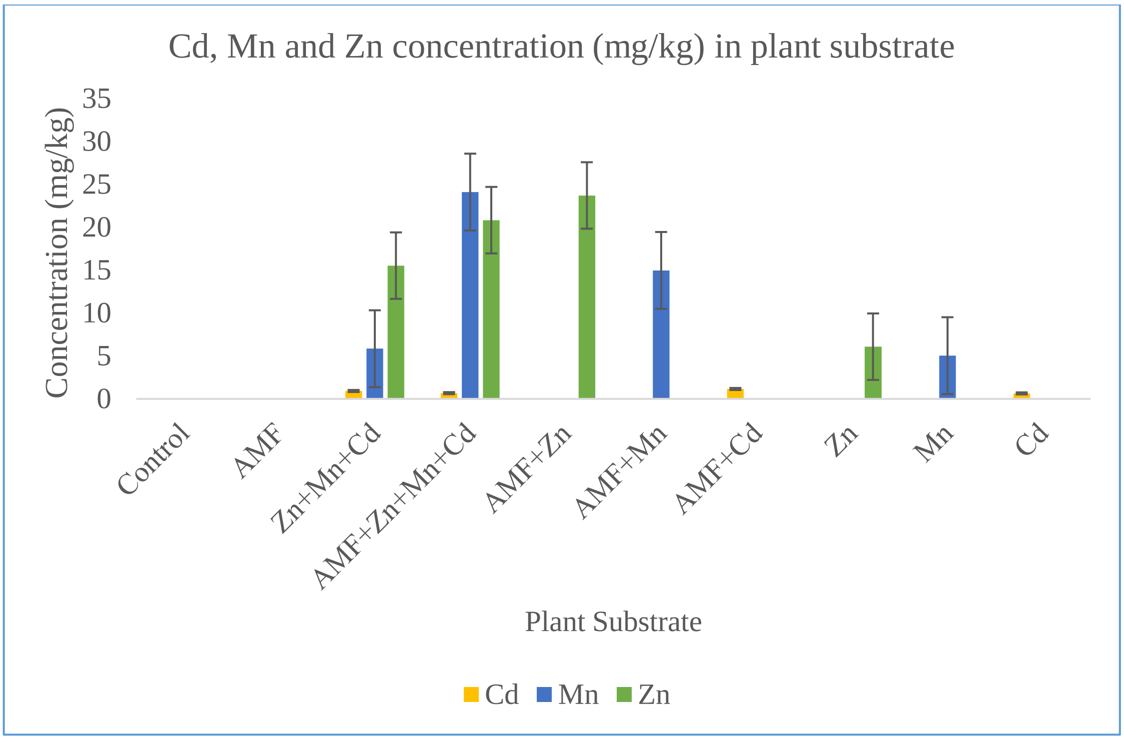

Figure 14: HM concentration (mg/kg) in plant substrate

4.5 Heavy metal removal

The total concentration (ppb) of Cd as shown in Figure 15 varied based on the treatments. Cd concentration ranged between 0.31 – 2.14 ppb in Zn+Mn+Cd treatment, 0.21 – 0.54ppb in AMF+Zn+Mn+Cd treatment, 0.13 – 0.49ppb in AMF+Cd treatment and 0.16 – 4.95ppb in Cd only treatment. The highest Cd concentration (ppb) was observed in Cd only treatment, while the least was recorded in AMF+Cd treatment. AMF significantly decreased Cd concentration by 98% in Zn+Mn+Cd with AMF than without AMF and by 83% in Cd with AMF than without AMF. Variations were observed among the treatment in terms of average removal efficiency (%) of Cd as illustrated in Figure 16. Cd removal efficiency (%) ranged between 99.61 – 99.98% in Zn+Mn+Cd treatment, 99.91 – 99.97% in AMF+Zn+Mn+Cd treatment, 99.91 – 99.99% in AMF+Cd treatment and 99.10 – 99.98% in Cd only treatment. The highest Cd removal efficiency (%) was observed in AMF+Cd treatment, while the least was recorded in Cd only treatment. AMF significantly increased Cd removal efficiency (%) by 0.07% in Zn+Mn+Cd with AMF than without AMF and by 0.32% in Cd with AMF than without AMF.

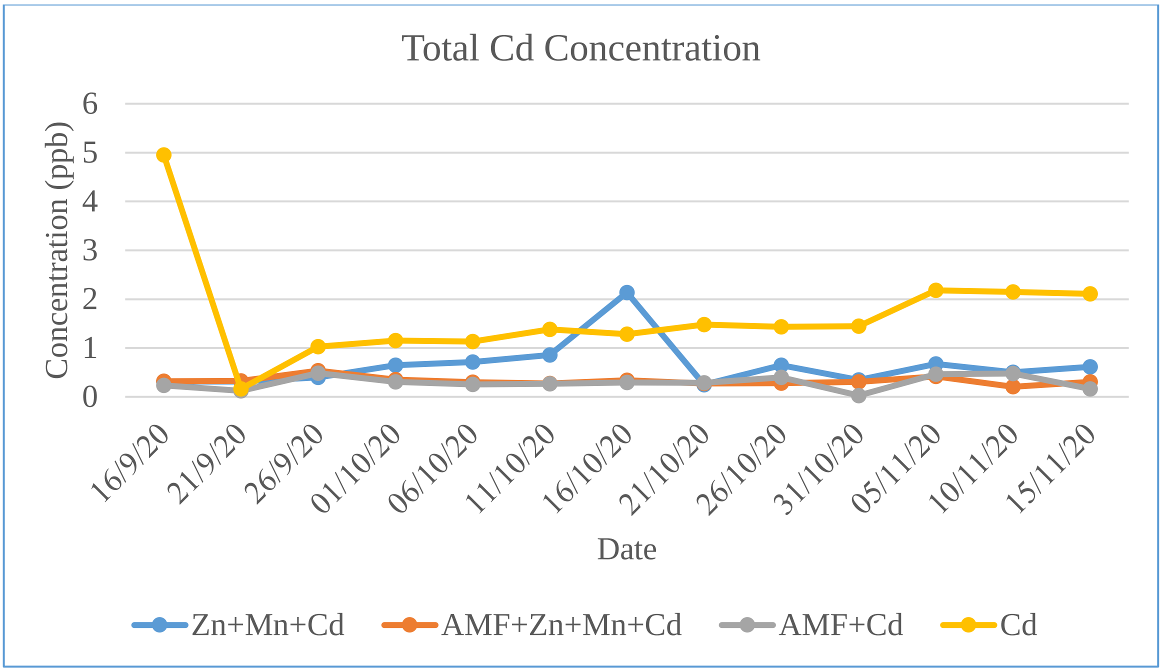

Figure 15: Total Cd Concentration

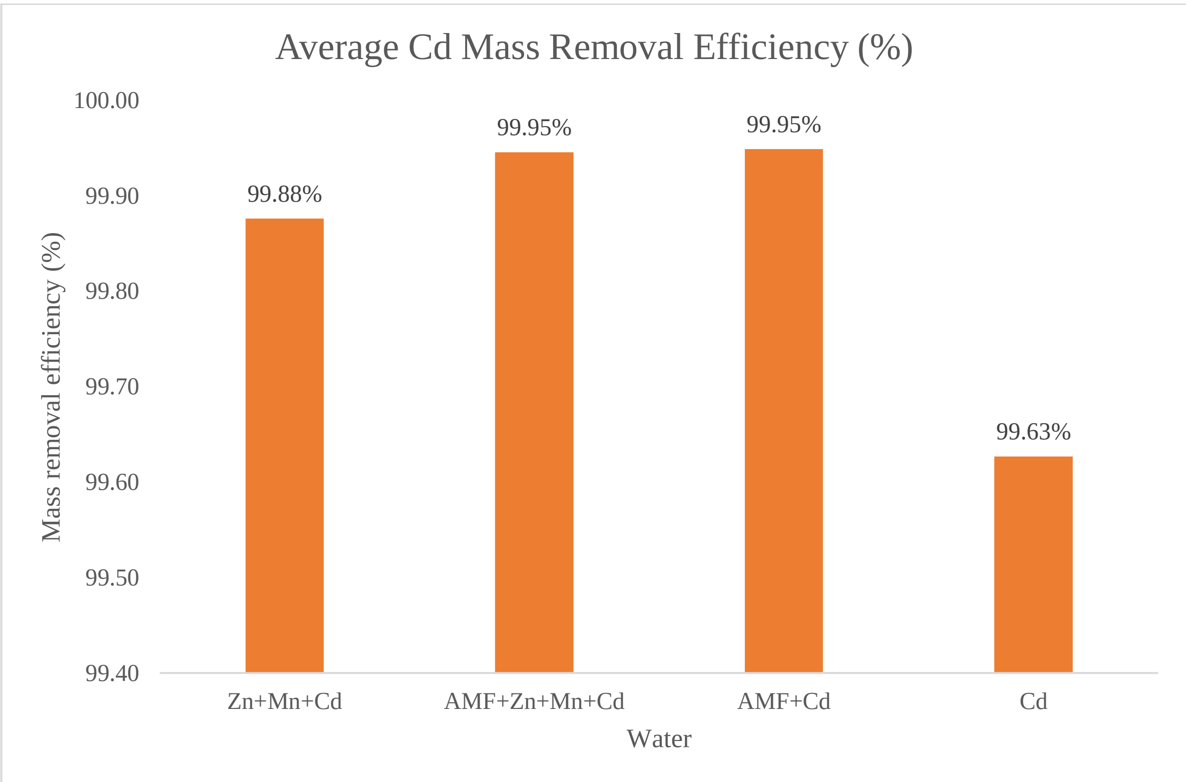

Figure 16: Average mass removal efficiency (%) of Cd

The total concentration (ppb) of Zn as illustrated in Figure 17 varied among the treatments. Zn concentration ranged between 4.12 – 35.38ppb in Zn+Mn+Cd treatment, 7.00 – 15.38ppb in AMF+Zn+Mn+Cd treatment, 4.90 – 34.38ppb in AMF+Zn treatment and 12.88 – 76.75ppb in Zn only treatment. The highest Zn concentration (ppb) was observed in Zn only treatment, while the least was recorded in AMF+Zn+Mn+Cd treatment. AMF significantly decreased Zn concentration by 51% in Zn+Mn+Cd with AMF than without AMF and by 48% in Zn with AMF than without AMF. Differences were also observed among the treatments in terms of average removal efficiency (%) of Zn as illustrated in Figure 18. Zn removal efficiency (%) ranged between 99.49 – 99.98% in Zn+Mn+Cd treatment, 99.82 – 99.98% in AMF+Zn+Mn+Cd treatment, 99.48 – 99.99% in AMF+Zn treatment and 98.64 – 99.97% in Zn only treatment. The highest Zn removal efficiency (%) was observed in AMF+Zn treatment, while the least was recorded in Zn only treatment. AMF significantly increased Zn removal efficiency (%) by 0.14% in Zn+Mn+Zn with AMF than without AMF and by 0.33% in Zn with AMF than without AMF.

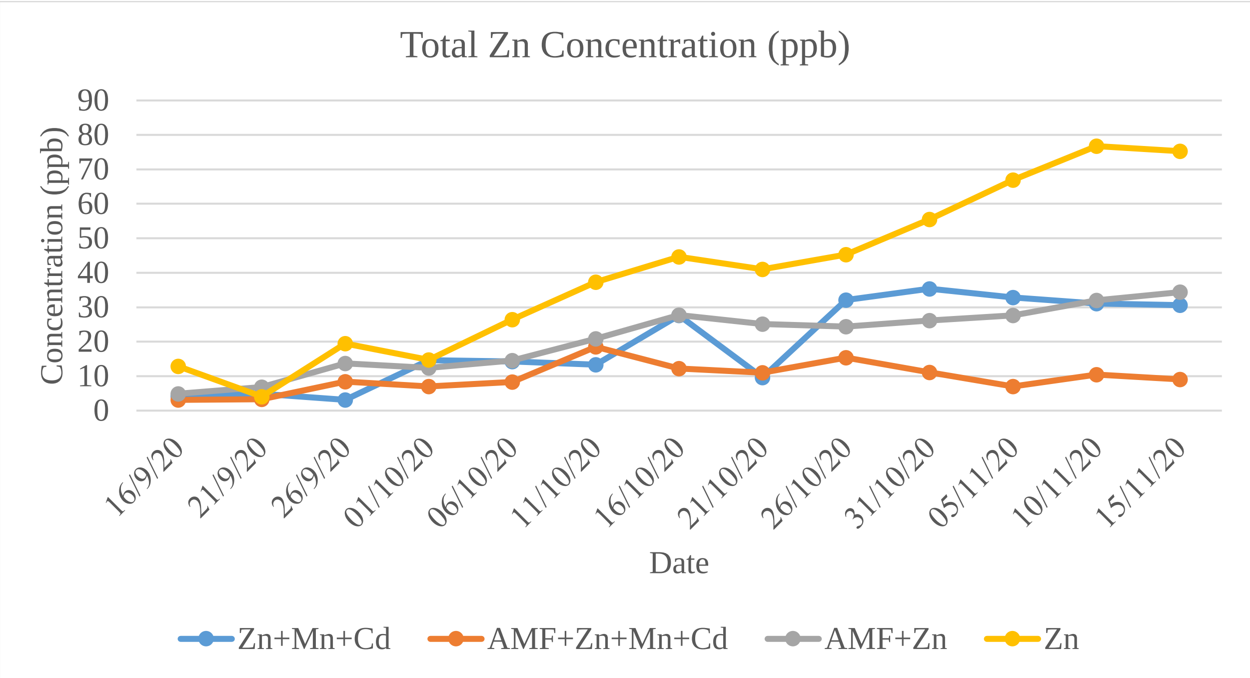

Figure 17: Total Zn Concentration (ppb)

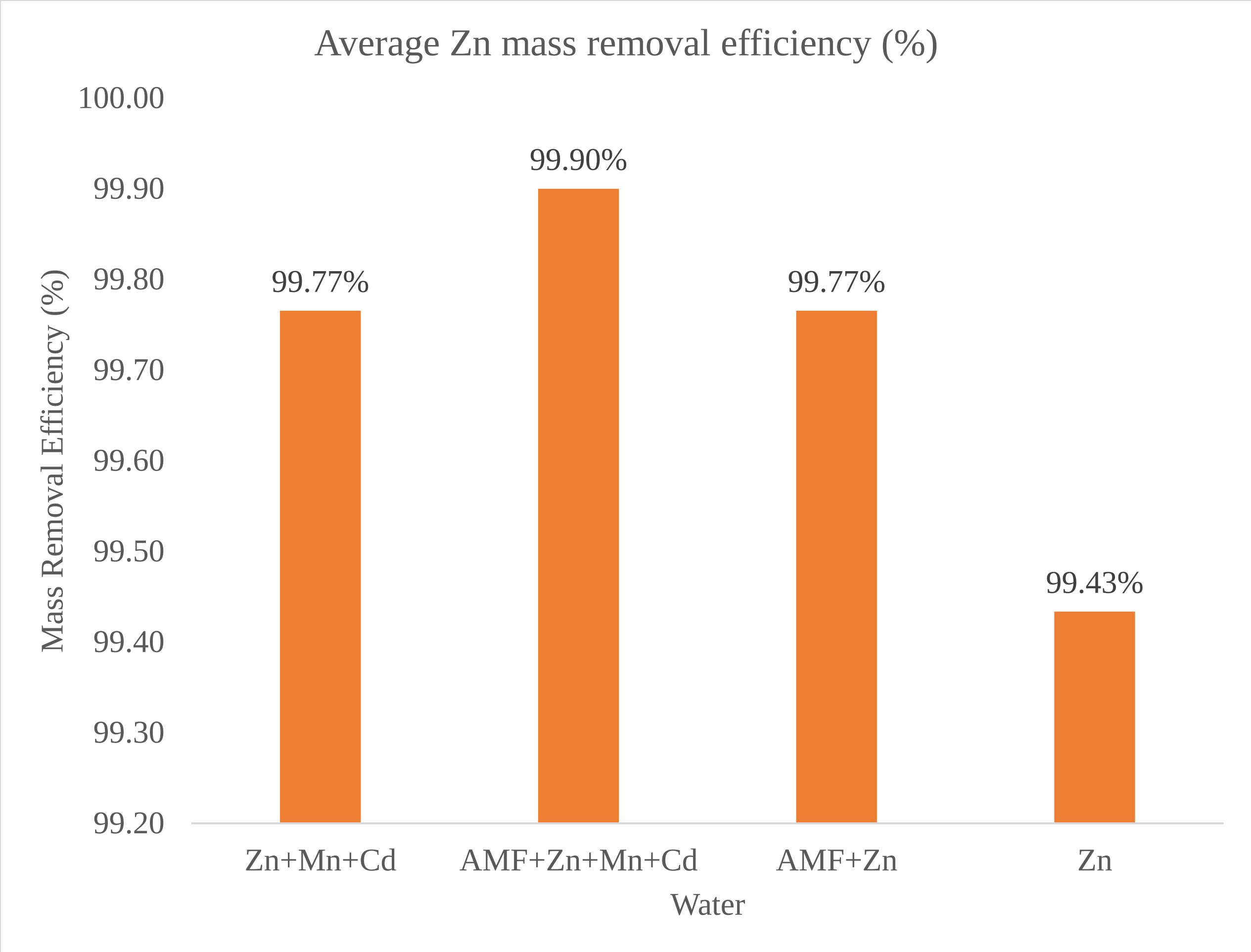

Figure 18: Average Zn mass removal efficiency (%)

The total concentration (ppb) of Mn as shown in Figure 19 revealed variations among the treatments. Mn concentration ranged between 25.3 – 60.46ppb in Zn+Mn+Cd treatment, 15.67 – 51.69ppb in AMF+Zn+Mn+Cd treatment, 24.62 – 48.94ppb in AMF+Mn treatment and 49.55 – 244.7ppb in Mn only treatment. The highest Mn concentration (ppb) was observed in Mn only treatment, while the least was recorded in AMF+Zn+Mn+Cd treatment. AMF significantly decreased Mn concentration by 19% in Zn+Mn+Cd with AMF than without AMF and by 71% in Zn with AMF than without AMF. Differences were also observed among the treatments in terms of average removal efficiency (%) of Mn as illustrated in Figure 20. Mn removal efficiency (%) ranged between 99.09 – 99.92% in Zn+Mn+Cd treatment, 99.24 – 99.91% in AMF+Zn+Mn+Cd treatment, 99.37 – 99.86% in AMF+Mn treatment and 96.23 – 99.55% in Mn only treatment. The highest Mn removal efficiency (%) was observed in Zn+Mn+Cd treatment, while the least was recorded in Mn only treatment. AMF significantly increased Mn removal efficiency (%) by 0.12% in Zn+Mn+Zn with AMF than without AMF and by 1.44% in Mn with AMF than without AMF.

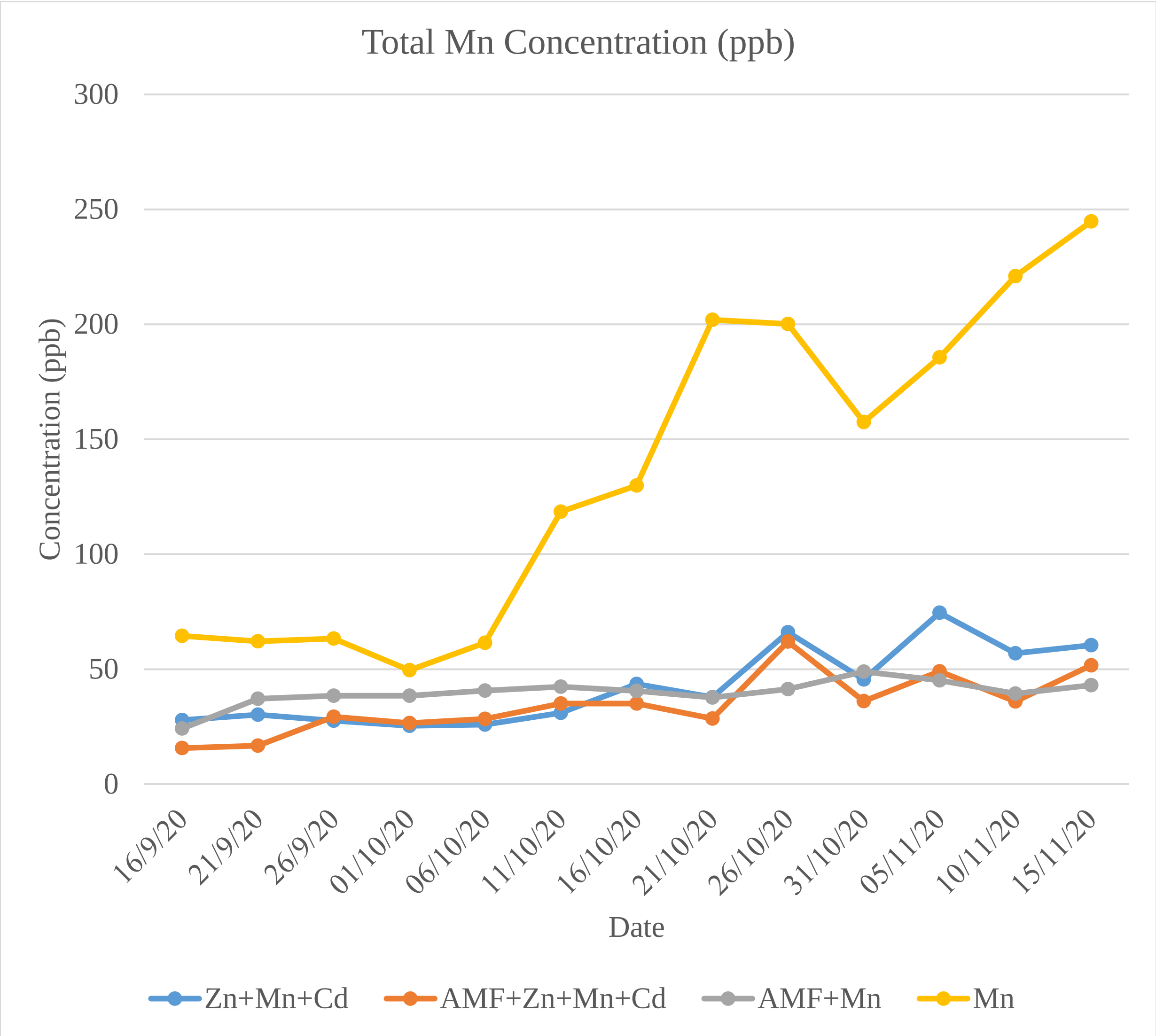

Figure 19: Total Mn Concentration (ppb)

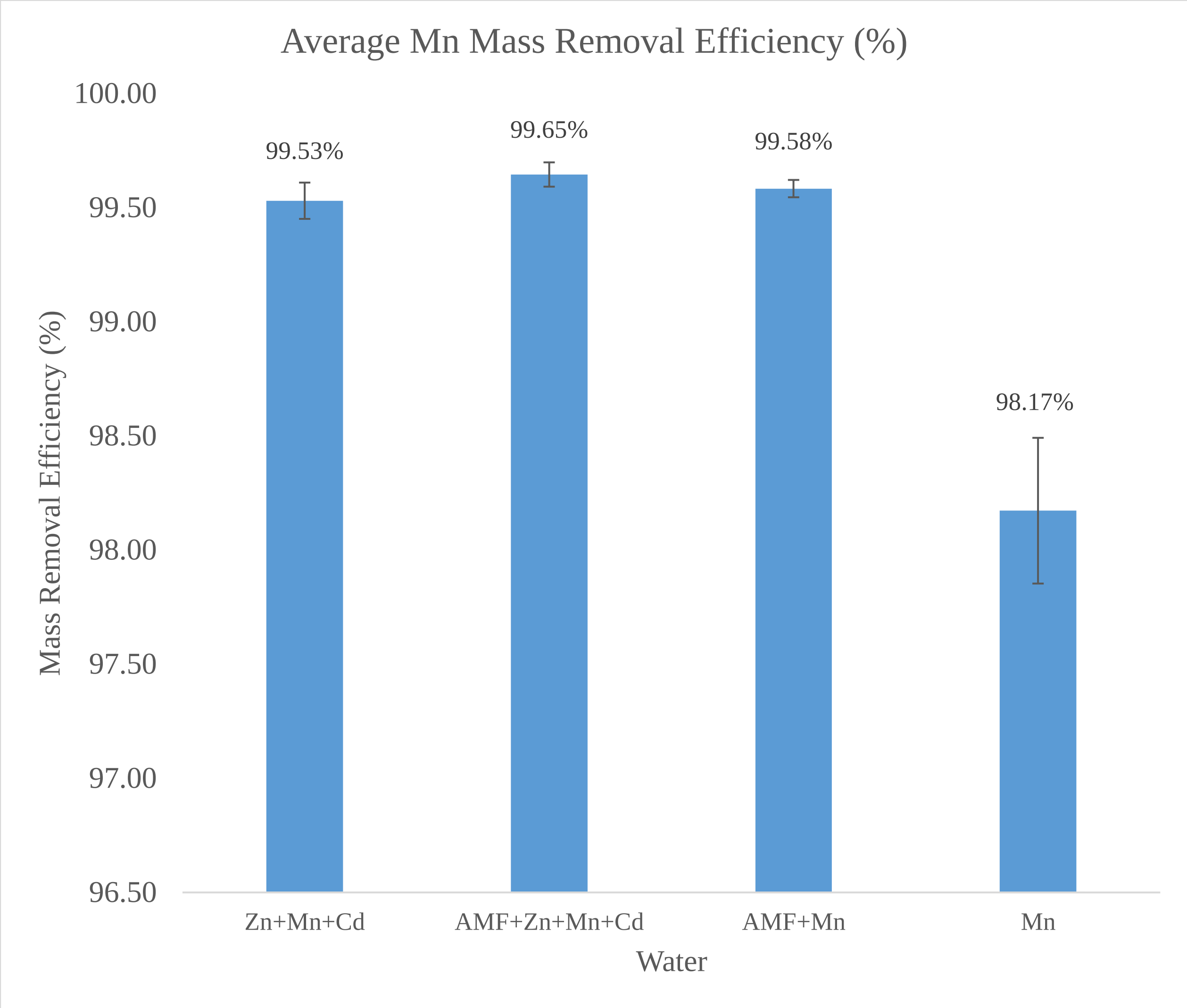

Figure 20: Average mass removal efficiency (%) of Mn

4.6 Water Loss

Various degrees of variation were noted among the treatments in terms of water loss (L) as illustrated in Figure 21 and Figure 22. Water loss ranged between 6.7%– 86.7% in Control treatment, 14.0% - 80.0% in AMF+Zn+Mn+Cd treatment, 28.7% - 100% in AMF only treatment, 28.0% - 83.3% in AMF+Cd treatment, 18.7% - 80.0% in AMF+Zn treatment, 26.7% - 80.0% in AMF+Mn treatment, 20.0% - 76.7% in Cd only treatment, 20% - 90% in Zn only treatment, 33.3% - 86.7% in Mn only treatment and 35.3% - 86.75% in Zn+Mn+Cd treatment. The highest average water loss (L) was observed in Mn only treatment (67.7%), while the least was recorded in Cd only treatment (51.1%) AMF increased water loss by 29% in the blank with AMF than without AMF and by 3% in Cd with AMF than without AMF. However, water loss was reduced by AMF by 17% in Zn+Mn+Cd with AMF than without AMF, by 16% in Zn with AMF than without AMF and by 20% in Mn with AMF than without AMF.

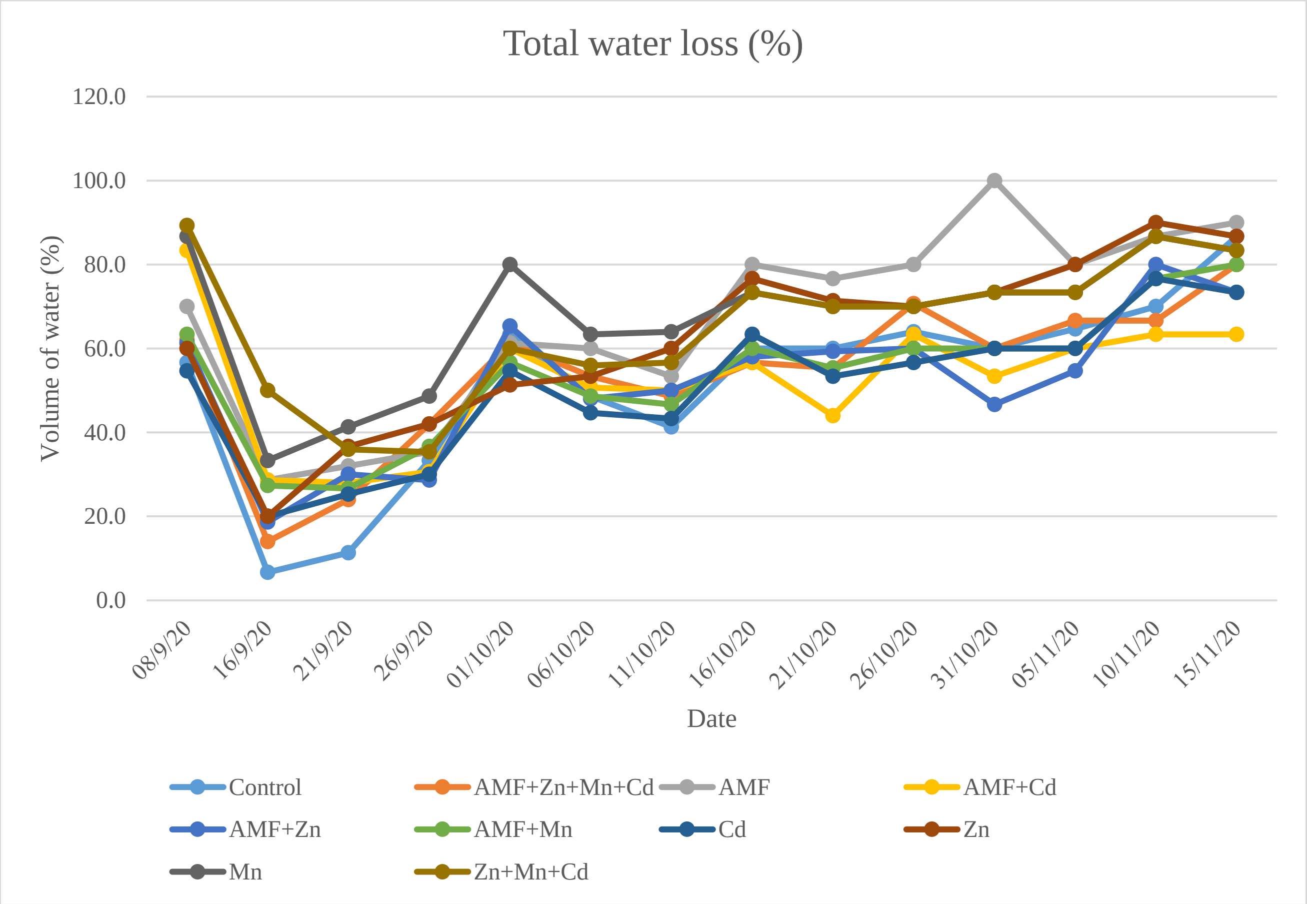

Figure 21: Total water loss (%)

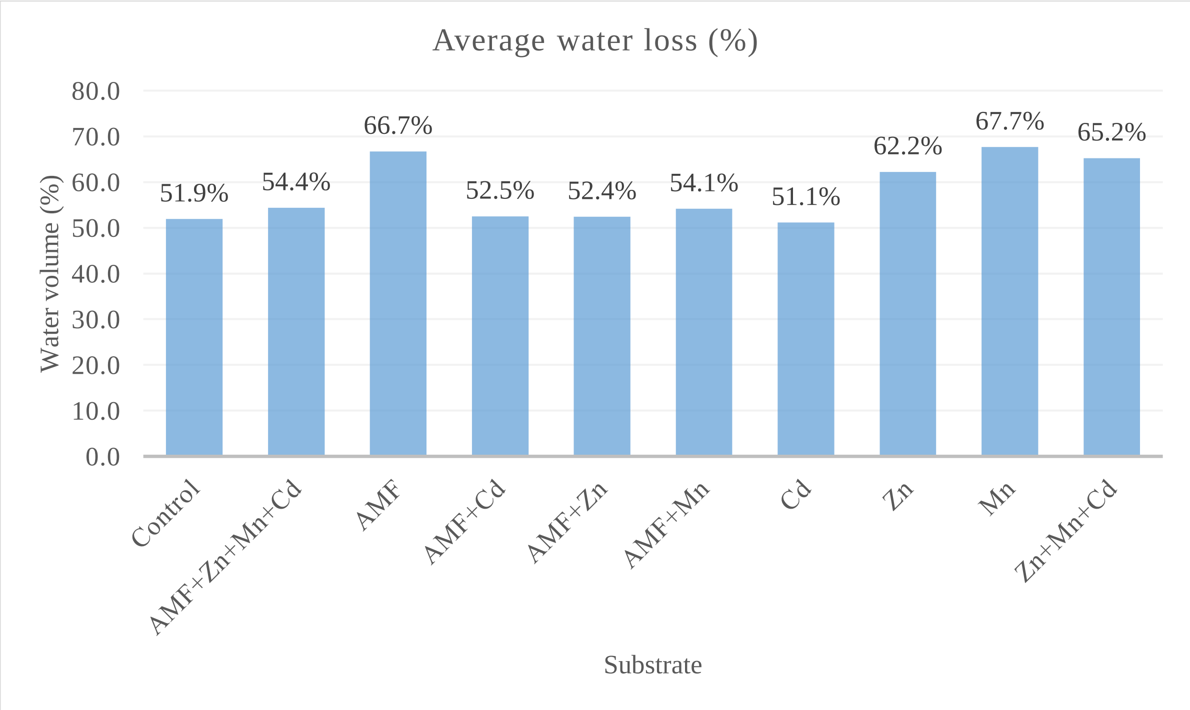

Figure 22: Average water loss (%)

4.7 pH Values

The pH values of the materials varied among the treatments as illustrated in Figure 23. pH values ranged between 6.99 – 7.21 in Control treatment, 6.79 – 7.19 in AMF+Zn+Mn+Cd treatment, 7.11 – 7.45 in AMF only treatment, 6.99 – 7.11 in AMF+Cd treatment, 7.05 – 7.19 in AMF+Zn treatment, 7.01 – 7.17 in AMF+Mn treatment, 6.99 – 7.30 in Cd only treatment, 7.01 – 17.16 in Zn only treatment, 6.93 – 7.14 in Mn only treatment and 7.11 – 7.61 in Zn+Mn+Cd treatment. The highest average pH value was obtained in the Control treatment (7.21), while the least was recorded in AMF only treatment (6.95). AMF slightly increased pH in Cd with AMF than without AMF and in Zn with AMF than without AMF. However, pH value was reduced by AMF in blank with AMF, in Zn+Mn+Cd with AMF, and in Mn with AMF.

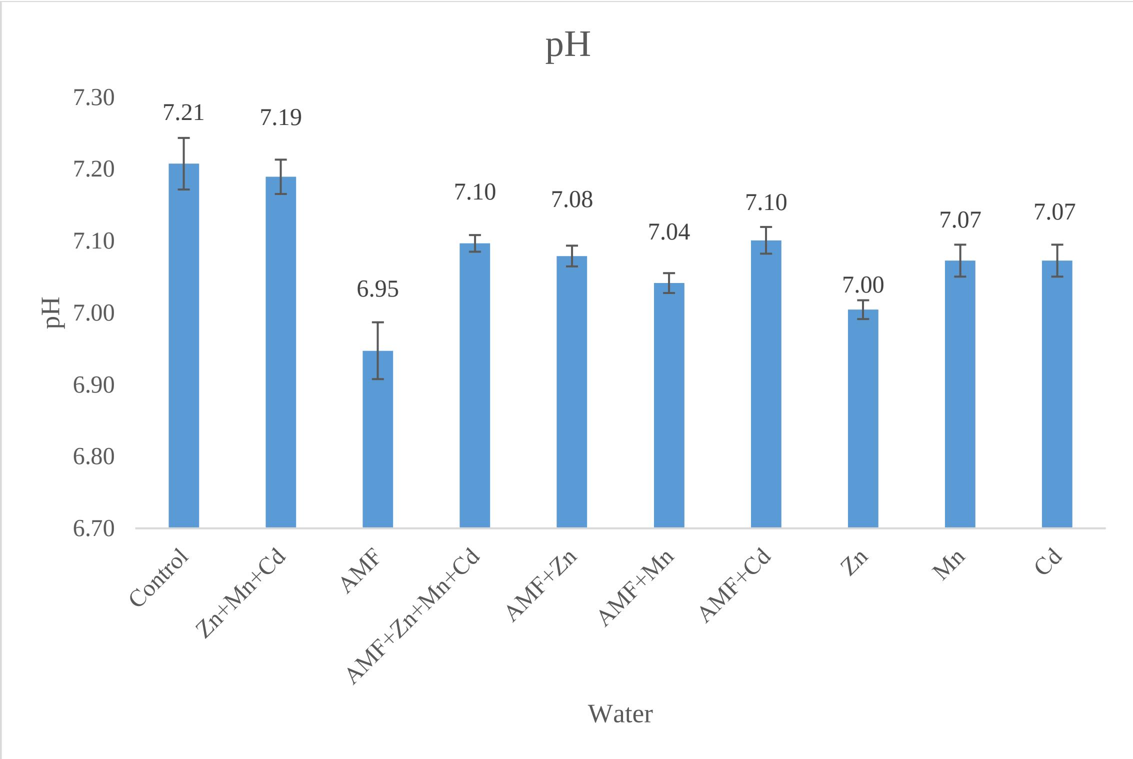

Figure 23: pH values

4.8 Oxidation-reduction Potential (ORP)

The oxidation-reduction potential values (mV) of the materials varied among the treatments as illustrated in Figure 24. ORP values ranged between 126.40 – 313.10mV in Control treatment, 138.40 – 316.20mV in Zn+Mn+Cd treatment, 135.7 – 307.6mV in AMF only treatment, 139.2 – 304.2mV in AMF+Zn+Mn+Cd treatment, 139.2 – 243.9mV in AMF+Zn treatment, 124.1 – 241.5mV in AMF+Mn treatment, 105.3 – 219.5mV in AMF+Cd treatment, 77.3 – 217.4mV in Zn only treatment, 177.5 – 218.4mV in Mn only treatment and 182.0 – 214.3mV in Cd only treatment. The highest average ORP value was obtained in Zn+Mn+Cd treatment (221.03mV), while the least was recorded in AMF+Zn treatment (185.86mV). AMF slightly increased ORP in Zn with AMF and blank with AMF than without AMF with 1% However, ORP value was reduced by AMF in Zn+Mn+Cd with AMF by 2%, in Mn with AMF.by 8% and in Cd with AMF by 7%.

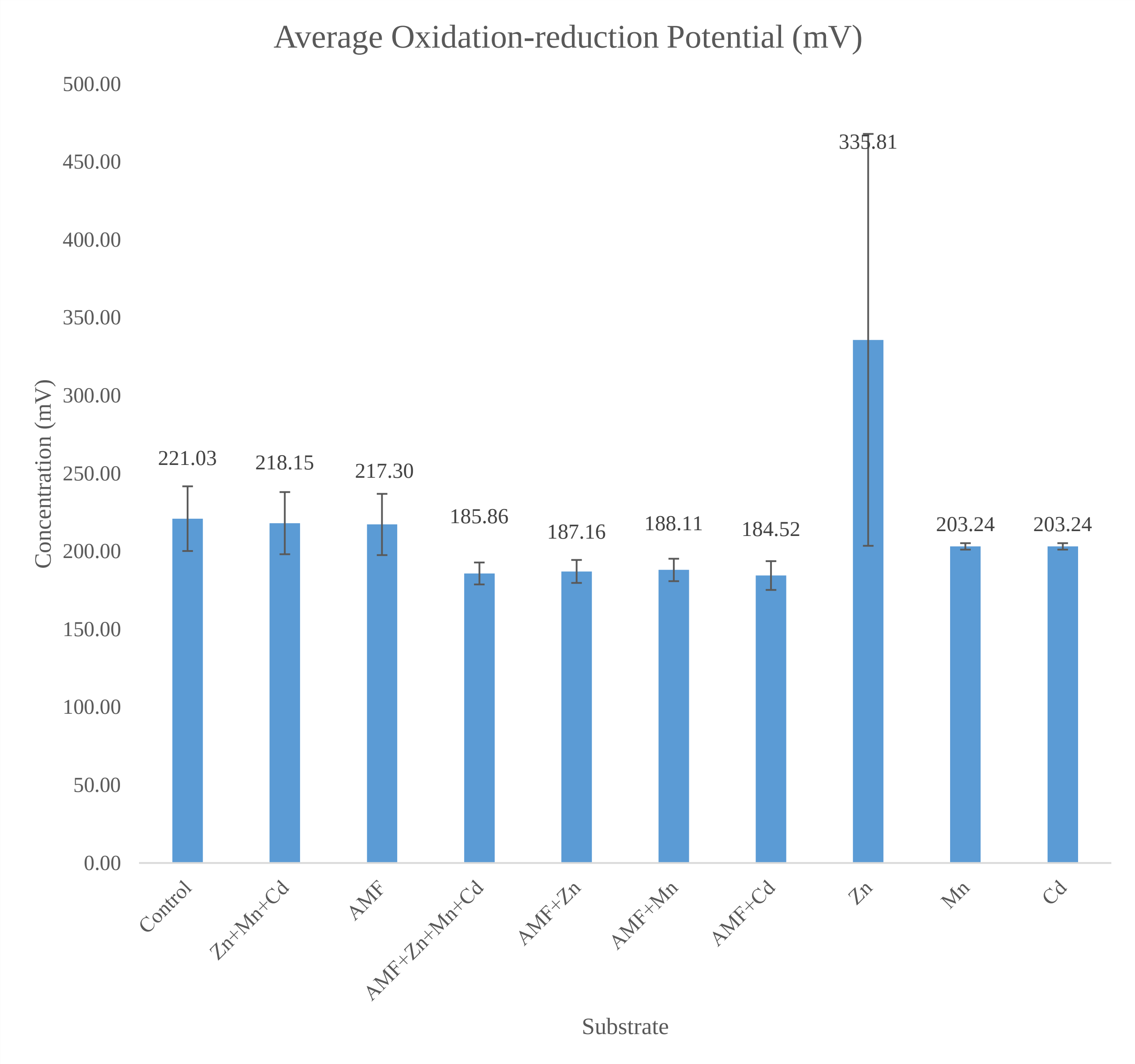

Figure 24: Oxidation-reduction Potential (mV)

4.9 Nitrogen Removal

The total concentration (mg/l) of NH_4^+ N as shown in Figure 25 revealed significant differences among the treatments. NH_4^+ N concentration ranged between 0.12 – 0.65mg/L in Control treatment, 0.04 – 1.94mg/L in Zn+Mn+Cd treatment, 0.13 – 0.41 in AMF only treatment, 0.27 – 0.39mg/L in AMF+Zn+Mn+Cd treatment, 0.08 – 0.36mg/L in AMF+Zn treatment, 0.03 – 0.78mg/L in AMF+Mn treatment, 0.05 – 0.52mg/L in AMF+Cd treatment, 0.23 – 1.36 in Zn only treatment, 0.28 – 1.82mg/L in Mn only treatment and 0.11 – 1.17mg/L in Cd only treatment. The highest NH_4^+ N concentration (mg/L) was observed in Zn+Mn+Cd treatment

(1.94mg/L), while the least was recorded in AMF+Mn treatment. AMF significantly decreased NH_4^+ N concentration by 20% in the blank with AMF than without AMF, by 49% in Zn+Mn+Cd with AMF than without AMF, by 67% in Zn with AMF than without AMF, by 45% in Mn with AMF than without AMF and by 55% in Cd with AMF than without AMF.

Variations were also observed among the treatments in terms of average removal efficiency (%) of NH_4^+ N as shown in Figure 26. NH_4^+ N removal efficiency (%) ranged between 84.03 – 99.75% in Control treatment, 51.91 – 99.83% in Zn+Mn+Cd treatment, 89.98 – 98.71% in AMF only treatment, 91.37 – 97.52% in AMF+Zn+Mn+Cd treatment, 91.76 – 99.51% in AMF+Zn treatment, 82.52 – 99.69% in AMF+Mn treatment, 88.43 – 99.46% in AMF+Cd treatment, 63.52 – 97.09% in Zn only treatment, 53.12 – 97.00% in Mn only treatment and 69.85 – 98.70% in Cd only treatment. The highest average NH_4^+ N removal efficiency (%) was observed in AMF+Zn treatment (96.19%), while the least was recorded in Mn only treatment (81.19%). AMF significantly increased NH_4^+ N removal by 0.3% in the blank with AMF than without AMF, by 8% in Zn+Mn+Cd with AMF than without AMF, by 12% in Zn with AMF than without AMF, by 13% in Mn with AMF than without AMF and by 10% in Cd with AMF than without AMF.

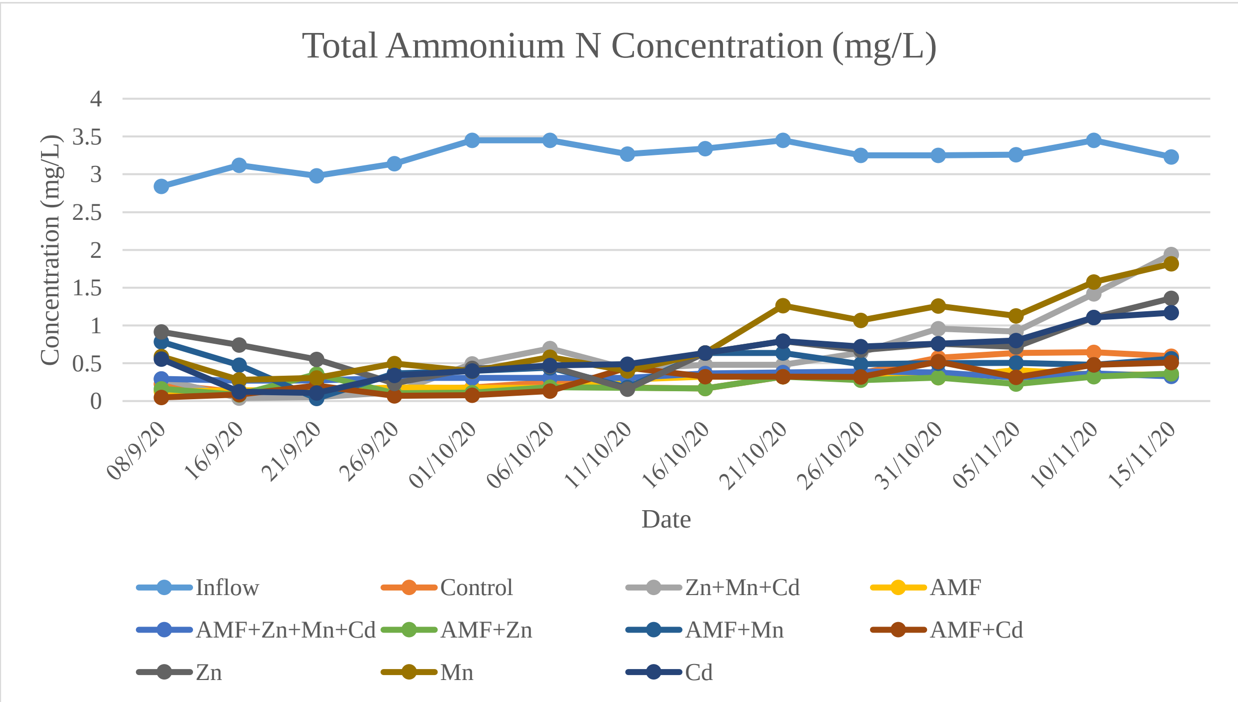

Figure 25: Total Ammonium N Concentration (mg/L)

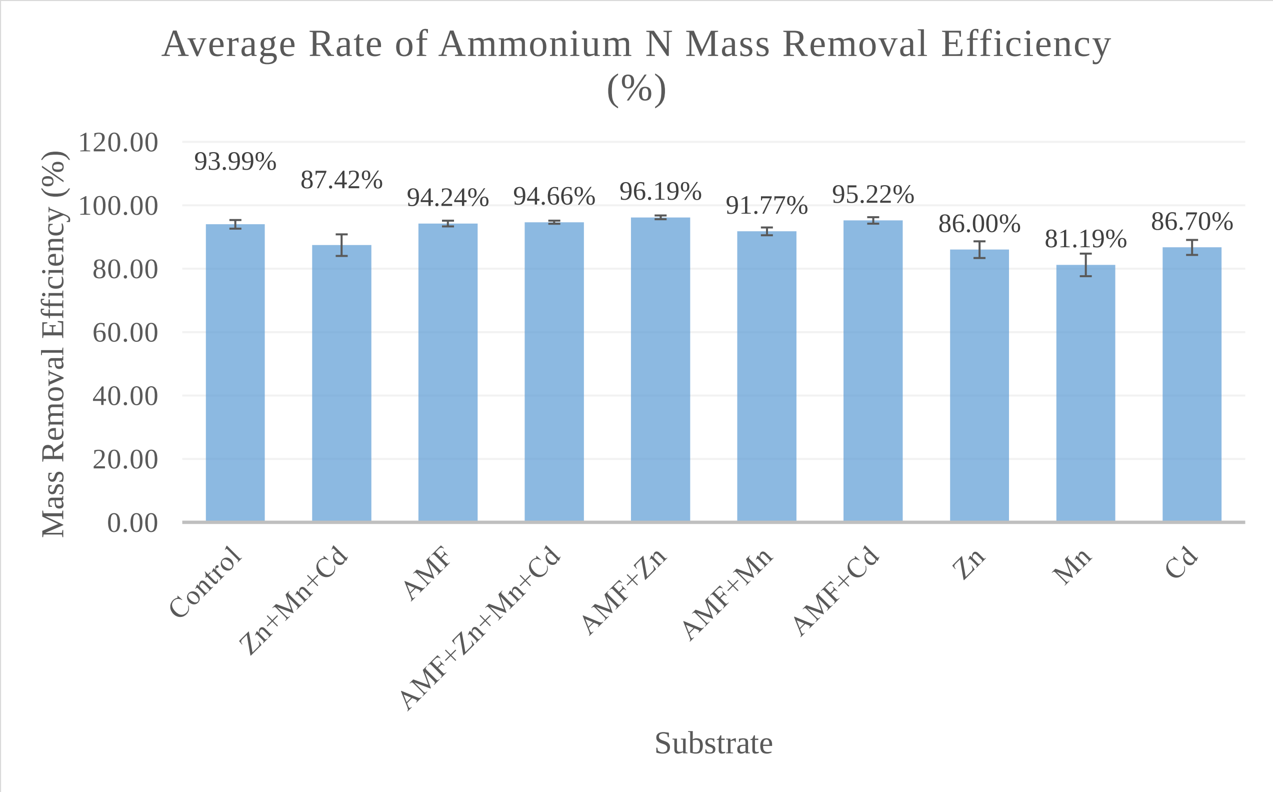

Figure 26: Average rate of Ammonium N mass removal efficiency (%)

The total concentration (mg/l) of total N illustrated in Figure 27 revealed significant variations among the treatments. Total N concentration ranged between 9.74 – 22.95mg/L in Control treatment, 29.66 – 46.39 mg/L in Zn+Mn+Cd treatment, 3.29 – 21.51mg/L in AMF only treatment, 11.98 – 33.69 mg/L in AMF+Zn+Mn+Cd treatment, 8.56 – 35.22mg/L in AMF+Zn treatment, 7.33 – 20.47 mg/L in AMF+Mn treatment, 7.95 – 27.05mg/L in AMF+Cd treatment, 10.83 – 31.56mg/L in Zn only treatment, 9.52 – 37.67 mg/L in Mn only treatment and 26.21 – 38.71 mg/L in Cd only treatment. The highest total N concentration (mg/L) was observed in Zn+Mn+Cd treatment (46.39mg/L), while the least was recorded in AMF only treatment (3.29mg/L). AMF significantly decreased total N concentration by 26% in blank with AMF than without AMF, by 50% in Zn+Mn+Cd with AMF than without AMF, by 30% in Zn with AMF than without AMF, by 9% in Mn with AMF than without AMF and by 36% in Cd with AMF than without AMF.

The treatments were also different in terms of average removal efficiency (%) of total N as shown in Figure 28. Total N removal efficiency (%) ranged between 67.07 – 97.43% in Control treatment, 36.06 – 84.79% in Zn+Mn+Cd treatment, 73.11 – 95.89% in AMF only treatment, 56.71 – 86.45% in AMF+Zn+Mn+Cd treatment, 71.96 – 96.92% in AMF+Zn treatment, 50.98 – 96.51% in AMF+Mn treatment, 63.52 – 93.79% in AMF+Cd treatment, 48.39 – 96.22% in Zn only treatment, 34.08 – 94.46% in Mn only treatment and 30.99 – 77.90% in Cd only treatment. The highest average total N removal efficiency (%) was observed in AMF+Zn treatment (84.76%), while the least was recorded in Zn+Mn+Cd treatment (57.31%). AMF significantly increased total N removal by 1% in the blank with AMF than without AMF, by 40% in Zn+Mn+Cd with AMF than without AMF, by 18% in Zn with AMF than without AMF, by 18% in Mn with AMF than without AMF and by 39% in Cd with AMF than without AMF.

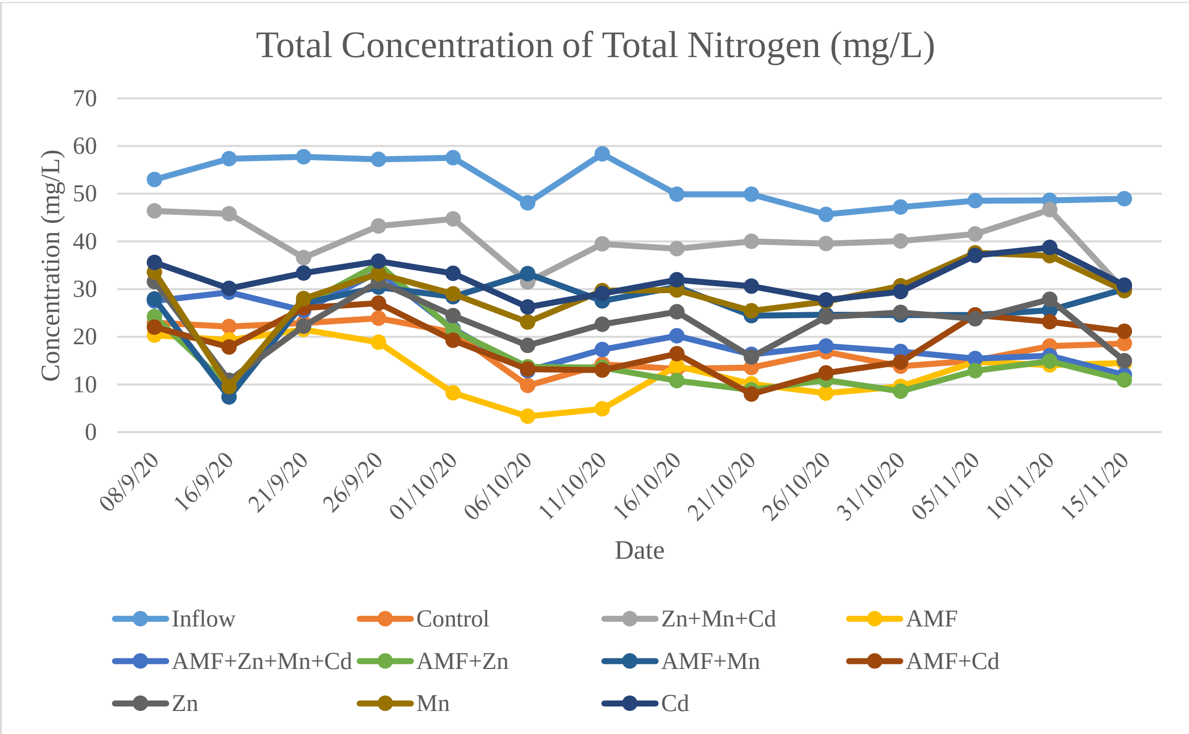

Figure 27: Total concentration of Total N (mg/L)

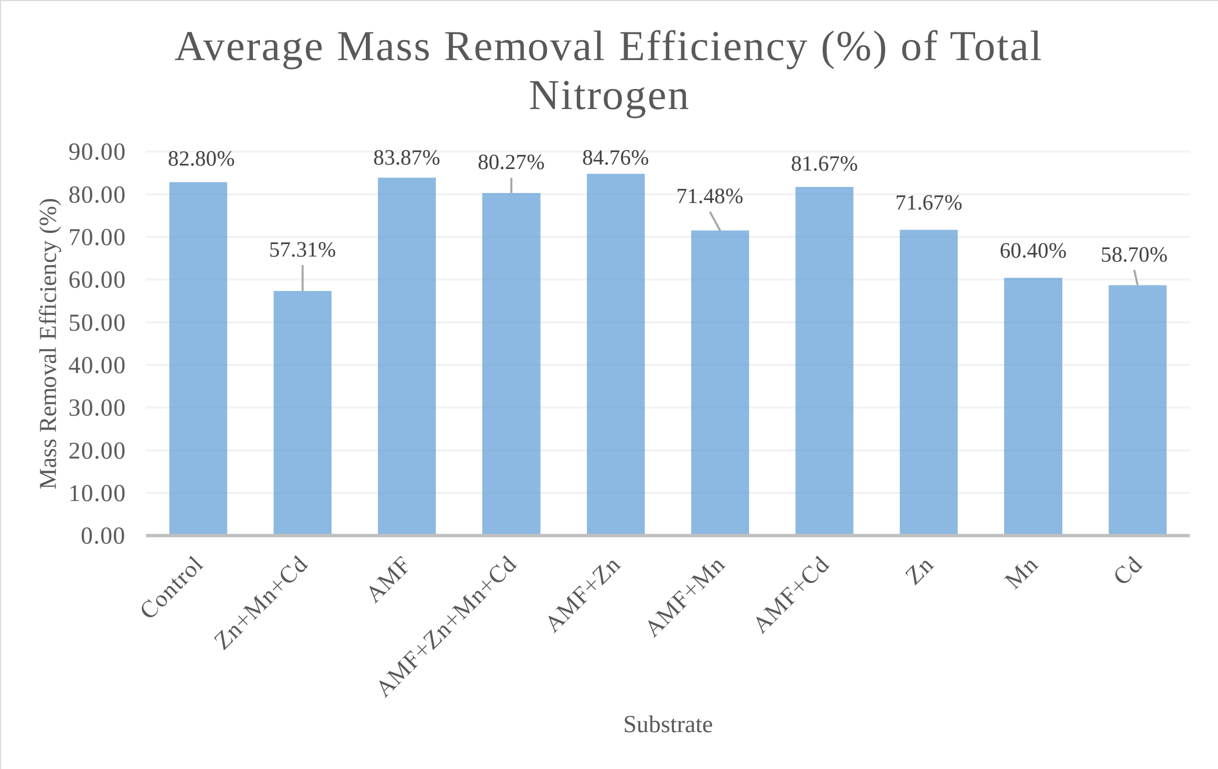

Figure 28: Average mass removal efficiency (%) of total N (%)

The average concentration (mg/l) of Nitrate N illustrated in Figure 29 revealed significant differences among the treatments. Total Nitrate N concentration ranged between 9.56 – 21.79mg/L in Control treatment, 13.21 – 88.22mg/L in Zn+Mn+Cd treatment, 1.74 – 18.15mg/L in AMF only treatment, 30.89 – 41.84mg/L in AMF+Zn+Mn+Cd treatment, 20.02 – 29.92mg/L in AMF+Zn treatment, 24.31 – 30.94mg/L in AMF+Mn treatment, 18.67 – 34.08mg/L in AMF+Cd treatment, 4.67 – 85.22 mg/L in Zn only treatment, 21.79 – 30.28 mg/L in Mn only treatment and 8.86 – 28.31mg/L in Cd only treatment. The highest average Nitrate N concentration (mg/L) was observed in AMF+Zn+Mn+Cd treatment (36.45mg/L), while the least was recorded in AMF only treatment (11.69mg/L). AMF significantly decreased average Nitrate N concentration by 19% in the blank with AMF than without AMF, while it increased average Nitrate N concentration by 60% in Zn+Mn+Cd with AMF than without AMF, by 21% in Zn with AMF than without AMF, by 6% in Mn with AMF than without AMF and by 74% in Cd with AMF than without AMF.

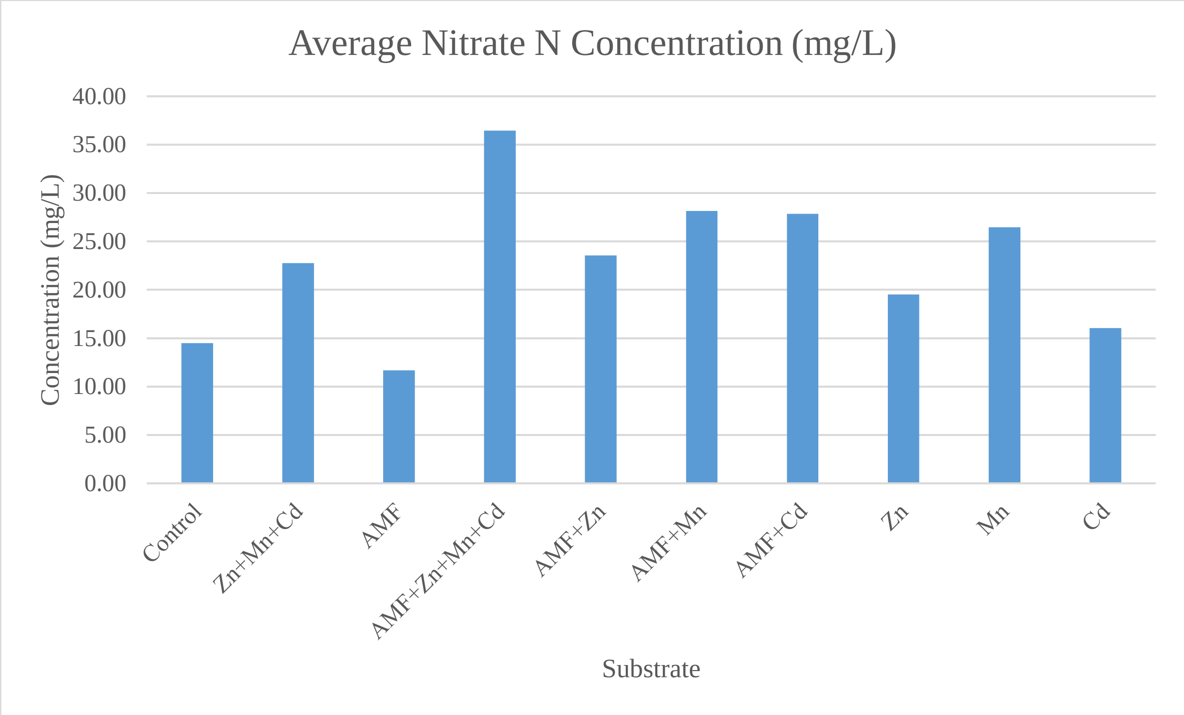

Figure 29: Average nitrate N concentration (mg/L)

4.10 Carbon removal

The concentration (mg/l) of total carbon (TC) shown in Figure 30 revealed variations among the treatments. Total C concentration ranged between 56.29 – 71.57mg/L in Control treatment, 40.95 – 64.18 mg/L in Zn+Mn+Cd treatment, 40.09–43.72mg/L in AMF only treatment, 31.58 –43.38mg/L in AMF+Zn+Mn+Cd treatment, 19.48–44.82mg/L in AMF+Zn treatment, 30.32 – 50.75mg/L in AMF+Mn treatment, 28.94 – 45.14mg/L in AMF+Cd treatment, 29.33 – 57.57mg/L in Zn only treatment, 32.31 – 46.37mg/L in Mn only treatment and 26.10 – 58.43 mg/L in Cd only treatment. The highest Total C concentration (mg/L) was observed in the control treatment (71.57mg/L), while the least was recorded in AMF only treatment (19.48mg/L). AMF significantly decreased Total C concentration by 33% in the blank with AMF than without AMF, by 33% in Zn+Mn+Cd with AMF than without AMF, by 26% in Zn with AMF than without AMF, by 4% in Mn with AMF than without AMF and by 25% in Cd with AMF than without AMF.

There were notable differences in terms of average mass removal efficiency (%) of Total C as shown in Figure 31. Total C mass removal efficiency (%) ranged between 49.34 – 97.11% in Control treatment, 53.5 – 94.2% in Zn+Mn+Cd treatment, 57.2 – 90.9% in AMF only treatment, 75.6 – 92.3% in AMF+Zn+Mn+Cd treatment, 67.2 – 97.3% in AMF+Zn treatment, 68.1 – 94.2% in AMF+Mn treatment, 73.9 – 95.7% in AMF+Cd treatment, 51.8 – 89.2% in Zn only treatment, 62.4 – 89.7% in Mn only treatment and 48.2 – 90.2% in Cd only treatment. The highest average Total C mass removal efficiency (%) was observed in AMF+Cd treatment (83.0%), while the least was recorded in the control treatment (69.7%). AMF significantly increased Total C mass removal efficiency by 6% in the blank with AMF than without AMF, by 14% in Zn+Mn+Cd with AMF than without AMF, by 17% in Zn with AMF than without AMF, by 7% in Mn with AMF than without AMF and by 17% in Cd with AMF than without AMF.

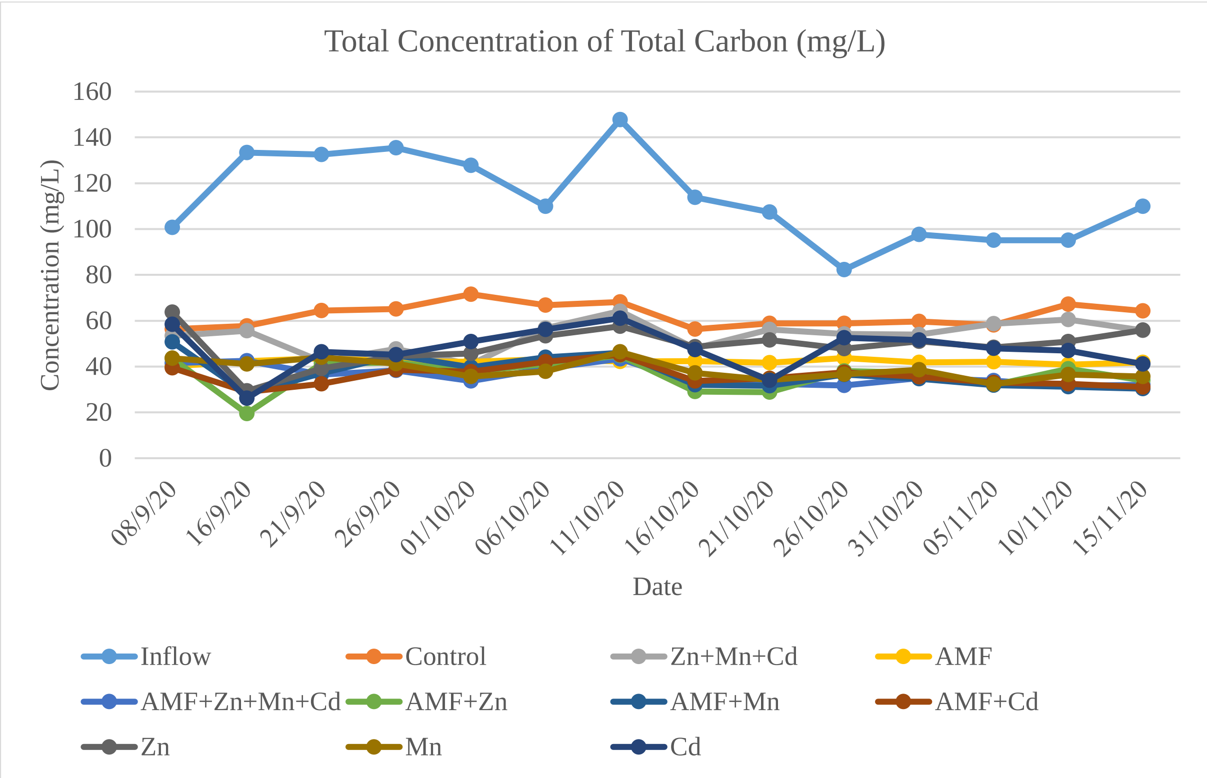

Figure 30: Total concentration of total carbon (mg/L)

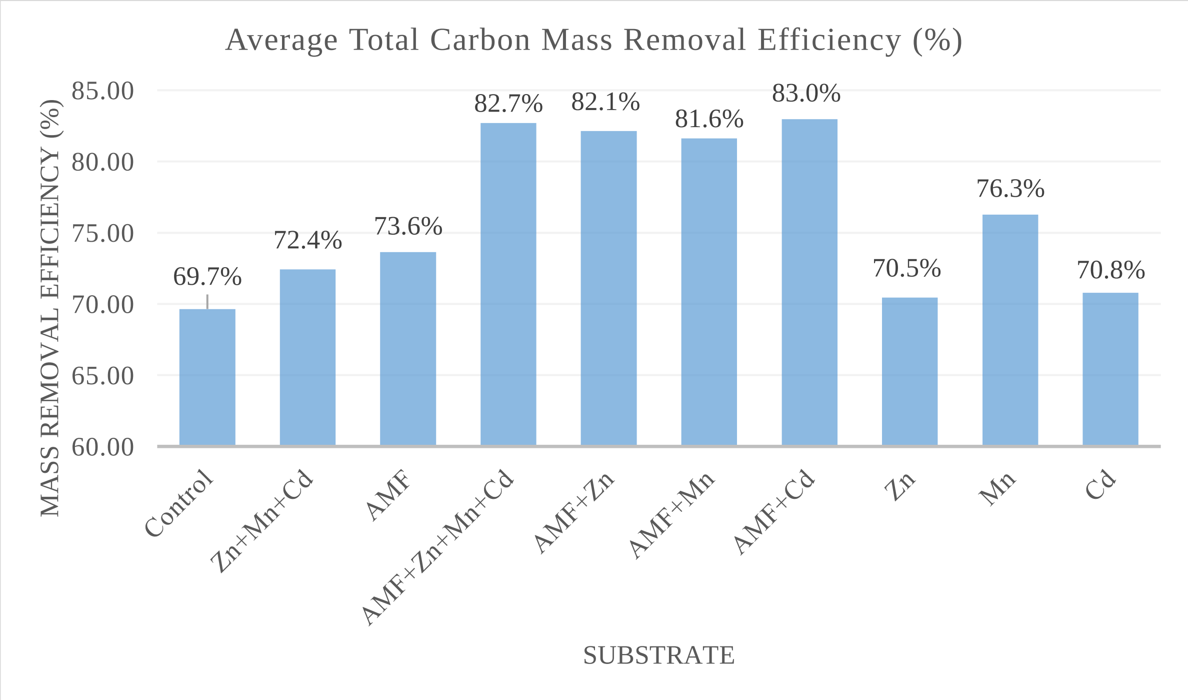

Figure 31: Average total carbon mass removal efficiency (%)

The concentration (mg/l) of Total Organic Carbon (TOC) illustrated in Figure 32 showed differences among the treatments. Total Organic C concentration ranged between 4.55 – 15.73mg/L in Control treatment, 3.47 – 17.31mg/L in Zn+Mn+Cd treatment, 0.30–8.76mg/L in AMF only treatment, 2.55 – 10.52mg/L in AMF+Zn+Mn+Cd treatment, 3.26 – 6.93mg/L in AMF+Zn treatment, 4.22 – 7.18mg/L in AMF+Mn treatment, 4.62 – 7.76mg/L in AMF+Cd treatment, 6.58 – 9.58 mg/L in Zn only treatment, 7.79 – 14.84mg/L in Mn only treatment and 9.03– 15.36mg/L in Cd only treatment. The highest Total Organic C concentration (mg/L) was observed in Zn+Mn+Cd treatment (17.31mg/L), while the least was recorded in AMF only treatment (0.30mg/L). AMF significantly decreased Total Organic C concentration by 36% in the blank with AMF than without AMF, by 44% in Zn+Mn+Cd with AMF than without AMF, by 43% in Zn with AMF than without AMF, by 49% in Mn with AMF than without AMF and by 49% in Cd with AMF than without AMF.

The average mass removal efficiency (%) of Total Organic C varied among the treatments as shown in Figure 33. Total Organic C mass removal efficiency (%) ranged between 83.85 – 99.18% in Control treatment, 80.52 – 98.65% in Zn+Mn+Cd treatment, 88.35 – 99.59% in AMF only treatment, 89.85 – 98.09% in AMF+Zn+Mn+Cd treatment, 93.97 – 98.58% in AMF+Zn treatment, 92.59 – 98.37% in AMF+Mn treatment, 92.14 – 98.20% in AMF+Cd treatment, 84.72 – 98.05% in Zn only treatment, 81.62 – 95.61% in Mn only treatment and 82.33 – 94.70% in Cd only treatment. The highest average TOC mass removal efficiency (%) was observed in AMF +Zn treatment (96.20%), while the least was recorded in Cd only treatment (89.02%). AMF significantly increased TOC mass removal efficiency by 2% in blank with AMF than without AMF, by 5% in Zn+Mn+Cd with AMF than without AMF, by 5% in Zn with AMF than without AMF, by 7% in Mn with AMF than without AMF and by 7% in Cd with AMF than without AMF.

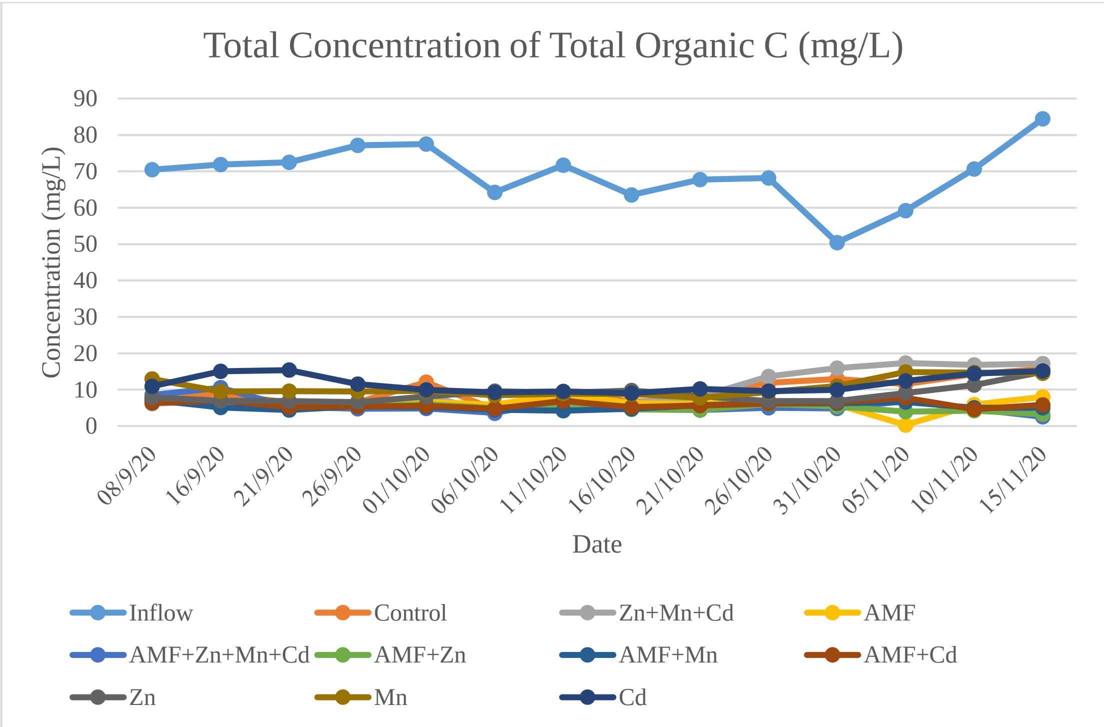

Figure 32: Total concentration of TOC (mg/L)

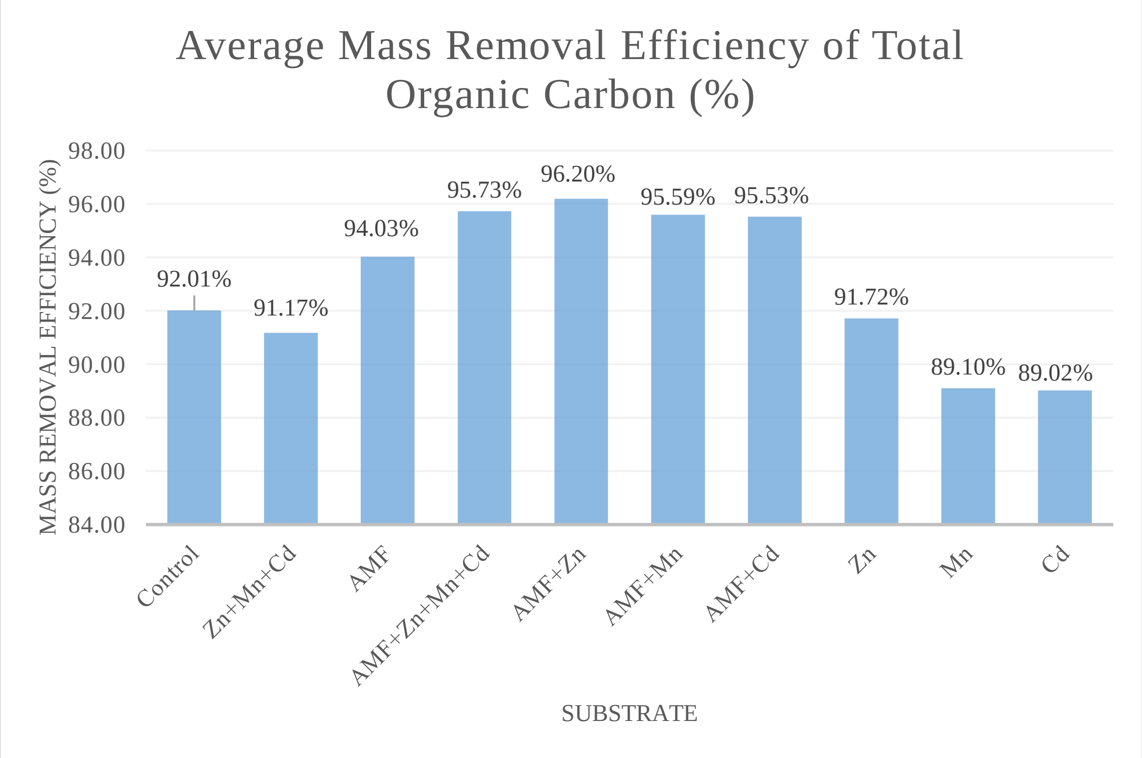

Figure 33: Average mass removal efficiency of TOC (%)

4.11 Phosphate (P) removal

The total concentration (mg/l) of P as shown in Figure 34 showed differences among the treatments. Phosphate concentration ranged between 1.55 – 2.94mg/L in Control treatment, 2.10 – 4.64mg/L in Zn+Mn+Cd treatment, 0.56–1.27mg/L in AMF only treatment, 2.50 – 3.09mg/L in AMF+Zn+Mn+Cd treatment, 2.22 – 4.49mg/L in AMF+Zn treatment, 2.28 – 4.31mg/L in AMF+Mn treatment, 1.64 – 4.99mg/L in AMF+Cd treatment, 3.15 – 6.42 mg/L in Zn only treatment, 2.60 – 5.62mg/L in Mn only treatment and 3.32– 5.22mg/L in Cd only treatment. The highest P concentration (mg/L) was observed in Zn only treatment (6.42mg/L), while the least was recorded in AMF only treatment (0.56mg/L). AMF significantly decreased P concentration by 55% in the blank with AMF than without AMF, by 23% in Zn+Mn+Cd with AMF than without AMF, by 12% in Zn with AMF than without AMF, by 33% in Mn with AMF than without AMF and by 18% in Cd with AMF than without AMF.

The average mass removal efficiency (%) of P varied among the treatments as shown in Figure 35. Phosphate mass removal efficiency (%) ranged between 84.60 – 97.42% in Control treatment, 65.50 – 97.16% in Zn+Mn+Cd treatment, 89.20 – 98.06% in AMF only treatment, 78.77 – 93.72% in AMF+Zn+Mn+Cd treatment, 69.38 – 96.40% in AMF+Zn treatment, 72.65

– 94.58% in AMF+Mn treatment, 65.82 – 96.97% in AMF+Cd treatment, 65.52 – 94.18% in Zn only treatment, 47.41 – 90.70% in Mn only treatment and 58.61 – 89.40% in Cd only treatment. The highest average P mass removal efficiency (%) was observed in AMF only treatment (93.26%), while the least was recorded in Mn only treatment (66.43%). AMF significantly increased P mass removal efficiency by 5% in the blank with AMF than without AMF, by 7% in Zn+Mn+Cd with AMF than without AMF, by 7% in Zn with AMF than without AMF, by 24% in Mn with AMF than without AMF and by 11% in Cd with AMF than without AMF.

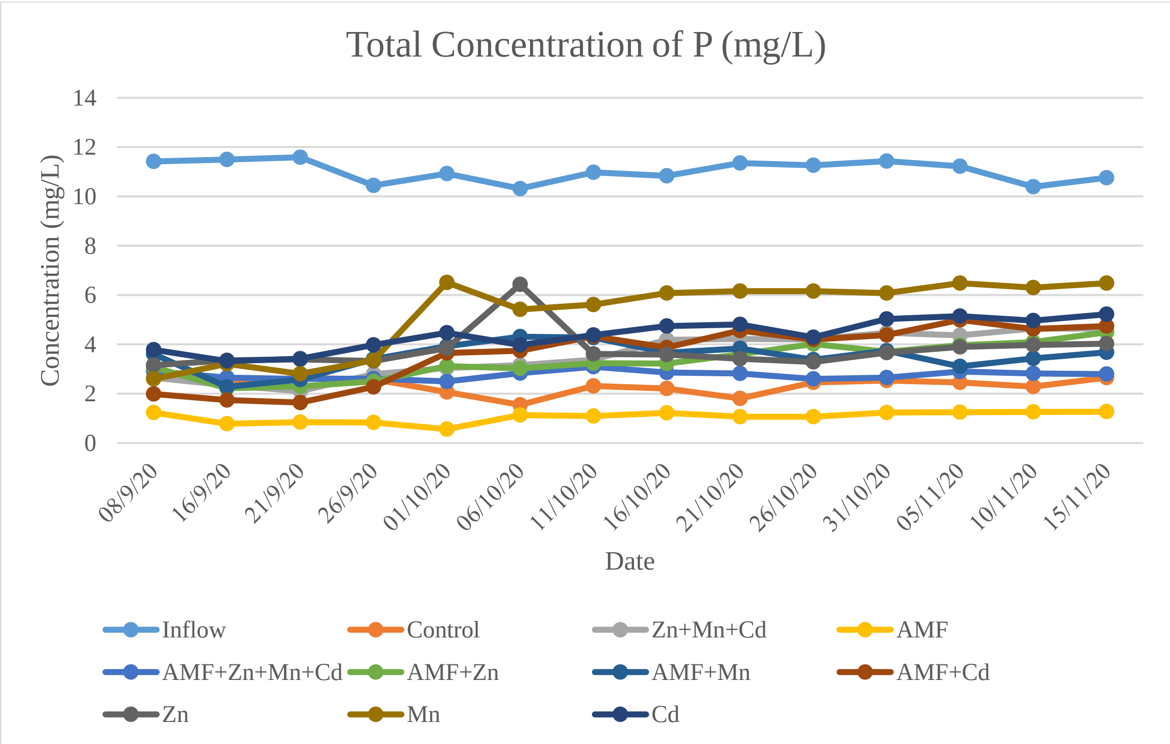

Figure 34: Total concentration of Phosphate P (mg/L)

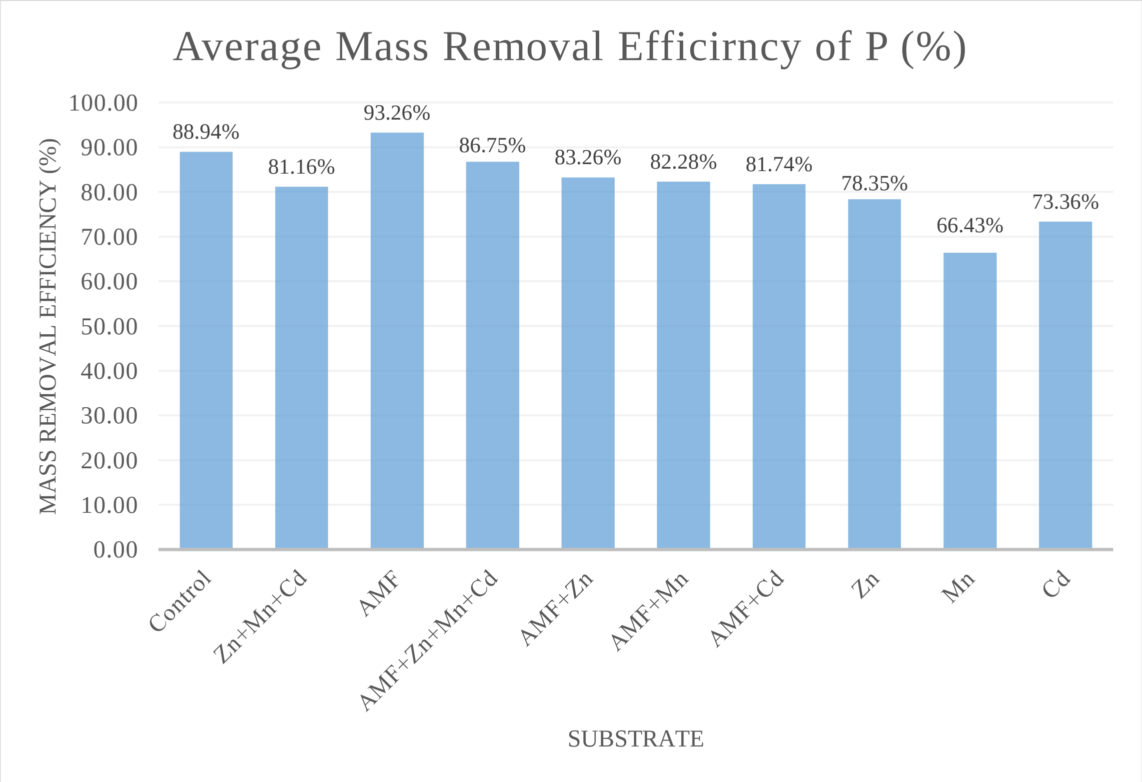

Figure 35: Average mass removal efficiency of Phosphate P (%)

5.0 DISCUSSION AND CONCLUSION

5.1 AMF colonization (M%) and Arbuscule abundance (A%)

The presence of AMF in metal-contaminated soils and their ability to make an efficient mycorrhiza symbiosis are extensively investigated by several researchers (Da Silva *et al*. 2003). AMF colonization was significantly higher in treatment inoculated with AMF without HM than in treatments inoculated with AMF and HM. Treatments with a single HM had significantly higher AMF colonization than a combination of the three HMs. This finding is in conformity with Zhang *et al*. (2019) and Yang *et al*. (2015) who observed AMF colonization decreased with heavy metal, and Ning *et al*. (2019) who reported that Cd addition decreased AMF colonization. It has also been reported that accessibility and retention of carbon, nitrogen and phosphorus, along with pH and oxygen availability were important defining factors of AMF colonization (Xu *et al*. 2016; Hu *et al*. 2020).

Ray and Inouye (2006) studied the effect of intermittent flows on AMF colonization and proposed that the length of the unflooded period shows a positive or direct correlation with the hyphal and AMF colonization. This was attributed to the oxygenation of the rhizosphere during the exchange of wet and dry periods (Liang *et al*., 2018), which provides suitable oxygen for the expansion of AMF colonization in CWs. Furthermore, plants can be colonized by AMF since the aerenchyma structure in wetland plants can provide active ventilation of the roots and rhizomes, and thus maintain favorable oxygen conditions for AMF growth (Dickopp and Kazda, 2011). However, because of the limited oxygen in wetland ecosystems, the regularly oxygen-free conditions always lead to negative effects on the processes of fungal root colonization. Due to this, AMF colonization in most wetland plant roots remained at a low level (< 25%) (Wang *et al*., 2018).

5.2 Biomass

AMF significantly increased biomass accumulation in plants. The is evident in how AMF inoculated treatments had higher root and shoot dry weight than non-inoculated treatments. AMF increased root dry weight by 7% with Zn+Mn+Cd, by 49% with Zn, by 12% with Mn and by 1% with Cd. AMF also increased shoot dry weight by 31% with Zn+Mn+Cd, by 10% with Mn and by 2% with Zn. On the other hand, AMF decreased shoot dry weight by 18% with Cd. This shows that heavy metals have inhibiting effects on plant biomass accumulation. Therefore, AMF had a positive effect on the expansion and growth of wetland plants even under heavy metal concentrations. Zhang *et al*. (2019) and Yang *et al*. (2015) both observed higher dry weight of shoots and roots with AMF under all Pb treatments as well. Similarly, Hu *et al*., (2020) showed that the biomass of inoculated wetland plants was significantly higher ($p < 0.05$) in inoculated plants than that of the corresponding non-inoculated plants.

5.3 Nutrients in plant

This study revealed that AMF significantly increased P concentration (mg/kg) in the plant root by 13% - 40% and K by 20% - 98%. AMF also increased K concentration (mg/kg) in the shoot by 72% - 152% and P concentration by 10%-72%. However, K concentration was slightly reduced by AMF in Zn and Mn by 6% and 3% respectively. AMF had been reported to increase the nutrition uptake and absorption for plants by increasing the root surface (Zhang *et al*. 2020). In general, AMF symbiosis contributes to the beneficial effect on plant growth under heavy metal concentration Various studies also obtained higher P contents with AMF under HM concentration in shoots and roots compared with non-inoculated plants (Chen *et al*. 2015; Zhang *et al*. 2020). Solaiman and Hirata (1997) also observed increased P transfer through hyphae from soils to roots or shoots. Therefore, AMF could increase nutrition like K and P uptake for plants to increase Pb resistance, and this might be a reason for AMF increasing plant growth. Additionally, previous studies indicated that AMF could deliver up to 42% N and 80% P into the plant to aid host plant growth (Marschner and Dell, 1994).

5.4 Heavy metals in plant

This study also showed that AMF reduced Zn (10% - 13%), Mn (35% - 55%) concentration and mass content in the root of plants induced with HM and AMF than without AMF. However, AMF increased Cd concentration by 38% - 278% and mass content by 47% - 283%. Several researches also shows that AMF intensified Cd concentrations in shoots of *Solanum nigrum, Lotus japonicas*, *Phragmites australis* (Liu *et al*., 2015; Zhang, Chen & Ohtomo, 2015; Wang *et al*., 2017). AMF reduced Cd (11%), Mn (34% - 37%) and Zn (24%) in the shoot of plants induced with HM and AMF than without AMF. Additionally, AMF reduced Cd concentration in plant substrate but increased Zn and Mn concentration. Heavy metal accumulation was higher in roots than in shoots. Roots usually accumulate a much higher amount of a heavy metal than shoots, which may be due to heavy metal precipitation in vacuoles and root cells, which is thought to be a detoxification mechanism (Congeevaram *et al*. 2007).

Studies have shown that plants cultivated on soils enriched with Cd and Zn exhibit significant repression in shoot and root growth, leaf chlorosis, and even death (Moghadam, 2016). There are several reports within the literature on realizing the AMF-influenced effects on the spread of metals in plants (Souza *et al*., 2012). Heavy metals can be immobilized in the fungal hyphae of internal and external origin (Ouziad *et al*., 2005) that can fix heavy metals in the cell wall and store them in the vacuole or may chelate with other substances in the cytoplasm (Punamiya *et al*., 2010) and thus reduce the toxicity of metals in the plants.

AMF is widely believed to support plant establishment in soils contaminated with heavy metals, because of their potential to strengthen the defence system of the AMF mediated plants to promote growth and development. Mycorrhizae can interupt the uptake of various metals into plants from the rhizosphere and their movement from the root parts to the aerial parts (Dong *et al*., 2008; Li *et al*., 2015). Mycelia of diverse AMF have high absorption of metals capability and cation exchange (Takács and Vörös, 2003). Metal non-adapted AMF settles the polluted soils and reduces uptake and accumulation of heavy metals, as noticed in perennial ryegrass (*Lolium perenne*) in artificially polluted soil with various elements like Ni, Zn, and Cd (Takács and Vörös, 2003). In rice, AMF was functional in lowering the levels of Cd in both the vacuoles and cell wall, led to Cd detoxification (Li *et al*., 2016). Wang *et al*. (2012) noticed that AMF-mediated boosted Cd tolerance in alfalfa (Medicago sativa L.) was possible due to the modification of chemical forms of Cd in various plant tissues.

5.5 Heavy metals removal

This study showed that removal efficiency (%) ranged between 99.91% - 99.99% for Cd, 98.64% - 99.99% for Zn and 96.23% - 99.92% for Mn. AMF increased Cd removal efficiency by 0.07% - 0.32%, Zn removal efficiency by 0.14% - 0.33% and Mn removal efficiency by 0.12% - 1.44%. The findings of this study agree with Xu *et al*. (2018) and reported that the roots of *P. australis* plants occupying two CWs for the treatment of metal-contaminated water concealed different species of AMFs, and these fungi seemed to play a vital role in metal removal from contaminated water. Four mechanisms have effects on metal removal in wetlands (Lesage *et al*. 2007): (1) adsorption to fine-textured sediments and organic matter (Gambrell 1994), (2) precipitation as insoluble salts (mainly sulfides and oxyhydroxides), (3) absorption and prompted changes in biogeochemical cycles by plants and bacteria (Kadlec and Knight 1996), and (4) deposition of suspended solids because of low flow rates. All these reactions cause accumulation of metals in the wetlands. Out of the mechanisms stated above, microorganisms, such as free-living, plant growth-promoting rhizobacteria, as well as symbiotic bacteria, played an important role in HM removal in CW by enhancing certain metabolic characteristics of their host (e.g., atmospheric N fixation and so on) and producing hormones and siderophores (Xu *et al*., 2018).

5.6 Nitrogen Removal

Mechanisms for nitrogen removal in constructed wetlands are manifold and include volatilization, ammonification, nitrification/denitrification, plant uptake and matrix adsorption. Numerous studies have shown that the primary removal mechanism in most of the constructed wetlands is microbial nitrification/denitrification (Vymazal *et al*., 2002). In addition, the metals (Cd, Pb, etc.) seem to show some inhibitory effect on nitrogen uptake by cattail plants (Lim *et al*., 2003). As with the removal of other pollutants and organic compounds, the expulsion of nitrogen from constructed wetlands is governed by numerous complex biological and mechanical pathways that can be enhanced by factors such as temperature, pH, carbon availability and operational factors of CWs. Nitrogen removal is deemed especially important as an increased release into the surrounding environment causes eutrophication of waters consequently leading to depleted oxygen levels and the death of numerous aquatic species (Grinberga and Lagzdins 2017). NH_4^+ N concentration in this study ranged between 0.03mg/L – 1.79mg/L. Generally, Total N and NH_4^+ N concentrations were higher in CWs induced with heavy metals without AMF inoculation compared to their counterparts with AMF. This result shows that AMF reduced NH_4^+ N concentration between 20%-67% and total N between 9%-36%. However, NH_4^+ N removal efficiency was increased by AMF by 0.3% - 13% and total N by 1%-40%. Hamel (2004) also found out that AMF can take up and transport NH_4^+N to their host plants. This contribution might be important as NH_4^+ reacts with the soil cation exchange complex and is less mobile than NO_3^-. The improved foraging ability of a mycorrhizal root will probably increase the uptake of NH_4^+ released from the mineralization of organic residues in soil. Furthermore, AMF-enhanced NH_4^+ uptake by plants could increase the soil nitrification rate. Tand *et al*., (2020) reported that the removal of nitrogen in CWs mainly relies on absorption by plants and the nitrification and denitrification of microorganisms. Some nitrogen is removed by plant uptake in CWs, and plants prefer ammonium when nitrate and ammonium coexist (Almeida *et al*., 2019).

5.7 Carbon Removal

This study showed that AMF reduced total C concentration by 3% - 33% and TOC by 36% - 49%. The average removal efficiency of total C was however increased by 6%- 17% and TOC by 2% - 7%. This may be attributed to the fact that AMF remains entirely dependent on plants for their carbon demand, and it has been reported that AMF used 4.3% of recently planting fixed C in just a single day (Tome *et al*. 2015). Sometimes this demand is high enough to suppress the early plant growth (Jakobsen 1999; Graham 2000; Ryan and Graham 2002). However, it is also hypothesized that AMF creates C sink strength and maintains the higher photosynthetic rate by regulating the product feedback inhibition mechanism and compensate the plant C use by AMF (Kaschuk *et al*. 2009; Schweiger *et al*. 2014). This source-sink interplay in plant-mycorrhizal symbiosis will seriously impact C fixation through photosynthesis and its belowground availability under future climatic conditions (Fatichi *et al*. 2014; Finzi *et al*. 2015). However, earlier it was uncertain whether enhanced C assimilation in mycorrhizal plants is due to greater nutrient supply or downward C flow to mycorrhizal roots or by other alternative mechanisms. Schweiger *et al*. (2014) stated that substantial extent of metabolic changes (at least 50%) and higher C assimilation in mycorrhizal *Plantago major* were not dependent on nutrient availability (P concentration). For more clarification, another mechanism proposed that higher gas exchange and source-sink interplay in plant mycorrhizal symbiosis could be as a result of aboveground source to above or below the ground sink (Godbold *et al*. 2006; Moyano *et al*. 2007; Kaschuk *et al*. 2009).

5.8 Phosphorus removal

Phosphorus is one of the key compounds which cause eutrophication of surface waters and its removal from wastewater using CWs has been of great interest (Prochaska and Zouboulis 2006).

The findings of this study revealed that AMF decreased P concentration in constructed wetlands inoculated while increasing mass removal efficiency. Several processes such as uptake by plants, adsorption, and precipitation within substrates play a major role in phosphate removal (Wang *et al*. 2013). The initial potential of each constructed wetland system to remove phosphorus is limited and dependent on plants as well as symbiotic microbes (Arias and Brix 2005). Plant uptake is generally seen as one of the main mechanisms by which phosphates are removed, however, this represents only a fraction of the total amount of the compound that is usually present in wastewater and is only a temporary mechanism by which phosphates are stored (Brix *et al*. 2001; Gupta *et al*. 2016). Also, microbial uptake of phosphates is not considered substantial and only temporary as phosphates are released back into the environment following the decay of organisms (Vymazal 2007).

5.9 Conclusion

Constructed wetlands (CWs) have been successfully used to improve the quality of various types of water and are considered versatile systems that provide ecosystem services. They integrate several components that include plants and associated organisms. This study established the relationship between the capacity of arbuscular mycorrhizal fungi (AMF) in the removal of heavy metals from AMF assisted constructed wetlands (CWs). AMF community was successfully established in the roots of plants induced with Zn, Mn and Cd confirming the importance of AMF symbiosis with wetland plants. AMF improved plant biomass and nutrient concentration by reducing Zn, Mn and Cd concentration in the plant shoot, root and substrate and increasing their removal efficiency. Meanwhile, AMF colonization can remove conventional pollutants in CWs with the removal efficiency of NH_4^+ N, TN, TC, TOC, total phosphate in AMF inoculated plants. Overall, this study provides encouraging evidence that the introduction of AMF into CWs can enhance the removal of heavy metals and improve plant performance.

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