CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Environmental Sciences

# The effect of nitrogen source on phytoremediation of heavy metals in plant-sand systems

## Bachelor Thesis

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2022

# CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Environmental Sciences

# BACHELOR THESIS ASSIGNMENT

Filip Bokros

Environmental Engineering

Thesis title

**Effect of nitrogen source on phytoremediation of heavy metals in plant-sand systems**

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### Objectives of thesis

1) Assess whether the removal of heavy metals can be affected by different nitrogen sources.
2) Evaluate the effects of nitrogen sources on the distribution of heavy metals.

### Methodology

Eight plant-sand systems will be established in this study, the systems will be carried out in pots with a dimension of 80×22×18 cm (length × width × height). The selected plant is Iris pseudacorus. Each system has a 15 cm sand layer. The experiment will be divided into four treatments with different nitrogen sources, including urea, NH4Cl, NaNO3 and skim milk. Each system will feed with Hoagland solution every 4 days in the whole experimental period. After the establishment of AMF symbiosis (about two months), heavy metals (Cr, Cu and Pb) will be added to each system. This experiment will be conducted at the Czech University of Life Sciences Prague.

**The proposed extent of the thesis**

50

**Keywords**

Nitrogen, heavy metals, plant-sand systems, phytoremediation

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**Recommended information sources**

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**Expected date of thesis defence**

2021/22 SS – FES

**The Bachelor Thesis Supervisor**

doc. Zhongbing Chen

**Supervising department**

Department of Applied Ecology

**Advisor of thesis**

Bo Hu

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|---|---|
| Electronic approval: 3. 2. 2022 | Electronic approval: 10. 2. 2022 |
| **prof. Ing. Jan Vymazal, CSc.** | **prof. RNDr. Vladimír Bejček, CSc.** |
| Head of department | Dean |

Prague on 28. 03. 2022

## Recognition:

I'd want to express my gratitude to the doc. Dr. Ing. Zhongbing Chen for his remarks on the completed work once it was submitted. My gratitude also goes to Ph.D. applicant Ing. Bo Hu, MSc. for his valuable guidance, willingness, and patience during the preparation of this thesis.

## Declaration:

*I hereby declare that I have independently elaborated the bachelor/final thesis with the topic of: The effect of nitrogen source on phytoremediation of heavy metals in plant-sand systems and that I have cited all of the information sources that I used in the thesis as listed at the end of the thesis in the list of used information sources.*

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

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*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. The student adds the date and place of the statement and signs it (Appendix No. 1).*

*In Prague, 2022*

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# Abstract:

As human civilization develops and living standards increase, so do demands, technical advancements, and environmental consequences. Contamination of the environment with heavy metals is among the most important sorts of contaminants. Heavy metals, as well as numerous herbicides, insecticides, and low-cost fertilizers, are an issue in the environment. Plants are used as a source of food, fuel, and fibber, but their potential to deal with pollution and actively participate in its cleanse has been discovered just lately. Artificial plant-based systems, such as constructed wetlands, have been successfully developed to treat wastewater as a biological filter that removes heavy metals and other organic compounds. Our study investigates the effects of several nitrogen sources (Urea, $NH_4^+$-N, $NO_3$-N) on the phytoremediation of heavy metals and other organic compounds by using laboratory size built reactors. Further analysis of water samples proceeded in the laboratory with measurement of proposed parameters for TOC, IC, TC, TN, $NH_4^+N$, $NO_3\ ^-N$. Plant samples were dried for determination of the amount of heavy metals concentration which was obtained from CECs measurement. When the initial concentrations of HMs in the soil solution emerged, plant growth rates were reduced. In the reactors without HMs, $NO_3^-N$ produced the highest rate of biomass, followed by urea, while $NH_4^+$-N produced the lowest rate of biomass. In the case of HMs reactors, the highest average rate was achieved while using an $NH_4^+$-N source, followed by urea, and eventually $NO_3^-N$. Under the N source, plants demonstrated the ability to develop significant biomass concentrations while also removing HMs from the substrate. The favorable influence of various N forms on lowering heavy metal concentrations in the substrate was reflected in heavy metal concentrations on the observed plant *Iris Wilsonii*. As a result, it can be determined that using phytoremediation in wastewater treatment can result in a more cost-effective and environmentally friendly using nitrogen technology.

**Keywords:** phytoremediation, heavy metals, nitrogen source, constructed wetlands, removal efficiencies

## Abbreviations:

CWs- Constructed wetlands

HMs- Heavy metals

OC- Organic Carbon

IC- Inorganic Carbon

TC- Total Carbon

TOC- Total Organic Carbon

TN- Total Nitrogen

UV-Ultra-Violet

WWTPs- Wastewater treatment plants

AMF- Arbuscular Mycorrhizal Fungi

IES- Ion exchange substrates

# Table of Contents

# 1 Introduction

Environmental pollution caused by heavy metals has been already present for several decades since the times of industrialization, urbanization, use of raw materials, mining, metal, and fabrics production. All these processes have made our living conditions easier, but in some ways, they reduce the quality of conditions of the environment we live in. Their every contact with living organisms brings undesirable and toxicological effects and a certain burden for the environment in which we live. Their gradual expansion distributes to people through food, industry, water, and pharmaceuticals. Research shows increasing concertation levels of heavy metals present in human bodies over the last 50 years due to elevated use of products that contain pollutants (Kizeková et al. 2017).

The increasing quantity of foreign and harmful compounds in the environment is causing people to reconsider their behaviours, attempting to limit risk and avert the threat posed by prior ill-considered actions. More and more hazardous compounds have been released into the environment in recent decades, affecting all living organisms on the planet, including people. Mining and the following processing of ores and other minerals are two of the most significant sources of heavy metal pollution.

All green plants can collect dangerous (contaminating) and toxic substances that are occurring in water, soil, wetlands, and the atmosphere. Subsequently, with the help of certain microorganisms, they act in the process of phytoremediation where, with the help of fixation, accumulation, and subsequent degradation, they remove toxic substances from the environment. It is not for nothing that the higher green plants are called the green liver of nature. The name was given not only for their ability to metabolize and degrade many pollutants but also for the potential to accumulate toxic heavy metals in their tissues, in large quantities (Pauková et al., 2020).

Today, we already know several ways to deal with the environmental issue to remove contaminants, but to achieve optimal results, the process is either very complex or expensive. On the contrary, phytoremediation is, in contrast to other methods and technological approaches for extracting these metals and pollutants from contaminated soil, low-cost and environmentally friendly, which opens up the possibility of establishing it in a much wider spectre for use (Tangahu et al., 2011).

The positive effects of phytoremediation on the removal of heavy metals have been reported already by many studies. However, knowledge about the effect of nitrogen sources on the phytoremediation of heavy metals has been poorly studied so far. Therefore, this project will be focused on the distribution of heavy metals (Cr, Cu, and Pb) in plant-sand systems under different nitrogen sources. Four nitrogen sources, including urea, $NH_4Cl$, $NaNO_3$, will be used in this study to set different nitrogen source conditions.

## 1.1 Heavy Metals

In definition, heavy metals (HMs) are metallic elements with high atomic weight, large thermal conductivity, and readily yield electrons and density, which is about five times greater when compared to water. HMs are considered to have a natural origin and most of their source can be found in the earth's crust processes, such as weathering of rocks and volcanic erosion. However, most of the ecological burden and risk for human health comes from non-natural origin due to industrialization and urbanization such as agriculture, domestic sewage water, pharmaceutics, mining, and industry. It may be said that nearly the majority of products we use during the day are a potential source of heavy metal pollution in the environment, beginning with the creation of electricity and heat in nuclear and coal power plants, textiles, plastics, petroleum, electronic devices to wood and paper production as shown on **Figure 1.**

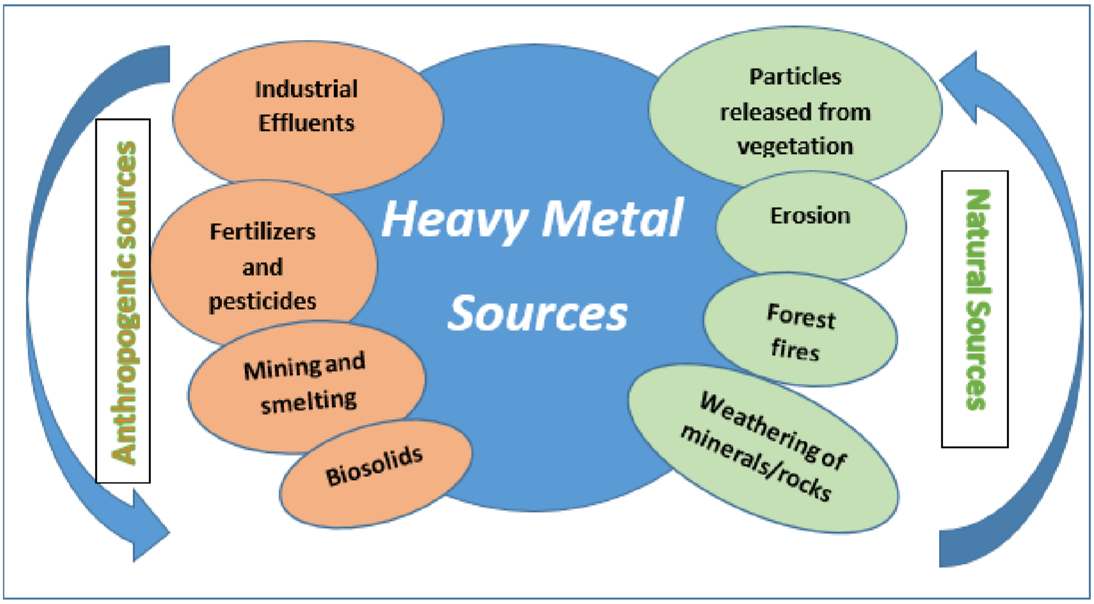

**Figure 1** – Heavy metal sources in the environment (Athar, Waris, Nisar, 2018)

Part of metals is determined as essential nutrients, which means that they figure as important catalyzers (cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se) and zinc (Zn). Deficiency and shortage can possibly lead to health disorders. The other part is considered non-essential, so the organism does not require its presence. They do not represent that much threat in low concentrations but from the certain amount they are toxic (aluminum (Al), antinomy (Sb), arsenic (As), barium (Ba), beryllium (Be), bismuth (Bi), cadmium (Cd), gallium (Ga), germanium (Ge), gold (Au), indium (In), lead (Pb), lithium (Li), mercury (Hg), nickel (Ni), platinum (Pt), silver (Ag), strontium (Sr), tellurium (Te), thallium (Tl), tin (Sn), titanium (Ti), vanadium (V) and uranium (U)) (Tchounwou et al., 2012).

One of the major pathways to how heavy metals get distributed in the environment is by the hydrological circulation of water. When we talk about transport processes of these metals, important movement is by an aquatic pathway in the water resources, leaching from the soil vertically down followed by the accumulation up to the food chain. The process that leads to it, is adsorption and absorption onto the various substrates, soils, sediments, particulates, or water where solubility plays an important role for heavy metals. When we take a closer look into the transport process in the groundwater where the water resides this process depends on the particles because water has to percolate through them. Smaller pore size creates a lower velocity of the water with the plume of the contaminant. A lot more dilated pore will essentially have a much higher velocity along with the contaminant.

The distribution of the aquifer property is called hydraulic conductivity. When the hydraulic conductivity is high, the mass will proceed in a much longer distance so the contaminant with the water will move further distance into the layer. Guidance by which concentration of the metal will move through the aquifer will depend on an aquifer characteristic (Bielski et al., 2020).

### 1.1.1 Bioavailability of metals

Heavy metals in soil can be present in form of soluble ions, insoluble salts, or as components from the rock from which soil arises. The metallic ions may have different valences such as trivalent arsenic, pentavalent arsenic. They may be present in soil solution or adsorbed on the clays particles and organic soil matter. All these forms of heavy metals are in dynamic equilibrium with each other. Metals present in the soil solution are directly present for uptake by plant roots. This bioavailability is influenced by factors such as soil pH, amount of clay, and organic matter in soil and redox conditions. Besides soil pH, the acidic organic matter forms stable complexes with metals, thus affecting their bioavailability. Copper ions are known to form strong coordination complexes with organic matter in the soil, decreasing the bioavailability to plants in soils that are rich in organic matter. Redox potential in the soil also changes the speciation and solubility of many elements and creates new compounds. The redox potential can affect metal solubility such as zinc, copper, cadmium, and lead which form soluble salts and stable complexes with hydrous oxides in a reducing environment but may participate in oxidizing conditions. Similarly, manganese exists as a soluble divalent cation under reducing conditions. Still, it may form insoluble oxides of trivalent manganese under oxidizing conditions, hence under some conditions which represent a reducing environment the availability of many heavy metals increases in the soil solution, making them toxic for many plant species (Li et al., 2021).

### 1.1.2 Ecotoxicity of Heavy metals

There is a growing demand for agriculture to produce an increasing amount of supplies to satisfy the market every year. Meaning, that farmers increase the use of inorganic and phosphate fertilizers among herbicides and pesticides, which are needed to control and prevent diseases of crops, grains, and vegetables possibly resulting in fluctuating and many times higher values of metals held in grains and vegetables of Ni, Zn, Pb, Cd, As and Cr. HMs inputs to agricultural land from excessive fertilizer use are raising concerns about their potential environmental impact (Ali et al., 2020).

Arsenic, mercury, cadmium, and lead have a significant role in bioaccumulation due to their pathophysiological significance and they can cause harm to important organs in the human body. Most affected are mainly reproduction, gastrointestinal tract, nervous system, and mucous tissues. There are still exact unknown processes of their workings but results from several studies show that their overload aggregation and exposure lead to the creation of free radicals and subsequent oxidative stress. HMs also form complexes with cellular compounds containing oxygen, nitrogen, and sulphur. Their ability is to advance cellular dysfunction by modulation of protein structures and crucial enzyme systems (Singh et al., 2017).

Non-essential heavy metals show different toxicity symptoms when accumulated in plants. Zinc is a component that deficiency empires diverse metabolic processes. Excess zinc availability misplaces other metal ions from example magnesium from enzymes like rubisco and leads to an inhibition of photosynthesis. Symptoms of zinc toxicity are similar to leaf chlorosis caused by magnesium or iron deficiency. Due to the cationic properties, metals interact with the molecular oxygen present in chloroplast and mitochondria and lead to the generation of reactive oxygen species, causing oxidative damage to cell components.

Metals are known to inhibit respiration and photosynthesis as they act at various sites of photosynthetic electron transport systems thus disrupting the generation of ATP. Some metals such as Cadmium, Chromium, and Mercury are also known to show genotoxic effects and cause DNA damage (Singh et al., 2016).

### 1.1.3 Heavy metals detoxification and tolerance mechanisms

Plants can cope with heavy metals either by restricting uptake and transport of the metal or by evolving various tolerance mechanisms. The Association of rhizobacteria with plant roots have been reported to restrict the movement of heavy metals to root cells either by adsorption or absorption of fungal mycelia or by selective immobilization of metals in root tissues colonized by mycorrhizal fungus. The binding of metals to cell wall components such as pectin is one of the mechanisms excluding the metal in the apoplast and the high cation exchange capacity of cell walls correlated to better metal tolerance by plants. Root exudation of phosphorus or organic acids such as malic acid has been shown as an exclusion mechanism against aluminium stress where the aluminium in soil is either precipitated as phosphate or chelated as organic salt. Organic acids and metallothioneins help to keep metals in the non-reactive state once they get into the cytoplasm. A decreasing root to shoot transport is achieved by transporting the metal to vacuoles in root cells. Metals may be also bound to chelators in the xylem thus reducing their toxicity. Tolerance in heavy metal stress involves mechanisms ranging from increased activity of antioxidant enzymes to prevent oxidative damage to the synthesis of organic acids or amino acids to form organometallic complexes and transport of these complexes to vacuoles. Besides vacuoles leaf trachoma are also known to store heavy metals often as metallothionein complexes. Shedding of leaves into which heavy metal has been deposited for storage, enables removal of the metal from the plant. This strategy has been noted in many species under metal-induced stress (Yang et al., 2020).

Hyper accumulator plants are plants, with encrypted tolerance mechanisms against metals and are able to hyper accumulate metals. These plants are adapted to soil containing large numbers and amounts of metals. Three steps in hyperaccumulation in such plants include 1.) Metal active transport across plasma membranes and roots 2.) Metal entry in the plant during translocation from root to shoot 3.) Metal chelation and sequestration in specific cell compartments within leaves.

Vacuoles serve as storage organelles for metals in many hyperaccumulation plant species and are able to accumulate much higher amounts without showing any symptoms of toxicity. Soil bacteria play an important role in affecting the bioavailability of metals. Rhizosphere bacteria in the hyperaccumulators are metal resistant and reported to lower soil pH, produce organic acids, metal chelating agents, and plant hormones. That plays a role in increasing root plant biomass.

Hyperaccumulation of heavy metals is taught to play a role as a defense mechanism along with secondary metabolites against possible enemies as are herbivores. Since plants do not synthesize heavy metals, but their source is from the soil, hyperaccumulation may constitute a metabolically inexpensive defense mechanism. Metals accumulated in plants can also induce locally increased metal concentration of soil due to the shedding of leaves loaded with higher amounts of metals which will have unfavorable effects on the metal-sensitive plants in the same area. For the mentioned reason, these plants are being exploited in metal concentrated soils, and this phytoremediation strategy is compared to other chemical methods to clean up contaminated soils much more economically (Neilson, Rajakaruna, 2021).

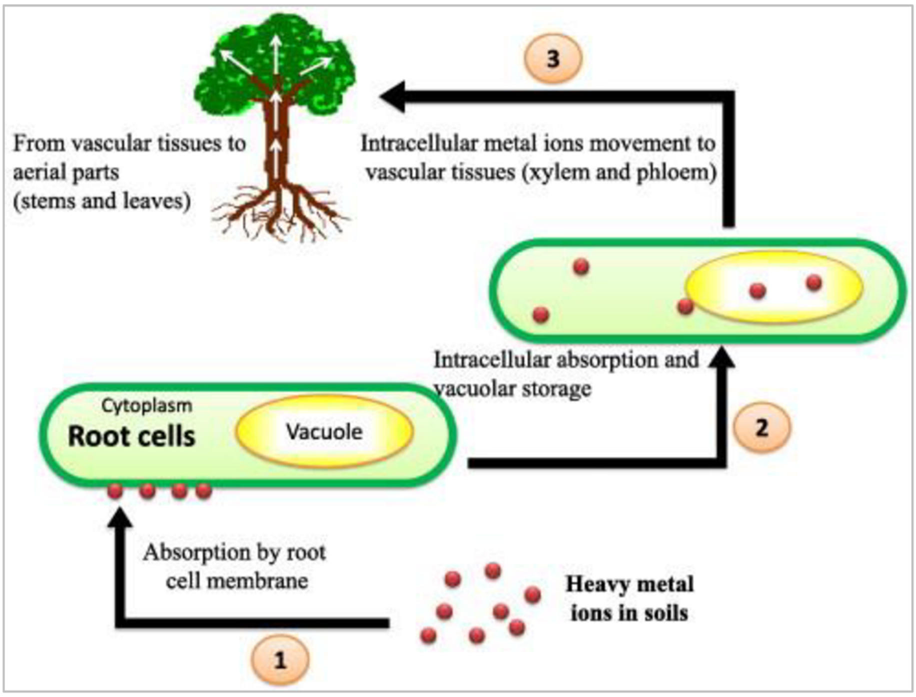

**Figure 2** – Graphical presentation of the movement of heavy metals in plants (Munees, 2014).

a) **Accumulation more in aboveground organs (stems and leaves) -** silver, chromium, lead, tin,
b) **Accumulation more in underground organs (roots and rhizomes) -** cadmium, cobalt, copper, iron, molybdenum,
c) **Accumulation evenly in aboveground and underground organs -** manganese, nickel, zinc. (Pauková, 2020).

Various HMs are likely to accumulate in distinct parts of plants; however, their allocation can be influenced by plant type.

### 1.1.4 Cadmium effect on plants

Cadmium is a silver-white metal element is known for its harmful and toxic effects on the whole ecosystem. Of all other heavy metals, Cd is considered to be one of the most toxic along with mercury and lead. In nature, its most commonly found as an impurity addition in zinc and lead ores. In case of presence in the soil, the element can easily be transported into all plant organs due to its fluidity and water solubility. Among the most serious symptoms that this element originates to a plant is chlorosis, which causes yellowing of leaves due to the lack of chlorophyll, growth suppression, carbon and nitrogen assimilation, and browning of roots, followed by the death of the plant. The higher content of this element manipulates the water content and conflicts with the transport and use of elements such as potassium (K), calcium (Ca), magnesium (Mg), and phosphorus (P). Stated regular concentrations in a plant are normally in the range of 0.2-0.8 mg $kg^{-1}$, and toxic is considered as 5-30 mg $kg^{-1}$. Regarding the amount of Cadmium concentration in the plant, we can distinguish them by classification into the following groups: 1.) Cadmium accumulators 2.) Cadmium avoiders (Ackova, 2018).

Some plants can accumulate higher cadmium concentrations in their parts or seeds, even though the cadmium levels in the soil are within limits. Therefore, in such special cases, it is necessary to replace Cd-tolerant cultivars with Cd-avoider ones to reduce the risk of intoxication to humans and animals. Cadmium poses a health risk for animals and humans in a plant tissue concertation that is commonly not phytotoxic, so the plants may not show any indication of toxicity but they possess segments exceeding safe concertation levels for humans with the likely possibility to cause health issues. Due to the renal capability of kidneys to build up metallothionein, which is very likely to bond with Cd, exceeding intake can cause serious damage to kidneys.

The amount of cadmium that the plant draws from the soil, water, or possibly a small amount from the atmosphere through the root cells can modulate factors such as pH and soil properties and organic acids' presence in the rhizosphere. In the oilseed rape study, it was found that the cadmium content in the heavily purified soil was relatively higher at soil pH 4 as opposed to the pH 5 sample and the metal level uptake was also higher in sandy soil as opposed to clay soil. It is also important to mention, that this heavy metal occurs in the acidic environment in the form of free ions $Cd^{2+}$, but in more alkaline soils at pH about 6-7, it forms other forms such as cadmium chloride ( $CdCl^{-}$), hydrated cadmium carbonate $CdCO_3$ and different complexes.

### 1.1.5 Lead Effect on plants

Lead is a blue-white coloured, soft, non-essential element of anthropogenic origin that quickly coats with a layer of oxide when exposed to the air. It combines directly with fluorine, chlorine, bromine, iodine, sulphur, selenium, tellurium, and polonium at elevated temperatures. Lead has been known for its phytotoxic and negative blow on the morphology of the plant even though, it does not have any biological function. All negative impacts of higher lead concentrations are observable during the long time exposure. The element affects all plant species, mainly by interacting with sulphydryl groups which are a source of enzyme function blockage (Ackova, 2018).

Lead is one of the most common heavy metals in the environment with a negative impact. It easily accumulates in the water and soil and is harmful to all living creatures in even low concentrations. Plants have created several instruments that will run when exposed to lead. Most commonly they are influenced through the first organ interacting with various parts of the rhizosphere- the root system. That's the reason why the majority of greenery contains around 90% of the total lead volume in their roots. The primary responsibility of the plant is by the synthesis and setting of callose, a specialized polysaccharide, in between plasma membrane and cell wall. It works as a blockade for Pb to be absorbed in higher concentrations and its further separation of dead tissue from living tissue in vacuoles followed by the influence of the root extension and branching or in the case of hyperaccumulator plants, the possibility of translocation to the higher sections of the plant.

There are various ways this metal gets distributed in the environment. Volcanic activity, erosion, and weathering of rocks contribute as the natural source spreading lead into the soil and water in nature. Research shows that volcanic eruption can be a source for up 10 000 tons of Pb into nature.

During past years, industrial use of lead has increased enormously affecting its cycle on a global scale, as Pb is included in the composition of acid batteries, fusible alloys, synthetic fertilizers, bullets, fuels, and fusible alloys.

Time retention in the soil is stated up to 5000 years. Lead is not biodegradable, which makes it very difficult and expensive to senate from the environment due to its low movability. Factor affecting the uptake of Pb by soil connected with increased concertation level is cation exchange capacity, OC content, and increased pH level (Founa et al., 2013).

### 1.1.6 Copper effect on plants

Copper functions as an important essential micronutrient for plant development and its growth functioning as a catalyst during respiration and photosynthesis. Another very important role is informing complex organic polymer in the cell wall called lignin. Besides its positive effects, copper may originate chlorosis, leaf discolouration, inhibition of root growth, necrosis in higher concentrations, and become highly toxic for plants which flowingly exhibits the plant to oxidative stress due to overproduction of reactive oxygen species. This leads to disruption of the main processes associated with photosynthesis.

The accumulation of copper in soils has a predominantly anthropogenic origin due to mining or agro-industrial activities. Copper is used as a broad-spectrum bactericide and fungicide mainly in its inorganic form in viticulture and horticulture as it´s very effective and low cost.

As the copper is very persistent in soil and highly toxic to terrestrial and aquatic organisms, a regulatory process has been placed on copper compound among the candidates for substitution.

The use of products containing copper salts (e.g., pesticides applied in vineyards and orchards) has caused a high level of accumulation in the upper layer around 0-20 cm of agricultural land in countries such as Spain, Italy, Portugal, and France (Pietrini et al., 2019).

## 1.2 Constructed wetlands

Already our ancestors observed that water, after passing through the wetland area outflowed as purified and cleaner. That´s why wetlands have been known as a very beneficial way of treating wastewater for hundreds of years. Over the last two decades, we could witness a dramatic development of many constructed wetland types which allow us to observe the treatment of highly loaded wastewater or sludge from a wide range of municipal and industrial sources. Simultaneously, there has been a rise in public awareness of man's worldwide environmental effect, especially with global sustainability and the threat of changes in the climate. As a result, the perception and value of constructed wetland treatment systems have shifted from curiosity to a highly relevant water treatment solution for our time, as they provide no or low energy usage, easy operation, minimal carbon footprint, biodiversity, robustness and can be naturally implemented into the existing landscape as well as consistent and with impressive performance (Treatment Wetlands - Constructed Wetlands, 2022).

As a result of their affordable value, wetlands have a lot of promise for use in underdeveloped nations. Their capability can be used as an alternative to world water treatment plants (Almuktar et al., 2018).

A constructed wetland is simply an excavation filled with organic or inorganic particle medium and reeds. To confine polluted water and so preserve groundwater and nearby subsoil, they are usually coated with a rubber (butyl) or plastic (HDPE) substance. Constructed wetlands can also be sealed with clay-based materials, offering a more natural option. Effluent can be supplied into the system in batches or as a continuous flow, depending on the treatment needs. The effluent may travel horizontally across or vertically up or down through the medium. Microbial degradation, or processing, of pollutants is the primary method of treatment, and it is carried out by bacterial communities that form biofilms on the particle media's surface (Treatment Wetlands - Constructed Wetlands, 2022).

As already deducted, constructed wetlands have a high absorption capacity. As a result, the quality of the effluent is usually relatively consistent. On the other hand, low temperatures are likely to have negative consequences, such as inhibiting N-removal, peak flows (washing out particles), and clogging of subsurface-flow systems. Temperature, hydraulic residence time (HRT), and loading rate influence removal percentages, which are extremely variable amongst systems.

Depending on the design specifications, constructed wetlands can be used to successfully treat wastewater influent as a primary, secondary, or tertiary treatment stage, as shown in **Figure 3**. Constructed wetlands have been shown to be the most effective secondary and tertiary treatment processes for water recycling, and they are now commonly used for the treatment of sewage and industrial effluents (Rosseau, 2008).

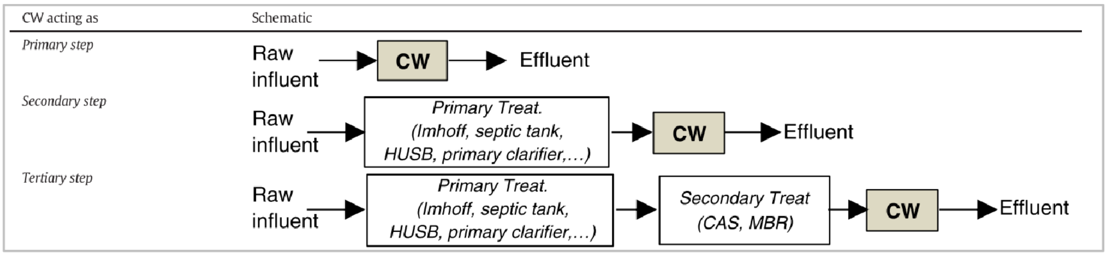

**Figure 3** – Constructed wetlands as primary, secondary, and tertiary treatment steps (Verlicchi and Zambello, 2014).

Water, a wetland plant species, a substrate of choice, and a natural microbial ecosystem are the main components of constructed wetlands. Based on the movement of water, constructed wetlands have traditionally been divided into two main categories: surface flow and subsurface flow. In contrary to subsurface flow, water flows above the surface in surface flow however in wetlands where water runs below the gravel and rock layer, reducing the human and ecological direct risk of exposure. Because subsurface flow wetlands have better rates of pollutant removal per unit of land than surface flow wetlands, they may be built smaller while still removing the same amount of pollutants (Halverson, Nancy, 2004).

According to Vymazal (2010), there are four forms of CWs for wastewater treatment: free water surface (FWS), horizontal subsurface flow (HSSF), vertical subsurface flow (VF), and combination systems (or so-called hybrid systems) that combine different types of CWs.

Free water surface constructed wetlands are a shallow sealed basin or succession of basins with 20–30 cm of rooting soil and a water depth of 20–40 cm is common along with emergent macrophytes. A considerable portion of the surface is covered by dense emergent vegetation, generally more than 50%. (Vymazal, 2010) FWS CWs have been commonly utilized in developing nations to reduce pollution from both point and non-point sources. (Guo, Cui, 2022) Aquatic macrophytes are better for wastewater treatment than terrestrial plants because they grow quicker, produce more biomass, and have higher pollution absorbing capacity (Kovrov, 2020).

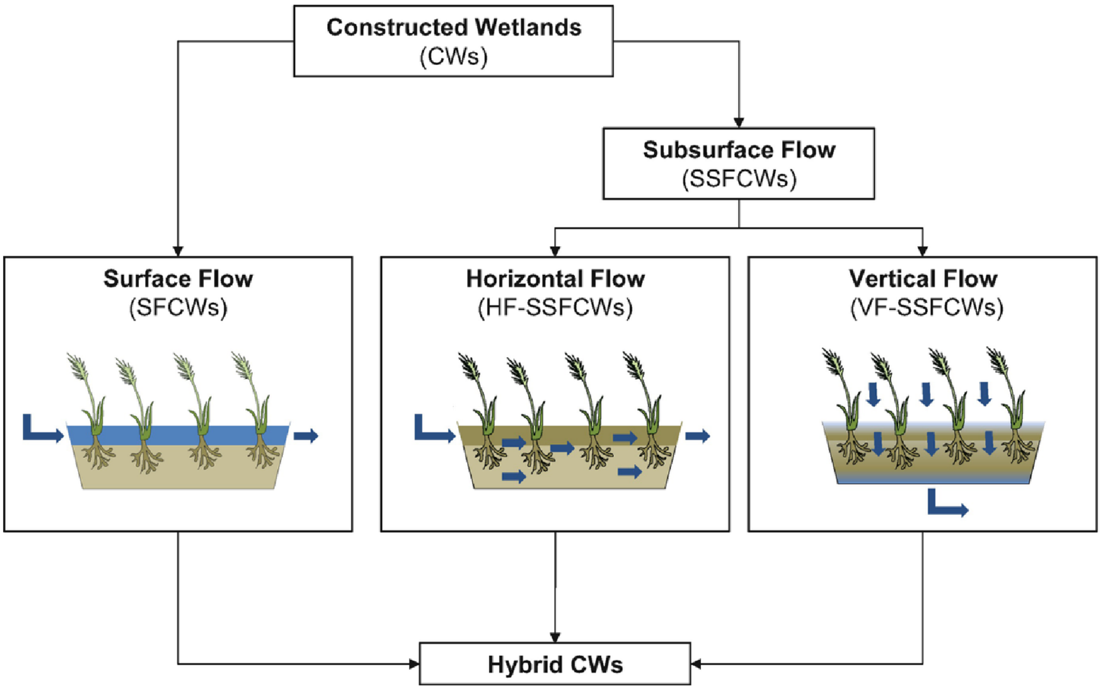

**Figure 4** – Scheme of most common CW configurations (Casas, Matamoros, 2020).

Constructed Wetlands with Horizontal Subsurface Flow dispose of with gravel or rock beds, are sealed by an insulating layer, and seeded with wetland plants. The wastewater enters from the intake and travels horizontally through the porous material beneath the bed's surface until it reaches the discharge section, where it is collected and released. Pollution is removed from the filter beds by microbial degradation, chemical, and mechanical processes in a network of aerobic, anoxic, and anaerobic zones, with aerobic zones limited to places near roots where oxygen escapes to the substrate. (Vymazal, 2010) A large number of research activity studies have found, as constructed wetlands operate, clogging of various degrees gradually appears in HFCWs along the direction of greywater due to physical, chemical, and biological mechanisms, which can seriously affect wetland treatment efficiency and shorten operational lifespan (Zhang et al., 2021).

Due to their inability to supply both aerobic and anaerobic conditions simultaneously, single-stage constructed wetlands cannot achieve effective total nitrogen removal. Vertical flow-constructed wetlands successfully remove ammonia-N, although denitrification is quite limiting in these systems. On the other hand, horizontal-flow constructed wetlands provide favorable conditions for denitrification, but their capacity to nitrify ammonia is reduced. As a result, several types of artificial wetlands may be mixed to take use of the distinct features of each system (Vymazal, 2007).

### 1.2.1 Enhanced CW approaches for HMs removal

#### Substrate selection

The substrate is an important design feature for CWs because it interacts with pollutants and offers an environment and medium for microorganisms and plants to carry out their typical metabolic activities (Yu et al., 2022). Finding an appropriate, cost-effective substrate that not only favors plant and microbe development but also effectively assists in pollution removal is a significant problem that may either boost or reduce the efficiency of the overall microenvironment. To determine CW effectiveness in removing pollutants, physical characteristics of substrates such as surface area, particle size, porosity, hydraulic retention time, electrical conductivity, and biological and chemical properties such as charge, toxicity, and chemical stability of electron donors and acceptors must be assessed (Yang et al., 2018). The substrate takes up nearly the whole volume of the CW construction, which is a key distinction between a manmade and natural wetland. Substrates not only offer physical and chemical support for wetland plants, surface areas and nutrients for microbial attachment, and hydraulic conditions for sewage flow, but they also directly remove pollutants through filtration, adsorption, and precipitation, among other beneficial functions (Wu et al., 2015).

### Organic enriched substrates (OES)

Fillers or partial fillers (combined with conventional substrates like gravel or other functional substrates in CWs) are widely used to improve heavy metal contaminated water treatment effectiveness by 6–34%. Peat and compost are common OES in CWs, as are organic wastes like cow manure and walnut shell. These substrates increase adsorption, complexation, plant uptake, and precipitation, altering physicochemical characteristics. Wetland fillers give organics that aid in the removal of heavy metals, particularly metalloids (Yu et al., 2022).

### Alkaline substrate

In the treatment of acidic heavy metal polluted water, alkaline substrates are usually employed to raise the pH and give anions for the CW column such as acidic mine drainage where pH values can be low. The most extensively utilized alkaline substrate is limestone (primarily composed of calcium carbonate), which has efficiently removed metals such as Fe, Mn, Cu, Zn, As, and B (recovery rates of 40–99 percent and 54.2-100 percent, respectively). Furthermore, certain OESs can also raise the pH or alkalinity of acidic water, such as bamboo chips (Yu et al., 2022).

### Ion exchange substrates

Ion exchange occurs in the water column between cations or functional groups of substrates and HMMs and is influenced by metal ion characteristics. Zeolite is a typical IES (with a cation exchange capacity (CEC) of 239.13 cmol $\cdot kg^{-1}$) that has been widely tested in CW for HMM elimination studies. According to studies, zeolite-based VFCW has a 92 percent removal efficiency for As and an 86% removal efficiency for Fe, compared to gravel, which only has a 43% for As and is unable to extract Fe (Yu et al., 2022). It was also discovered that CW made up of zeolite-dominated lava sand accumulates Fe, Mn, Zn, Ni, Cu, and Pb better than fluviatile sand (Huang et al., 2017). Above mentioned substrates are summarized in **Table 1.**

**Table 1** – Summary of substrates (organic enriched substrates, alkaline substrates, other substrates) used in CWs for enhancing the removal of HMMs.

| ***CW Type*** | ***Enhancement (vs. Control)***[a] | ***HMMS*** | $C_{in}^{b}$ ***(mg/L)*** | ***Remark*** |
|---|---|---|---|---|
| | OES | | | |
| HFCW | Compost | Zn | 2.3 | RE:67.5% |
| HFCW | Cow manure and bamboo chips | Fe, Co, Ni, Cr | 100, 1, 1, 1 | RE: 91.6%, 93.7%, 97.8%, 99.7% |
| VFCW | Compost vs. gravel | Fe, Cu, Zn | 8, 4.5, 4 | RE (Re increase value with the control experiment)[a]:48 (11)%, 56(15)%, 61(16)%, |
| VFCW | Cocopeat vs. gravel | Fe, B | 107 | RE:46.7(54.3)%, 9.4(6.3)% |
| VFCW | Peat vs. gravel | Ni, Zn | 1-5 | RE:99.3(32.8)%, 94.7(20.7)% |
| VFCW | Walnut shell vs. gravel | Zn, Cu, Cd, Cr | 5.0, 4.9, 5.3, 6.8 | RE:25.0(12.3)%, 68.5(34.1)%, 27.7(13.2)%, 93(17.6)% |
| UFCW | Peat vs. sand | B | 2,65-5.65 | RE: 91 (8)% |
| | ***Alkaline substances*** | | | |
| HFCW | Limestone | As, Fe, Pb, Zn | 2.14-3.72,<br>56.95-49.26,<br>0.9-0.88,<br>12.26-7.43, | RE:>96%,>96%,>94%,>40%,<br>Raises the pH from 2 to 7.1 |
| VFCW | Limestone vs. gravel | As, Fe | 3.1, 107 | RE:99(54.2)%, 98(100)%<br>Raises pH from 2.6 to 6.7 |
| VFCW | Shell grits | Cu, Fe, Pb, Zn | 4, 160, 1.57, 12.09 | RE:99.4%, 99.1%, 95.7%, 97.4%<br>Raises pH from 2.6 to 7.8 |
| | ***Other Substrates*** | | | |
| VFCW | Manganese ore vs. gravel | Mn | 0.16-2.24, 0.11-2.23 | RE: 95 (49) %, Manganese oxide surface could support manganese-oxidizing strains |
| VFCW | ZN-LDH modified zeolite and quartz sand vs. Natural substrate | Cr | 4 | RE: 17-33 (5-7)%, Increase the number of functional group and adsorption sites and promotes the growth of functional microbes |
| VFCW | Biocahr vs. sand | Cd, Zn, Cu | 5 | Contains abundant exchangeable groups and functional ions. |

Note: RE is removal efficiency, HFCW is horizontal subsurface flow CW, VFCW is vertical subsurface flow CW, UFCW is upflowed VFCW, LDH is layered double hydroxides, - [a] Valid description in brackets only if the control exists in the literature; otherwise, the RE of enhanced approaches are documented. The RE increase value is calculated by subtracting the RE of the control experiment from that of the enhanced experiment, same as the following text. - [b] Mean inflow concentration of HMMs (Yu, Wang, Chi, 2022).

### 1.2.2 Heavy metal removal in CWs

The following are the primary mechanisms for removing metal from industrial wastewater in constructed wetlands: (i) Filtration and sedimentation (ii) Precipitation (iii) Adsorption (iv) Uptake by the helophytes and microorganisms. In CWs, the major processes for removing heavy metals from wastewater are filtration and sedimentation. Sedimentation is a physical process that occurs after other mechanisms have gathered heavy metals into large enough particles to sink. Precipitation is affected by the metal's solubility product, the pH of the wastewater, the number of metal ions, and the presence of relevant anions. Precipitation occurs when the concentrations of cations and anions are sufficient that their product reaches a certain threshold. Biological removal, which includes plant and microbial absorption, is another key mechanism for heavy metal removal in the CWs. The pace at which plants remove metals varies greatly, depending on the plant's growth rate, species, and the percentage of heavy metals in the effluent (Choudhary et al., 2011). The HMs concentration in plant tissues may be used to assess the amount of plant absorption (Zhou et al., 2019).

A phytoremediation is a potential option for removing dioxane from polluted soils, and other hydrophilic pollutants are explored as well. Plants in built wetlands absorb industrial heavy pollutants. There is a study where Water Hyacinth in constructed wetlands was able to remove up to 95% of bioavailable mercury emitted inside the wetland system during a three-day period. The initial mercury content, chloride concentration, and pH value all impacted mercury bioavailability. Mercury has a natural propensity to accumulate in plant roots. Just for study comparison, the average mercury level in the roots of Water Hyacinths was 3.5 times higher than that of Reeds when the initial mercury concentration in solution was 50 ppb. The Water Hyacinth roots acquire 110.55 mg/g after three hours, but Reed's roots accumulate just 28.9 mg/g (Qasaimeh et al., 2015).

CWs are also known for their outstanding pathogen removal capabilities which consist of (i) Physical factors (aggregation, filtration, sedimentation, UV exposure) (ii) Chemical factor (adsorption, oxidative damage) (iii) Biological mechanisms (natural deaths, ingestion by nematodes and protozoans) (Choudhary, Kumar, Sharma, 2011).

### 1.2.3 Techniques and strategies of phytoremediation

In recent years, scientists and engineers have begun to develop cost-effective ways for cleaning contaminated regions that incorporate the utilization of microorganisms/biomass or living plants. Phytoremediation is a new approach for cleaning up polluted places that is cost-effective, has aesthetic benefits, and may be used for a long time. Phytotransformation, phytostabilization, phytoextraction, and rhizofiltration are the best applications for sites with shallow contamination of organic, nutrient, or metal pollutants that are open and receptive to one of the five applications. (Jadia, Fulekar, 2009) Plant species must fulfill the following characteristics in order to be acceptable for phytoextraction: (i) metal tolerance to hazardous quantities of elements, (ii) rapid biomass output, and (iii) efficient HM accumulation in easily harvestable areas (Suman et al., 2018).

#### Phytoextraction

Phytoextraction is also known as phytoaccumulation or phytosequestration and is the uptake of contaminants from soil or water by plant roots, their translocation to and accumulation in aboveground biomass. Roots and shoots are subsequently collected to remove the pollutants from the soil as shown in **Figure 5** (Jadia, Fulekar, 2009).

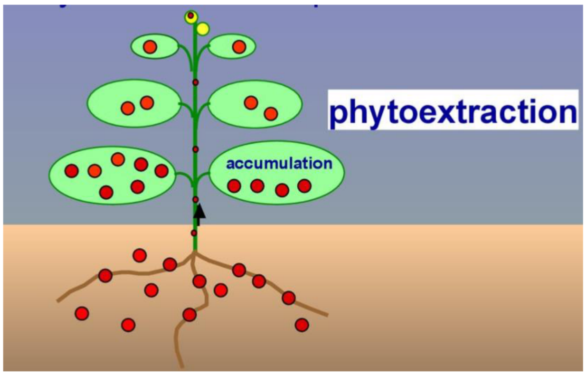

**Figure 5** – Shows the phytoextraction process- accumulation and translocation of HMs. (Kovrov, 2020).

#### Phytostabilization

Phytostabilization, also known as phytoimmobilization, is the process of using specific plants to stabilize pollutants in polluted soil. It is used to minimize the mobility and bioavailability of contaminants in the environment, preventing them from migrating into groundwater and entering the food chain. The primary goals of the plant are to (i) reduce the amount of water percolating through the soil matrix, which could lead to the formation of hazardous leachate; (ii) act as a barrier to prevent direct contact with contaminated soil; and (iii) prevent soil erosion and the spread of toxic metal to other areas. Plants can immobilize heavy metals in soil by root sorption, precipitation, and rhizosphere complexation of metal valence reduction (Jadia, Fulekar 2009).

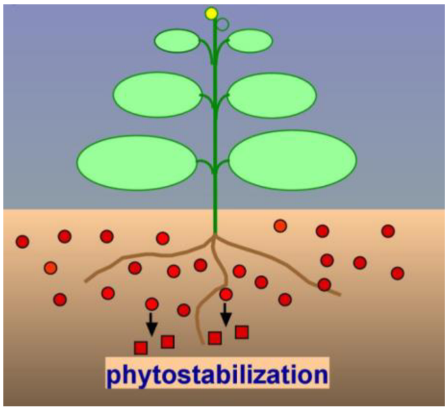

**Figure 6** – Shows the phytostabilization process- lowering mobility of contaminants (Kovrov, 2020.).

### Rhizofiltration

Rhízofiltration is a process of removing pollutants from water by plant roots in a hydroponic system. Pb, Cd, Cu, Ni, Zn, and Cr, which are largely retained inside the roots, can be filtered via rhizofiltration. Contaminants do not have to be transported to the shoots, which is a benefit. As a result, species that aren't hyperaccumulators can be utilized. Terrestrial plants are preferred because their root systems are fibrous and considerably longer, providing a higher root surface. Karkhanis (et al., 2006) used pistia, duckweed, and water hyacinth *(Eichornia crassipes)* in a greenhouse experiment on rhizofilteration to remediate an aquatic habitat damaged by coal ash containing heavy metals. Coal ash is rhizofiltered at a rate of 0, 5, 10, 20, 30, and 40%. Simultaneously, physicochemical properties of leachate were examined and researched in order to better understand leachability. The findings revealed that pistia has a high potential capacity for heavy metal absorption (Zn, Cr, and Cu), and that duckweed, has also a high potential for heavy metal uptake. In comparison to pistia and duckweed, water hyacinth had decreased rhizofiltration of Zn and Cu (Kharkanis et al., 2006).

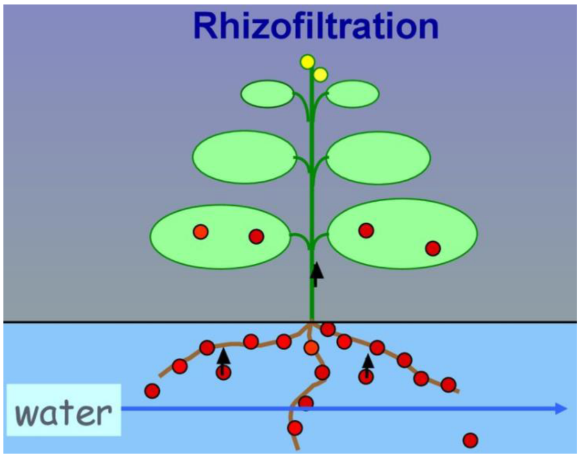

**Figure 7** – Shows the rhizofiltration process- removing pollutants by plant roots (Kovrov, 2020).

**Phytovolatilization**

Phytovolatilization is the process of using plants to absorb pollutants from the soil, convert them to volatile forms, and exhale them into the atmosphere. The principal metal contamination for which this technique has been utilized is mercury. This approach has the benefit of transforming the pollutant, mercuric ion, into a less harmful compound (elemental Hg). The negative is that mercury released into the atmosphere is likely to be recycled by precipitation and subsequently redeposited in lakes and seas, thereby repeating the anaerobic bacteria's creation of methyl-mercury.

## 1.3 Nitrogen interaction with heavy metals in plants

Nitrogen is required in considerable amounts as a component of cellular constituents, nucleic acids, and proteins and for plant metabolism. Its absence is one of the most prevalent constraints for plant growth. Plants require a lot of nitrogen since it makes up 1.5-2.0% of their total dry mass (Hussain et al., 2020). The most prevalent and readily accessible forms in soil are nitrate ($NO_3^-$) and ammonium ($NH_4^+$). Plants better metabolize nitrate as a consequence of bacterial nitrification. However, due to the suppression of these organisms, the ammoniacal form may be the most prevalent depending on soil conditions. It is well known that different sources of nitrogen can have varying effects on plant metabolism once they reach the leaves. The build-up of $NH_4^+$ can reduce photosynthesis, but an excess of $NO_3^-$ can cause the development of reactive species, resulting in oxidative stress (Nascimento et al., 2021). Nitrogen together with phosphorus is the most important macrobiogenous element. Natural sources of this element are mainly sewage water, wastewater from agriculture, decomposition of biomass of dead organisms. Anthropological occurrence is caused by runoff from agriculture areas fertilized by inorganic fertilizers, atmospheric water, and wastewater from coal processing.

Uptake of Nitrogen followed by reduction and assimilation in plants is the only way plants may convert inorganic N into organic form. Organic (urea, amino acids, etc.) and inorganic ($NH_4^+$, $NO_3^-$, dinitrogen) chemicals, as well as the plant's surroundings, influence the accessible N forms (Hussain et al., 2020).

### 1.3.1 Metal action on the plant N uptake

Heavy metal stress is considered to produce a number of changes, both direct and indirect, during the active and passive uptake of both inorganic forms of N. Physiological research imply that metal toxicity disrupts the constitutive and inductive components of the nitrate transportation system, affecting $NO_3^-$ and $NH_4^+$ absorption (Globus et al., 2002). The impact was demonstrated in cucumber seedlings where metal toxicity with Cd, Pb, Cu, and Ni blocked the $NO_3^-$ transporters with higher affinities. A similar impact was seen when high-affinity ammonium transporters were inhibited. The direct interaction of metal ions with both low and high affinities $NH_4^+$ and $NO_3^-$ transporter proteins could explain the restricted N absorption. Furthermore, heavy metals such as Cu, Cd, and Ni have been shown to speed up the formation of free radicals, damaging various important organic compounds. Some essential metal ions, such as Cu, Pb, Hg, Cd, Ni, and Zn, are thought to interact with membrane components, influencing the net ion transport system in the plant body, as one of the indirect impacts of heavy metal stress on ion uptake in plants. This membrane interaction comprises changed membrane lipids, total lipid amount, composition, and saturation; the main harm is caused by lipid peroxidation in this process. Heavy metals, particularly Cu, Hg, Cd, Zn, and Al, cause potassium leakage, enhancing cell membrane permeability. As a result, the indirect effect of heavy metal stress on $NO_3^-$ and $NH_4^+$ absorption is as mentioned, a change in membrane permeability (Hussain et al., 2020).

## 1.4 Objectives

Plants are a crucial aspect of the Earth's "organism". They are an essential aspect of our planet's life cycle. They create oxygen, provide a vital source of livelihood and energy, and they also help to complete our environment. They can also contribute significantly to environmental clean-up in addition to these benefits. Of course, it isn't that easy. It's crucial to figure out which plants are best for a specific sort of pollution, as well as their ability to gather, stabilize, evaporate, and, in some cases, decompose some of the world's most dangerous toxins.

As previously stated, constructed wetlands provide a natural, sustainable, and low-cost option for eliminating persistent toxins released into the environment by humans. The utilization of plants in constructed wetlands is a very broad and important use for the proper and subsequent handling of such plants. However, the multiple processes and factors underlying the elimination of heavy metals and nitrogen source's effect on phytoremediation in built wetlands are understudied, and knowledge is very minor about possible nitrogen sources and their effect on the distribution of heavy metal in plants and soil.

# 2 Methods and Materials

On the premises of the Czech University of Life sciences in Prague, small-scale laboratory sub-surface vertical built wetlands were created. In total, six constructed wetland systems (CWs) were established in this study. The CW systems were carried out in PVC-U materials columns with a dimension of 150 ×550 mm (diameter × height). The details are shown in **Figure 8**. The selected plant is *Iris Wilsonii*, a blooming plant that is common throughout China and belongs to the Iridaceae family. Long and drooping grey-green leaves, hollow stems, and two fragrant yellow, pale yellow, or yellow/white flowers characterize this rhizomatous herbaceous perennial. The experiment has two heavy metal treatments (with or without heavy metals) and three nitrogen treatments where we added different nitrogen sources, including urea, $NH_4Cl$, and $NaNO_3$. Each system will feed with synthetic wastewater every 5 days in the whole experimental period (**Table 2**). The experimental setup's initial phase lasted 3 months, and it was aimed to see if plants could successfully adjust to their new environment. Plants were fed a low-concentrated nutrient solution intermittently during a four-day cycle during this time. After CWs were stable, from June to August, heavy metals (Cu, Zn, and Pb) were mixed with wastewater and added into each system from September to November. The added concentration of heavy metals was 15 mg/L of Cu, 20 mg/L of Zn, mg/L of 5 mg/L Pb.

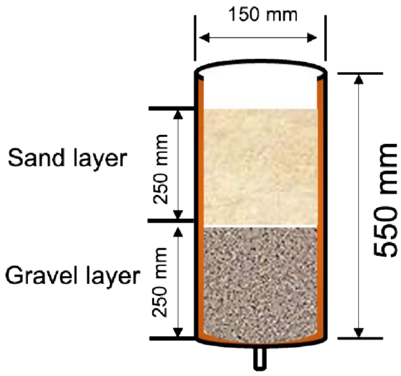

**Figure 8** – The composition of the constructed wetland system

**Table 2** – The characteristics of simulated wastewater with concentration (mg/L) of nutrients.

| **Reagents** | **mg/L** | **Reagents** | **mg/L** |
|---|---|---|---|
| *Same composition in CWs* | | | |
| ***Urea*** | ***104*** | $CuSO_4\cdot 5H_2O$ | ***0.01*** |
| $NH_4Cl$ | ***16*** | $FeSO_4\cdot 7H_2O$ | ***0.45*** |
| $CH_3COONa\cdot 3H_2O$ | ***255*** | $MnSO4\cdot H_2O$ | ***0.02*** |
| $KH_2PO_4$ | ***41*** | $Pb(NO_3)_2$ | ***0.02*** |
| $NaHCO_3$ | ***25*** | $H_3BO_3$ | ***0.04*** |
| $MgSO_4\cdot 7H_2O$ | ***41*** | $Na_2MoO_4\cdot 2H_2O$ | ***0.02*** |
| $CaCl_2\cdot 6H_2O$ | ***28*** | $KCr(SO_4)_2\cdot 12H_2O$ | ***0.02*** |
| *Different nitrogen sources for controls* | | | |
| ***Urea treatments*** | | ***Urea*** | ***205.71*** |
| $NaNO_3$ ***treatments*** | | $NaNO_3$ | ***364.29*** |
| $NH_4Cl$ ***treatments*** | | $NH_4Cl$ | ***229.29*** |

Sampling proceeded every 5 days by the following process. The effluent was taken from the bottom of the plastic pots, and the amount was measured in 0,5 L plastic containers. 25ml of the water was taken into testing tubes for subsequent analysis in the laboratory. The water volume of each sample was recorded and then discarded. Reactors of sub-surface constructed wetlands setup are shown in figure n.

s

**Figure 9** – The sub-surface constructed wetland setup simulation in the Czech University of Life Sciences, Prague

Following the further analysis of water samples in the laboratory started with measurement of pH and conductivity. All samples were analyzed directly from the bottle of the collected sample with special digital analysis equipment separately proposed for measurements of desired parameters. Measurements were taken using the multi-parameter device Multi 3620 IDS SET C for field measurements, with two-channel input. Set with IDS electrodes: digital pH electrode SenTix® 940, digital conductivity cell TetraCon® 925 displayed in **Figure 10**.

After inserting a probe into water, the stabilization procedure initializes as shown below in figure n. Subsequently, when stable parameters were obtained, results were recorded, for conductivity data were measured in µS/m. Following each measurement probes were rinsed using deionized water.

**Figure 10** – Multi-parameter device Multi 3620 IDS SET C / Determination of pH and conductivity of water samples.

An indophenol method was used to detect ammonia ions using an Agilent Technologies Cary 60 UV-Vis spectrophotometer. An alkaline and a coloring agent solution had to be created in order to quantify each sample. 16 g sodium hydroxide (NaOH) was dissolved in 250 mL deionized water to prepare the alkaline solution, and 1 g sodium dichloroisocyanurate dihydrate ($C_3N_3O_3CL_2Na2H_2O$) was added after a period of incubation until the solution reached room temperature. The solution was kept in a dark container and maintained in the refrigerator once it was dissolved. To make the dyeing solution, 250 ml deionized water was mixed with 32.5 g sodium salicylate ($C_7H_5O_3Na$) and 32.5 g sodium citrate dihydrate ($Na_3C_6H_5O_7$ $2H_2O$). After the dissolution, sodium nitroprusside dihydrate ($Na_2[Fe(CN)_5NO)).2H_2O$ - 0.238 g was added to the solution and waited till dissolved. The coloring agent, the same as the alkaline solution, was placed in a dark container and kept in the refrigerator. Each sample (700 µl) was pipetted into a reaction tube with an alkaline solution and a coloring agent and left to stand for 60 minutes after the reagent solutions were prepared. Likewise, the same amount of influent was applied. Each sample was measured using a 1 cm cuvette using the spectrophotometer at a predetermined wavelength of 655 nm.

The automatic SKALAR Formacs TOC/TN analyzer provided a convenient way to measure total organic carbon (TOC), inorganic carbon (IC), and nitrogen (TN). Following placement into a high-temperature reactor (750-950 °C), each sample is placed on a spinning carousel that acts as an autosampler. $PO_4^{3-}$ -P, $NO_3^-$ -N, and $NO_2$

-N were analyzed by 883 Basic IC plus (Metrohm, Switzerland). $NH_4^+$-N was determined by the standard method.

All plants (with intact root systems) were carefully removed from CW systems at the end of the experiment, rinsed with deionized water, and then split into shoots and roots segments. The root length and shoot height of the fresh plants were measured directly after the harvest. Meanwhile, the analysis was carried out using a fresh sample of plant tissues. To prepare dry samples, plants were put in a 40 °C oven for 120 hours. The PrimacsSN analyzer was used to measure the proportion of total carbon (TC) and nitrogen (TN) in dry plant samples. Determination of the amount of heavy metals concentration was obtained from CECs measurement. Mass removal efficiencies of pollutants were calculated based on the effective mass balance of pollutants in the influent (1500 ml) and effluent by the equation.

$$Mass\ removal\ efficiency\ (\%) = \frac{V_{in}\ x\ C_{in} -\ V_{out}\ x\ C_{out}}{V_{in}\ x\ C_{in}} x100$$

# 3 Results and discussion

## 3.1 Plant growth

The results of our experiment, which lasted several months and began in late August and ended in November, were collated and analyzed using the measuring procedures specified in the previous, methodology section. The dry biomass of planted *Iris Wilsonii*, which includes the weight (g) of shoots and roots is represented in Graph 1 and Graph 2 may have major implications for nutrient intake, pollutant removal, and microbial processes within the soil. In comparison, the use of arbuscular mycorrhizal fungi (AMF) has a visibly positive effect that promotes plant growth and total plant biomass while using different Nitrogen sources (Urea, $NH_4^+$-N, $NO_3$-N). The majority of land plants have facultative symbionts, meaning they benefit from AM fungus but can also survive without them, although at a lower fitness level. Mycorrhizal plants are assumed to have a selection advantage over non-mycorrhizal individuals of the same species in most natural environments, which are characterized by mineral nutrient deficiencies and diverse abiotic stress conditions. As a result, AM has the potential to promote intraspecific competition and favor mycorrhizal plants (Chen et al., 2018).

Plants' physiological and biochemical functions are adversely affected by HMs, with the most noticeable consequences being growth inhibition, chlorosis, necrosis, leaf rolling, altered stomatal action, and lowered water potential. The obtained results in Figure 11 confirm that heavy metals in plants reduce their growth and biomass compared to those without heavy metal treatment. The average weight of dry biomass for plants treated for the model without AMF the average weight of dry biomass without heavy metals is 12,2g and with heavy metals is 11,38g.

Results show, that Nitrate Nitrogen ($NO_3$-N) was observed as more favorable for the growth of *Iris Wilsonii* which enhanced the biomass level in Non-MHs reactors. In the study of *Botryococcus sp., the poor growth rates of ammonium (NH4+) N sources were recorded, likely attributable to* ammonia ($NH_3$) in the culture media in the form of ammonium ions. It is well known that $NH_3$ exists in the $NH_4^+$ form under acidic circumstances (< pH 7) and that algal cells cannot directly assimilate ammonium. As the pH of the media rises during culture, it's more likely that $NH_3$ may volatilize, preventing alga cells from developing. Urea, which is quite cost-effective in comparison to other nitrogen sources, may be converted to NH4 + and bicarbonate by microalgae and then easily assimilated. Botryococcus sp., a wastewater-born green alga, also developed effectively in the presence of urea (Ndayambaje et al., 2019).

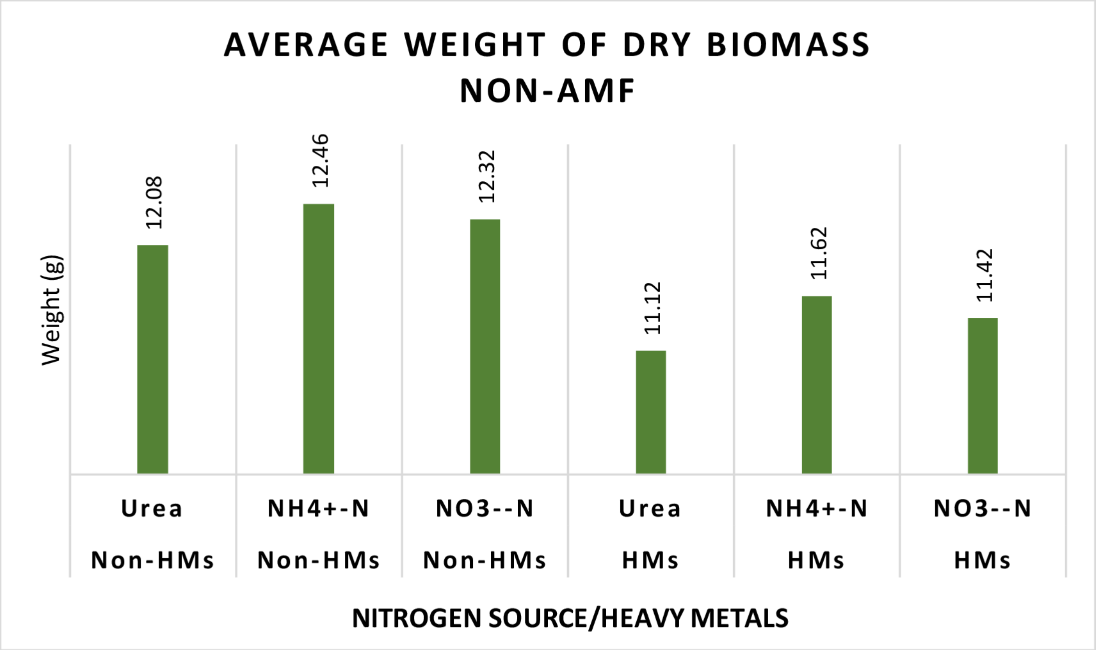

**Figure 11** – Average weight of dry biomass (g) without the use of arbuscular mycorrhizal fungi.

## 3.2 Nitrogen removal

Nitrate ($NO_3^-$) is an important nitrogen source for plant growth and metabolism. It is the most widely used nitrogen fertilizer in agriculture along with ammonium ($NH_4^+$) and urea ($CO(NH_2)_2$) (Noguero, Lacombe, 2016). Several studies confirmed that nitrogen fertilization can increase plant yields and N uptake compared with no N fertilization. Although adsorption, plant absorption, and assimilation all play a role in nitrogen transformation, ammonification, nitrification, and denitrification are the most common mechanisms for nitrogen transformation and removal. Both organic and inorganic nitrogen can be found in wastewater. Amino acids, urea, uric acids, and purine are all examples of organic nitrogen. Ammonium ($NH_4^+$), nitrite ($NO_2^-$), nitrate ($NO_3^-$), nitrous oxide ($N_2O$), and dissolved elemental nitrogen or nitrogen gas are the inorganic forms of nitrogen ($N_2$). Nitrogen gas ($N_2$), nitrous oxide ($N_2O$), nitric oxide ($NO_2$), and free ammonia ($NH_3$) are all examples of gaseous nitrogen. Only ammonia volatilization, denitrification, plant absorption (with biomass harvesting), ammonia adsorption, ANAMOX, and organic nitrogen burial are mechanisms that finally remove nitrogen from wastewaters. Other processes (such as ammonification or nitrification) transfer nitrogen between different nitrogen forms and do not remove nitrogen from wastewater (Vymazal, 2007).

Water eutrophication is mostly caused by nitrogen pollution. Urine currently accounts for 80 percent of the nitrogen and 10% of the COD load in municipal sewage. Several treatments were developed to meet the nitrogen and COD discharge standards, including chemical treatment and extended hydraulic stay duration, both of which significantly increase treatment time, floor area, and energy consumption. On the other hand, Urea is an abandoned hydrogen source that has been overlooked. Since the oxidation reaction of urea requires strong alkaline conditions, it is difficult to convert urea to N2 while simultaneously producing H2 in a neutral solution, but due to the large specific surface area, copper foam (CF) produces H2. It is considered a suitable cathode substrate with low loss, excellent conductivity and stability, and excellent rapid removal effect of nitrates and nitrites in solution as a copper-based catalyst. In response to these findings, a self-driving nano photoelectrocatalytic (PEC) device was designed to efficiently create H2 and remove TN for urine treatment at neutral pH (Wang et al., 2021).

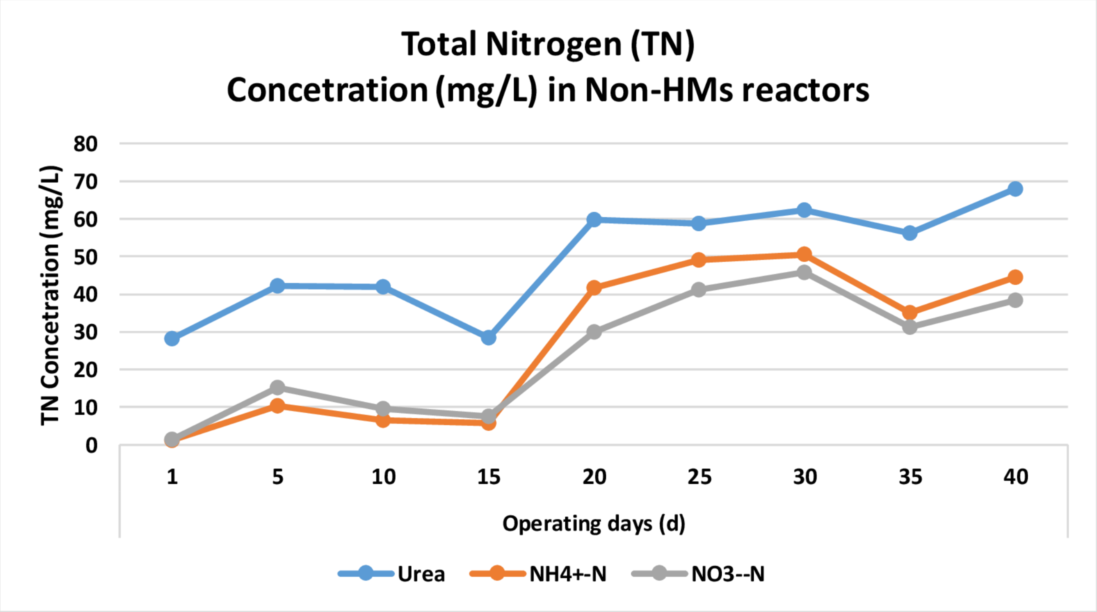

**Figure 12** – Total Nitrogen concertation in Non-HMs reactors

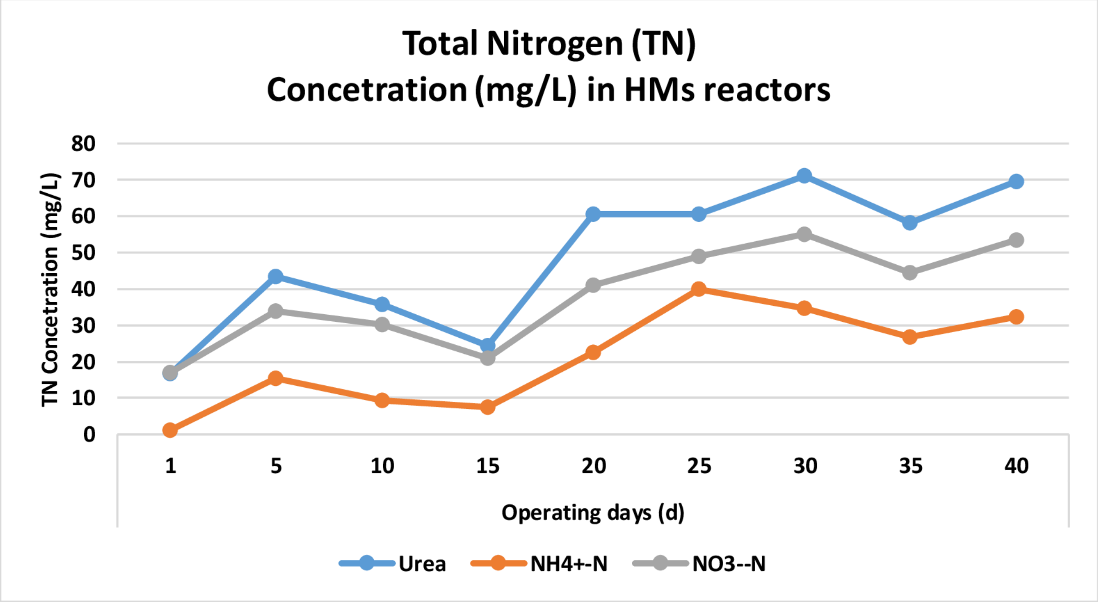

**Figure 13** – Total Nitrogen concertation in HMs reactors

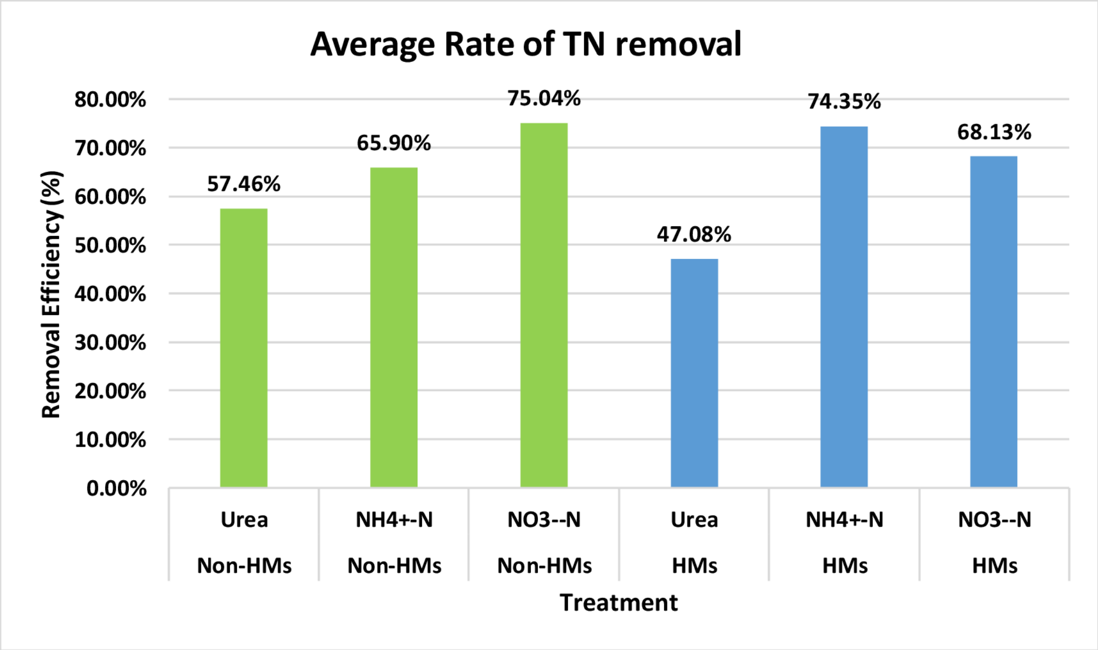

**Figure 14** – Average rate of removal for total nitrogen (TN) in HMs and Non-HMs reactors.

The highest removal rate (75%) was observed in the Non-HMs reactor with $NO_3^-$-N treatment. Traditional biological nitrogen removal procedures in WWTPs include nitrification and denitrification. The removal rates of TN from $(NH_2)_2CO$ were found to be lower, with 57.46% and 47.08% respectively. Ammonium, nitrite, organic nitrogen, and ammonia are just a few of the components that can contribute to total nitrogen. Algae have been found to be capable of assimilating a variety of inorganic nitrogen compounds (nitrate, nitrite, and ammonia). The fact that the lower removal efficiency of TN was eliminated in this research for urea $(NH_2)_2CO$ indicates that there were probably still some organic compounds in the culture fluid that could not be converted to $NO_3$-N or assimilated (Ndayambaje et al., 2019).

Our result can be based on process, where Ammonia ($NH_4^+$ -N) can be oxidized to nitrite ($NO_2$ -N) in the first stage by ammonia-oxidizing bacteria (AOB), and then to nitrate ($NO_3$ -N) in the second step by nitrite-oxidizing bacteria (NOB). Finally, denitrification bacteria use organic carbon as an electron source to convert $NO_3$-N to nitrogen gas ($N_2$). However, this process is in need of a carbon supply and significant oxygen use, especially in order to achieve stricter criteria (Peng, Fang, Du, 2022). From HMs reactors´ $NH_4^+$-N treatment has the highest removal rate (74%) of TN. The influence of heavy metals generally decreased TN removal, the only exception is in the reactor of $NH_4^+$-N treatment.

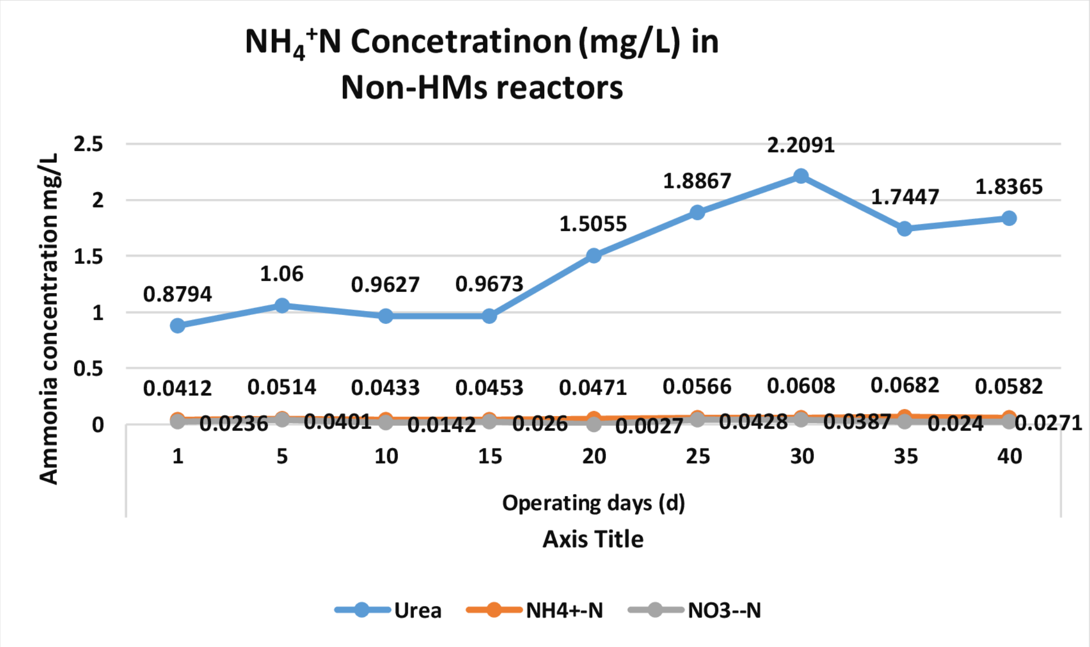

**Figure 15** – Ammonia Concentration (mg/L) in Non-HMS reactors.

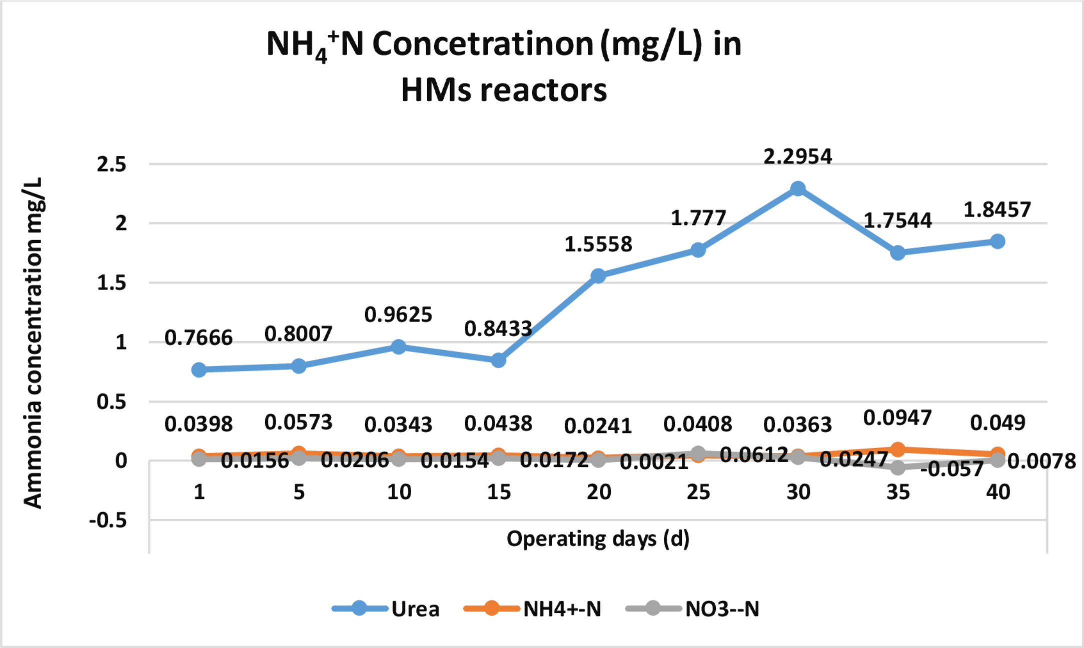

**Figure 16** – Ammonia Concentration (mg/L) in HMS reactors.

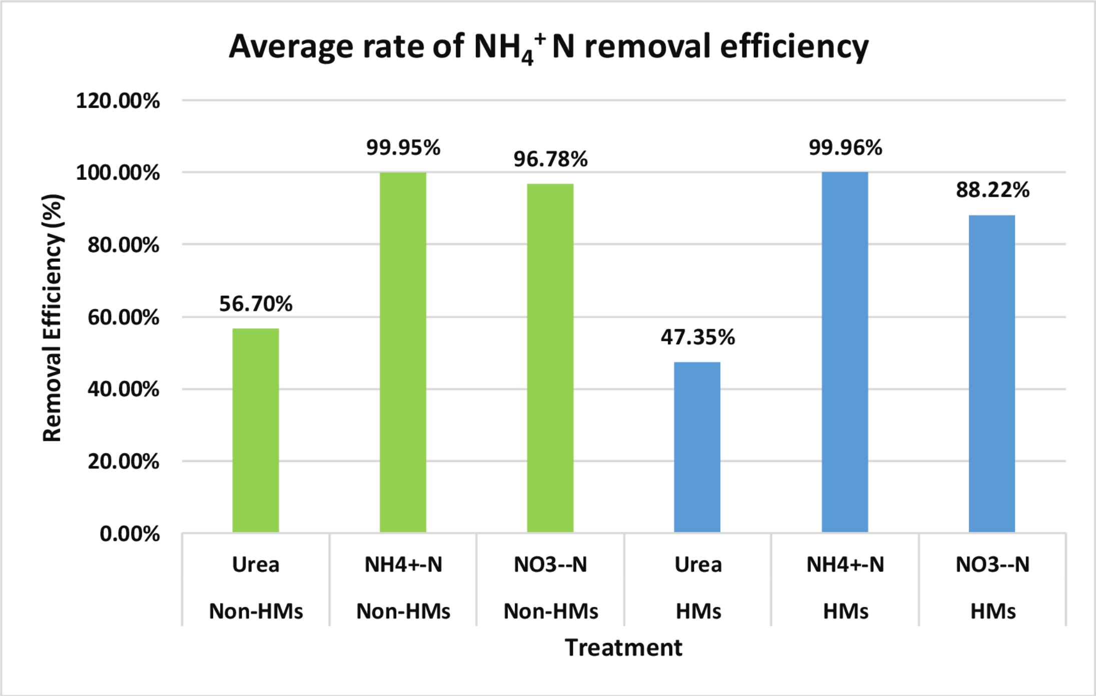

**Figure 17** – Average rate of removal for ammonia in Non-HMs and HMs reactors.

Application of NH$_4^-$ based N fertilizers can increase soil acidity due to the release of H ions during hydrolysis. The results of our experiment show, that concertation of ammonia (NH$_4^+$N) as can be seen in **Figure 14** (Non-HMs reactors) and **Figure 15** (HMs reactors) differ depending on the type of treatment employed. Removal efficiency (**Figure 16**) ranges from 56.70 – 99.95% in Non-HMs reactors and 47.35 – 99.96% respectively. Treatment with ammonia (NH$_4^+$N) and nitrate-nitrogen (NO$_3$-N) are proved to be more beneficial for ammonia removal in comparison to urea where removal efficiency ranges at 47 – 56.7%. In both most effective treatment types of reactors, their success may be caused by faster metabolism of nitrifying bacteria which convert $NH_4^+N$ to nitrite ($NO_2$-N) and then to nitrate $NO_3$-N (Ndayambaje, 2019).

Another study shows, the chemical species of available N, as well as the pH of the growing media, drove differences in *M. aeruginosa* growth rates. pH did not affect nitrate growth rates. On ammonium, the fastest growth rate was reported at a pH of 8.2, but this growth rate did not persist at higher or lower pH values. With an 8.2 percent growth rate, urea was the third-best option (Krausfeldt et al., 2019)

Increases in nitrate concentrations can be seen as a result of nitrification, as shown in **Figure 17** and **Figure 18**. The Nitrate nitrogen ($NO_3^-$—N) mean concertation in the effluent of Non-HMS treatment by urea, ammonia, and nitrite treatment was 171.83 mg/L, 122.46 mg/L, and 104.07 respectively and in reactors with HMs 185.64 mg/L, 106 mg/L, and 137.67 mg/L respectively. These concentrations were higher than ones in the influents in the case of urea and ammonia treatment (27.43 mg/L, 29.48 mg/L).

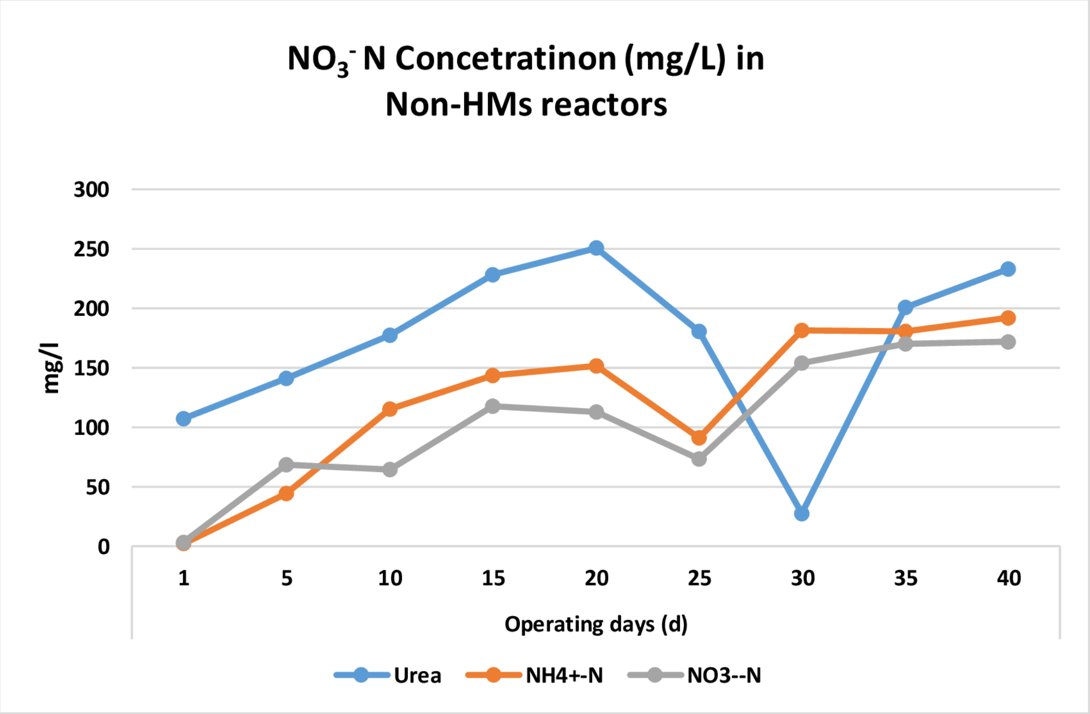

**Figure 18** – Nitrate Concentration (mg/L) in Non-HMS reactors.

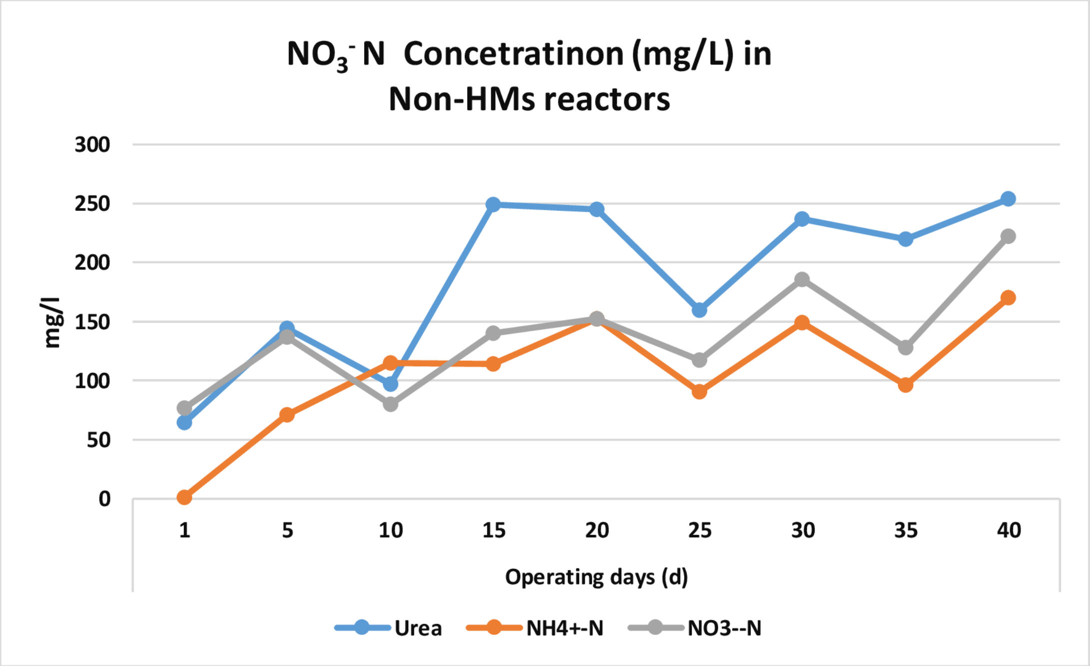

**Figure 19** – Nitrate Concentration (mg/L) in HMS reactors.

Total Carbon Removal (TOC)

To raise plant biomass output and the quantity of residue returned to the soil, nitrogen fertilizer can boost soil organic C. In many temperate and boreal habitats, the availability of soil inorganic nitrogen (N) is a crucial controller of plant productivity and carbon (C) sequestration. Microbial development on the media surfaces removes soluble organic molecules, which are then connected to the roots and rhizomes of plants. Organic matter includes around 45 to 50 percent carbon (C), which is used as a source of energy by a variety of microorganisms. Helophytes in the root zone provide oxygen for this process, which converts organic carbon to carbon dioxide (Choudhary, Kumar, Sharma, 2011). Some nitrogen sources, like urea, produce both $CO_2$ and $N_2O$. Through $NH_3$ volatilization and $NO_3$-N leaching, nitrogen fertilizers also release $N_2O$ indirectly. Our observations of different concentrations of total carbon (TC) are represented in **Figure 19** and **Figure 20**. The highest concertation values of TC were recorded under $NO_3^-$N treatment in both Non-HMs (129.4mg/L) and HMs (117.5mg/L) reactors.

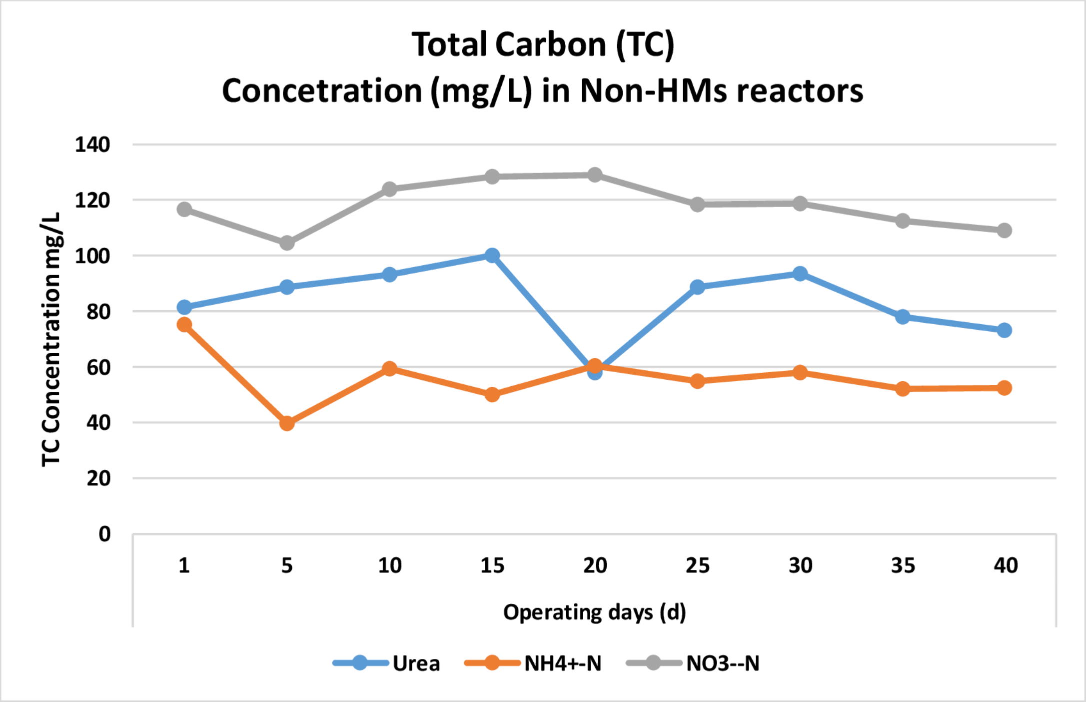

**Figure 20** – Total Carbon Concentration (mg/L) in Non-HMS reactors.

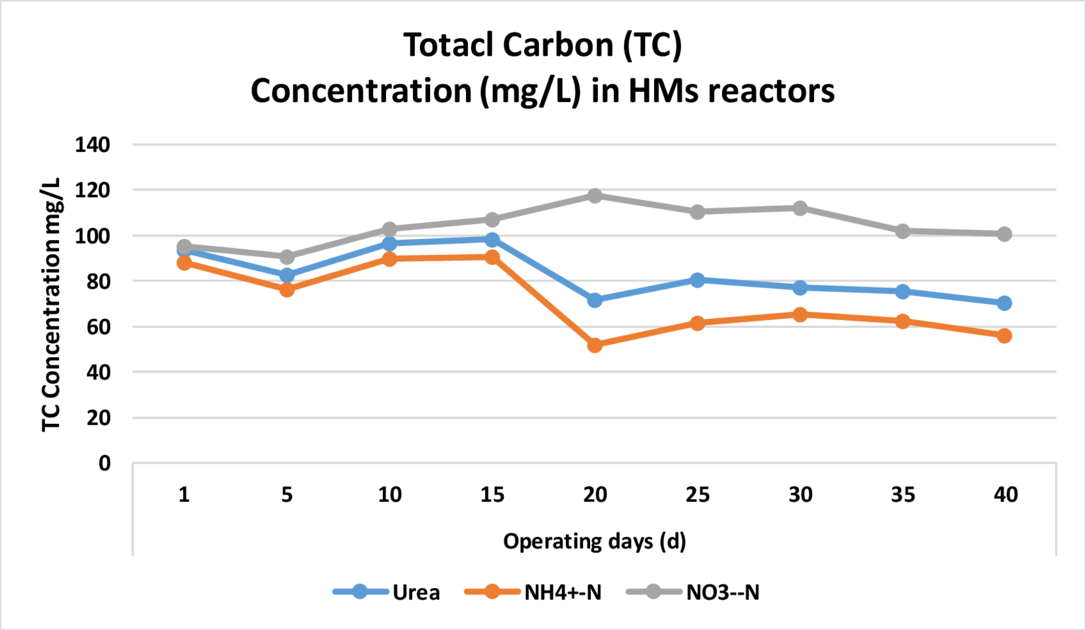

**Figure 21** – Total Carbon Concentration (mg/L) in HMS reactors.

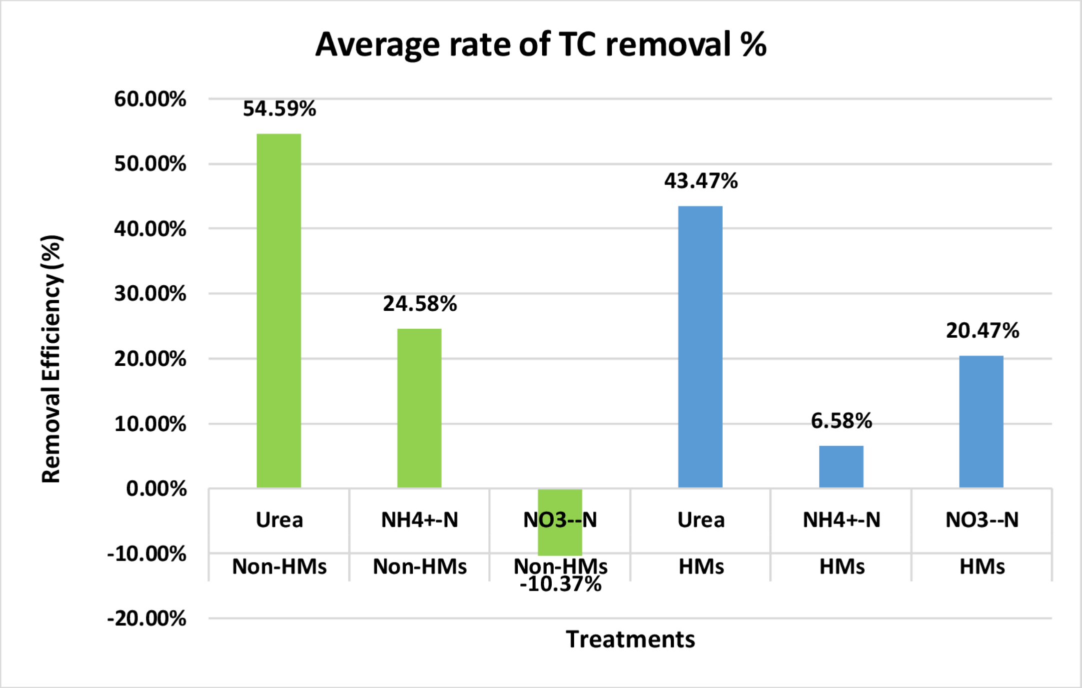

**Figure 22** – Average rate of removal for Total Carbon in Non-HMs and HMs reactors.

Removal efficiency illustrated by **Figure 21** was found to be 43.47 – 54.59% for Urea, 6,58 - 24.58% for Ammonia, and -10.37 - 20.47% for $NO_3^-N$ processing. The highest removal rate was attributed to Urea. This high removal efficiency score in comparison to other types of nitrogen treatments can be based on the possibility of urea producing $CO_2$ and $N_2O$, so the TC concertation, by the use of this nitrogen source along with soil respiration, decreases in the water. Microorganisms in the rhizosphere are primarily responsible for carbon transformation and removal in the soil, as evidenced by the fact that unplanted CWs had greater carbon removal efficiency than planted mesocosms (Baptista, 2003).

Heavy metals do have an impact on the removal efficiency of the TC values. $NO_3^-$ N treatment in Non-HMs reactor has negative removal value (-10.37%) where on the contrary HMs reactor has a positive (20.47%) the result was probably influenced by the higher volume of effluent in the firstly mentioned reactor. Respiration activity is a component of the mineralization of organic matter in the soil, as well as other metabolic activities that release $CO_2$. In the research was deducted, that the amount of heavy metals in the soil did not correlate with baseline respiration activity even though possibly can appear lowering activity of soil respiratory rate. However, many studies are not unified in conclusion (Friedlová, 2010).

Values for total organic carbon (TOC) are found in **Figure 22** and **Figure 23**. All treatments in both types of reactors are shown to be effective for TOC removal with its rate ranging from 72.10– 90.05% as shown in **Figure 24**. Urea was found to have the highest removal efficiency with 90.05% in Non-HMs reactor and 87.89% in HMs reactor and considered to be most suitable for the TC and TOC removal in contrary to N removal were figured as least effective. Heavy metals have shown to have a very slight influence on the decrease of removal rates of carbon.

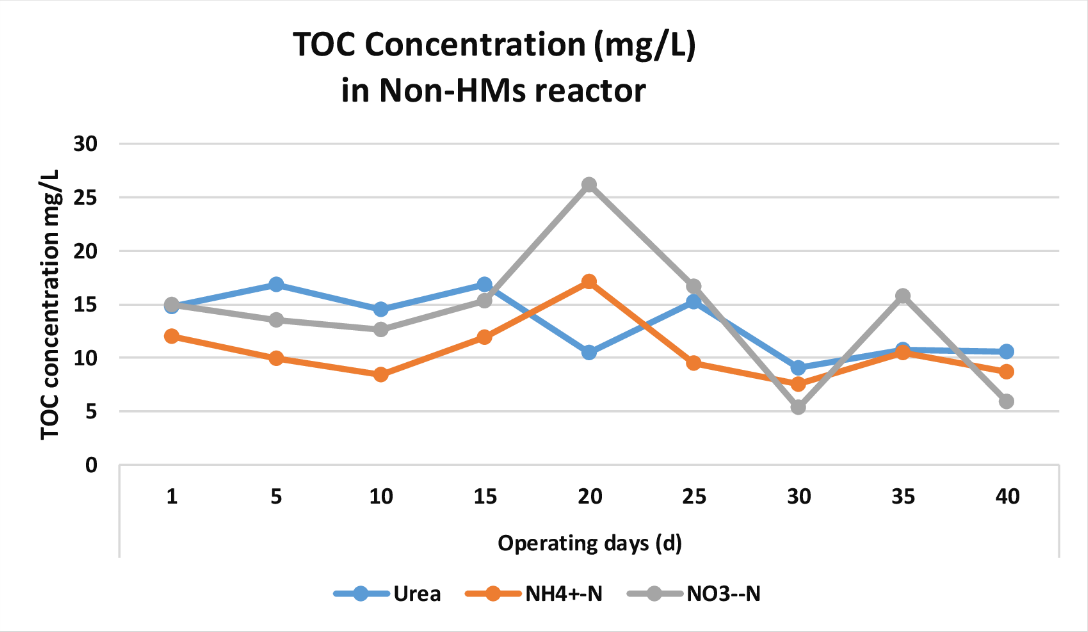

**Figure 23** – Total Organic Carbon Concentration (mg/L) in Non-HMS reactors.

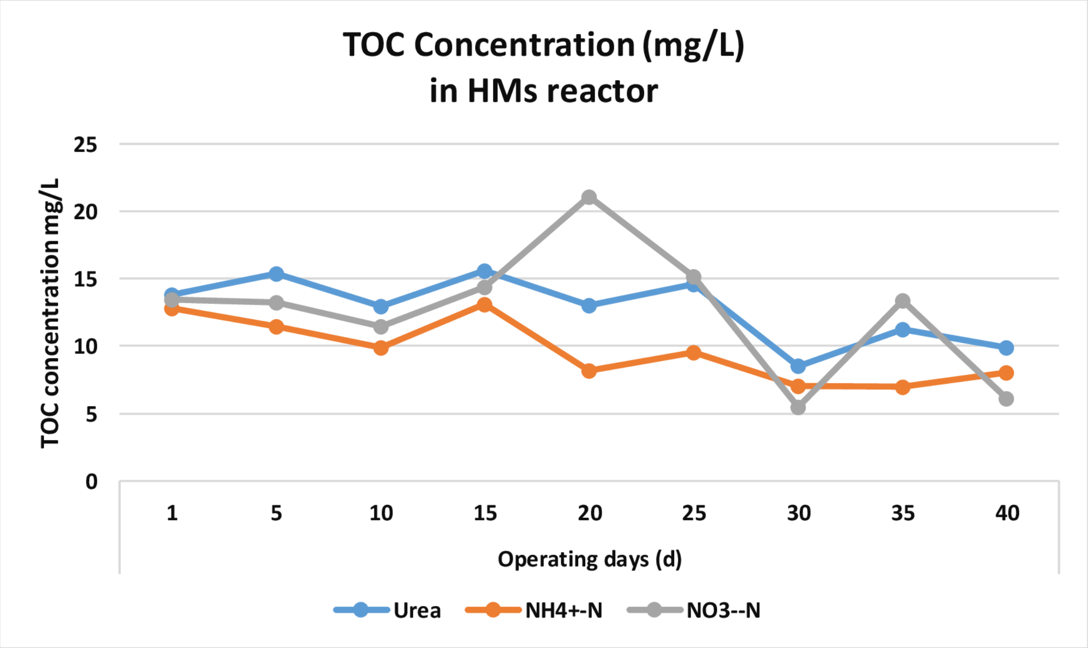

**Figure 24** – Total Organic Carbon Concentration (mg/L) in HMS reactors.

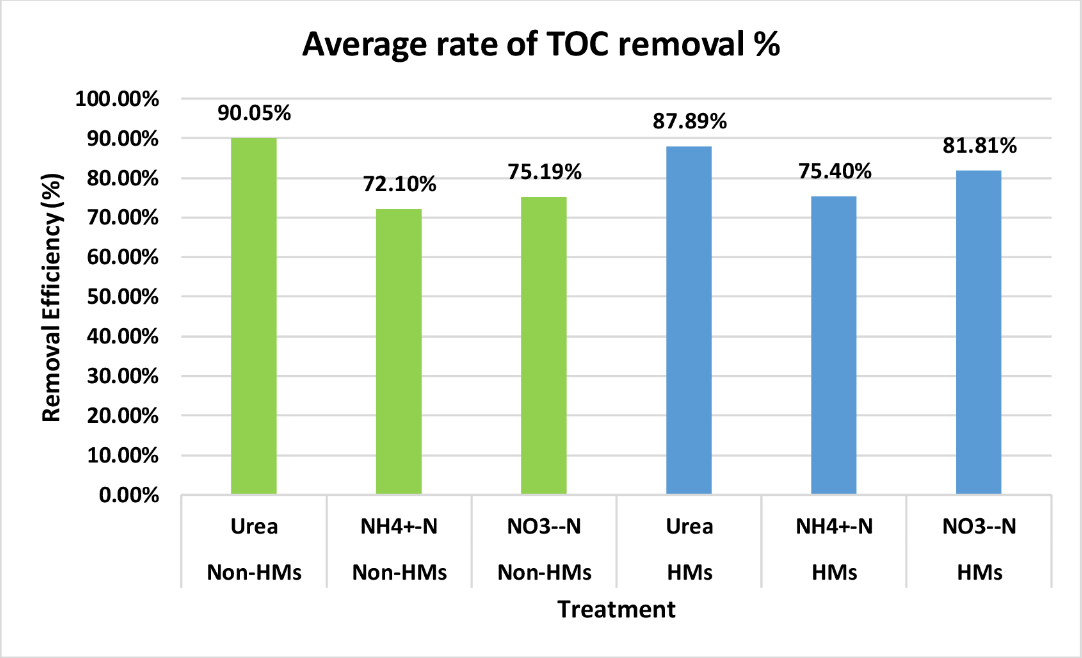

**Figure 25** – Average rate of removal for Total Organic Carbon in Non-HMs and HMs reactors.

For inorganic carbon (IC) concentrations increases among influent and effluent were recorded in both types of reactors under nitrogen sources. Soil nitrogen transformation (e.g., nitrification) and plant N absorption both release protons, causing soil acidification. $CO_2$ is released when the acidity in carbonate-containing soils is neutralized. As a result, N fertilization should be precisely estimated based on plant requirement, with over-fertilization avoided not only because N is a source of local and regional eutrophication, but also because global acidification continues to release $CO_2$ (Zamanian et al., 2018). The average rate of IC removal is negative in all reactors as shown in **Figure 25**. A possible explanation of this case could be that the soil matrix may have been entirely saturated by carbon due to the high carbon content of the substrate, making it unable to extract it as efficiently. However, mechanisms by which different nitrogen fertilizers influence the removal of carbon are still poorly studied and understood.

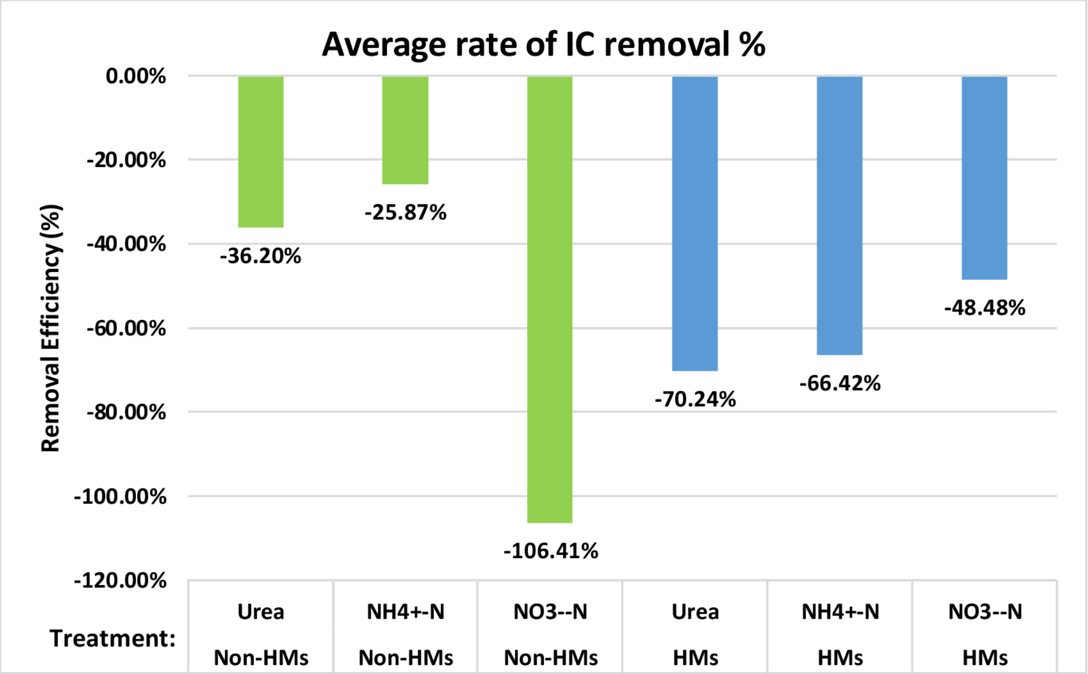

**Figure 26** – Average rate for removal of inorganic carbon (IC) in HMs and Non-HMs reactors.

## 3.3 Heavy Metals in plant tissues

Our samples of plants´ tissues containing heavy metals were analyzed by X-ray fluorescence spectrometers (XRF). **Figures 26 and 27** show that the amount of HMs of two plant parts increases with the increased concentration of HMs in the soil solution. Copper was found to be more likely to accumulate in the shoot part of the plant. The best visible result is seen under urea addition where in roots part accumulated 67 mg/kg, in shoots part, it was 126 mg/kg of Cu, which differs from the research of (Pauková, 2020), where the accumulation of copper was exceeded in the rhizosphere. Our data shows that lead (Pb) for instance is more likely accumulated in roots although the difference with shoots mass volume of lead is not that dramatic. This may be owing to the fact that lead is unable to enter the endodermis, preventing it from moving or transmitting. Lead is absorbed negatively by root hairs and retained to a significant degree in root cells and intracellular vacuoles, with just a smaller amount being transported to the leaves (Al-Qasi et al., 2021). This demonstrates our results that the root system has more lead than the shoot. As shown in **Figure 11**, it is clear that the biomass concentrations of *Iris Wilsonii* decreased as the initial concentrations of HM in the plant increased.

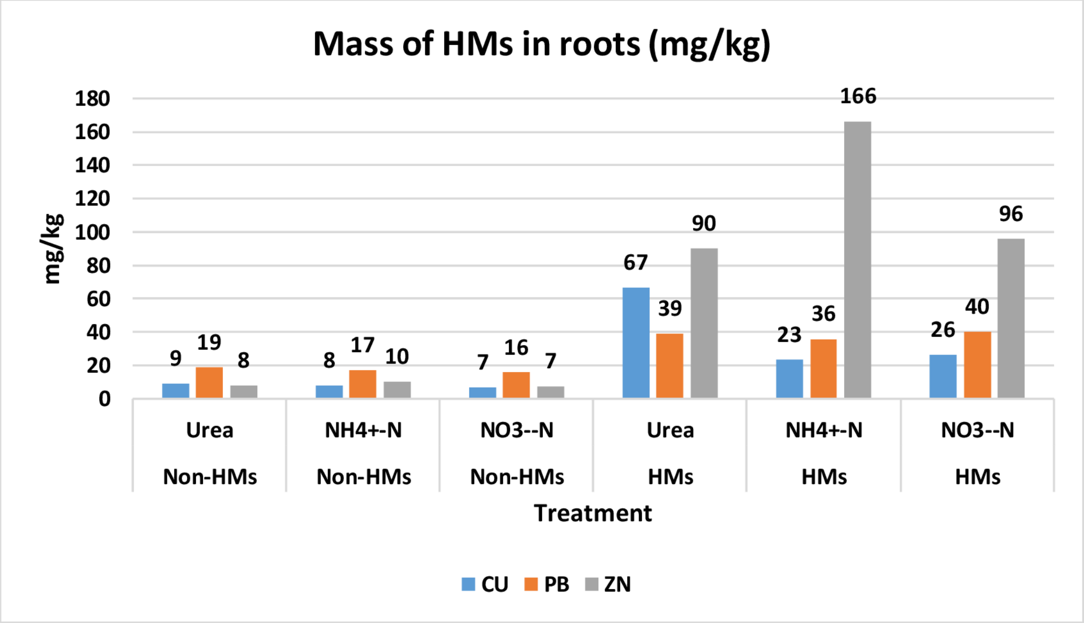

**Figure 27** – Average mass of HMs located in roots of the plant.

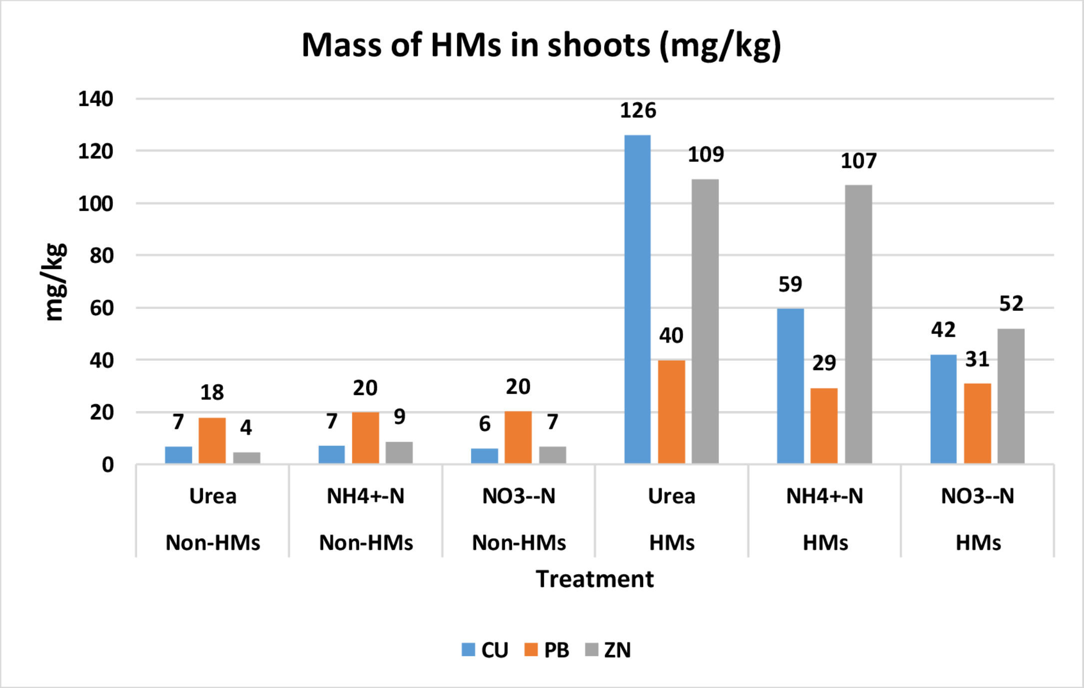

**Figure 28** – Average mass of HMs located in shoots of the plant.

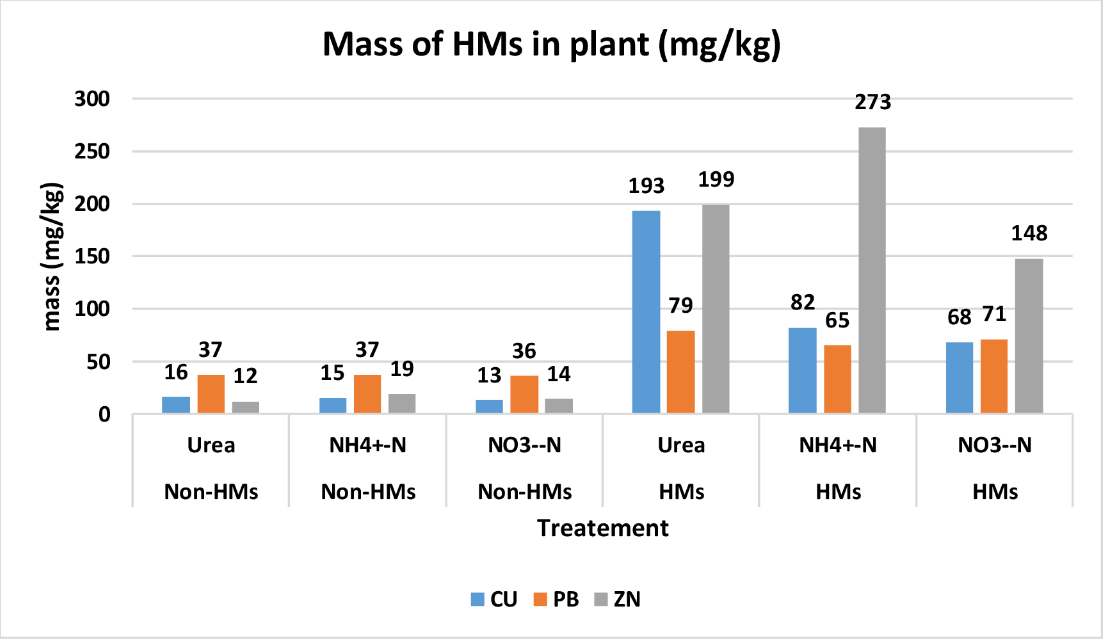

**Figure 29** – Average mass of HMs located throughout the whole plants

Nitrogen figures as an important component of plants, since it is used to generate a variety of secondary metabolites and is also necessary for the production of chlorophyll, a fundamental component of photosynthesis. Desorption and adsorption, chemical transformation, dilution impact, and transportation have all been documented as effects of N utilization on soil–HMs dynamics, all of which influence HMs absorption by plants. Adding various N compounds ($NH_4^+$-N and $NO_3^-$N) in different combinations and doses has been found to alter plant growth and biomass in several studies (Maqbool et al., 2020). Observed reactors show the convincing influence and ability of plants to accumulate Copper (Cu) in their body parts as shown in **Figure 28**. All HMs reactors under different nitrogen sources show a higher mass concentration of Cu when compared to Non-HMs reactors. Samples under urea influence have the highest mass content of Cu with 193mg/kg followed by ammonia (82mg/kg) and nitrogen-nitrate (68mg/kg). Similar results are observed with Lead (Pb), however, the mass portion measurements in the Non-HMs reactors and HMs reactors are not that significant. In the case of Pb, the urea reactor also contains the largest amount of lead mass (79 mg/kg), slightly higher than nitrogen-nitrate (71 mg/kg). Zinc (Zn), as the third observed element reports the highest accumulation values in reactors treated with ammonia source (273 mg/kg) followed by urea (199 mg/kg) and nitrogen nitrate (148 mg/kg). A different study shows, that values represented in **Figure 28** can differ based on the use of different substrates. The constructed wetland's capacity to remove pollutants may be affected by the selection of substrate, rate of removal by plants linked with their growth, and concentration of heavy metals in the environment.

Research dedicated to observation on cadmium (Cd) phytoextraction enhanced by urea on Solanum nigrum L. concluded that urea-treated plants showed the highest growth rate compared to other nitrogen sources. Furthermore, the urea-treated plants collected a greater Cd content. The use of nitrogen fertilizers reduced oxidative stress and increased antioxidant enzymatic activity. It is concluded that urea may benefit S. nigrum development under Cd stress (Maqbool, Ali, Rizwan, 2020). Dilution effect and ammonium ions promote cell membrane potential depolarization, resulting in the inflow of NH4 + into the cytoplasm of root cells, lowering Cd absorption (Sharkawi, Zayed, 2012).

# 4 Conclusion

In this study, the effect of different nitrogen sources on the uptake of HMs in the plant *Iris Wilsonii* was evaluated. Plant growth rates reduced when the initial concentrations of HMs in soil solution emerged. $NO_3$-N resulted in the highest rate of biomass in the reactors without HMs followed by urea while $NH_4^+$-N obtained the lowest rates of biomass. Vice versa for HMs reactors highest average rate was under $NH_4^+$-N source followed by urea and lastly $NO_3$-N. Plants presented the capacity to produce high biomass concentrations under the N source, and remove HM and nutrients from the substrate. The treatment removal efficiency of TN, $NH_4^+$-N, TC, was reduced with the presence of HMs in the substrate of the reactor under different N sources. The positive impact of adopting different N forms in decreasing heavy metals in the substrate was reflected in heavy metals concentration on *Iris WIlsonii*. Nitrogen treatment can aid in the phytoremediation of HMs while also promoting plant recovery. Using nitrogen technology with phytoremediation in wastewater treatment can result in a more cost-effective and ecologically friendly solution, however, further studies are required. This topic is very little studied in research papers and I hope, that my bachelor's thesis will also contribute to increasing interest and awareness in the use of specific plants and different methods for the phytosanation of areas contaminated by heavy metals.

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# 6 Table of figures

# 7 Table of tables