# Performance of Vertical Flow Constructed Wetlands as Quaternary Treatment Systems

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# Performance of Vertical Flow Constructed Wetlands as Quaternary Treatment Systems

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# 1 Contents

**List of Figures**

**List of Tables**

# 1. Introduction

## 1.1 Contamination of emerging concern

Over the past few decades, contaminants of emerging concern (CECs), also called micropollutants, have become an important topic in water quality research and environmental management (Pal et al., 2010; Petrie et al., 2015). These compounds include many types of substances, such as pharmaceuticals, personal care products, pesticides, and industrial chemicals. They are continuously released into the environment because of human activities and consumption patterns and because wastewater treatment plants do not remove them efficiently (Aus Der Beek et al., 2015). These contaminants are particularly challenging because they are widespread, persistent, and toxic once they enter aquatic environments.

Several studies have shown that even trace levels of these compounds can affect aquatic organisms, particularly in terms of endocrine disruption, reproduction, and behavioral changes (Aus Der Beek et al., 2015; Wilkinson et al., 2022).

Micropollutants also rarely occur in isolation. In real wastewater systems, they are typically found as complex mixtures, and their combined effects are still not fully understood This so-called "mixture effect" makes environmental risk assessment more difficult because several compounds are present together and their combined effects are still not fully understood (Petrie et al., 2015).

The increasing consumption of pharmaceuticals and personal care products, together with population growth and urbanization, has further increased the continuous release of these contaminants into aquatic systems (Venditti et al., 2024).

In general, these findings highlight the growing need to understand the behavior of micropollutants in water.

Most micropollutants reach natural water bodies through wastewater systems. This makes wastewater treatment plants (WWTPs) an important link between human activities and the environment.

## 1.2 Removal of Organic Micropollutants in Conventional Wastewater Treatment Plants

Conventional WWTPs are designed to remove organic matter, nutrients, and suspend solids from wastewater before discharge into the environment. They generally include preliminary treatment, primary settling, biological treatment, and secondary clarification processes. These systems usually achieve high removal efficiency for conventional pollutants such as biodegradable organic matter and suspended solids. However, they were not specifically designed to remove trace organic contaminants such as micropollutants (Petrie et al., 2015; Verlicchi & Zambello, 2014).

The limited removal of micropollutants in conventional WWTPs is mainly related to the original design of these treatment systems. Biological processes such as activated sludge treatment are highly efficient for biodegradable compounds, but many micropollutants are only partially biodegraded or remain resistant to conventional biological treatment. As a result, micropollutant removal often depends on the physicochemical properties of each compound, including biodegradability, solubility, and affinity for solids (Petrie et al., 2015).

Several processes can influence the fate of micropollutants during wastewater treatment. Some compounds may undergo partial biodegradation, while others remain dissolved in the aqueous phase or adsorb onto sludge. For example, diclofenac can be partly biodegraded during conventional biological treatment, whereas carbamazepine is frequently reported as one of the most persistent pharmaceutical compounds and often shows poor removal efficiencies in activated sludge systems (Petrie et al., 2015; Verlicchi & Zambello, 2014). Consequently, removal efficiencies can vary considerably between compounds and operating conditions (Verlicchi & Zambello, 2014).

Recent studies have confirmed that treated wastewater effluents remain an important pathway for micropollutant release into aquatic environments (Venditti et al., 2022).

A well-known example is carbamazepine, a pharmaceutical compound frequently detected in treated wastewater because of its persistence and low biodegradability. Several studies reported that carbamazepine is only partially removed during conventional wastewater treatment processes. Because of its persistence and low biodegradability, it is frequently used as an indicator compound in micropollutant studies (Clara et al., 2004; Petrie et al., 2015).

Another important aspect is the continuous discharge of treated wastewater into receiving water bodies. Even when micropollutants are present at low concentrations, their continuous release may still contribute to long-term exposure of aquatic ecosystems (Pal et al., 2010). In addition, biological treatment processes can sometimes generate transformation products with different physicochemical properties and toxicity profiles, which further complicates the assessment of treatment efficiency (Luo et al., 2014).

These limitations have increased interest in additional treatment approaches capable of improving micropollutant removal before wastewater discharge into the environment (Eggen et al., 2014; Verlicchi & Zambello, 2014). This limited performance is mainly related to the design and goals of conventional wastewater treatment systems. These systems were mainly developed to remove large amounts of pollutants, not trace organic contaminants found at low concentrations (Venditti et al., 2022).

In general, biological treatment processes rely on microbial activity to degrade organic matter. While this approach works very well for easily biodegradable substances, it is often less effective for micropollutants, many of which are chemically stable and resistant to biodegradation (Verlicchi & Zambello, 2014).

Another key limitation is the lack of specific removal mechanisms that are known to be effective for micropollutants. For example, advanced oxidation processes or engineered adsorption systems are not typically included in conventional treatment designs. As a result, Removal performance remains variable and strongly depends on the specific compound (Eggen et al., 2014).

Several European countries have introduced requirements for post-treatment systems capable of achieving around 80% removal of selected micropollutants, a level that cannot be reached by conventional WWTPs alone (Venditti et al., 2022). At the same time, the distribution of micropollutants between water and sludge adds another level of complexity. Some compounds tend to attach to sludge, which can lower their concentration in the treated water. However, this does not mean they are completely removed, because they may build up in the sludge, creating concerns about sludge treatment and disposal (Sipma et al., 2010). In addition, recent research has shown that treatment performance can strongly depend on environmental conditions and system characteristics. For instance, factors such as substrate properties, redox conditions, and operational parameters can significantly influence micropollutant removal pathways (Dotro et al., 2017; Muniz Sacco et al., 2025).

## 1.3 Micropollutant legislation (EU UWWTD and Luxembourg)

At the European level, the Water Framework Directive (WFD) established a comprehensive framework for the protection of surface water and groundwater resources, with the objective of achieving good ecological and chemical status of water bodies. As scientific evidence accumulated regarding the occurrence, persistence, and environmental impacts of micropollutants, the need for more targeted monitoring programs and advanced treatment requirements became increasingly evident. The inclusion of micropollutants in European monitoring strategies represented an important step toward linking scientific knowledge with environmental policy and improving the protection of aquatic ecosystems.

Recent studies have demonstrated that conventional wastewater treatment plants are often unable to completely remove many pharmaceutical compounds, personal care products, and other trace organic contaminants from wastewater effluents (Venditti et al., 2022). Consequently, the continuous release of micropollutants into receiving water has become an important environmental concern and has contributed to the development of stricter European regulations. In response to these challenges, the revised Urban Wastewater Treatment Directive (UWWTD) introduced new requirements for micropollutant management in urban wastewater treatment systems. Directive (EU) 2024/3019 requires the implementation of quaternary treatment in selected wastewater treatment plants and establishes a minimum average removal target of 80% for a set of indicator micropollutants. The directive aims to reduce the discharge of hazardous and persistent contaminants into aquatic environments and further supports the application of the polluter-pays principle through Extended Producer Responsibility (EPR) mechanisms (Directive (EU) 2024/3019, 2024).

In addition to European regulatory initiatives, several countries have developed national approaches to reduce micropollutant emissions through the upgrading of existing wastewater treatment facilities and the implementation of advanced treatment technologies. In Luxembourg, research projects investigating nature-based wastewater treatment systems have focused on representative micropollutants such as carbamazepine, diclofenac, tris(chloropropyl) phosphate (TCPP), and mecoprop (MCPP). These compounds were selected because of their environmental relevance, persistence, and frequent detection in wastewater systems.

The revised UWWTD identifies twelve indicator micropollutants that are used to evaluate the performance of quaternary wastewater treatment processes. An overview of these indicator compounds and the micropollutants considered in Luxembourg-based research initiatives is presented in Table 1.1.

Table 1.1 Indicator micropollutants included in the revised Urban Wastewater Treatment Directive (UWWTD) (Directive (EU) 2024/3019, 2024)

| **Compound** | **Category** |
| --- | --- |
| Amisulpride | Pharmaceutical |

| | |
|---|---|
| Carbamazepine | Pharmaceutical |
| Citalopram | Pharmaceutical |
| Clarithromycin | Pharmaceutical |
| Diclofenac | Pharmaceutical |
| Hydrochlorothiazide | Pharmaceutical |
| Metoprolol | Pharmaceutical |
| Venlafaxine | Pharmaceutical |
| Benzotriazole | Industrial chemical |
| Candesartan | Pharmaceutical |
| Irbesartan | Pharmaceutical |
| 4-/5-Methylbenzotriazole | Industrial chemical |

Most of the indicator compounds included in the revised UWWTD are pharmaceuticals frequently detected in wastewater effluents, including analgesics, antibiotics, antidepressants, and cardiovascular drugs. The list also includes industrial chemicals such as benzotriazole and methylbenzotriazoles, which are commonly associated with urban and industrial activities. Their inclusion reflects growing concerns regarding the persistence of micropollutants in aquatic environments and the need for advanced treatment technologies capable of reducing their discharge into receiving waters (Directive (EU) 2024/3019, 2024).

## 1.4 Quaternary treatment approaches

Quaternary treatment is generally applied as a final polishing step to improve the removal of micropollutants that are not sufficiently eliminated during conventional wastewater treatment (Eggen et al., 2014).

Quaternary treatment represents an important development in wastewater management because it specifically targets trace organic contaminants that are not sufficiently removed by conventional treatment processes (Eggen et al., 2014; Petrie et al., 2015). The increasing implementation of these treatment stages is mainly driven by growing scientific concern regarding micropollutants and by evolving environmental regulations at the European level (Directive (EU) 2024/3019, 2024; Petrie et al., 2015). Despite their high removal efficiencies, the implementation of these technologies is often associated with higher energy demand, increased operational costs and greater technical complexity**.** However, in real wastewater treatment conditions, achieving consistent removal efficiency remains difficult, especially for persistent compounds. Recent studies have highlighted this issue and further support the need for improved quaternary treatment strategies (Venditti et al., 2022). These technologies are also linked to higher energy use, increased operational costs, and more complex system management. In practice, they often require specialized infrastructure and technical expertise, which can limit their use, especially in smaller or decentralized wastewater treatment plants. This creates an important question: how can micropollutants be removed effectively while keeping treatment systems affordable and simple to operate? To address this issue, interest in alternative approaches, including nature-based systems, has increased. These systems aim to achieve similar treatment performance while lowering operational costs and environmental impact (Muniz Sacco et al., 2025; Venditti et al., 2024). Nature-based systems, such as constructed wetlands, depend on the interaction of physical, chemical, and biological processes within the same system. This combination of processes is an important characteristic that allows flexible and reliable treatment performance under different conditions (Dotro et al., 2017). As a result, constructed wetlands have gained increasing attention as possible options for quaternary treatment, especially where sustainability and low-tech / low-cost operation are important considerations.

## 1.5 Quaternary treatment technologies overview

In response to the growing concern about micropollutants and the limitations of conventional wastewater treatment plants, several advanced technologies have been developed and implemented as part of quaternary treatment strategies. These technologies are specifically designed to remove trace organic contaminants that are not sufficiently eliminated during conventional wastewater treatment processes (Petrie et al., 2015; Verlicchi & Zambello, 2014).

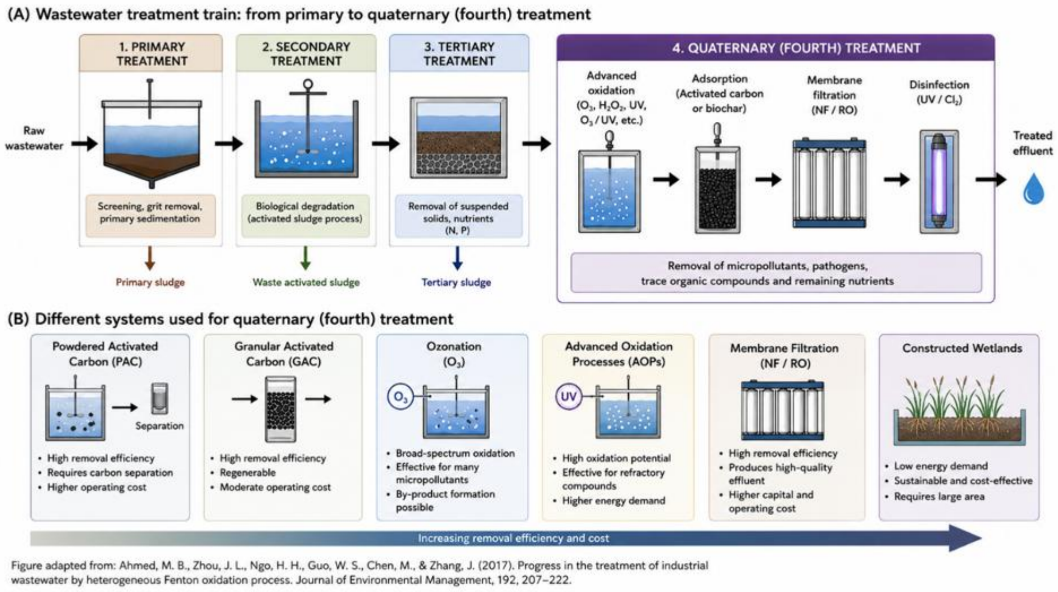


Figure 1.0.1 Main stages of wastewater treatment and principal technologies used in quaternary treatment for micropollutant removal.

The most widely applied quaternary treatment technologies include activated carbon adsorption, membrane filtration, ozonation, and advanced oxidation processes (AOPs). Each technology relies on different removal mechanisms and therefore presents specific advantages and limitations depending on the target contaminants and treatment objectives (Petrie et al., 2015; Verlicchi & Zambello, 2014). Activated carbon adsorption is one of the most implemented technologies for micropollutant removal. This process relies on the large surface area and porous structure of carbon materials to retain organic molecules from wastewater (Carvalho et al., 2011). Activated carbon can be applied in powdered activated carbon (PAC) or granular activated carbon (GAC) forms. Its performance depends on several factors, including contact time, carbon properties, and wastewater composition Membrane technologies, such as nanofiltration and reverse osmosis, remove micropollutants by acting as physical barriers that retain dissolved contaminants These systems can achieve high removal efficiencies and produce high-quality effluents. However, they are often associated with high energy demand, elevated operational costs, and the generation of concentrated reject streams requiring further management (Verlicchi & Zambello, 2014).

Ozonation is one of the most widely applied quaternary treatment technologies, particularly in full-scale wastewater treatment plants. It is based on the strong oxidizing power of ozone, which reacts with a broad range of organic compounds and promotes their transformation or degradation (Eggen et al., 2014). Ozonation has demonstrated high removal efficiencies for numerous pharmaceuticals and personal care products (Petrie et al., 2015). Nevertheless, it may generate transformation products that require further toxicological evaluation (Eggen et al., 2014).

Advanced oxidation processes (AOPs), including UV/$H_2O_2$ and photocatalytic systems, rely on the generation of highly reactive radicals capable of degrading persistent organic contaminants (Petrie et al., 2015). Compared with ozonation, UV/$H_2O_2$ systems do not require ozone generation and may reduce the formation of some ozonation by-products. However, the efficiency of AOPs can be reduced by radical scavengers naturally present in wastewater, such as bicarbonates, carbonates, and natural organic matter. Like ozonation, these technologies may also generate intermediate transformation products requiring additional assessment (Eggen et al., 2014). Despite their proven effectiveness, quaternary treatment technologies are frequently associated with increased operational costs, energy demand, and technical complexity (Venditti et al., 2022). These limitations become particularly important for large-scale applications and decentralized treatment systems where economic feasibility remains a major consideration (Muniz Sacco et al., 2025).

## 1.6 Constructed wetlands

### 1.6.1 Overview of different CW-configurations

Constructed wetlands (CWs) are engineered systems developed to reproduce treatment processes naturally occurring in wetland environments (Vymazal, 2011). These systems are increasingly investigated as sustainable solutions for wastewater polishing and micropollutant removal because they combine physical, chemical, and biological processes within the same treatment unit (Dotro et al., 2017; Nivala et al., 2013).

Different wetland configurations have been developed for wastewater treatment applications, including free water surface wetlands, horizontal subsurface flow wetlands, and vertical flow constructed wetlands (VFCWs). These configurations differ mainly in hydraulic operation, oxygen transfer, and dominant treatment mechanisms (Dotro et al., 2017).

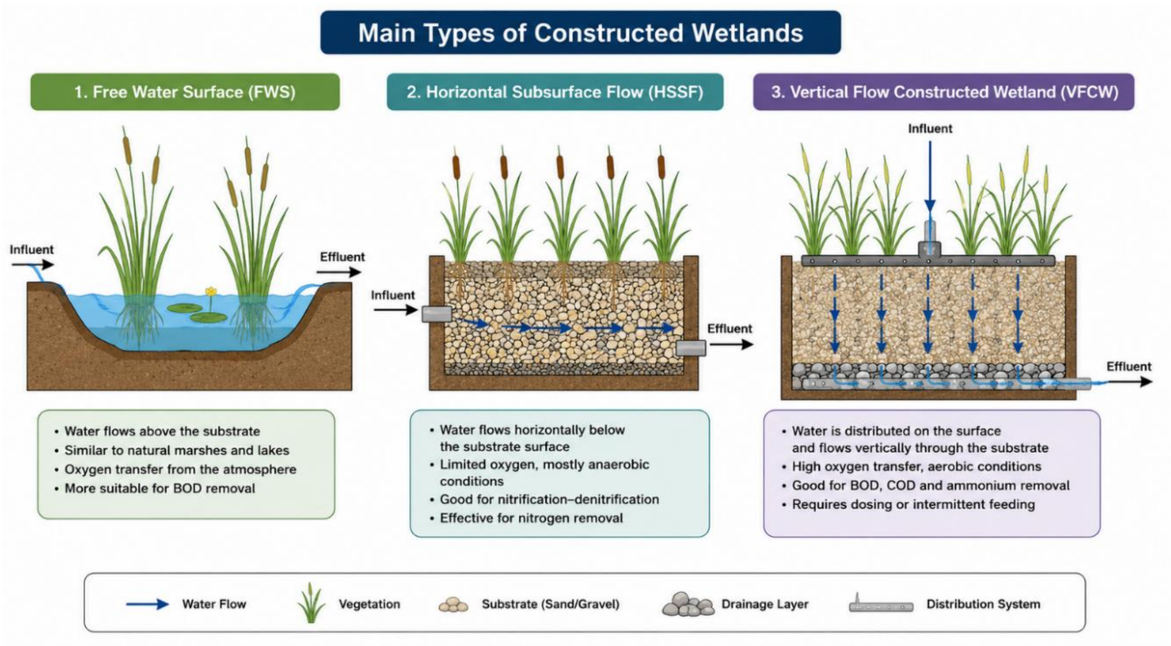


Figure 1.2 Schematic representation of the main constructed wetland configurations: free water surface (FWS), horizontal subsurface flow (HSSF), and vertical flow constructed wetland (VFCW).

Among these configurations, VFCWs have received increasing attention for micropollutant removal applications. In VFCWs, wastewater is intermittently distributed onto the surface of the filter bed and percolates vertically through the substrate. Between feeding events, air re-enters the pore spaces, improving oxygen availability within the media and supporting aerobic microbial activity (Dotro et al., 2017; Nivala et al., 2013).

In constructed wetlands, micropollutant removal usually results from several mechanisms occurring simultaneously within the system, including filtration, sedimentation, adsorption, plant-related processes, and microbial degradation (Vymazal, 2011). The contribution of each mechanism depends on contaminant properties, substrate composition, hydraulic conditions, and oxygen availability (Dotro et al., 2017).

Despite these promising results, treatment efficiency remains strongly influenced by wetland design and operational conditions. Parameters such as hydraulic loading rate, feeding strategy, vegetation, substrate characteristics, and environmental conditions can significantly affect pollutant behavior and removal performance within the systems (Dotro et al., 2017).

Compared with advanced quaternary treatment technologies such as ozonation, membrane filtration, or advanced oxidation processes, constructed wetlands usually operate with lower energy demand and simpler infrastructure requirements (Nivala et al., 2013; Vymazal, 2011).

Treatment mainly relies on natural processes occurring inside the substrate and rhizosphere rather than on continuous chemical addition or high-energy oxidation systems.

A typical constructed wetland consists of three main components: substrate, vegetation, and hydraulic distribution systems. Together, these elements contribute to pollutant removal through filtration, adsorption, microbial degradation, and plant-associated processes (Dotro et al., 2017). The substrate supports water flow and provides surfaces for biofilm development and adsorption processes. Vegetation contributes indirectly by supporting rhizosphere microbial activity and promoting localized oxygen transfer around the roots (Vymazal, 2011). Hydraulic operation additionally controls wastewater distribution, retention time, and oxygen renewal within the system.

Because these systems operate with limited external energy demand and relatively simple maintenance requirements, constructed wetlands are increasingly considered attractive options for decentralized and sustainable wastewater treatment applications (Nivala et al., 2013).m

Recent studies reported that VFCWs can achieve promising removal efficiencies for several emerging contaminants, particularly under intermittent feeding conditions that enhance oxygen transfer and biodegradation processes (Venditti et al., 2022). More recently, increasing attention has also been directed toward substrate amendments such as activated biochar. Because of its high surface area and adsorption capacity, biochar may improve the retention of selected micropollutants inside the filter media (Venditti et al., 2024).

### 1.6.2 CW removal mechanisms

Pollutant removal in constructed wetlands results from the interaction of several physical, chemical, and biological mechanisms occurring simultaneously within the system (Dotro et al., 2017). Physical processes mainly include sedimentation and filtration. Suspended particles can be retained while wastewater percolates through the substrate media. Chemical mechanisms are largely associated with adsorption processes, where contaminants interact with mineral surfaces or organic materials present within the substrate (Vymazal, 2011).

Biological processes are also essential for treatment performance. Microorganisms growing within the substrate and rhizosphere contribute to organic matter degradation, nutrient transformation, and micropollutant biodegradation. Depending on oxygen availability, both aerobic and anaerobic pathways may occur inside the same wetland system (Dotro et al., 2017).

The importance of adsorption processes has increased with the introduction of alternative substrate amendments such as activated biochar. Because of its porous structure and high surface area, biochar may improve the retention of several organic contaminants, especially during the early stages of operation when adsorption processes remain dominant (Venditti et al., 2024). At the same time, plant roots contribute indirectly to treatment by supporting rhizosphere microbial communities and influencing oxygen transfer within the substrate. These combined interactions between substrate, microorganisms, and vegetation are considered important for long-term wetland performance (Nivala et al., 2013; Vymazal, 2011).

#### 1.6.2.1 Role of plants in constructed wetlands

Plants contribute to treatment performance in constructed wetlands through both direct and indirect mechanisms. Nutrient uptake represents one direct contribution, particularly for nitrogen and phosphorus compounds. Some organic contaminants may also be absorbed by plant tissues, although this contribution generally remains limited for many micropollutants (Vymazal, 2011).

Plants additionally influence the hydraulic behavior of the systems through evapotranspiration, which can modify water balance and hydraulic retention time inside the wetlands (Nivala et al., 2013). Another contribution is linked to phytodegradation and rhizosphere interactions. Although direct degradation within plant tissues is usually limited, roots create favorable conditions for microbial development by releasing oxygen and organic compounds into the surrounding environment (Dotro et al., 2017). As a result, vegetation contributes to pollutant removal through uptake, evapotranspiration, phytodegradation, and the stimulation of rhizosphere microbial activity (Nivala et al., 2013; Vymazal, 2011).

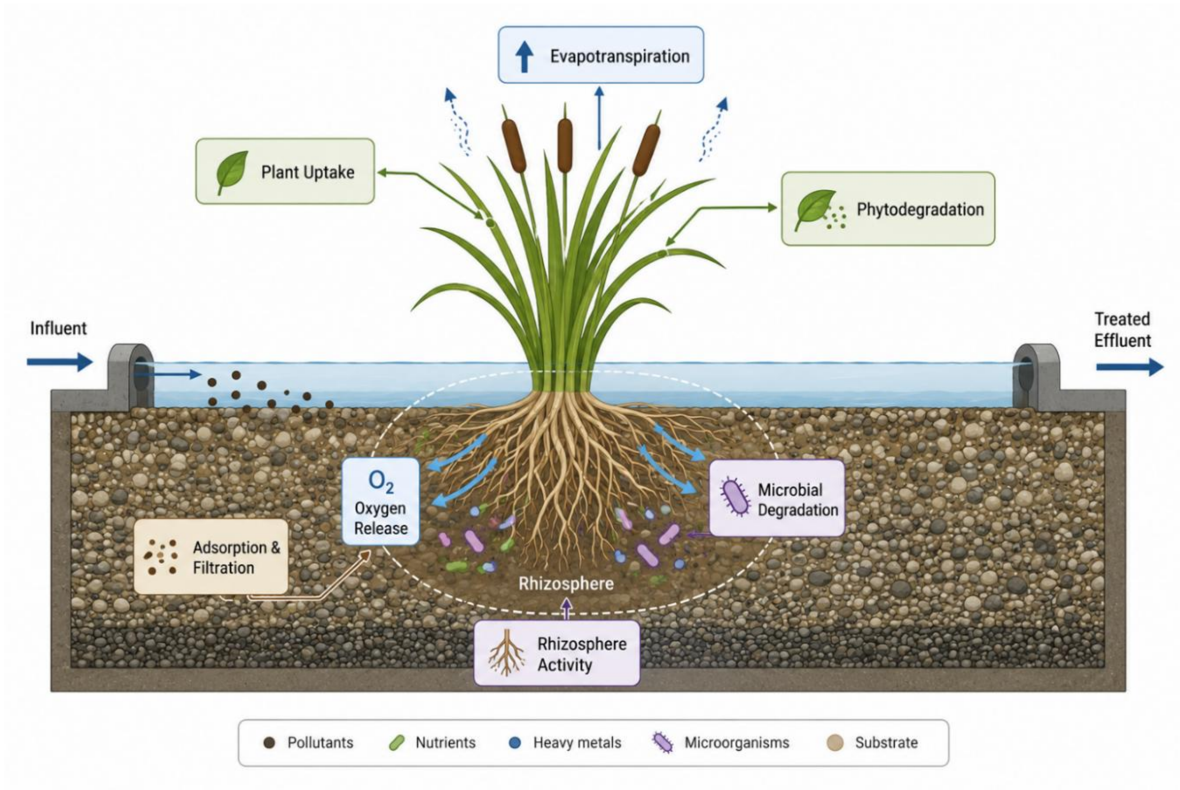


Figure 1.3 Conceptual illustration of phytoremediation mechanisms in constructed wetlands, showing plant uptake, evapotranspiration, phytodegradation, rhizosphere activity, microbial degradation, oxygen release, and substrate-based pollutant retention.

#### 1.6.2.2 Root oxygen transfer and rhizosphere effects

Wetland plants can transport oxygen from the atmosphere toward the root zone through specialized tissues. This oxygen release creates localized aerobic microenvironments within the substrate and supports aerobic microbial activity around the roots (Vymazal, 2011).

The rhizosphere, which corresponds to the area surrounding plant roots, is characterized by strong biological interactions between plants, microorganisms, and contaminants. Organic compounds released by roots can stimulate microbial metabolism and promote the development of active biofilms involved in pollutant degradation processes (Dotro et al., 2017).

Because vertical flow systems operate under intermittent feeding conditions, oxygen renewal is generally more efficient than in saturated horizontal flow wetlands. This combination of intermittent aeration and rhizosphere oxygen transfer can significantly enhance aerobic biodegradation processes inside VFCWs (Nivala et al., 2013; Venditti et al., 2022).

#### 1.6.2.3 Microbial processes

Microbial activity is considered one of the main drivers of pollutant removal in constructed wetlands. Microorganisms participate in organic matter degradation, nutrient transformation, and micropollutant biodegradation through different biochemical pathways (Dotro et al., 2017). Microorganisms are involved in several key processes including organic carbon degradation, nitrification, denitrification, and the biodegradation of selected micropollutants. For example, aerobic bacteria can oxidize ammonium into nitrate, whereas denitrifying microorganisms convert nitrate into nitrogen gas under oxygen-limited conditions (Dotro et al., 2017; Vymazal, 2011). In VFCWs, intermittent feeding improves oxygen transfer inside the substrate and promotes aerobic microbial processes such as nitrification and aerobic biodegradation (Nivala et al., 2013). At the same time, less oxygenated zones may support denitrification and other anaerobic processes, allowing several complementary removal pathways to coexist within the same treatment system (Vymazal, 2011).

The development of microbial communities is strongly influenced by substrate characteristics, oxygen availability, temperature, and hydraulic conditions. Biofilms attached to substrate particles provide large surfaces for microbial colonization and play a major role in long-term pollutant removal performance (Dotro et al., 2017; Vymazal, 2011).

### 1.6.3 Vertical vs horizontal constructed wetlands

Constructed wetlands are commonly divided into horizontal flow and vertical flow systems according to wastewater movement through the substrate (Vymazal, 2011).

In horizontal flow wetlands, wastewater moves laterally through saturated media. Oxygen transfer is generally limited, resulting in more reduced conditions within the system. These wetlands are therefore often associated with denitrification and anaerobic processes (Dotro et al., 2017). In contrast, vertical flow constructed wetlands operate with intermittent surface feeding. Wastewater infiltrates vertically through the substrate, while resting periods allow air to re-enter the pore spaces. This operation significantly improves oxygen transfer and promotes more aerobic conditions within the filter media (Nivala et al., 2013). Because oxygen transfer is usually more efficient in VFCWs, these systems often show stronger nitrification and higher aerobic microbial activity than horizontal flow wetlands (Vymazal, 2011). Several studies also reported improved micropollutant removal performances in vertical flow systems, particularly when combined with adsorptive materials such as biochar (Muniz Sacco et al., 2025; Venditti et al., 2022, 2024).

One of the main operational characteristics of VFCWs is the intermittent feeding regime, which promotes periodic air renewal within the substrate and enhances oxygen transfer compared with continuously saturated wetland systems. Between feeding events, partial drainage allows air to re-enter the substrate, further enhancing oxygen renewal within the system (Nivala et al., 2013). Improved oxygen availability supports aerobic microbial processes involved in nitrification and organic contaminant degradation. As a result, VFCWs often show better treatment performance for biodegradable pollutants and some micropollutants compared with more saturated wetland configurations (Vymazal, 2011).

Another advantage is that these systems remain relatively simple to operate while requiring limited external energy input. Because treatment mainly relies on natural processes occurring within the substrate and rhizosphere, VFCWs are increasingly considered promising alternatives for sustainable wastewater polishing and quaternary treatment applications (Venditti et al., 2022).

## 1.7 Evidence of better micropollutant removal in VFCW

In recent years, more studies have investigated the performance of vertical flow constructed wetlands (VFCWs) for micropollutant removal. Available results suggest that these systems can achieve moderate to relatively high removal efficiency for some compounds. However, performance strongly depends on the characteristics of the pollutants and the operating conditions (Nivala et al., 2013; Verlicchi & Zambello, 2014).

In general, compounds that are easier to biodegrade or adsorb are usually removed more effectively in VFCWs. This is mainly related to the higher oxygen availability in these systems. Improved oxygen conditions promote microbial activity and increase degradation processes. As a result, biological pathways responsible for the removal of many organic contaminants become more efficient (Vymazal, 2011).

Recent studies have shown that better aeration and intermittent feeding can improve the removal of several micropollutants. This is especially true when aerobic biodegradation is the main removal mechanism (Muniz Sacco et al., 2025).

At the same time, micropollutants can also be removed by attaching to the substrate, especially during the first stages of operation. Using materials with stronger adsorption properties can therefore improve treatment efficiency. However, adsorption by itself is not always enough to completely remove contaminants. For example, systems amended with biochar have shown better initial removal performance because of their higher adsorption capacity. This shows that the choice of substrate is an important factor for improving treatment performance (Venditti et al., 2024). Even though these results are encouraging, treatment efficiency can vary greatly between compounds. Some persistent substances, such as Carbamazepine, remain difficult to remove in both conventional treatment systems and constructed wetlands.

This highlights the importance of studying the behavior of each compound individually when assessing treatment performance (Clara et al., 2004; Verlicchi & Zambello, 2014).

Another important aspect is the dynamic behavior of constructed wetland systems. Over time, microbial communities grow and vegetation becomes more established. As this happens, the contribution of different removal mechanisms can change. This means that system performance is not constant but evolves over time. Therefore, long-term monitoring is important for a proper evaluation (Nivala et al., 2013).

This behavior has been observed in recent studies, where the system gradually changed from adsorption-dominated processes to biologically driven removal as it matured (Muniz Sacco et al., 2025). This shift can be explained by the gradual development of microbial communities and more stable redox conditions within the system. These factors are known to influence degradation pathways (Dotro et al., 2017).

In general, these findings suggest that vertical flow constructed wetlands are a promising solution for wastewater treatment. However, they cannot completely remove all micropollutants. When applied as polishing steps after conventional treatment, VFCWs can significantly lower micropollutant concentrations. At the same time, they maintain relatively low energy demand and simple operational requirements (Venditti et al., 2022).

## 1.8 FORGOTTEN project context and research gaps

The present study was conducted within the framework of the FORGOTTEN project (Fungal BiOdegRadation of emerginG cOnTaminanTs in NaturE-based SolutioNs). The project aims to improve the understanding of contaminant transformation processes occurring in nature-based wastewater treatment systems. It combines experimental wetland operation with advanced microbial investigations to better characterize the biological processes involved in micropollutant removal (Venditti, 2025).

Within the project structure, the present thesis contributes to Work Package 1 (WP1), which focuses on the establishment and operation of laboratory-scale treatment systems. The data generated in this study provide experimental support for the broader objectives of the FORGOTTEN project (Venditti, 2025).

Despite the increasing number of studies investigating micropollutant removal in constructed wetlands, important knowledge gaps remain. The influence of wetland design choices on treatment performance is not yet fully understood, particularly regarding the role of vegetation and alternative substrate amendments (Venditti et al., 2022; Vymazal, 2011). In addition, the interactions between biological and physicochemical removal processes remain difficult to quantify under controlled experimental conditions because several removal mechanisms occur simultaneously within wetland systems (Dotro et al., 2017; Nivala et al., 2013).

Addressing these uncertainties is essential for improving the design and optimization of nature-based treatment systems. Therefore, the present study was developed to investigate specific research questions related to the performance of vertical flow constructed wetlands and the factors influencing micropollutant removal.

Based on these gaps, the following research questions were addressed:

- How effectively can lab-scale vertical flow constructed wetlands remove selected micropollutants from synthetic treated wastewater?
- To what extent does vegetation influence micropollutant removal and overall treatment performance in vertical flow constructed wetlands?
- How does biochar amendment modify micropollutant removal processes compared with conventional substrate configurations?
- How do interactions between vegetation, substrate characteristics, and microbial processes contribute to micropollutant removal mechanisms?

## 1.9 Objective

The main objective of this study was to evaluate the performance of laboratory-scale vertical flow constructed wetlands treating synthetic wastewater containing selected micropollutants. More specifically, the study aimed to:

- Compare planted and unplanted systems.
- Evaluate the effect of biochar amendment on treatment performance.
- Investigate how vegetation and substrate characteristics influence micropollutant removal.
- Improve the understanding of the biological processes involved in pollutant transformation, including interactions with microbial communities within the FORGOTTEN project context.

# 2. Materials and Methods

## 2.1 Experimental setup

The experimental setup was developed and operated at the University of Luxembourg to investigate the performance of vertical flow constructed wetlands (VFCWs) for the removal of micropollutants from treated wastewater effluent. The experiment was carried out under controlled indoor laboratory conditions as part of the FORGOTTEN project. Six independent lysimeters were operated simultaneously during the study and were fed with synthetic treated wastewater containing selected micropollutants frequently found in wastewater treatment plant effluents (Venditti et al., 2022, 2024). Different vegetation and substrate configurations were tested to investigate the effect of plants and activated biochar on treatment performance under controlled operating conditions. The systems were operated under intermittent feeding conditions to reproduce the hydraulic behavior often observed in vertical flow constructed wetlands used as polishing steps after conventional wastewater treatment. Hydraulic loading conditions, feeding frequency, substrate composition, and monitoring procedures were maintained under controlled conditions throughout the experiment. The following sections describe the experimental site, laboratory-scale system design, and treatment configurations used during the study.

### 2.1.1 Experimental site and environmental conditions

The experimental setup consisted of six vertical flow constructed wetland lysimeters operated simultaneously under controlled laboratory conditions. The lysimeters were cylindrical columns with an internal diameter of approximately 28 cm and were designed to reproduce the operating principles of laboratory-scale vertical flow constructed wetlands. Each unit included a treatment compartment and a drainage section allowing vertical percolation of wastewater through the filter media.

### 2.1.2 Indoor laboratory-scale system design

The experimental setup consisted of six laboratory-scale vertical flow constructed wetland lysimeters operated simultaneously under controlled indoor conditions. The systems were designed to reproduce the main hydraulic and treatment processes occurring in full-scale vertical flow constructed wetlands. Detailed information regarding lysimeter construction, substrate composition, hydraulic operation, instrumentation, and treatment configurations is provided in the following sections. A schematic representation of the experimental setup is presented in Figure 2.1.

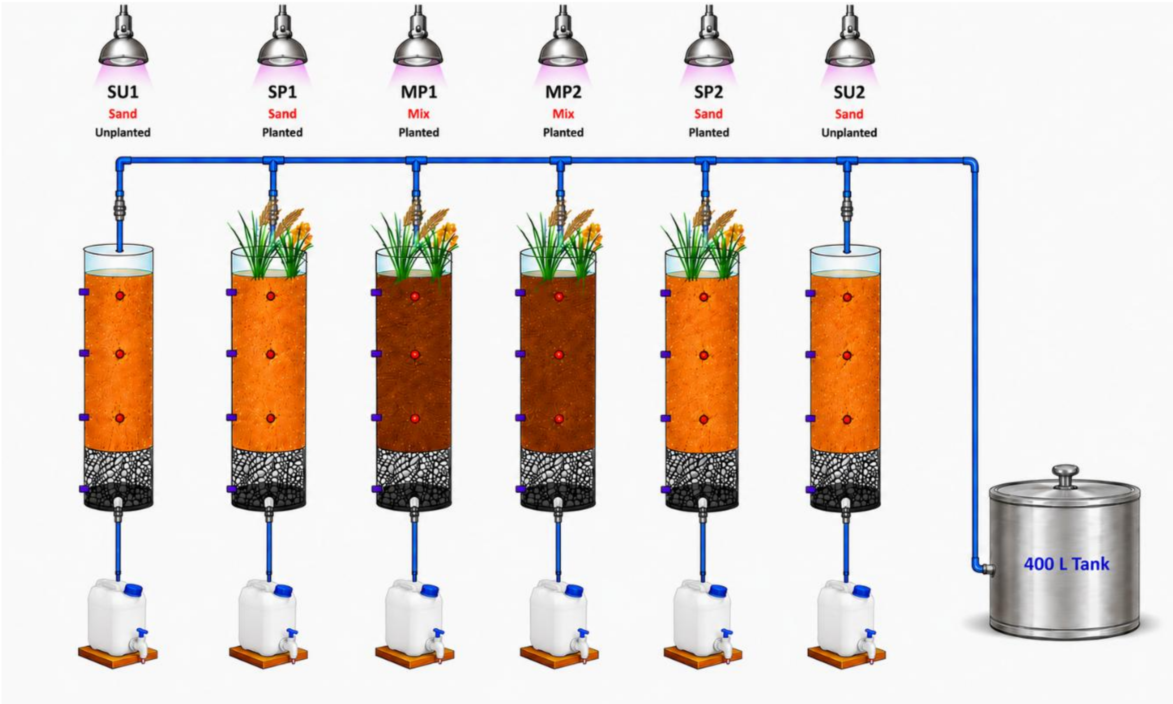


Figure 2.1 Schematic representation of the laboratory-scale vertical flow constructed wetland system

### 2.1.3 Experimental configurations and duplicate systems

The experimental design was established to investigate the influence of macrophytes and biologically activated biochar on the performance of laboratory-scale vertical flow constructed wetlands. Three treatment configurations were therefore implemented under controlled conditions and operated in duplicate throughout the experiment.

The first configuration consisted of unplanted lysimeters filled with expanded clay substrate. The second configuration consisted of planted lysimeters containing the same substrate. The third configuration consisted of planted lysimeters amended with 15 % activated biochar. The comparison between these configurations enabled the assessment of the individual and combined effects of vegetation and biochar amendment on system performance. All treatment units were operated under identical hydraulic and environmental conditions during the monitoring period (Venditti et al., 2022, 2024). A schematic overview of the treatment configurations is presented in Figure 2.2.

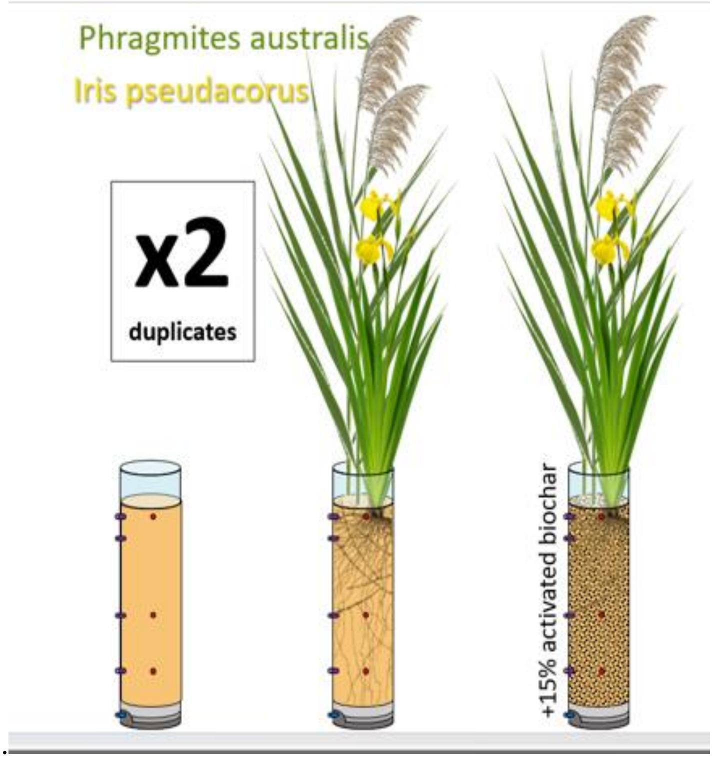


Figure 2.2 Overview of the three experimental configurations used in the study (unplanted, planted, and planted + biochar systems).

## 2.2 Substrates

The substrate plays a fundamental role in vertical flow constructed wetlands by providing physical support for plant development, promoting biofilm growth, and contributing to contaminant retention processes. In addition to influencing hydraulic behavior, substrate characteristics may affect the removal of nutrients and emerging contaminants through sorption and microbial interactions.

Expanded clay was selected as the main substrate material for all lysimeters. This material is commonly used in nature-based treatment systems because of its porous structure, favorable hydraulic properties, and low risk of clogging under long-term operation. The porous surface also provides suitable conditions for microbial colonization and biofilm development, which are important processes in wastewater treatment (Venditti et al., 2022).

To evaluate the potential benefits of substrate amendment, activated biochar was incorporated into one of the treatment configurations. Previous investigations demonstrated that biologically activated biochar can enhance the performance of constructed wetlands and improve the removal of emerging contaminants. In addition to its adsorption capacity, biochar provides a large surface area that can support microbial growth and increase contact between contaminants and active treatment surfaces (Venditti et al., 2022, 2024).

Detailed information regarding the composition and preparation of the substrate mixtures is provided in the following sections.

### 2.2.1 Expanded clay

Expanded clay was used as the main substrate material in all lysimeters. The material consisted of Liapor expanded clay with a particle size of approximately 0–3 mm. Expanded clay was selected because it combines good hydraulic conductivity with high porosity, allowing water infiltration while maintaining oxygen circulation inside the substrate profile. Its rough and porous surface can also support microbial biofilm development and contribute to pollutant retention processes inside vertical flow systems.

Before installation, the substrate material was distributed manually inside the lysimeters to obtain homogeneous conditions between the different experimental units. The expanded clay formed the main treatment layer above the gravel drainage section and was used either alone or mixed with activated biochar depending on the experimental configuration.

Expanded clay has previously been used in laboratory-scale vertical flow constructed wetlands treating wastewater effluent and emerging contaminants because of its hydraulic stability and suitability for aerobic treatment conditions (Muniz Sacco et al., 2025; Venditti et al., 2022).

### 2.2.2 Biochar supporting material

Activated biochar was added to selected lysimeters to investigate its potential to increase micropollutant removal. The amended treatment media contained approximately 15 % activated biochar mixed with 85 % expanded clay. According to the FORGOTTEN project setup, the biochar material was supplied by Klimatfarmer and biologically activated using microorganisms including *Lactobacillus*, *Rhodopseudomonas*, and *Saccharomyces*.

The incorporation of biochar was motivated by its porous structure and elevated adsorption capacity, characteristics that can favor the retention of persistent contaminants within the treatment media. Previous studies on biochar-amended vertical flow constructed wetlands reported improved removal of compounds such as carbamazepine and diclofenac after incorporation of activated biochar into the substrate mixture (Venditti et al., 2022, 2024). Before installation into the lysimeters, the activated biochar was mixed manually with the expanded clay to obtain a more homogeneous distribution within the treatment layer. The amended and non-amended configurations were then compared during operation to better understand the contribution of adsorption mechanisms to micropollutant removal inside the systems.

### 2.2.3 Layer configuration and substrate profile

Each lysimeter contained a multilayer substrate arrangement designed to maintain stable hydraulic conditions and facilitate vertical water percolation throughout the experiment. The bottom section of each column consisted of a 10 cm drainage layer composed of different gravel fractions arranged progressively from coarse to fine material. The lowest layer contained approximately 2 cm of coarse gravel (20–40 mm), followed by 3 cm of gravel (4–8 mm) and 5 cm of fine gravel (2–5 mm). This drainage arrangement was designed to facilitate water collection at the outlet and maintain appropriate flow conditions during operation.

Above the drainage layer, the main treatment zone consisted of approximately 90 cm of treatment media. The upper 10 cm of the treatment layer was inoculated with 1 % (v/v) microbial inoculum during system preparation. The inoculum was collected from a four-year-old pilot-scale constructed wetland located in Hersberg that had been continuously operated with real wastewater treatment plant effluent. The donor system contained the same plant species and a similar substrate composition as the experimental lysimeters. This inoculation step was performed to introduce an established microbial community into the newly constructed systems and promote the early development of biological treatment processes within the substrate.

During installation, the different materials were added progressively to the lysimeters to ensure consistent filling conditions and comparable substrate distribution among all experimental units. The vertical arrangement of the different layers was selected to reproduce substrate configurations commonly applied in vertical flow constructed wetlands treating wastewater effluents under intermittent loading conditions (Venditti et al., 2022, 2024).

### 2.2.4 Substrate characterization and analytical methods

Several analyses were performed to characterize the physical and physicochemical properties of the substrate materials used in the lysimeters. These analyses were conducted to better understand the properties of the treatment media and their potential influence on hydraulic behavior, oxygen distribution, microbial activity, and pollutant removal performance. Similar analytical approaches have previously been applied for the characterization of substrates used in constructed wetland systems.

Particle size distribution was determined using a mechanical sieve shaker equipped with a series of sieves covering different particle size classes. Prior to analysis, substrate samples were dried under laboratory conditions. The samples were then sieved for a fixed period, and the mass retained on each sieve was measured using an analytical balance. The percentage of each particle size fraction was subsequently calculated from the measured masses. These analyses were used to evaluate substrate structure and potential effects on water movement and aeration within the lysimeters.

Additional physicochemical analyses included pH, electrical conductivity (EC), cation exchange capacity (CEC), and base saturation. These parameters were measured to characterize the chemical properties of the treatment media and assess factors that may influence nutrient retention and microbial development within the substrate.

Proximate analyses were also performed to determine the mineral and organic fractions of the substrate materials. Samples were first dried at 103–105 °C until constant mass was achieved. Approximately 1 g of dried substrate was subsequently placed in ceramic crucibles and subjected to different thermal treatments in a laboratory furnace. Labile organic matter was estimated after heating at 250 °C for 4 h, ash content was determined after heating at 750 °C for 3.5 h, and volatile matter was measured after heating at 950 °C for 10 min. Samples were weighed before and after each thermal treatment using an analytical balance, and mass differences were used to calculate the corresponding fractions (Muniz Sacco et al., 2025).

Dissolved oxygen conditions within the treatment media were monitored using oxygen sensor spots (SP-PSt3-NAU) connected to a Fibox-4 oxygen meter. Sensors were installed at depths of 5, 50, and 95 cm within the lysimeters. Dissolved oxygen profiles were measured during three monitoring campaigns conducted on 13 February, 4 March, and 1 May 2026. These measurements were used to evaluate oxygen distribution throughout the substrate profile, compare aerobic conditions between treatment configurations, and identify potential limitations in oxygen transfer associated with substrate ageing or clogging development during operation (Venditti et al., 2022).

## 2.3 Plants

Plants were introduced into selected lysimeters to investigate the influence of vegetation on the treatment performance of the vertical flow constructed wetland systems. The planted units were used to evaluate the contribution of macrophytes to pollutant removal and biological activity within the treatment media under controlled laboratory conditions.

Macrophytes can influence treatment processes through several mechanisms. Their root systems provide surfaces for microbial colonization and promote the development of an active rhizosphere within the substrate. Oxygen released by the roots can improve aerobic conditions in the treatment media and support microbial processes involved in the degradation of organic matter and nutrient transformation. Plants also contribute to water uptake through evapotranspiration. In addition, the presence of rigid stems above the substrate surface can improve air circulation and help limit the formation of surface crusts that may develop under intermittent top-feeding conditions in vertical flow constructed wetlands (Venditti et al., 2022). The plant species used in the experiment and the rationale for their selection are described in the following section.

### 2.3.1 Plant species selection and rationale

The planted lysimeters contained a polyculture composed of *Phragmites australis* and *Iris pseudacorus*. These macrophyte species were selected because they are widely used in constructed wetland applications and are well adapted to permanently wet conditions. Both species develop extensive root and rhizome networks that promote oxygen transfer, microbial colonization, and nutrient uptake within the treatment media (Beral et al., 2023).

*Phragmites australis* is characterized by dense and deep rhizomes associated with important root biomass, whereas *Iris pseudacorus* develops coarse roots and fleshy rhizomes. These root structures can favour rhizosphere activity and support aerobic degradation processes within the wetland media. Similar macrophytes have previously been used in vertical flow constructed wetlands treating wastewater effluent and emerging contaminants under controlled laboratory conditions (Beral et al., 2023; Venditti et al., 2022). The polyculture configuration was selected to reproduce vegetation conditions frequently observed in constructed wetlands and increase plant diversity within the systems. The association of both species was also intended to promote more heterogeneous root development throughout the treatment layer and support microbial activity in the rhizosphere zone.

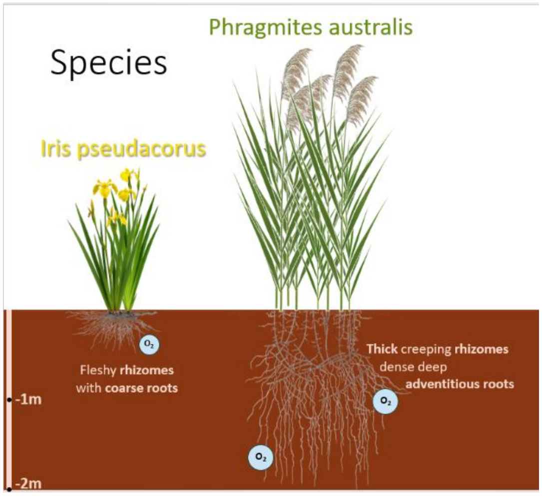


Figure 2.3 Phragmites australis and Iris pseudacorus used in the experiment and schematic representation of their root systems.

## 2.3.2 Plant origin, supplier, and initial condition

The plants were introduced into the lysimeters during the setup phase of the experiment. Prior to transplantation, the root systems were carefully washed with tap water to remove attached soil particles. A soap bath was subsequently applied to eliminate potential pests and contaminants before planting.

After cleaning, the plants were weighed to determine their initial fresh biomass and distributed between the planted lysimeters according to the experimental design. Comparable plant density and initial biomass were maintained between duplicate units to ensure similar starting conditions.

Prior to planting, the upper 10 cm of treatment media was inoculated with substrate and rhizosphere material collected from the Hersberg pilot-scale constructed wetland. This system had been operated for approximately four years using treated wastewater effluent and contained the same plant species and a similar substrate composition as the experimental lysimeters. The inoculum was incorporated into the upper substrate layer before plant installation to introduce an established microbial community into the newly constructed systems. Following inoculation, the plants were transplanted directly into the treatment layer and arranged according to the experimental configuration (Beral et al., 2023; Venditti et al., 2022).

### 2.3.3 Plant monitoring and maintenance

Plant development and health conditions were monitored regularly throughout the experimental period. Visual observations were carried out to follow plant growth, leaf condition, color changes, and overall vegetation development inside the planted lysimeters.

During the first weeks after transplantation, particular attention was given to plant acclimation under indoor laboratory conditions. The condition of both *Phragmites australis* and *Iris pseudacorus* was monitored throughout the study period, and no significant plant mortality was observed after planting.

Artificial lighting was maintained for 14 h $d^{-1}$ throughout the experiment using red–blue–white horticultural LED lamps specifically developed for plant cultivation. The lighting system was used to support plant growth and maintain stable indoor cultivation conditions during the study. Fresh biomass was measured before transplantation and again at the end of the experimental period to evaluate plant development during operation. The vegetated lysimeters were also inspected regularly for signs of stress, reduced growth, chlorosis, or abnormal plant development (Beral et al., 2023; Venditti et al., 2022).

## 2.4 Synthetic wastewater

Synthetic treated wastewater was used mimic treated municipal wastewater effluent. Synthetic wastewater allowed stable hydraulic operation and more homogeneous influent composition throughout the study, particularly for TOC, nutrient concentrations and micropollutant levels. Similar synthetic wastewater approaches have previously been used in laboratory-scale vertical flow constructed wetland studies investigating wastewater polishing and micropollutant removal under controlled operating conditions (Muniz Sacco et al., 2025; Venditti et al., 2022).

### 2.4.1 Synthetic wastewater preparation

The synthetic treated wastewater was prepared using tap water according to an adapted OECD 303A formulation used to simulate treated municipal wastewater effluent (Venditti et al., 2022)

The influent was prepared in a 400 L stainless-steel storage tank with a working volume of approximately 350 L connected to the lysimeter feeding system.

Table 2.1 Composition of the synthetic treated wastewater used during the experiment.

| Compound | Formula | CAS Number | Amount added to 350 L (g) | Final concentration (mg $L^{-1}$) |
|---|---|---|---|---|
| **Peptone ex meat** | — | 73049-73-7 | 15.75 | 45 |
| **Meat extract** | — | N/A | 12.25 | 35 |
| **Urea** | $CH_4N_2O$ | 57-13-6 | 2.80 | 8 |
| **Potassium dihydrogen phosphate ($KH_2PO_4$)** | $KH_2PO_4$ | 7778-77-0 | 1.05 | 3 |

The formulation was designed to reach target concentrations of approximately 60 mg $L^{-1}$ COD, 15 mg $L^{-1}$ TN, 2.5 mg $L^{-1}$ $PO_4$-P and 5 mg $L^{-1}$ $NO_3$-N.
Before the preparation of a new batch, the storage tank was emptied, rinsed with tap water and disinfected with ethanol to limit microbial growth and algae development inside the storage system. Continuous mixing was maintained during operation to preserve homogeneous wastewater composition throughout the feeding cycles. New batches were prepared regularly during the experimental period to maintain stable operating conditions for all lysimeters (Venditti et al., 2022).

### 2.4.2 Micropollutant selection and stock solution preparation

Three micropollutants were selected during the experiment: carbamazepine, diclofenac, and tris(chloropropyl) phosphate (TCPP). These compounds were chosen because they are frequently detected in treated wastewater effluents and represent different classes of emerging contaminants relevant to environmental monitoring and European regulatory frameworks. In addition, validated analytical methods and documented transformation products are available for all three compounds. The selected micropollutants also exhibit contrasting biodegradation behaviors and transformation pathways. Carbamazepine is considered a persistent pharmaceutical with limited biodegradation and numerous reported transformation products. Diclofenac presents intermediate biodegradation potential with several known transformation pathways, whereas TCPP represents a persistent organophosphate flame retardant with documented biological and oxidative degradation products. The combination of these compounds allowed the evaluation of treatment performance for micropollutants displaying different environmental fates and removal mechanisms (Venditti et al., 2022, 2024). Stock solutions of each micropollutant were prepared separately in methanol and stored in amber glass bottles at −20 °C until use. During influent preparation, 20 mL of stock solution was added to the synthetic wastewater storage tank to obtain a final concentration of 5 μg $L^{-1}$ for each compound in the influent. After addition, continuous mixing was maintained to ensure homogeneous distribution of the micropollutants throughout the wastewater before hydraulic distribution to the lysimeters. The selected concentration was intended to reproduce environmentally relevant levels commonly reported for micropollutants in treated wastewater effluents while remaining suitable for controlled laboratory-scale investigations (Venditti et al., 2022).

**Carbamazepine**

Carbamazepine was selected as a representative persistent pharmaceutical compound frequently detected in wastewater treatment plant effluents. Due to its low biodegradability and limited removal during conventional wastewater treatment, it is commonly used as a reference compound in micropollutant removal studies. Numerous transformation products have been reported for carbamazepine, making it a useful indicator for evaluating contaminant fate during biological treatment processes.

**Diclofenac**

Diclofenac was selected because it is one of the most frequently reported anti-inflammatory pharmaceuticals in wastewater effluents and aquatic environments. Previous studies have shown variable removal efficiencies depending on operating conditions, vegetation, and substrate characteristics. Several transformation products and biodegradation pathways have been described, making diclofenac suitable for assessing treatment performance under different wetland configurations.

**TCPP**

Tris(chloropropyl) phosphate (TCPP) was selected as a representative organophosphate flame retardant commonly detected in wastewater and environmental waters. TCPP is considered a persistent contaminant, but documented transformation products and degradation pathways have been reported under biological and oxidative conditions. Its inclusion allowed the assessment of treatment processes acting on contaminants with physicochemical properties different from those of pharmaceutical compounds (Venditti et al., 2022, 2024).

### 2.4.3 Hydraulic loading and intermittent feeding operation

The lysimeters were operated under intermittent feeding conditions throughout the experiment. Wastewater distribution was controlled using programmable peristaltic pumps connected to the synthetic wastewater storage tank. The feeding system was configured to reproduce the hydraulic functioning commonly applied in vertical flow constructed wetlands treating wastewater effluent under aerobic conditions (Venditti et al., 2022).

Each lysimeter received 7.07 L $d^{-1}$ distributed through three feeding cycles per day. Feeding events were programmed at 09:00, 13:00, and 18:00. Each feeding event lasted 30 min and corresponded to a hydraulic loading rate of 100 L $d^{-1}$ $m^{-2}$. During each programmed cycle, wastewater was transferred from the storage tank to the different lysimeters through the feeding network.

Intermittent loading was selected because resting periods between feeding events can promote oxygen renewal inside the treatment media and support aerobic degradation processes within vertical flow systems (Venditti et al., 2022, 2024).

The hydraulic functioning of the systems was monitored throughout the experiment. Regular inspections were performed to verify pump operation, wastewater distribution between the lysimeters, and the proper functioning of the feeding network during the different loading cycles. The influent storage tank was also checked during operation to maintain stable feeding conditions and homogeneous wastewater composition before distribution to the systems (Venditti et al., 2022).

## 2.5 Sampling strategy

Different sampling procedures were carried out throughout the experiment in order to monitor the treatment performance and operating conditions of the vertical flow constructed wetland systems.

Influent and effluent water samples were collected throughout the experimental period. During the first month of operation, water sampling was performed on a weekly basis to monitor the start-up phase of the lysimeters and verify stable system functioning. After the first month, water sampling was performed once per month until the end of the experiment. Water quality analyses included measurements of temperature, dissolved oxygen, electrical conductivity, oxidation-reduction potential, chemical oxygen demand, total organic carbon, inorganic carbon, total nitrogen, ammonium, phosphate, and pH.

Samples were collected from the influent storage tank and from the outlet of each lysimeter using identical procedures for all treatment configurations. This approach allowed direct comparison between planted and unplanted systems as well as between standard and biochar-amended substrates under the same hydraulic operating conditions.

Substrate samples were collected for granulometric and physicochemical characterization of the treatment media. Rhizosphere samples were collected for DNA and RNA extraction analyses to evaluate microbial presence and activity within the root zone. Plant observations were conducted throughout the experiment to assess vegetation establishment, growth, and adaptation to the experimental conditions.

Dissolved oxygen profiles were measured within the treatment media using optical oxygen sensors installed at different depths in the lysimeters. Measurements were performed at depths of 5 cm, 50 cm, and 95 cm to evaluate oxygen distribution throughout the treatment layer.

The same sampling and analytical procedures were applied to all experimental units throughout the study to ensure comparability between treatment configurations (Venditti et al., 2022).

### 2.5.1 Water sampling

Water samples were collected from both influent and effluent streams throughout the experiment to evaluate treatment performance in the different lysimeter configurations. During the first month of operation, water sampling was performed weekly to monitor system stabilization and evaluate the first treatment responses. After the start-up phase, water sampling was performed once per month until the end of the experiment.

Influent samples were collected from the dedicated sampling barrel providing a 24 h composite sample representative of the synthetic wastewater supplied to the lysimeters. Effluent samples were collected independently from the outlet of each lysimeter after vertical percolation through the substrate profile. The same sampling procedure was applied to all experimental units to compare the different configurations under identical operating conditions (Venditti et al., 2022).

Influent and effluent volumes were recorded during each monitoring campaign to evaluate the hydraulic performance and water balance of the systems. Water samples were collected directly in 1 L amber glass bottles according to the analytical requirements. Depending on the analytical procedure, samples were analyzed immediately after collection or stored under refrigerated conditions before laboratory analysis.

### 2.5.2 Dissolved oxygen profiling

Dissolved oxygen (DO) profiles were monitored throughout the experiment to evaluate oxygen distribution within the treatment media under intermittent feeding conditions. Oxygen measurements were performed using SP-PSt3-NAU oxygen sensor spots connected to a Fibox-4 oxygen meter. The sensor spots were installed directly inside the lysimeters, allowing non-destructive measurements within the substrate profile.

The oxygen sensor spots were positioned at depths of 5 cm, 50 cm, and 75 cm from the top of the treatment layer (Figure 2.X). These positions corresponded to the upper, intermediate, and lower sections of the substrate profile. DO values were recorded directly inside the columns without disturbing the surrounding media.

Measurements were performed during each monitoring campaign in all lysimeters throughout the experimental period. The same measurement procedure was applied to all treatment configurations, including planted and unplanted systems as well as standard and biochar-amended substrates. The resulting DO profiles were used to evaluate vertical oxygen distribution within the treatment layer and to compare oxygen conditions between the different wetland configurations. (Venditti et al., 2022). Position of dissolved oxygen sensor spots is presented in Figure 2.4.

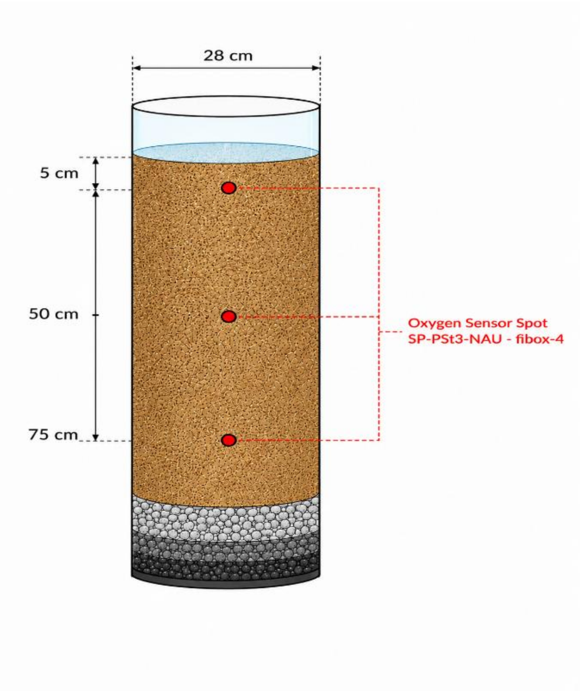


Figure 2.4 Position of dissolved oxygen sensor spots installed within the lysimeter at depths of 5, 50 and 75 cm.

### 2.5.3 Substrate and rhizosphere sampling

Substrate and rhizosphere samples were collected at the beginning of the experiment (T0) before the start of wastewater feeding. Sampling was performed to characterize the physical, chemical, and microbiological properties of the materials used in the lysimeters.

Sampling procedures were carried out using sterile tools and gloves to minimize contamination. Before sampling, equipment was cleaned with a bleach solution and rinsed several times with deionized water. Rhizosphere samples were collected directly from the root zone of the planted lysimeters, whereas bulk substrate samples were collected from the substrate material used in the experimental units. A total 15 g of material was collected for each sample.

Immediately after collection, samples intended for microbiological analyses were transferred into sterile sampling bags, preserved on dry ice, and stored at −80 °C until DNA and RNA extraction. Additional substrate samples were collected for granulometric analysis, pH determination, and furnace-based physicochemical characterization (Venditti et al., 2022).

### 2.5.4 Plant sampling and biomass monitoring

Plant monitoring was performed monthly throughout the experiment to evaluate vegetation development inside the planted lysimeters. Visual observations were carried out during operation to follow plant growth, leaf condition, color changes, and the overall health status of *Phragmites australis* and *Iris pseudacorus* under indoor laboratory conditions.

During the first weeks after transplantation, regular observations were performed to verify successful acclimation of the plants inside the wetland systems. Before plantation, the remaining soil attached to the roots was removed carefully using tap water according to the starting protocol. Fresh biomass measurements were also recorded during plant preparation before installation into the lysimeters.

Vegetation development was followed throughout the monitoring period to identify possible signs of stress, reduced growth, leaf degradation, or mortality within the systems. Observations were performed simultaneously for the duplicate lysimeters to maintain comparable vegetation conditions between the different wetland configurations during operation.

Monitoring plant development was considered important because vegetation growth and rhizosphere activity can influence pollutant removal inside vertical flow constructed wetland systems (Beral et al., 2023; Venditti et al., 2022).

## 2.6 Analytical methods

Water, substrate, rhizosphere, and plant samples collected during the experiment were analysed to evaluate the performance of the vertical flow constructed wetland systems. Water samples were used to monitor physicochemical conditions, organic matter removal, nutrient dynamics, suspended solids retention, and selected micropollutants. Substrate and rhizosphere samples were analysed to characterize physicochemical properties and microbial indicators associated with the different wetland configurations.

The monitored parameters included temperature, pH, dissolved oxygen (DO), electrical conductivity (EC), oxidation-reduction potential (ORP), total suspended solids (TSS), chemical oxygen demand (COD), total organic carbon (TOC), inorganic carbon (IC), total nitrogen (TN), NO3, NH4, PO4, dissolved oxygen profiles within the substrate, substrate physicochemical properties, and DNA/RNA-related microbial analyses. The analytical procedures applied for each parameter are described in the following sections.

### 2.6.1 Water analysis

Physicochemical parameters measured in water samples included pH, dissolved oxygen (DO), electrical conductivity (EC), oxidation-reduction potential (ORP), and temperature.

pH measurements were performed using a WTW pH 3310 portable meter equipped with a pH electrode. Electrical conductivity (EC), oxidation-reduction potential (ORP), and temperature measurements were carried out using a WTW pH/Cond 3320 portable meter equipped with the corresponding probes. Dissolved oxygen concentrations in influent and effluent water samples were measured using a HANNA Instruments HI 9146 dissolved oxygen meter.

Chemical oxygen demand (COD) concentrations were determined in influent and effluent water samples collected throughout the experiment.

Before analysis, samples were homogenized manually. COD measurements were performed using HACH cuvette tests. Following digestion, the cuvettes were cooled to room temperature prior to spectrophotometric determination. COD concentrations were subsequently measured using a HACH LANGE DR 3900 spectrophotometer according to standard wastewater analytical procedures (Venditti et al., 2022).

Total suspended solids (TSS) analyses were carried out using filtration and gravimetric procedures Glass fibre filters (45 µm pore size) were dried under controlled laboratory conditions and weighed using analytical balances to determine the initial filter mass. Known sample volumes were subsequently filtered through the pre-weighed filters using a laboratory vacuum filtration system connected to an ILMVAC vacuum pump. During filtration, suspended particles were retained on the filter surface while the liquid phase passed through the filter.

After filtration, the filters containing suspended solids were dried and weighed again. TSS concentrations were calculated from the difference between the initial and final filter masses relative to the filtered sample volume (Venditti et al., 2022).

Nutrient analyses were performed to determine concentrations of TOC, IC, TN, NO3, NH4, and PO4 in influent and effluent samples. Before analysis, 45 mL of water samples were filtered using glass fiber filters (45 µm pore size) to remove suspended particles and transferred into analytical vials according to laboratory procedures.

Anion analyses were performed using a Thermo Scientific Dionex ion chromatography system equipped with a conductivity detector and a Dionex AS-DV autosampler. The remaining sample volume was used for TOC, IC, and TN determination using a Shimadzu TOC-L CPN analyzer. Depending on the analytical procedure, dilution and sample handling steps were performed using calibrated micropipettes and Milli-Q water to ensure suitable analytical conditions before injection (Venditti et al., 2022, 2024).

Water samples intended for micropollutant analysis were collected and preserved according to laboratory procedures. Following filtration and preparation, the samples were transferred into dedicated LC–MS vials and stored until analysis.

The samples will be injected into the LC–MS system in September; therefore, micropollutant results are not presented in this thesis (Muniz Sacco et al., 2025; Venditti et al., 2022, 2024).

### 2.6.2 Granulometric and physicochemical characterization of substrates

The physical properties of the substrates were characterized through granulometric and physicochemical analyses prior to the start of the experiment. Granulometric analysis was performed to determine particle size distribution and substrate homogeneity. Approximately 200 g of dried substrate were placed in a mechanical sieve shaker (Retsch AS 200, Germany) equipped with a series of sieves of decreasing mesh sizes. The samples were sieved for 5 min at an amplitude of 45 Hz, and the mass retained on each sieve was recorded to determine the particle size distribution of the substrates (Muniz Sacco et al., 2025).

Physicochemical characterization focused on the determination of organic labile matter, ash content, volatile matter, and bulk density. Moisture content was first removed by drying the samples at 103–105 °C until constant weight was achieved. Approximately 1 g of dried substrate was then placed in ceramic crucibles and subjected to successive thermal treatments in a muffle furnace (Carbolite ELF 11/14B). Labile organic matter was determined after heating at 250 °C for 4 h, ash content after combustion at 750 °C for 3.5 h, and volatile matter after heating at 950 °C for 10 min in closed crucibles. Mass losses recorded after each thermal treatment were used to calculate the corresponding fractions. Bulk density was determined gravimetrically using dried substrate samples (Muniz Sacco et al., 2025).

### 2.6.3 Soil analysis

DNA and RNA were extracted using the RNeasy PowerSoil Total RNA Kit and the RNeasy Power Soil DNA Elution Kit (Qiagen) following the manufacturer's protocol.

The physical properties of the substrates were characterized through granulometric and physicochemical analyses prior to the start of the experiment. Granulometric analysis was performed to determine particle size distribution and substrate homogeneity. Approximately

200 g of dried substrate were placed in a mechanical sieve shaker (Retsch AS 200, Germany) equipped with a series of sieves of decreasing mesh sizes. The samples were sieved for 5 min at an amplitude of 45 Hz, and the mass retained on each sieve was recorded to determine the particle size distribution of the substrates (Muniz Sacco et al., 2025).

Physicochemical characterization focused on the determination of organic labile matter, ash content, volatile matter, and bulk density. Moisture content was first removed by drying the samples at 103–105 °C until constant weight was achieved. Approximately 1 g of dried substrate was then placed in ceramic crucibles and subjected to successive thermal treatments in a muffle furnace (Carbolite ELF 11/14B). Labile organic matter was determined after heating at 250 °C for 4 h, ash content after combustion at 750 °C for 3.5 h, and volatile matter after heating at 950 °C for 10 min in closed crucibles. Mass losses recorded after each thermal treatment were used to calculate the corresponding fractions. Bulk density was determined gravimetrically using dried substrate samples (Muniz Sacco et al., 2025).

### 2.6.4 Batch experiments for Indigo Carmine removal

Pollutant removal kinetics were investigated through duplicate batch. The experiments were conducted under controlled laboratory conditions using substrate materials collected from the different treatment configurations. Approximately 2 g of dried substrate material were transferred into 250 mL Erlenmeyer flasks. Subsequently, 200 mL of Milli-Q water produced using a Thermo Scientific Smart2Pure Pro UV/UF purification system were added to each flask. Indigo Carmine was used as a model pollutant at an initial concentration of 5 mg $L^{-1}$. Blank controls without substrate were prepared and analysed in parallel under identical conditions.

The flasks were placed on a Thermo Scientific MaxQ 2000 orbital shaker and continuously agitated at 150 rpm at room temperature throughout the experiment. Samples were collected after 0, 0.5, 2, 6, 24, 48, 74, 86, 172, and 264 h of contact time to monitor concentration changes over time. At each sampling event, samples were collected using adjustable micropipettes and filtered through 0.45 μm syringe membrane filters prior to analysis. Indigo Carmine concentrations were determined using a VWR UV-6300PC double-beam spectrophotometer operated at 608 nm. Calibration was performed using standard Indigo Carmine solutions prepared over the concentration range used during the experiments. The amount of dye remaining in solution was determined from the absorbance measurements, and concentration profiles were used to compare pollutant removal behaviour between the different substrate materials. All gravimetric measurements required during sample preparation were performed using an OHAUS analytical balance. (Muniz Sacco et al., 2025).

### 2.6.5 Evapotranspiration measurement

Evapotranspiration (ET) was monitored throughout the experimental period to evaluate water losses occurring within the vertical flow constructed wetland systems. The influent volume supplied to each lysimeter was known from the feeding protocol, while the effluent volume was determined by collecting the outflow in individual collection tanks positioned beneath each lysimeter. Before each measurement, the empty collection tanks were weighed using a KERN DE 60K5N laboratory balance. After the monitoring period, the tanks containing the collected effluent were weighed again and the net water mass was calculated by subtracting the empty tank weight from the total weight. Assuming a water density of 1 kg $L^{-1}$, the measured mass was converted directly into water volume. Evapotranspiration was subsequently estimated using a water balance approach based on the difference between the known influent volume and the measured effluent volume according to Equation X (Beral et al., 2023).

$$ET = | V_{in} - V_{out} |$$

where ET is the evapotranspiration (L), Vin is the influent volume supplied to the lysimeter (L), and Vout is the measured effluent volume collected at the outlet (L). Higher ET values indicated greater water losses through evaporation and plant transpiration within the lysimeters.

## 2.7 Data analysis

Experimental data obtained during monitoring campaigns were compiled and organized using Microsoft Excel and RStudio (version 4.4.3). Data from influent and effluent samples were grouped according to sampling campaigns and lysimeter configurations before statistical analysis.

Descriptive statistics, including mean values and standard deviations, were calculated for all monitored parameters. Graphical representations were generated using RStudio and included boxplots, temporal evolution plots, QQ plots, and dissolved oxygen profile plots to visualize treatment performance and compare the different wetland configurations.

Dataset normality was evaluated using QQ plots and Shapiro–Wilk tests. For parameters that did not meet normality assumptions, log transformation was applied prior to statistical analysis. Differences between treatment configurations were evaluated using Repeated measures ANOVA. When significant differences were detected ($p < 0.05$), Tukey post-hoc tests were performed to identify pairwise differences between treatment groups.

The statistical analysis focused on comparing planted and unplanted systems as well as expanded clay and biochar-amended treatment media operated under identical hydraulic conditions.

In addition to concentration data, removal efficiencies were calculated for selected water quality parameters to facilitate comparison of treatment performance among the different wetland configurations. Removal efficiency was determined using influent and effluent concentrations according to the following equation:

$$Removal\ efficiency\ (\%) = \frac{Cinfluent - Ceffluent}{Cinfluent} \times 100$$

where Cinfluent is the influent concentration and Ceffluent is the corresponding effluent concentration. The calculated removal efficiencies were used only for graphical visualization and comparison of overall treatment performance.

# 3. Results

## 3.1 Substrate Characterization

### 3.1.1 Granulometric distribution

The particle size distribution of the sand and biochar-amended substrates is presented in Figure 3.1.

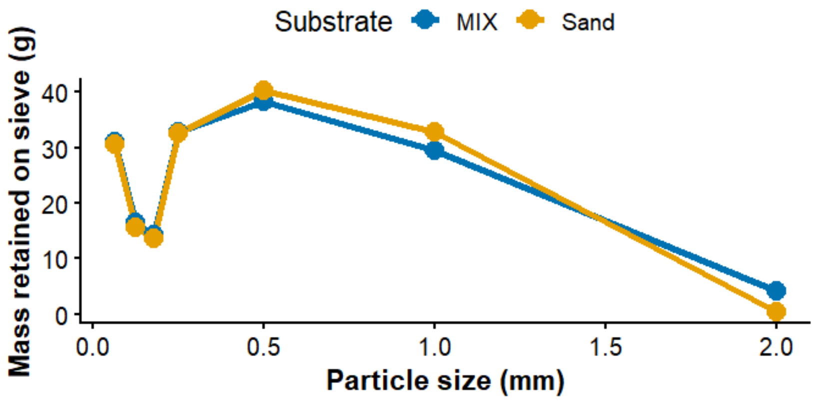


Figure 3.0.1 Particle size distribution of the sand and biochar-amended substrates.

Both substrates exhibited a similar particle size distribution pattern. The highest proportion of material was retained in the intermediate particle size fractions, particularly around 0.5 and 1.0 mm. Lower proportions were observed in both the finest and coarsest fractions. Only minor differences were observed between the two substrates across the different sieve sizes. Overall, the addition of biochar did not substantially modify the granulometric distribution of the substrate.

### 3.1.2 Physicochemical Properties

The physicochemical properties of the sand and biochar-amended substrates were evaluated to assess the influence of biochar addition on substrate characteristics. Organic matter fractions and bulk density measurements were used to compare the two substrate types. The results are presented in Figures 3.2A and 3.2B.

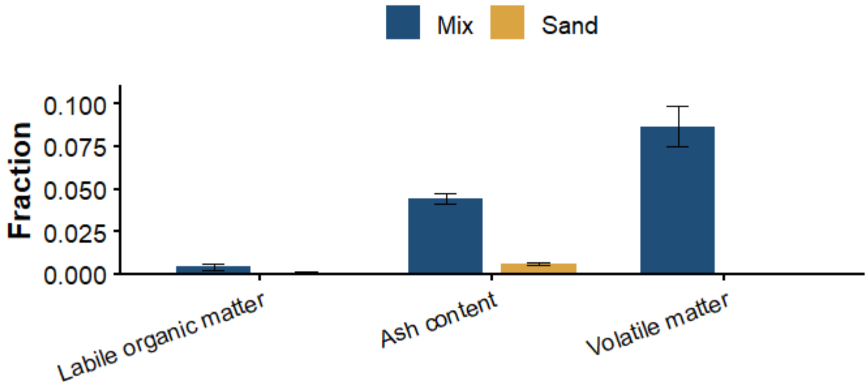


Figure 0.2A Organic fractions of the sand and biochar-amended substrates, including labile

The biochar-amended substrate (Mix) exhibited higher values of labile organic matter, ash content and volatile matter than the sand substrate. The largest difference was observed for volatile matter, which was substantially higher in the biochar-amended substrate. These results reflect the higher organic matter content introduced by the addition of biochar.

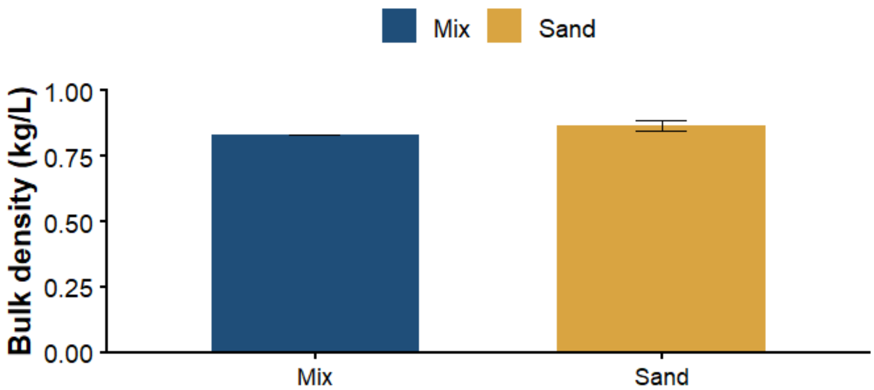


Figure 3.2 Bulk density of the sand and biochar-amended substrates.

Bulk density was slightly lower in the biochar-amended substrate than in the sand substrate.

### 3.1.3 Indigo Carmine removal kinetics in sand and mixed substrates

The evolution of inorganic carbon (IC) concentrations during the batch experiment is presented in Figure 3.3 IC concentrations decreased progressively in both substrate types throughout the monitoring period. However, the reduction occurred more rapidly in the mixed substrate than in the sand substrate.

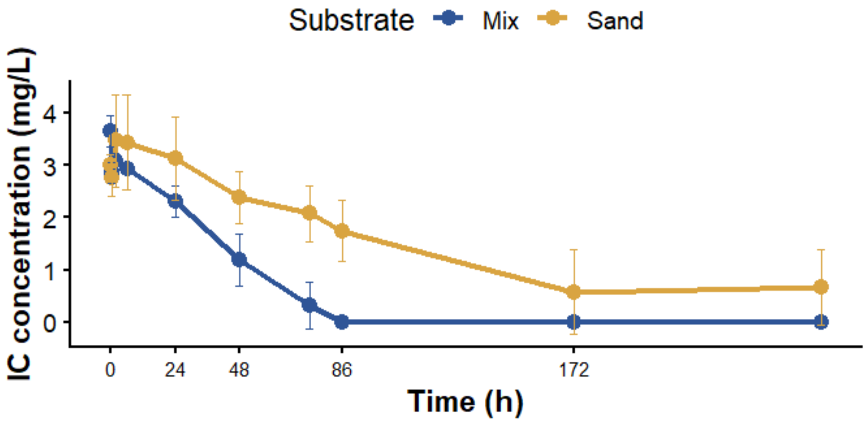


Figure 3.3 Evolution of Indigo Carmine concentrations in sand and mixed substrates during the 172-h batch experiment.

In the mixed substrate, Indigo Carmine concentrations decreased rapidly during the first 48 h and reached values close to zero after approximately 86 h. Thereafter, concentrations remained near zero until the end of the experiment. In contrast, a more gradual decrease was observed in the sand substrate. Residual Indigo Carmine concentrations were still detected after 172 h, indicating a slower removal process compared with the mixed substrate. Overall, the mixed substrate exhibited a higher removal efficiency and faster Indigo Carmine depletion throughout the monitoring period.

## 3.2 Microbial Results

DNA and RNA concentration extracted are presented in Figure 3.4.

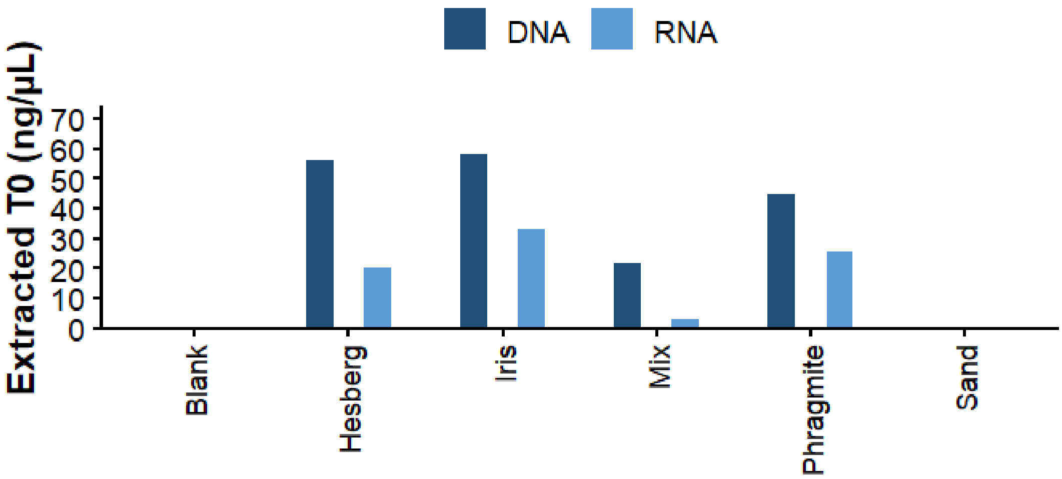


Figure 3.4 DNA and RNA concentrations measured in the different samples.

Detectable concentrations of both DNA and RNA were obtained in all samples except for the sand substrate. DNA concentrations were consistently higher than RNA concentrations across all investigated sample types. The highest DNA concentrations were measured in the Iris and Hesberg samples, whereas lower concentrations were observed in the Mix sample. RNA concentrations followed a similar pattern, with the highest values measured in the Iris and Phragmite samples. No detectable DNA or RNA was observed in the blank sample.

## 3.3 Water Quality and Treatment Performance

The treatment performance of the different lysimeter configurations was evaluated using physicochemical parameters, carbon fractions, nutrient concentrations and evapotranspiration measurements. The following sections present the results obtained during the first five months of monitoring. Statistical analyses were performed to compare the different treatment configurations, and the significance of the observed differences is reported for each parameter where applicable.

### 3.3.1 Temperature

The distribution of temperature values measured during the monitoring period is presented in Figure 3.5.

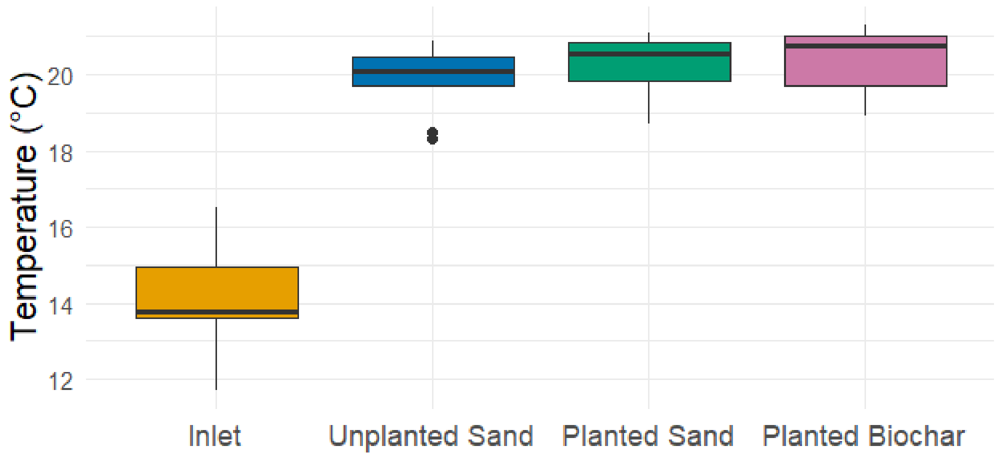


Figure 3.5 Temperature values measured in the influent and effluents during the monitoring period.

The influent exhibited the lowest temperature values, with a median temperature of approximately 14 °C. Higher temperatures were observed in all lysimeter effluents, with most values ranging between 19 and 21 °C. Temperature distributions were relatively similar among the three treatment configurations. Variability within each treatment configuration was limited throughout the monitoring period.

Statistical analysis revealed significant differences between the monitored groups (ANOVA, $p < 0.001$). Tukey post-hoc comparisons indicated that the planted sand and planted biochar systems differed significantly from the influent and unplanted sand groups.

### 3.2.2 Dissolved Oxygen (DO)

The distribution of dissolved oxygen (DO) concentrations measured during the monitoring period is presented in Figure 3.6.

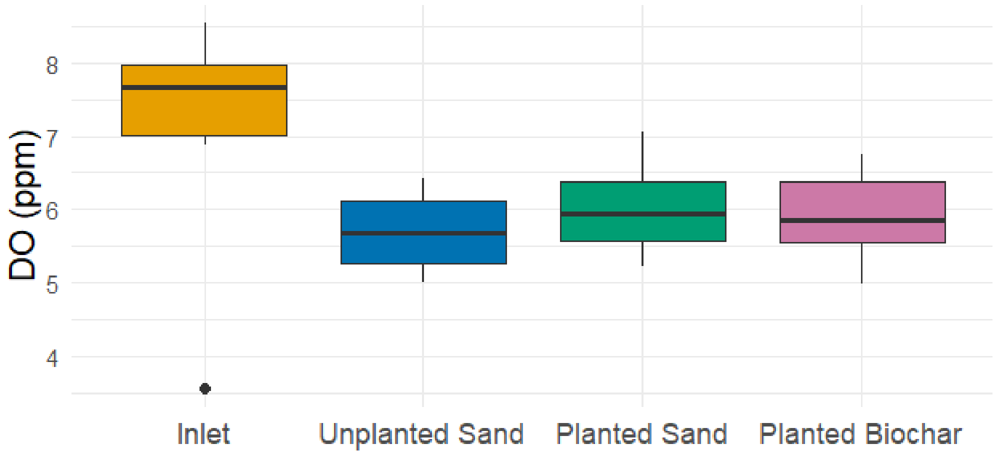


Figure3.6 Distribution of dissolved oxygen (DO) concentrations in influent and lysimeter effluents.

Influent samples exhibited the highest dissolved oxygen concentrations, with median values close to 8 mg $L^{-1}$. Lower DO concentrations were measured in all treatment systems, where median values generally ranged between approximately 5.5 and 6.2 mg $L^{-1}$. Similar DO distributions were observed among the unplanted sand, planted sand and planted biochar treatments, although slightly higher values were recorded in the planted systems. Variability within the treatment groups remained relatively limited throughout the monitoring period.

Statistical analysis revealed significant differences between treatment groups (ANOVA, $p < 0.05$). Tukey post-hoc comparisons indicated that the influent differed significantly from the unplanted sand treatment, whereas the planted sand and planted biochar treatments showed intermediate values.

### 3.3.3 Electrical Conductivity (EC)

The distribution of EC values measured during the monitoring period is presented in Figure 3.7.

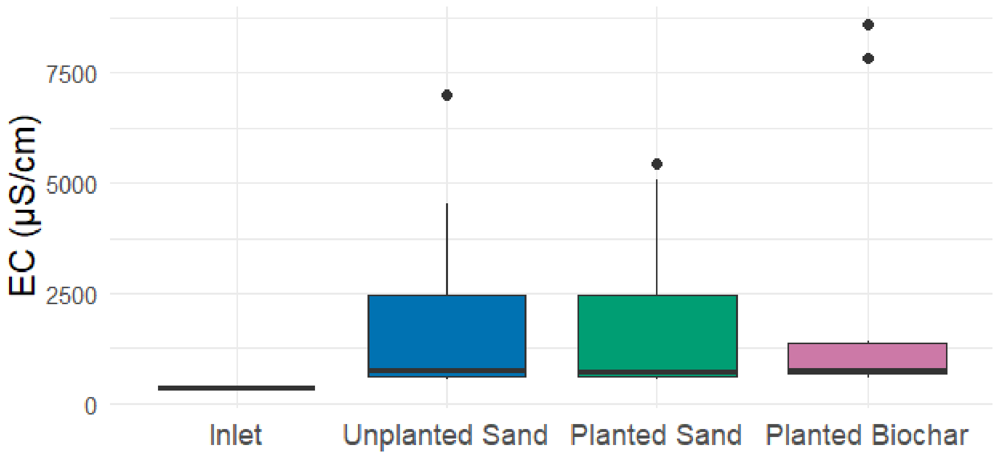


Figure 3.7 Distribution of EC values in influent and lysimeter effluents.

The influent exhibited the lowest EC values, with limited variability throughout the monitoring period. Higher EC values were observed in all lysimeter effluents. Median values were relatively similar among the unplanted sand, planted sand and planted biochar systems. Greater variability was observed in the treatment units compared with the influent, and several elevated values were recorded during the monitoring period. The highest individual EC values were observed in the planted biochar system, while similar overall distributions were recorded in the planted and unplanted sand treatments.

Statistical analysis showed no significant differences in EC between the treatment configurations (ANOVA, $p = 0.26$).

### 3.3.4 Oxidation–Reduction Potential (ORP)

The distribution of ORP values measured during the monitoring period is presented in Figure 3.8.

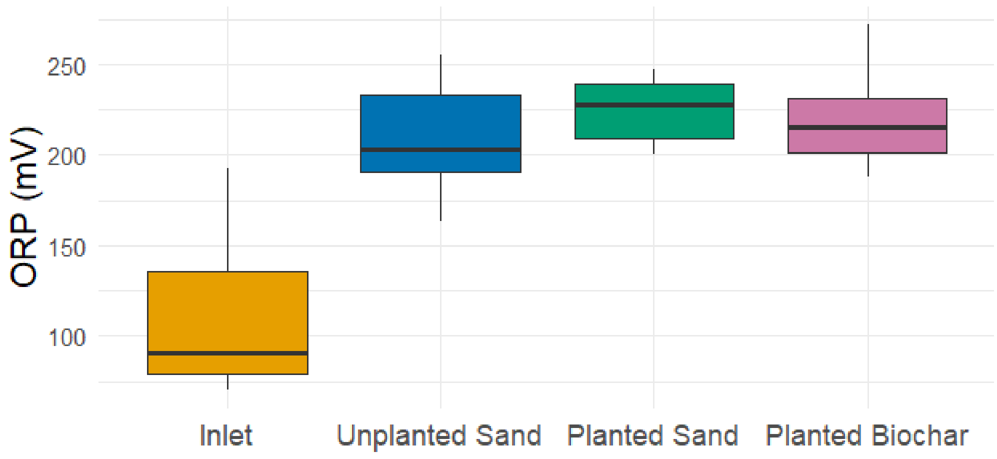


Figure 3.8 Distribution of oxidation–reduction potential (ORP) values in influent and lysimeter effluents.

ORP values were markedly higher in the treated effluents than in the influent. Median influent values were below 100 mV, whereas median effluent values ranged between approximately 200 and 230 mV. Similar ORP distributions were observed among the unplanted sand, planted sand and planted biochar systems. Positive ORP values were maintained in all treatment units throughout the monitoring period, indicating predominantly oxidative conditions within the lysimeters.

Statistical analysis revealed significant differences between the influent and the treatment systems (ANOVA, $p < 0.001$). Tukey post-hoc comparisons showed that all treatment configurations differed significantly from the influent, whereas no significant differences were detected among the three lysimeter treatments.

### 3.3.5 Chemical Oxygen Demand (COD)

The distribution of chemical oxygen demand (COD) concentrations measured during the monitoring period is presented in Figure 3.9.

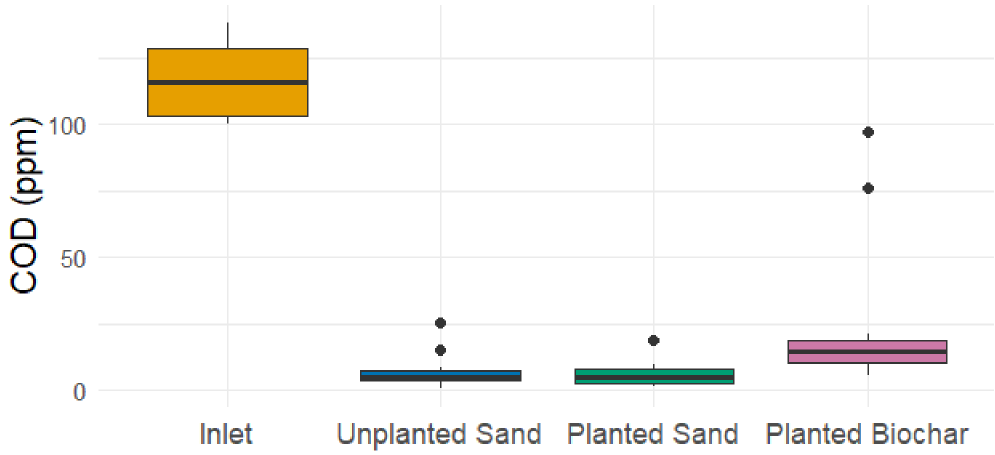


Figure 3.9. Distribution of chemical oxygen demand (COD) concentrations in influent and lysimeter effluents.

Influent samples exhibited the highest COD concentrations during the monitoring period, with median values above 100 mg $L^{-1}$. Lower COD concentrations were observed in all treatment systems. Median values in the unplanted sand, planted sand and planted biochar lysimeters were substantially lower than those measured in the influent. Although some elevated values were recorded in the planted biochar treatment, the overall distribution of COD concentrations remained considerably lower than in the influent.

Statistical analysis revealed significant differences in COD concentrations among the monitoring groups (ANOVA, $p < 0.001$). Tukey post-hoc comparisons showed that all treatment systems differed significantly from the influent, whereas no significant differences were detected among the three lysimeter configurations

### 3.3.6 Total Organic Carbon (TOC)

The distribution of TOC concentrations measured during the monitoring period is presented in Figure 3.10.

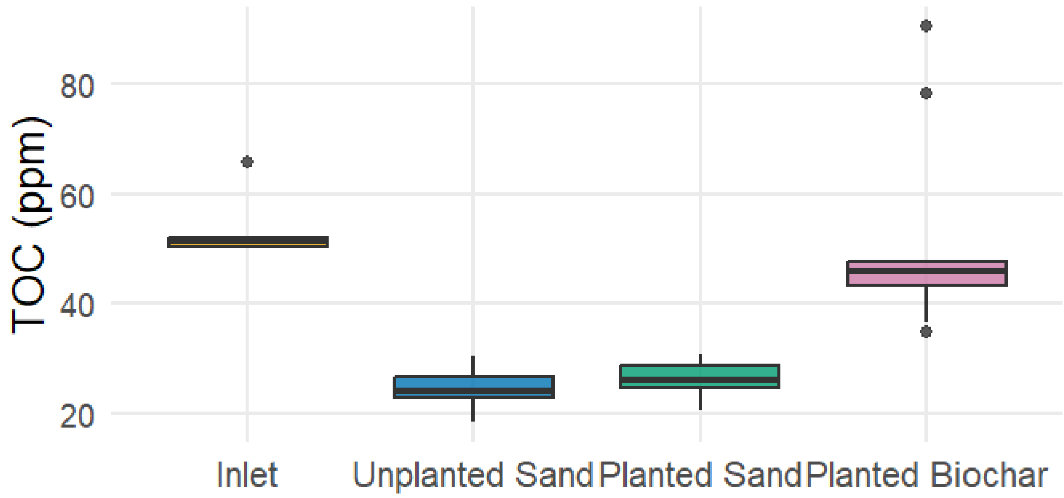


Figure 0.10 Distribution of total organic carbon (TOC) concentrations in influent and lysimeter effluents.

TOC concentrations were generally higher in the influent than in the treated effluents. Median influent concentrations were close to 50 mg $L^{-1}$. Lower TOC concentrations were observed in the unplanted sand and planted sand systems, where most values ranged between approximately 20 and 30 mg $L^{-1}$. Higher TOC concentrations were recorded in the planted biochar treatment, with median values closer to those measured in the influent. The planted biochar system also exhibited greater variability and several elevated measurements during the monitoring period.

### 3.3.7 Inorganic Carbon (IC)

The distribution of IC concentrations measured during the monitoring period is presented in Figure 3.11.

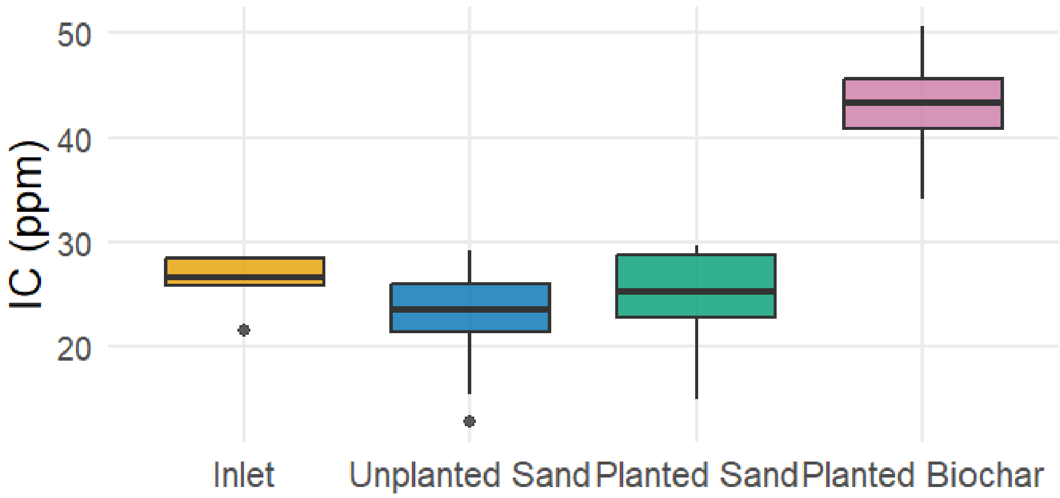


Figure 0.11 Distribution of inorganic carbon (IC) concentrations measured in influent and lysimeter effluents.

IC concentrations varied among the different treatment configurations. Influent samples exhibited median concentrations of approximately 27 mg $L^{-1}$. Similar IC distributions were observed in the unplanted sand and planted sand systems, with median values ranging between approximately 24 and 26 mg $L^{-1}$. Higher IC concentrations were measured in the planted biochar treatment, which exhibited the highest median values and the widest distribution of observations. Most measurements in this treatment ranged between approximately 35 and 50 mg $L^{-1}$.

### 3.3.8 Total Nitrogen (TN)

The distribution of TN concentrations measured during the monitoring period is presented in Figure 3.12.

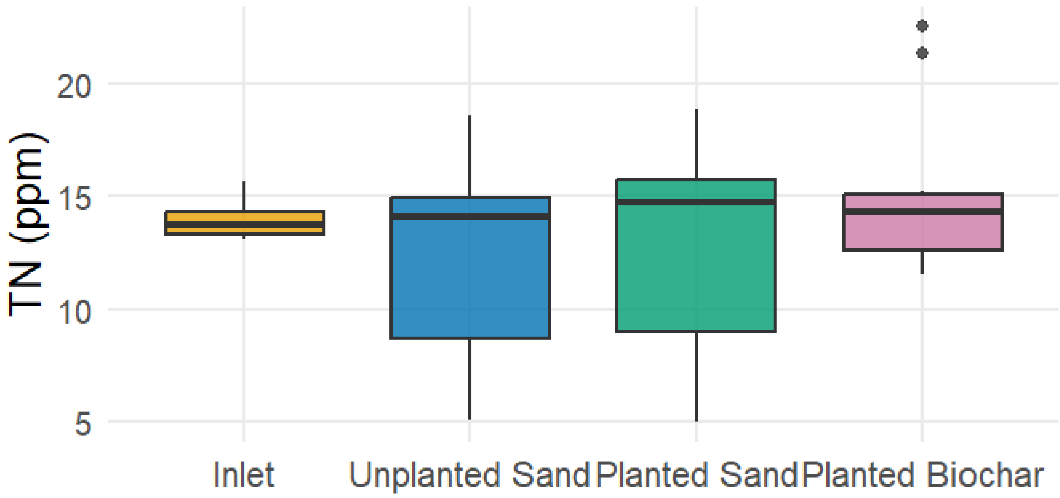


Figure 3.12 Distribution of total nitrogen (TN) concentrations measured in influent and lysimeter effluents.

TN concentrations showed variability throughout the monitoring period. Similar median concentrations were observed in the influent and in all treatment systems, with values generally ranging between approximately 14 and 15 mg $L^{-1}$. The unplanted sand and planted sand configurations exhibited the widest distributions, whereas influent values were more narrowly distributed around the median. The planted biochar system showed a relatively narrow distribution for most observations, although several elevated values were recorded during the monitoring period. Overall, TN concentrations remained within a comparable range across the different treatment configurations. Nevertheless, Repeated measures ANOVA indicated significant differences between treatments ($p < 0.001$).

### 3.3.9 NH4

The distribution of NH4-N concentrations recorded in the different treatment configurations is presented in Figure 3.13.

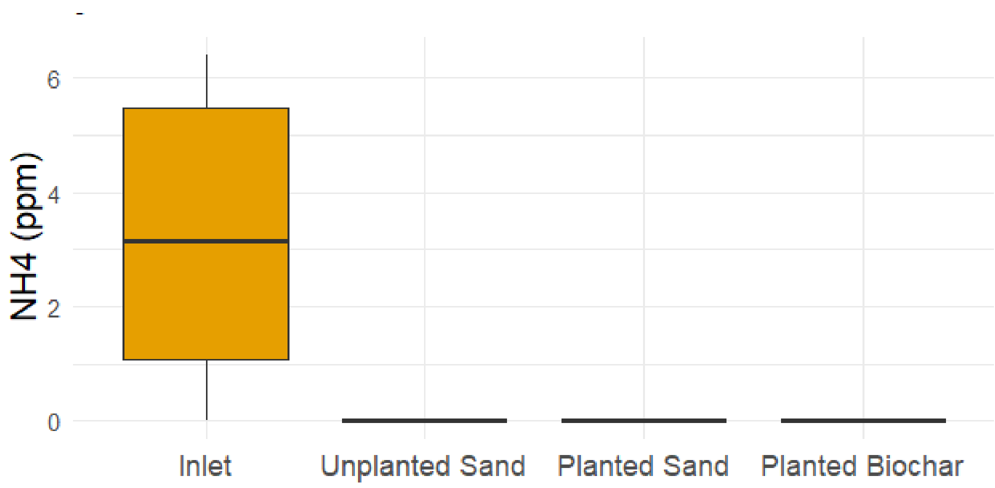


Figure 0.13 Distribution of NH4 concentrations measured in influent and lysimeter effluents.

The highest NH4-N concentrations were observed in the influent samples, with values ranging from near 0 to approximately 6.5 mg $L^{-1}$. In contrast, NH4-N concentrations in all treated effluents remained close to zero throughout the monitoring period. Similar distributions were observed in the unplanted sand, planted sand and planted biochar systems, where only very low concentrations were detected. Overall, NH4-N concentrations in the lysimeter effluents remained substantially lower than those measured in the influent samples during the experiment.

Repeated measures ANOVA indicated significant differences between treatments ($p < 0.001$). Tukey post-hoc comparisons showed that all treatment systems differed significantly from the influent, whereas no significant differences were observed among the unplanted sand, planted sand and planted biochar treatments.

### 3.3.10 NO3

The distribution of NO3 concentrations recorded in the different treatment configurations is presented in Figure 3.14.

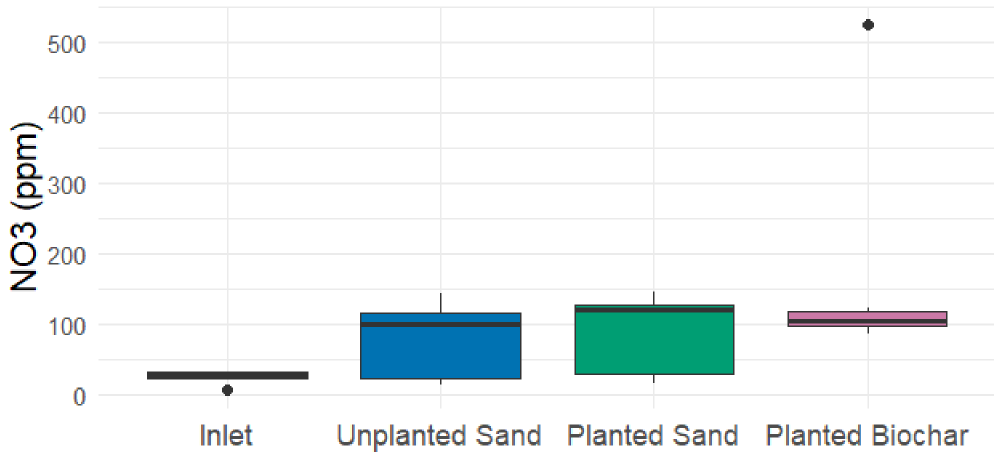


Figure 0.14 NO3 concentrations measured in the influent and treated effluents of the different lysimeter configurations during the monitoring period.

NO3 concentrations were substantially higher in all treatment configurations compared with the influent. The lowest concentrations were observed in the influent, whereas the unplanted sand, planted sand and planted biochar systems all exhibited elevated NO3 levels. The planted biochar treatment showed the highest median concentrations, while the unplanted sand and planted sand treatments displayed similar distributions. Greater variability was observed in the treatment systems than in the influent, particularly in the planted sand and planted biochar configurations. The increase in NO3 concentrations relative to the influent coincided with the reduction in NH4-N concentrations observed in the treatment units.

Repeated measures ANOVA indicated significant differences between treatments ($p < 0.05$). Tukey post-hoc comparisons showed that the planted biochar treatment differed significantly from the influent, whereas no significant differences were observed among the influent, unplanted sand and planted sand treatments.

### 3.3.11 $PO_4$

The distribution of $PO_4$ concentrations recorded in the different treatment configurations is presented in Figure 3.15.

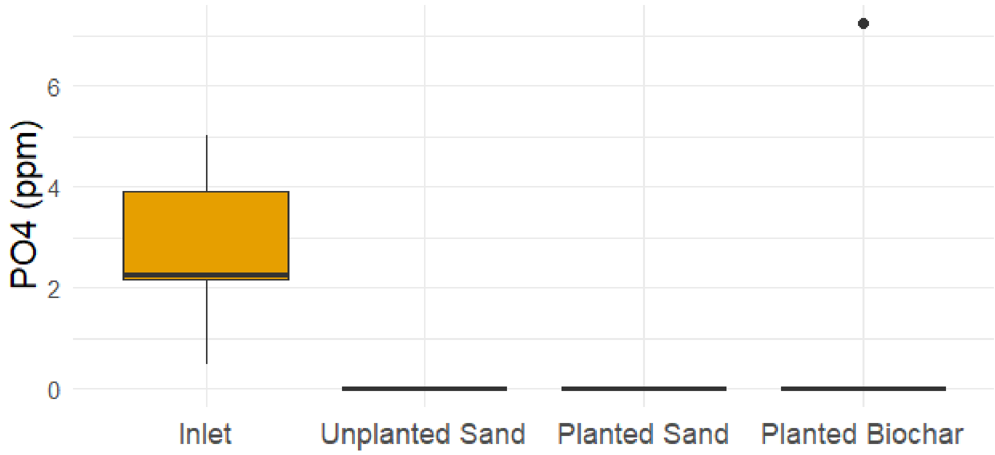


Figure 0.15 Distribution of orthophosphate ($PO_4$) concentrations measured in influent and lysimeter effluents.

The highest PO4 concentrations were observed in the influent samples, with values generally ranging between approximately 2 and 5 mg L−1. In contrast, PO4 concentrations in all treated effluents remained close to zero throughout most of the monitoring period. Similar distributions were observed in the unplanted sand, planted sand and planted biochar systems, although one elevated value was recorded in the planted biochar treatment. Overall, PO4 concentrations were substantially lower in all treatment systems than in the influent. Repeated measures ANOVA indicated significant differences between treatments ($p < 0.001$). Tukey post-hoc comparisons showed that all treatment configurations differed significantly from the influent, whereas no significant differences were observed among the unplanted sand, planted sand and planted biochar treatments.

### 3.3.12 pH

The distribution of pH values measured during the monitoring period is presented in Figure 3.16.

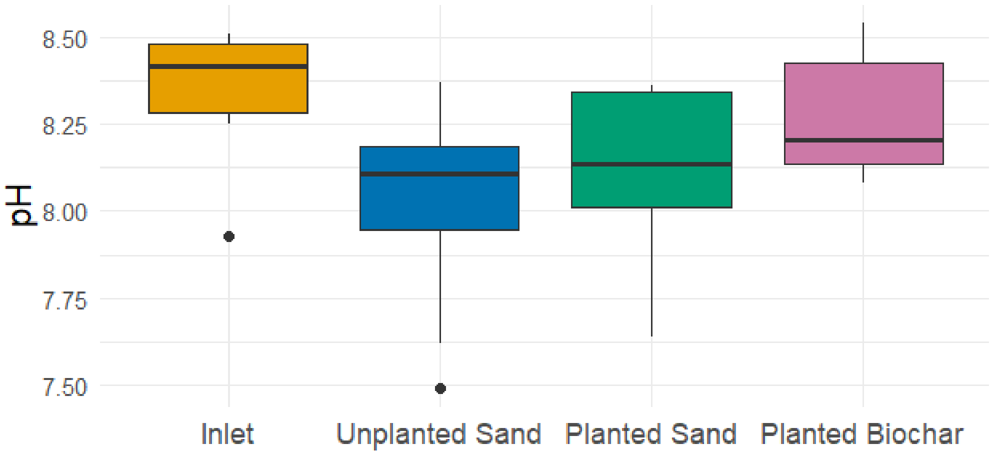


Figure 3.16 Distribution of pH values in influent and lysimeter effluents.

The influent exhibited the highest pH values, with median values around 8.4. Lower pH values were observed in all lysimeter effluents. Median values in the unplanted sand, planted sand and planted biochar systems ranged between approximately 8.1 and 8.2. Similar pH distributions were observed among the three treatment configurations, although slightly greater variability was recorded in the planted sand system.

Statistical analysis indicated a significant overall effect of treatment on pH (ANOVA, $p = 0.0139$). However, Tukey post-hoc comparisons did not identify significant pairwise differences between individual treatment groups.

### 3.3.13 Removal efficiencies of COD, N and P

The removal efficiencies of COD, N and P measured in the different treatment configurations are presented in Figure 3.17.

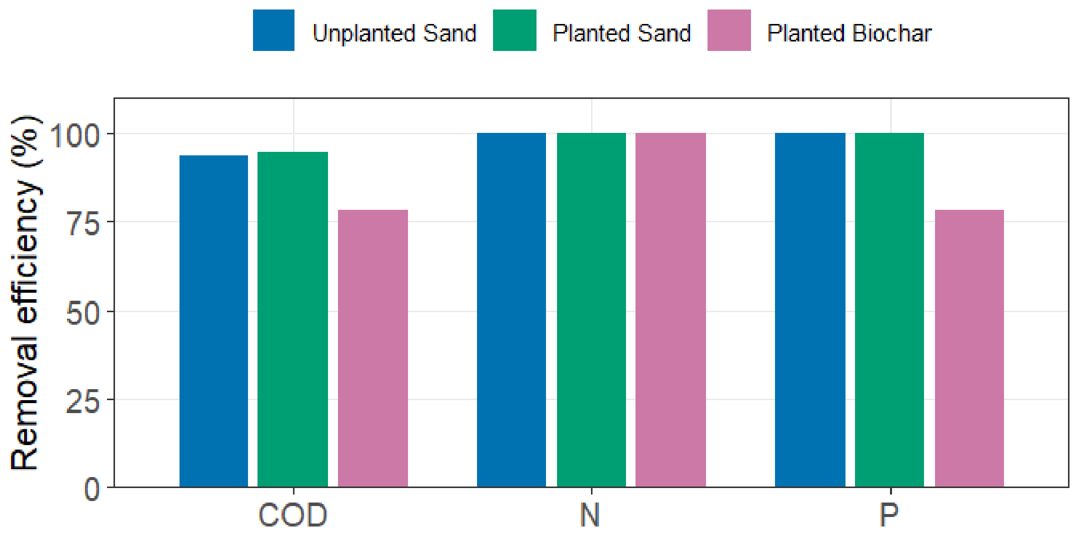


Figure 3.17 Removal efficiencies (%) of COD, N and P in the unplanted sand, planted sand and planted biochar systems, calculated from mean influent and effluent concentrations measured during the monitoring period.

High removal efficiencies were observed for all monitored parameters. COD removal exceeded 90% in the unplanted sand and planted sand systems, whereas lower values were observed in the planted biochar system. Nitrogen removal remained close to 100% in all treatment configurations. Phosphorus removal exceeded 99% in the unplanted sand and planted sand systems and was lower in the planted biochar treatment.

## 3.4 Dissolved Oxygen Profile

Dissolved oxygen was measured at three depths within the lysimeters (5, 50 and 75 cm). The results are presented in Figure 3.18.

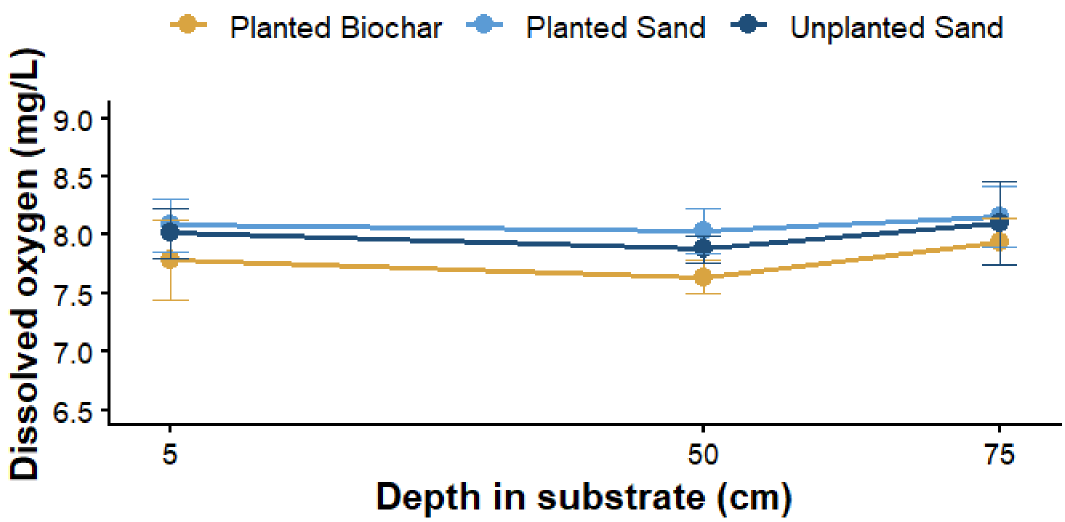


Figure 3.18 Dissolved oxygen profiles measured at 5, 50 and 75 cm within the lysimeters.

Dissolved oxygen concentrations remained relatively stable throughout the substrate profile in all treatment configurations (Figure 3.1). Values ranged between approximately 7.5 and 8.5 mg $L^{-1}$, with only minor differences observed between the monitored depths (5, 50 and 75 cm). No pronounced oxygen depletion was detected with increasing depth, indicating that aerobic conditions were maintained throughout the substrate profile during the monitoring period. Similar dissolved oxygen concentrations were measured in planted and unplanted systems as well as in biochar-amended and non-amended substrates. These results suggest that oxygen availability was not a limiting factor within the lysimeters under the applied operating conditions.

## 3.5 Evapotranspiration (ET)

The distribution of evapotranspiration values is presented in Figure 3.19.

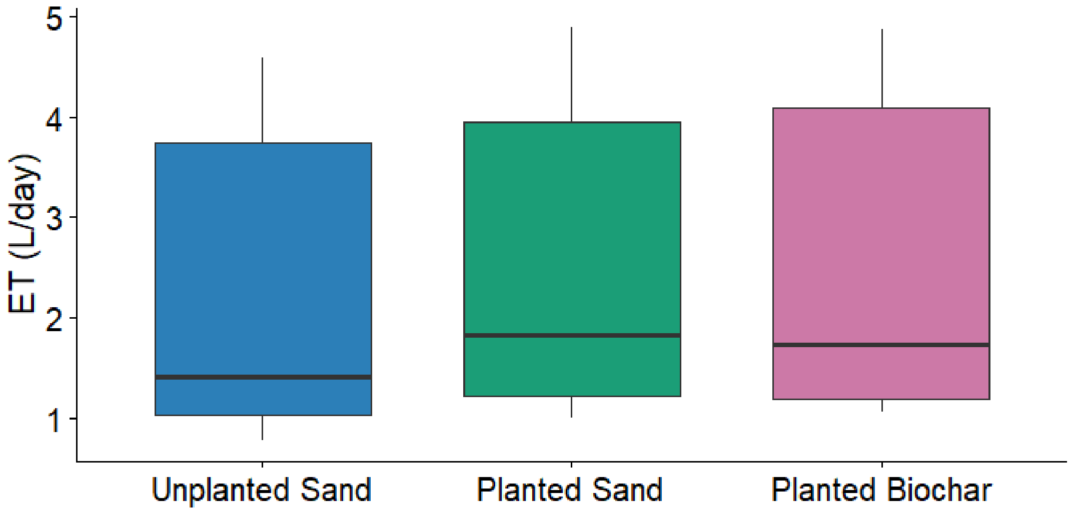


Figure 0.19 Distribution of evapotranspiration values measured in influent and lysimeter effluents.

Evapotranspiration (ET) values varied among the different treatment configurations during the monitoring period. Median ET values were slightly higher in the planted sand and planted biochar systems compared with the unplanted sand systems, although substantial overlap between the distributions was observed. Variability was present within all treatment groups, with ET values generally ranging between approximately 1 and 5 L $day^{-1}$. Overall, no clear separation between treatment configurations was observed based on the ET measurements.

## 3.6 Statistical analysis of treatment performance

Statistical analyses were performed using linear mixed-effects models to evaluate the influence of treatment configuration on the monitored water quality parameters. Parameters with p-values below 0.05 were considered significantly affected by treatment conditions and were subsequently examined using Tukey post-hoc comparisons to identify differences between treatment groups. Significant statistical differences identified during these analyses are reported within the corresponding results sections for each monitored parameter.

# 4. Discussion

## 4.1 Comparison with Literature

### 4.1.1 Substrate Characteristics

The granulometric analysis showed very similar particle size distributions in the sand and biochar-amended substrates, indicating that biochar addition did not substantially alter the physical structure of the treatment media. This observation is important because substrate granulometry directly influences hydraulic conductivity, water retention and oxygen transfer in vertical flow constructed wetlands. Previous studies have highlighted the importance of maintaining an appropriate particle size distribution to ensure efficient water percolation while reducing the risk of clogging and preserving aerobic conditions within the filter bed (Dotro et al., 2017; Kadlec & Wallace, 2009). Consequently, the comparable granulometric profiles observed in the present study suggest that differences observed later in treatment performance are unlikely to be primarily related to particle size effects.

In contrast, the addition of biochar clearly modified the physicochemical characteristics of the substrate. Higher contents of labile organic matter, ash and volatile matter were measured in the mixed substrate, with the most pronounced difference observed for volatile matter. These results reflect the incorporation of biochar into the substrate matrix and confirm that biochar influenced substrate composition more strongly than substrate structure. Similar observations have been reported in biochar-amended wetland systems, where biochar increased the organic and reactive fractions of the treatment media while maintaining the overall physical integrity of the substrate (Muniz Sacco et al., 2025; Venditti et al., 2024).

The mixed substrate also exhibited a slightly lower bulk density than the sand substrate. This difference is consistent with the lower density of biochar compared with mineral materials and further supports the influence of biochar on substrate characteristics. Overall, the results indicate that biochar primarily affected substrate composition rather than granulometric distribution. This distinction is important for interpreting the subsequent treatment results, as any differences observed between the sand and biochar-amended systems are more likely associated with the physicochemical properties introduced by biochar than with differences in particle size distribution. Similar findings have been reported in previous studies demonstrating that biochar can modify substrate characteristics while maintaining conditions suitable for the long-term operation of vertical flow constructed wetlands (Dotro et al., 2017; Muniz Sacco et al., 2025; Venditti et al., 2024).The faster Indigo Carmine removal observed in the mixed substrate suggests a greater adsorption capacity compared with the sand substrate alone. The incorporation of biochar likely increased the available surface area and the number of adsorption sites, thereby enhancing dye retention. Similar observations have been reported in studies investigating biochar-amended treatment media, where the addition of carbon-rich materials improved the removal of dissolved contaminants through adsorption and surface interactions.

The progressive decrease in Indigo Carmine concentrations over time indicates that adsorption was the dominant removal mechanism during the batch experiment. The rapid decline observed during the initial phase is consistent with the occupation of readily available adsorption sites, whereas the slower removal rates at later stages may reflect the gradual saturation of the substrate surface. Comparable adsorption patterns have been reported in laboratory-scale studies evaluating contaminant retention by porous substrates and biochar-based media.

The superior performance of the mixed substrates agrees with previous studies showing that biochar amendments can enhance pollutant retention compared with conventional mineral substrates. (Muniz Sacco et al., 2025). Similarly reported improved contaminant removal in substrates containing biochar, which was attributed to their higher sorption potential and more complex pore structure. These findings suggest that biochar incorporation may contribute to improved treatment performance and increased contaminant retention in constructed wetland substrates.

### 4.1.2 Microbial indicators

The detection of DNA and RNA in most investigated samples indicates the establishment of microbial communities within the experimental systems. The presence of RNA is particularly relevant because it is generally associated with metabolically active microorganisms, whereas DNA may also originate from inactive or dead cells. Therefore, the simultaneous detection of both nucleic acids suggests that biological processes were occurring within the substrates and plant-associated zones during the experiment.

Higher nucleic acid concentrations observed in samples associated with vegetation support the important role of the rhizosphere in constructed wetland functioning. Plant roots provide surfaces for biofilm development, release organic compounds that stimulate microbial growth, and contribute to the creation of favorable microenvironments for microbial activity. Similar interactions between plants, microorganisms, and substrate have been identified as key mechanisms supporting pollutant transformation and removal in vertical flow constructed wetlands.

The results are also consistent with recent findings showing that microbial processes become increasingly important during wetland maturation and may contribute substantially to pollutant removal alongside adsorption mechanisms. (Muniz Sacco et al., 2025) reported that long-term operation of vertical flow constructed wetlands promotes the development of active microbial communities and enhances biodegradation processes within the treatment media.

Consequently, the microbial indicators measured in this study provide additional evidence that biological processes contributed to treatment performance throughout the experimental period. These findings highlight the importance of microbe–plant–substrate interactions in supporting pollutant transformation within vertical flow constructed wetlands.

### 4.1.3 Physicochemical conditions

The lower temperatures observed in the influent were primarily related to the refrigerated storage conditions used for the synthetic wastewater tank. After entering the lysimeters, the wastewater was exposed to laboratory conditions and artificial lighting, resulting in slightly higher temperatures in the effluents. Therefore, the observed temperature increase cannot be attributed solely to treatment processes occurring within the wetlands.

Despite these differences between influent and effluent temperatures, only minor variations were observed among the three treatment configurations. This indicates that vegetation and substrate composition had a limited influence on thermal conditions within the systems. Similar studies on vertical flow constructed wetlands have reported that temperature mainly reflects operational and environmental conditions, while treatment performance is more strongly influenced by substrate properties, oxygen availability, and microbial activity (Dotro et al., 2017; Thalla et al., 2019). Consequently, temperature was not considered a limiting factor during the experimental period, and the differences observed in treatment performance were likely associated with biological and physicochemical processes rather than thermal effects.

Dissolved oxygen concentrations remained relatively high in all treatment configurations throughout the experiment, indicating that aerobic conditions were generally maintained within the vertical flow systems. The lower DO concentrations measured in the effluents compared with the influent suggest oxygen consumption by microbial degradation processes occurring during wastewater percolation through the substrate. Similar observations have been reported for vertical flow constructed wetlands, where oxygen is continuously consumed during the oxidation of organic matter and nitrogen compounds (Dotro et al., 2017; Thalla et al., 2019).

Only limited differences were observed between the unplanted sand, planted sand and planted biochar systems. Nevertheless, the slightly higher DO values recorded in the planted treatments may be associated with the presence of plant root systems, which can improve oxygen transfer and contribute to the maintenance of aerobic microenvironments in the rhizosphere. Similar effects of vegetation on oxygen availability have been described in planted wetland systems, although the magnitude of this influence depends on plant species, root development and operational conditions (Beral et al., 2023).

Overall, all configurations maintained suitable aerobic conditions. The limited differences between treatments suggest that intermittent feeding and vertical flow operation were the primary factors controlling oxygen availability, while the contribution of plants and biochar remained secondary under the conditions tested.

The absence of statistically significant differences between the treatment configurations suggests that vegetation and biochar amendment did not substantially modify the overall ionic environment within the systems. Similar observations have been reported in constructed wetland studies, where EC was found to be primarily influenced by mineralization processes and wastewater–substrate interactions rather than by vegetation effects alone (Dotro et al., 2017; Kadlec & Wallace, 2009). Nevertheless, the lower EC values generally observed in the planted biochar system may indicate a moderate influence of biochar on ionic dynamics within the treatment media. Previous studies have shown that biochar can alter physicochemical properties of wetland substrates and influence the retention of dissolved compounds through sorption processes (Venditti et al., 2022, 2024). Although this tendency was not statistically confirmed in the present study, it is consistent with the improved pollutant retention frequently reported for biochar-amended systems.

These findings suggest that EC was less sensitive to treatment-related differences than parameters directly associated with organic matter and nutrient transformations. Similar conclusions have been reported in previous studies, where substrate amendments had a stronger influence on contaminant removal than on bulk conductivity measurements. Therefore, parameters directly related to organic matter, nutrient, and micropollutant removal provide a more reliable indication of treatment efficiency than EC alone.

The consistently higher ORP values observed in all lysimeter effluents compared with the influent indicate that predominantly oxidative conditions were maintained within the treatment systems throughout the monitoring period. Positive ORP values are generally associated with oxygen availability and aerobic microbial activity, which are favorable for organic matter degradation and nitrification processes in vertical flow constructed wetlands. Similar observations have been reported in previous studies, where intermittent feeding and enhanced air exchange promoted oxidative environments within vertical flow systems (Dotro et al., 2017; Kadlec & Wallace, 2009). Although the planted sand and planted biochar systems exhibited slightly higher ORP values than the unplanted sand treatment, no significant differences were observed among the three treatment configurations. This suggests that the oxidative conditions were primarily controlled by the vertical flow design and intermittent loading regime rather than by plant presence or biochar amendment. Comparable findings were reported by (Venditti et al., 2022), who observed that oxidative conditions were maintained across different vertical flow wetland configurations, with only minor differences between treatment media.

The ORP results support the predominance of oxidative conditions within the lysimeters and are consistent with the efficient biological transformations observed during the experiment (Beral et al., 2023; Dotro et al., 2017; Kadlec & Wallace, 2009).

The pH values remained relatively stable throughout the monitoring period, indicating that the treatment systems maintained favorable chemical conditions for biological and physicochemical treatment processes. The slightly alkaline pH range observed in all configurations is consistent with values commonly reported for vertical flow constructed wetlands treating municipal and synthetic wastewaters (Kadlec & Wallace, 2009; Nivala et al., 2013). The slight decrease in pH observed in the treated effluents compared with the influent may be associated with nitrification processes occurring within the substrate. During nitrification, ammonium is oxidized to nitrate, releasing hydrogen ions and causing a moderate reduction in pH. The near-complete removal of $NH_4$-N together with the increase in $NO_3$ concentrations observed in the present study supports the occurrence of active nitrification. Similar relationships between ammonium oxidation, nitrate production and slight pH decreases have been reported in previous constructed wetland studies (Kadlec & Wallace, 2009; Vymazal, 2011). Despite minor differences among treatment configurations, pH values remained within a range considered suitable for microbial activity, organic matter degradation and nitrogen transformation processes. This suggests that stable environmental conditions were maintained throughout the experiment and that none of the treatment configurations generated pH conditions that could limit treatment performance.

### 4.1.4 Organic matter removal

The substantial reduction in COD concentrations observed in all treatment systems demonstrates the effective removal of biodegradable organic matter within the vertical flow constructed wetlands. The high dissolved oxygen and positive ORP values measured throughout the experiment indicate that aerobic degradation was likely the dominant mechanism responsible for organic matter removal. Similar relationships between aerobic conditions and efficient COD removal have been widely reported in vertical flow constructed wetlands treating municipal and synthetic wastewater (Dotro et al., 2017; Kadlec & Wallace, 2009). No significant differences were detected among the unplanted sand, planted sand and planted biochar systems, suggesting that the vertical flow configuration itself was sufficient to achieve high organic matter removal under the experimental conditions. This observation is consistent with the findings of (Venditti et al., 2022), who reported effective organic matter removal in laboratory-scale vertical flow systems regardless of substrate modification. Likewise, (Beral et al., 2023) observed that the contribution of vegetation may become less pronounced when oxygen transfer and hydraulic operation already provide favorable conditions for microbial degradation.

Although slightly higher residual COD concentrations were occasionally observed in the planted biochar treatment, overall removal performance remained high in all systems. The results therefore suggest that the high COD removal observed across all configurations was mainly related to the operational conditions of the vertical flow system rather than to vegetation or biochar amendment.

Similar conclusions have been reported in previous studies investigating vertical flow constructed wetlands operated under well-aerated conditions (Dotro et al., 2017; Kadlec & Wallace, 2009; Venditti et al., 2022). The reduction of TOC observed in the wetland systems confirms the capacity of vertical flow constructed wetlands to remove dissolved and particulate organic matter through a combination of filtration, adsorption and microbial degradation processes. According to Treatment Wetlands, organic carbon removal in vertical flow systems is primarily associated with aerobic biodegradation supported by intermittent feeding and enhanced oxygen transfer within the substrate matrix. These observations are consistent with oxidative carbon transformation processes reported for vertical flow wetlands (Dotro et al., 2017).

The lower TOC concentrations measured in the planted systems are consistent with the recognized contribution of vegetation to treatment performance. Plant roots promote microbial colonization, stimulate rhizosphere activity and improve oxygen transport within the substrate, thereby enhancing the degradation of organic compounds. Similar findings were reported by (Beral et al., 2023). who demonstrated that vegetated systems generally achieved greater pollutant retention and improved treatment performance compared with unplanted controls.

The behavior observed in the biochar-amended system suggests that biochar may influence carbon dynamics differently from conventional mineral substrates. Although biochar can provide additional surface area for microbial growth and pollutant adsorption, it may also contribute organic carbon release, particularly during the initial operational period or because of ageing processes (Muniz Sacco et al., 2025; Venditti et al., 2024). This could explain why TOC concentrations in the planted biochar treatment remained higher than those measured in the planted sand system despite the generally good treatment performance.

Similar organic matter removal performances have been reported in pilot- and laboratory-scale VFCWs, confirming the suitability of this technology as a polishing step for wastewater treatment (Thalla et al., 2019; Venditti et al., 2022).

The higher inorganic carbon (IC) concentrations observed in the planted biochar system may indicate an enhanced mineralization of organic matter within the wetland matrix. Under aerobic conditions, microbial degradation converts organic carbon into carbon dioxide, which subsequently dissolves in water as dissolved inorganic carbon species such as bicarbonate and carbonate. The positive ORP values and the well-oxygenated conditions measured throughout the experiment support the occurrence of these oxidative processes.

The simultaneous decrease in TOC and increase in IC suggests that part of the organic carbon pool was transformed rather than simply removed from the system.

Similar mechanisms have been described for vertical flow constructed wetlands, where efficient aeration promotes organic matter oxidation and carbon mineralization (Dotro et al., 2017). Biochar may further enhance these processes by providing a large specific surface area for microbial colonization and biofilm development, thereby stimulating microbial activity and carbon turnover (Venditti et al., 2024).

In addition, biochar-containing substrates can influence carbonate equilibria through their alkaline properties and mineral content, potentially contributing to higher dissolved inorganic carbon concentrations in the treated effluent. Studies investigating innovative biochar-based substrates in vertical flow constructed wetlands reported that biochar not only improves adsorption capacity but also modifies biogeochemical processes occurring within the substrate matrix (Muniz Sacco et al., 2025; Venditti et al., 2024).

Overall, the elevated IC concentrations measured in the planted biochar treatment are consistent with intensified organic matter transformation processes and may reflect a more advanced degree of carbon mineralization compared with the conventional sand-based systems.

### 4.1.5 Nutrient transformation

#### 4.1.5.1 Nitrogen dynamics

Total nitrogen removal remained relatively limited across all wetland configurations. Although some reduction compared with the influent was observed, TN concentrations in the effluents remained within a comparable range. This behavior is consistent with the operation of vertical flow constructed wetlands, where nitrification is generally favored by aerobic conditions, while complete nitrogen removal may be constrained by the limited availability of anoxic zones required for denitrification (Kadlec & Wallace, 2009; Nivala et al., 2013).

The predominance of aerobic conditions within the lysimeters likely favored nitrification, while limiting denitrification and therefore complete nitrogen removal.

(Dotro et al., 2017; Luo et al., 2014). No clear advantage of the planted biochar configuration was observed for total nitrogen removal. This suggests that under the operational conditions applied in this study, the presence of biochar and vegetation had a limited influence on overall nitrogen elimination. Similar findings have been reported in several constructed wetland studies where improvements in organic matter removal did not necessarily translate into substantially higher TN removal efficiencies. The near-complete removal of $NH_4$-N observed in all treatment configurations indicates that favorable conditions for nitrification were maintained throughout the experimental period. Similar behavior has been widely reported in vertical flow constructed wetlands, where intermittent loading enhances oxygen transfer and stimulates nitrifying microbial communities (Kadlec & Wallace, 2009; Nivala et al., 2013).

No clear differences were observed among the treatment configurations, suggesting that the overall system design and operating conditions were sufficient to promote ammonium oxidation regardless of vegetation or biochar addition. Similar findings have been reported by (Luo et al., 2014), who highlighted the dominant role of oxygen availability in controlling ammonium removal in aerobic treatment systems.

Although micropollutant concentrations were not available in the present study, $NH_4$-N removal may be considered a surrogate indicator of biological treatment activity. Efficient ammonium oxidation reflects the presence of active microbial communities and favorable aerobic conditions, which are also involved in the biodegradation of certain pharmaceutical compounds. Therefore, the high $NH_4$-N removal observed in this study suggests that the lysimeters provided environmental conditions that could support the transformation of biodegradable organic micropollutants.

However, caution is required when extrapolating $NH_4$-N removal to micropollutant behavior. Compounds such as diclofenac may be partially affected by aerobic biodegradation processes, whereas carbamazepine is generally considered highly persistent and poorly removed in both conventional wastewater treatment plants and constructed wetlands (Nivala et al., 2013; Verlicchi & Zambello, 2014). Moreover, TCPP removal is governed by different mechanisms and is not directly associated with nitrogen transformation processes. Consequently, $NH_4$-N removal should be regarded as an indirect indicator of favorable biological treatment conditions rather than direct evidence of micropollutant removal.

The increase in nitrate concentrations observed in the treated effluents is consistent with active nitrification within the vertical flow wetlands and confirms the efficient conversion of ammonium under the operating conditions applied.

Similar nitrate accumulation has frequently been reported in vertical flow constructed wetlands due to their enhanced oxygen transfer compared with horizontal flow systems (Kadlec & Wallace, 2009; Nivala et al., 2013). The intermittent feeding regime applied in the present study likely promoted aeration of the substrate and contributed to efficient ammonium oxidation.

The slightly higher nitrate concentrations observed in the planted biochar treatment may indicate enhanced microbial activity and improved oxygen diffusion within the biochar-amended substrate. Biochar has been reported to provide additional surface area for microbial colonization and to support the development of nitrifying communities (Luo et al., 2014). However, differences among the treatment configurations remained relatively limited, suggesting that the vertical flow operation itself was the dominant factor controlling nitrification performance.

Similar nitrogen transformation patterns have been widely reported in aerobic vertical flow wetlands treating municipal and synthetic wastewater (Kadlec & Wallace, 2009; Nivala et al., 2013).

#### 4.1.5.2 Phosphorus dynamics

The near-complete removal of orthophosphate observed in all treatment configurations indicates that phosphorus retention processes were highly effective throughout the experiment. Unlike nitrogen removal, which largely depends on biological transformations, phosphorus removal in constructed wetlands is mainly controlled by adsorption, precipitation and substrate retention mechanisms (Kadlec & Wallace, 2009).

The similarly low phosphate concentrations measured in the planted and unplanted systems suggest that the substrate played the dominant role in phosphorus removal. Previous studies have reported that mineral substrates can efficiently retain phosphate through sorption of metal oxides and other reactive surfaces, particularly during the early operational stages of wetland systems (Kadlec & Wallace, 2009; Vymazal, 2011).

The generally low phosphate concentrations observed in the biochar treatment are also consistent with studies showing that biochar may contribute additional sorption sites for phosphorus retention. However, the isolated elevated value recorded in the planted biochar system indicates that phosphorus retention may not be completely stable over time and can be influenced by temporary desorption processes or operational variability (Luo et al., 2014).

Similar phosphorus removal performances have been reported in vertical flow constructed wetlands treating municipal and synthetic wastewater, where substrate-related retention mechanisms play a dominant role in phosphate removal (Kadlec & Wallace, 2009; Nivala et al., 2013; Vymazal, 2011). The results obtained in the present study are consistent with these findings.

### 4.1.6 Removal efficiencies of COD, N and P

Similar removal efficiencies have been reported in previous studies investigating vertical flow constructed wetlands for wastewater treatment. (Kadlec & Wallace, 2009) highlighted that the combination of aerobic microbial degradation, filtration and physicochemical retention processes is largely responsible for pollutant removal in these systems. Likewise, (Nivala et al., 2013) reported efficient removal of conventional wastewater parameters in vertical flow wetlands operating under aerobic conditions.

The comparable treatment performances observed among the different configurations are consistent with findings reported by (Luo et al., 2014), who showed that treatment efficiency is governed by the interaction between biological, physical and hydraulic processes, making the contribution of individual design modifications difficult to isolate.

Although biochar amendments have been reported to enhance adsorption capacity and microbial habitat availability in some studies, their effects are not always reflected in higher removal efficiencies for conventional water quality parameters. Similar observations have been described in studies where biochar-related benefits were more evident for specific contaminants than for bulk indicators of wastewater quality (Beral et al., 2023; Luo et al., 2014).

### 4.1.7 Dissolved Oxygen Profile

The maintenance of aerobic conditions throughout the substrate supports the biological transformations observed in the lysimeters, particularly the efficient conversion of ammonium to nitrate. Similar relationships between dissolved oxygen availability and nitrogen transformations have been widely reported in vertical flow constructed wetlands (Kadlec & Wallace, 2009; Nivala et al., 2013). Although no pronounced differences were observed between planted and unplanted systems, phytoremediation studies have shown that species such as *Phragmites australis* can contribute to oxygen transfer into the rhizosphere, creating localized aerobic zones that support microbial activity and pollutant degradation. However, under the operating conditions of the present experiment, the intermittent feeding regime and vertical flow design appear to have played a greater role in maintaining oxygen availability than vegetation effects alone (Beral et al., 2023).

From the perspective of micropollutant removal, the observed oxygen profile is also relevant because aerobic microbial processes are frequently involved in the transformation and degradation of many pharmaceutical compounds and other contaminants of emerging concern. Therefore, oxygen availability was unlikely to be a limiting factor for biological treatment processes within the lysimeters during the monitoring period.

### 4.1.8 Evapotranspiration (ET)

The slightly higher evapotranspiration observed in the planted systems is consistent with previous findings for vegetated constructed wetlands, where plant water uptake and transpiration contribute to increased water losses compared with unplanted systems (Kadlec & Wallace, 2009; Venditti et al., 2022). The presence of *Phragmites australis* and *Iris pseudacorus* likely contributed to the observed evapotranspiration. Similar observations have been reported in vertical flow constructed wetlands, where vegetation influenced water balance and supported rhizosphere processes associated with treatment performance (Beral et al., 2023; Venditti et al., 2022). The absence of major differences between the planted sand and planted biochar systems suggests that vegetation rather than substrate amendment was the primary factor influencing evapotranspiration. Similar conclusions have been reported in previous studies, where evapotranspiration was found to be more strongly related to plant development and environmental conditions than to substrate composition (Kadlec & Wallace, 2009).

## 4.2 Plant and biochar effects

The results suggest that vegetation influenced internal treatment processes within the wetlands, although its effects were not always directly reflected in removal efficiencies. Previous studies have shown that the contribution of wetland vegetation is often indirect, occurring through rhizosphere development and plant–microbe interactions rather than through direct pollutant uptake alone (Beral et al., 2023; Kadlec & Wallace, 2009).

The relatively small differences between planted and unplanted systems suggest that treatment performance was primarily controlled by the vertical flow configuration. Similar observations have been reported by (Nivala et al., 2013), who found that the influence of vegetation may become less evident when favorable treatment conditions are already maintained within the substrate.

The effect of biochar was less evident when considering conventional water quality parameters alone. However, the biochar-amended systems exhibited differences in nitrate and inorganic carbon dynamics, suggesting that biochar may have influenced internal biogeochemical processes. Previous studies have reported that biochar can provide additional surfaces for microbial colonization, modify substrate physicochemical properties and affect carbon and nutrient cycling within treatment media (Venditti et al., 2022, 2024).

The absence of a consistent improvement in removal efficiency does not necessarily indicate that biochar was ineffective. Some authors have highlighted that the benefits of biochar are often more apparent for specific contaminants, including micropollutants, than for conventional wastewater quality indicators such as COD, nitrogen or phosphorus (Luo et al., 2014; Venditti et al., 2022). Therefore, part of the contribution of biochar may not be fully reflected by the parameters monitored in the present study.

The limited differences observed between treatments may also be related to the duration of the experiment, as the effects of vegetation and substrate amendments often become more pronounced during longer operational periods (Kadlec & Wallace, 2009; Venditti et al., 2024).

Overall, vegetation and biochar acted as complementary factors influencing specific biological and physicochemical processes within the wetlands, while the overall treatment performance remained high across all configurations.

## 4.3 Micropollutant removal mechanisms

Although micropollutant concentrations were not available for direct evaluation, several observations provide insight into the potential removal mechanisms operating within the wetland systems. Constructed wetlands remove micropollutants through a combination of biodegradation, adsorption, plant-associated processes and physicochemical transformations, with the relative importance of each mechanism depending on the characteristics of the target compound (Luo et al., 2014; Verlicchi & Zambello, 2014).

The near-complete removal of $NH_4$-N observed in all treatment configurations suggests the presence of active biological processes and environmental conditions favorable for micropollutant transformation.

However, the relationship between nitrogen transformations and micropollutant removal is compound-specific. Diclofenac has been reported to undergo partial biodegradation under aerobic conditions, whereas carbamazepine is generally recognized as one of the most persistent pharmaceutical compounds in wastewater treatment systems and constructed wetlands (Verlicchi & Zambello, 2014). Consequently, the favorable aerobic conditions observed in the present study may have supported diclofenac transformation to a greater extent than carbamazepine removal.

In the biochar-amended systems, adsorption may represent an additional removal mechanism. Previous studies have shown that biochar can increase the retention of organic contaminants by providing a large specific surface area and additional sorption sites (Venditti et al., 2022, 2024) Such mechanisms may be particularly relevant for compounds exhibiting limited biodegradability, where adsorption can complement biological removal processes.

Vegetation may also contribute indirectly to micropollutant attenuation through rhizosphere-mediated processes that enhance contaminant transformation and biodegradation (Beral et al., 2023; Kadlec & Wallace, 2009). Although direct measurements of carbamazepine, diclofenac and TCPP were not available, the environmental conditions observed in the present study are consistent with mechanisms previously associated with micropollutant attenuation in constructed wetlands. Therefore, the tested configurations likely provided favorable conditions for micropollutant removal processes, although the actual removal efficiencies could not be quantified.

# 5. Conclusion

The objective of this study was to evaluate the performance of laboratory-scale vertical flow constructed wetlands treating synthetic wastewater containing selected micropollutants and to investigate the influence of vegetation and biochar amendment on treatment processes.

The comparison between planted and unplanted systems showed that all configurations achieved high treatment performance for conventional wastewater parameters. Only limited differences were observed between vegetated and non-vegetated systems in terms of overall removal efficiencies. However, vegetation influenced internal wetland processes, as reflected by higher evapotranspiration rates and the presence of microbial indicators associated with plant-root environments. These findings suggest that the contribution of vegetation was mainly indirect and related to rhizosphere processes rather than direct pollutant uptake.

The evaluation of biochar amendment showed that biochar modified substrate physicochemical properties and enhanced Indigo Carmine adsorption during batch experiments. Differences in nitrate and inorganic carbon dynamics also suggested an influence of biochar on internal biogeochemical processes. Nevertheless, these effects were not consistently reflected in higher removal efficiencies for conventional water quality parameters, indicating that the benefits of biochar may be more evident for specific contaminants and long-term treatment processes than for bulk wastewater indicators.

Regarding nutrient removal, all systems achieved near-complete removal of $NH_4$-N and orthophosphate, while total nitrogen removal remained limited. The simultaneous decrease in ammonium and increase in nitrate concentrations indicated active nitrification within the wetlands. Dissolved oxygen and redox measurements further confirmed that aerobic conditions were maintained throughout the systems and supported biological treatment processes.

Although direct measurements of micropollutants were not available, the results provided valuable information regarding conditions potentially favorable for micropollutant attenuation. The efficient ammonium removal, maintenance of aerobic conditions, adsorption potential associated with biochar, and evidence of active microbial communities suggest that several mechanisms commonly involved in micropollutant removal were present within the investigated systems. However, the actual removal efficiencies of carbamazepine, diclofenac and TCPP could not be quantified and therefore remain uncertain.

Finally, the detection of DNA and RNA within the substrates and plant-associated zones confirmed the establishment of active microbial communities. These findings improved the understanding of biological processes occurring within the FORGOTTEN project systems and highlighted the importance of interactions between substrate, vegetation and microorganisms in supporting pollutant transformation.

Overall, the study demonstrated that laboratory-scale vertical flow constructed wetlands can provide efficient removal of conventional wastewater pollutants while maintaining environmental conditions favorable for biological and physicochemical treatment processes. Future work should include direct micropollutant analyses and longer operational periods to better evaluate the specific contributions of vegetation and biochar to micropollutant removal and long-term treatment performance.

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# Appendix A

Supplementary Statistical Results:

Table 3.1 Summary of statistical analyses (linear mixed-effects models) for the monitored water quality parameters.

| Parameter | p-value | Significant difference |
|---|---|---|
| **Temperature** | <0.001 | Yes |
| **DO** | <0.05 | Yes |
| **EC** | 0.26 | No |
| **ORP** | <0.001 | Yes |
| **COD** | <0.001 | Yes |
| **$NH_4$** | <0.001 | Yes |
| **$NO_3$** | <0.05 | Yes |
| **$PO_4$** | 0.0003 | Yes |
| **pH** | 0.0139 | Yes |

**Note:** Significant treatment effects were considered at $p < 0.05$. Parameters showing significant effects were further evaluated using Tukey post-hoc comparisons.

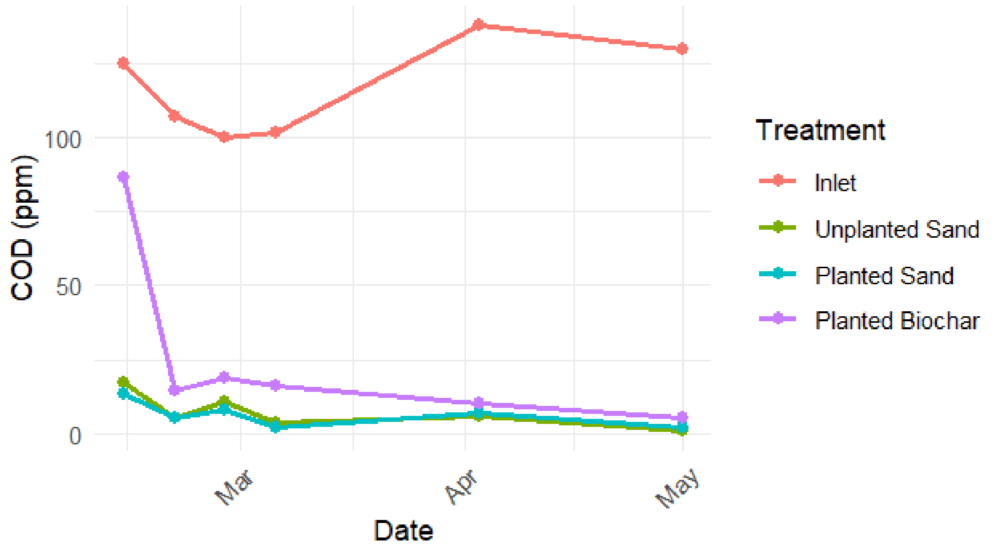
Table 3.2. Tukey post-hoc comparisons for parameters showing significant treatment effects.

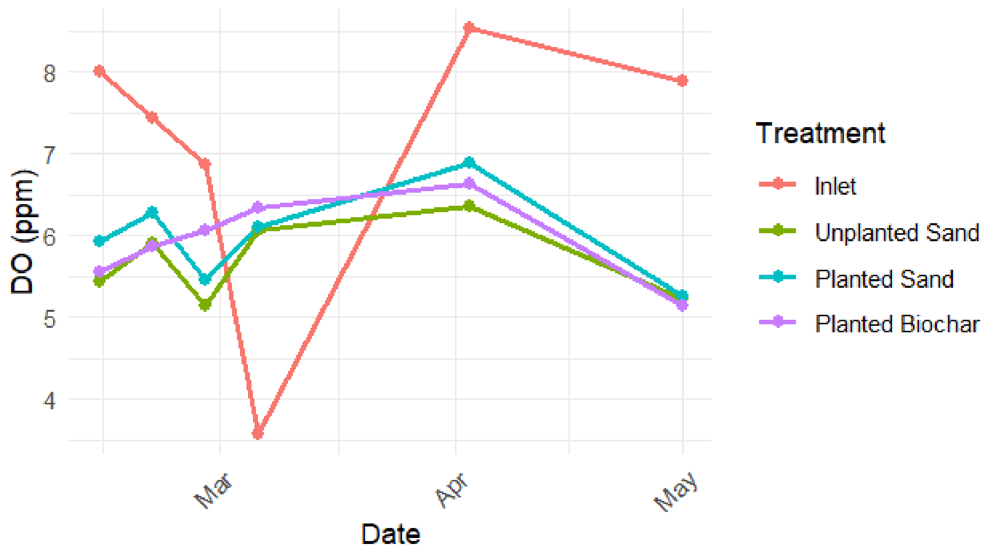
| Parameter | Inlet | Unplanted Sand | Planted Sand | Planted Biochar |
|---|---|---|---|---|
| Temperature | a | a | b | b |
| DO | a | b | ab | ab |
| EC | a | a | a | a |
| ORP | a | b | b | b |
| COD | a | b | b | b |
| $NH_4$ | a | b | b | b |
| $NO_3$ | a | a | a | a |
| $PO_4$ | a | b | b | b |
| pH | a | a | a | a |

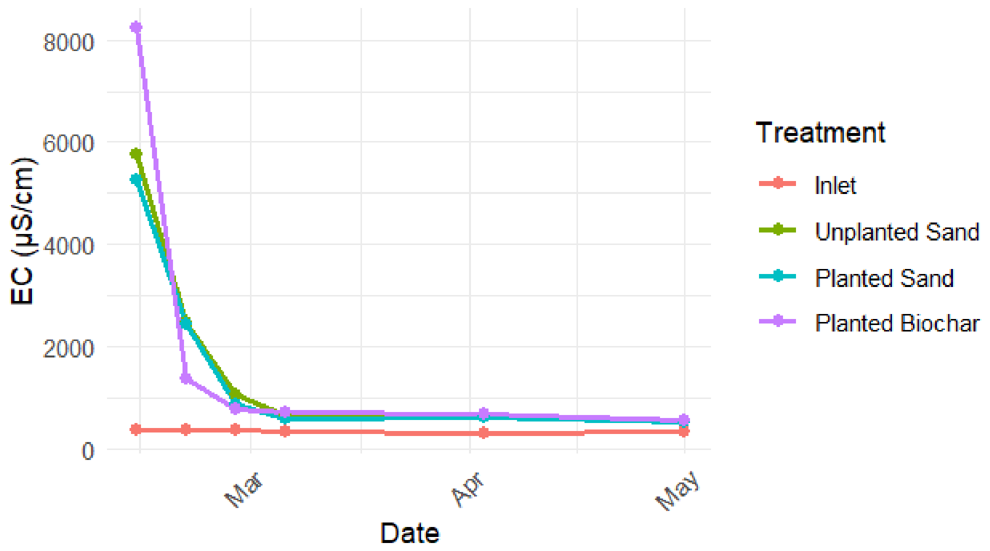
**Note:** Different letters (a, b) indicate significant differences between treatments according to Tukey's post-hoc test ($p < 0.05$). Treatments sharing at least one letter are not significantly different. Therefore, groups labelled "a" and "a" are statistically similar, groups labelled "a" and "b" are significantly different, and groups labelled "ab" are not significantly different from either "a" or "b".

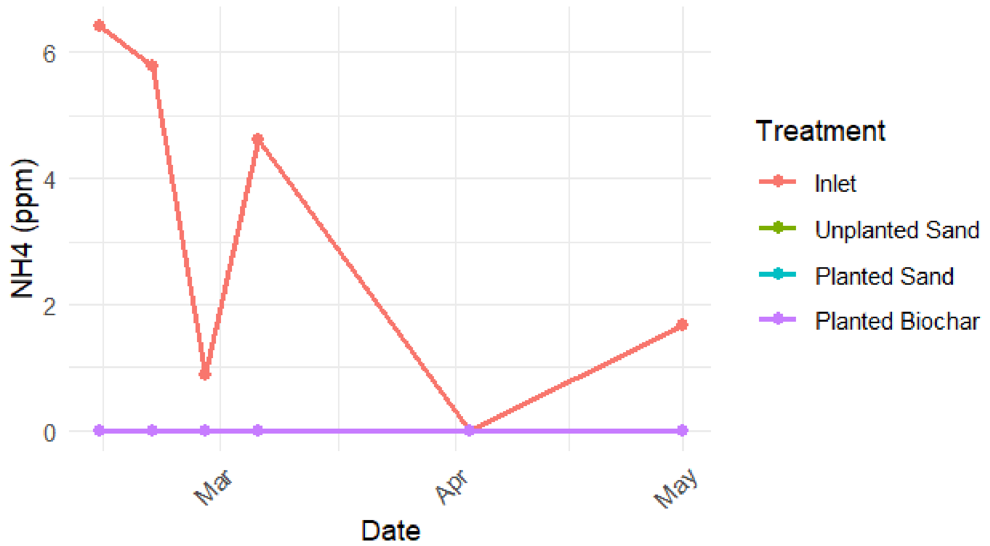
# Appendix B

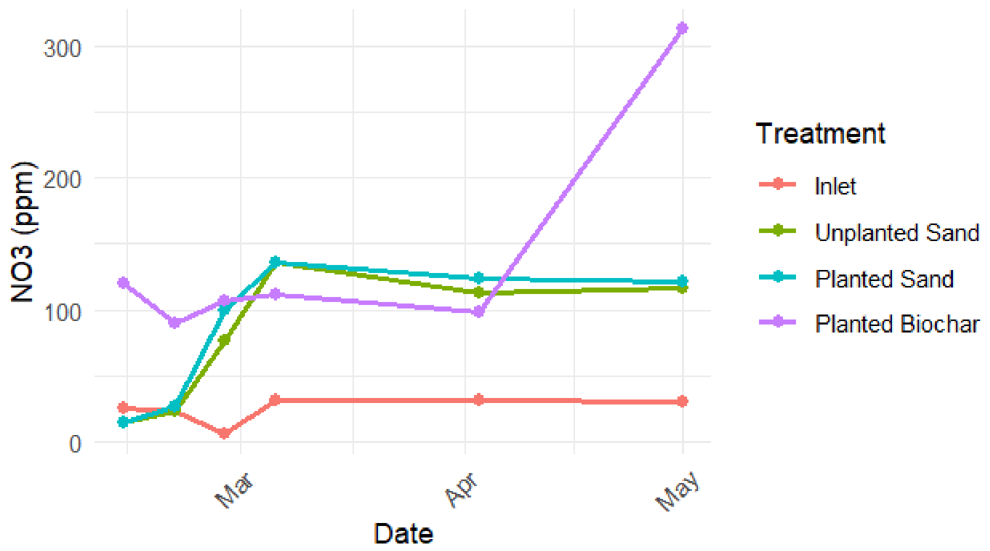
Additional figures:

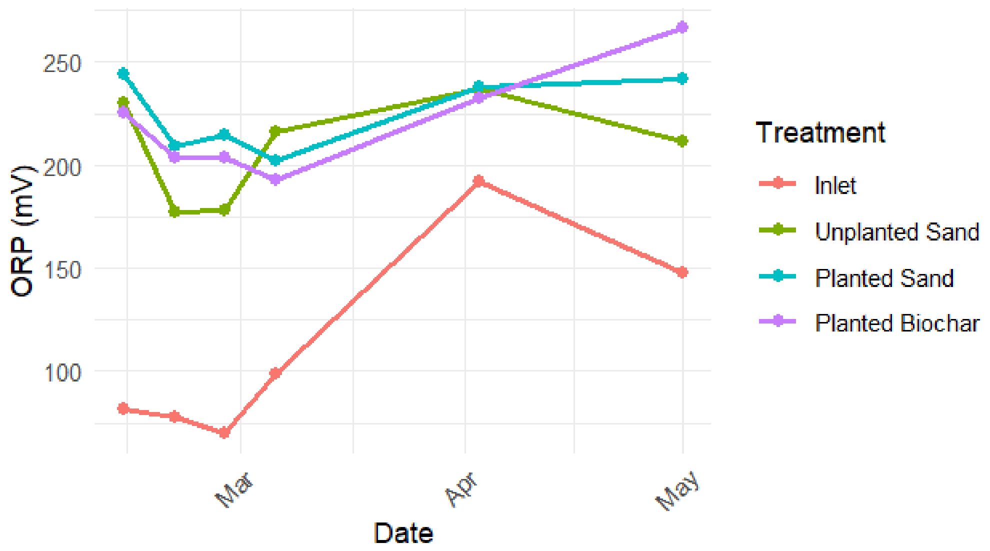


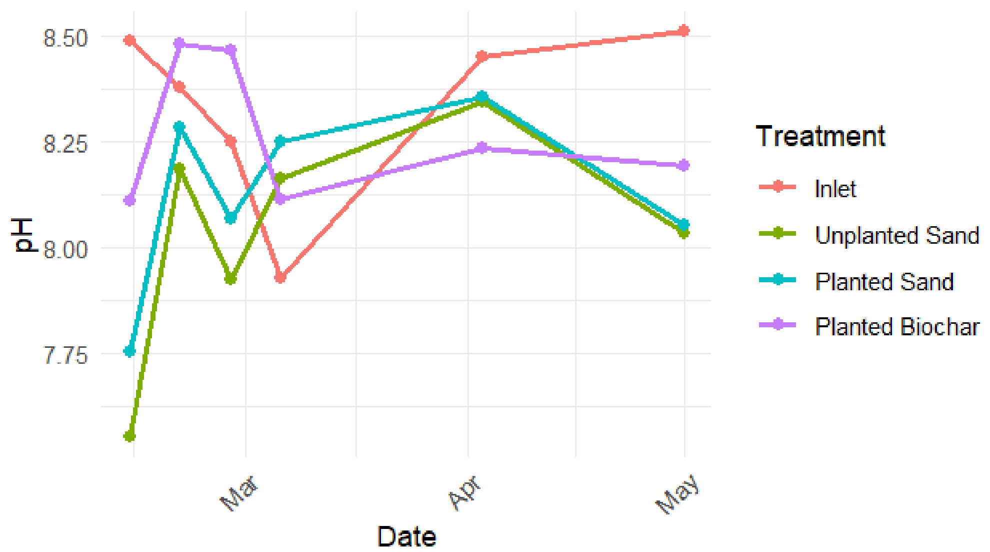

8000
6000
4000
2000
0
EC (µS/cm)
Mar
Apr
May
Date
Treatment
Inlet
Unplanted Sand
Planted Sand
Planted Biochar


6
4
2
0
NH4 (ppm)
Mar
Apr
May
Date
Treatment
Inlet
Unplanted Sand
Planted Sand
Planted Biochar

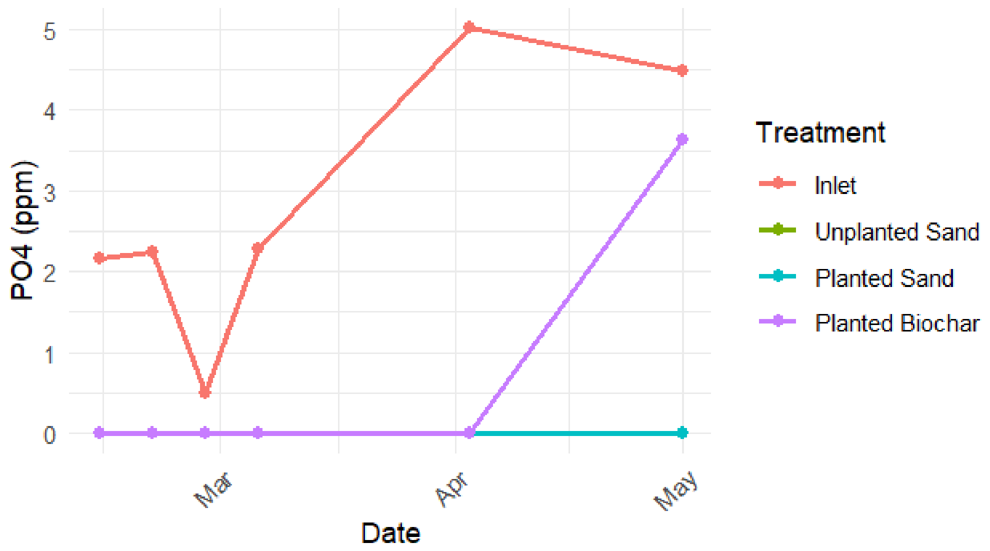

300
200
100
0
NO3 (ppm)
Mar
Apr
May
Date
Treatment
Inlet
Unplanted Sand
Planted Sand
Planted Biochar



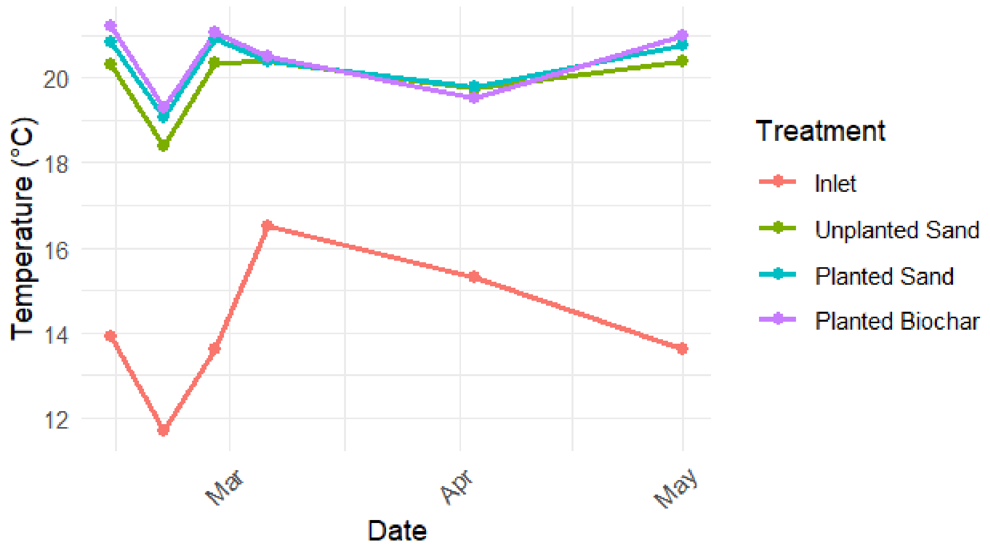
ORP (mV)
250
200
150
100
Mar
Apr
May
Date
Treatment
Inlet
Unplanted Sand
Planted Sand
Planted Biochar

pH
8.50
8.25
8.00
7.75
Mar
Apr
May
Date
Treatment
Inlet
Unplanted Sand
Planted Sand
Planted Biochar



PO4 (ppm)
5
4
3
2
1
0
Mar
Apr
May
Date
Treatment
Inlet
Unplanted Sand
Planted Sand
Planted Biochar

Temperature (°C)
20
18
16
14
12
Mar
Apr
May
Date
Treatment
Inlet
Unplanted Sand
Planted Sand
Planted Biochar