Long-term performance of a real scale constructed wetland for wastewater treatment

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Resumo

Devido à falta de infraestruturas adequadas, o tratamento de águas residuais nas zonas rurais e montanhosas é ainda considerado difícil. A utilização de leitos de plantas (LP) pode ajudar a apoiar uma abordagem mais eficiente a este problema. Estes sistemas mimetizam, de uma forma otimizada, os processos de fitorremediação que ocorrem nas zonas húmidas naturais. Eles incluem vegetação selecionada, um meio ou substrato de suporte e fauna e comunidades microbianas associadas.

Esta dissertação teve como objetivo avaliar a operação a longo prazo (12 anos) de um LP com fluxo subsuperficial horizontal, implementado numa unidade de turismo em contexto rural e a biodiversidade, mais especificamente a fauna, associada ao substrato.

A metodologia utilizada consistiu na amostragem periódica das águas residuais à entrada e saída do LP, com o objetivo de compreender a dinâmica do sistema de tratamento, nomeadamente no que diz respeito aos nutrientes (nitrito, nitrato, amónio), conteúdo orgânico (carência química de oxigénio), pH, condutividade e parâmetros microbianos (coliformes totais e *Escherichia coli*). A análise da fauna associada ao substrato foi feita através da montagem sazonal (outono e inverno) de armadilhas no LP. Os resultados foram avaliados através de testes ANOVA e índices ecológicos como: diversidade de Shannon-Wiener, riqueza de espécies e equitabilidade de Pielou. Os dados das águas residuais e substrato mostraram, em geral, que houve uma diminuição na carga de contaminantes desde a entrada até a saída do LP. Em termos de biodiversidade, foi encontrada maior variedade de taxa e número de espécies no outono, quando comparado ao inverno, e a análise estatística mostrou que, em relação aos índices ecológicos, não houve diferenças significativas entre os locais de amostragem ao longo das estações.

É possível concluir que o LP tem uma elevada capacidade de depuração, mesmo após operação a longo prazo, e promove a biodiversidade, em termos de fauna associada, como um importante serviço ecossistémico.

Palavras-chave: soluções baseadas na natureza, fitorremediação, biodiversidade, qualidade de água, água residual doméstica, fauna.

Abstract

Due to a lack of suitable infrastructures, the treatment of wastewater in rural and mountain areas is still considered difficult. The use of constructed wetlands (CW) can help to support a more efficient approach to this problem. These systems mimicin an optimized way the phytoremediation processes that occur in natural wetlands. They comprise selected vegetation, a supporting media or substrate and associated fauna and microbial communities.

This dissertation had as a goal to evaluate the long-term operation (12 years) of a horizontal subsurface flow CW, implemented in a tourism unit in a rural context and the biodiversity, more specifically the fauna, associated to the substrate.

The methodology used consisted the periodical sampling of the wastewater at the inlet and outlet of the CW, with the objective of understanding the dynamic of the treatment system, namely concerning to the nutrients (nitrite, nitrate, ammonium), organic content (chemical oxygen demand), pH, conductivity and microbial parameters (total coliforms and *Escherichia coli*). The analysis of the associated fauna of the substrate was done through the seasonal (autumn and winter) setup of pitfall traps in the CW. Results were evaluated through ANOVA tests and ecological indexes such as: Shannon-Wiener diversity, species richness and Pielou's evenness. Wastewater and substrate data showed, in general, that there was a decrease in contaminants load from the inlet to the CW outlet. In terms of biodiversity, a greater variety of taxa and number of species were found in the autumn, when compared to winter, statistical analysis showed that, in relation to the ecological indexes, there were not significant differences between the sampling sites through the seasons.

It is possible to conclude that the CW has a high depuration capacity, even after long term operation, and promotes biodiversity, in terms of associated fauna, as an important ecosystem service.

Keywords: nature-based solutions, phytoremediation, biodiversity, water quality, domestic wastewater, fauna.

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List of Abbreviations

FCUP	FACULDADE DE CIÊNCIAS DO PORTO
CW	CONSTRUCTED WETLAND
CO_2	CARBON DIOXIDE
PPCP	PHARMACEUTICAL AND PERSONAL CARE PRODUCT
FWS	FREE WATER SURFACE
COD	CHEMICAL OXYGEN DEMAND
BOD	BIOCHEMICAL OXYGEN DEMAND
NH_4^+-N	AMMONIUM - NITROGEN
NO_2^- -N	NITRITE - NITROGEN
NO_3^- -N	NITRATE - NITROGEN
PO_4^{3-} -P	PHOSPHATE - PHOSPHORUS
CFU	COLONY FORMING UNITS
P_2O_2	PHOSPHORUS OXIDE
K_2O	POTASSIUM OXIDE
CA	CALCIUM
MN	MANGANESE
MG	MAGNESIUM
MO	MOLYBDENUM
B	BORON
SO_3	SULPHUR TRIOXIDE
FE	IRON
NA	SODIUM
FAO	FOOD AND AGRICULTURE ORGANIZATION
TOTAL N	TOTAL NITROGEN
WHO	WORLD HEALTH ORGANIZATION

1. Introduction

Wetlands are transition areas between aquatic and terrestrial ecosystems, maintaining humid conditions with an abundance of water. They harbour high diversity of organisms, delivering a wide range of ecosystem services, including provisioning, regulating, supporting and cultural (Clarkson et al., 2013). Wetlands possess provisioning services due to being sources of food, such as rice or fish, fresh water and mineral products, for both personal and commercial use. Regulating services include improving water quality, trapping sediments, containing floods by forming a physical barrier, serving as reservoirs and holding the soil through the vegetation's roots and supporting carbon management, with natural wetlands having been used to treat domestic and industrial wastewater (Choi et al., 2016). Wetland supporting services are associated to primary production, serving as chemical sources, sinks and transformers and water storage. Through serving as nursery areas, maintaining lifecycles and gene pools, and biodiversity strongholds, with very rich fauna and flora, often exclusive to the area, habitat services are provided by these environments. Finally, cultural services are provided in the form of aesthetic, educational, cultural and spiritual activities, ranging from tourism to educational activities to archaeological sites (Clarkson et al., 2013; Mitsch and Gosselink, 2015; Bae and Lee, 2018; Jespersen, 2020). Wetlands include a variety of habitats such as marshes, floodplains, peatlands, lakes and rivers, found throughout all continents, with the exception of Antarctica. They possess soils, substrates, and biota adapted to flooding or/and waterlogging and associated conditions of restricted aeration (Vymazal, 2011).

Constructed wetlands (CWs) are engineered systems that are inspired and mimic many functions and physical, chemical, and biological processes that occur in natural wetlands (Lu et al., 2016). Constructed wetlands were first conceived to treat wastewater by Dr Käthe Seidel in the 1950s and put in full operational status in the 1960s. During the 1970s and 1980s, the knowledge of the technology was disseminated, increasing in popularity in the 1990s and 2000s and combining approaches, like vertical and horizontal flow, in response to the need for more effective removal of ammonia (NH_4^+) and total nitrogen (N). In recent times, CW are recognized as a reliable wastewater treatment technology and important forms of green infrastructures, and they represent a viable solution for the treatment of many types of wastewater, like agricultural runoff, domestic wastewater or landfill leachate (Vymazal, 2011; Zhang et al., 2020).

1.1. Constructed wetlands as nature-based solutions

Constructed wetlands are nowadays considered a nature-based solution (NbS) of excellence given the advantages that they pose, such as the flexibility of application they possess and the ecosystem services they provide (Calheiros et al., 2015; Jespersen, 2020).

Nature-based solutions, as defined by the European Commission, are *“solutions that are inspired and supported by nature, which are cost-effective, simultaneously provide environmental, social and economic benefits and help build resilience; such solutions bring more, and more diverse, nature and natural features and processes into cities, landscapes and seascapes, through locally adapted, resource-efficient and systemic interventions*.” (Commission et al., 2020).

Ecosystem services, in short, are the benefits that humans derive from nature (Mace et al., 2012). In the previous section, the ecosystem services that natural wetlands can offer were briefly described. Constructed wetlands are designed to provide mostly water treatment services being carbon sequestration also included (Aleissa and Bakshi, 2021). It was theorized that CWs focus on several goals, in which the CW should be multi-purpose for rainwater storage, retaining pollutants, recreation and wetland ecosystems, such as wildlife habitats, while following sustainable urban drainage concepts. Other ecosystem services that CWs can provide are temperature control, for example, contributing to reducing the heat island effect (Metcalfe et al., 2018) and regulation of greenhouse gas levels (such as CO_2) (Jamion et al., 2023).

Conventional sewage treatment plants are important, but possess drawbacks in their operation and maintenance, such as a high energy consumption, due to their need for electricity, pumping, oxygen injection and infrastructure requirements, resulting in low degradation rates for refractory pollutants. Constructed wetlands may support a sustainable and cost-effective approach, being a technology for wastewater treatment on the municipal and domestic level, storm water management and protection of coastal zones (Metcalfe et al., 2018; Liu et al., 2019; Horne, 2020).

Xenobiotics, such as drugs and pesticides, are degraded by the CWs with biological, physical and chemical processes contributions from rhizomes, microorganisms and components of the matrix (Dordio et al., 2007; Choi et al., 2016; Wang et al., 2022). Recently, the suitability of CWs for the removal of some pharmaceuticals and personal care products (PPCPs) has been assessed, with the processes of physicochemical decomposition, adsorption from plants and soil, photodegradation and biodegradation from microbial activity of the CW acting upon them (Liu et al., 2019).

1.1.1. Types of constructed wetlands

There are several types of CWs that can be classified based on their flow or based on the type of vegetation, which are depicted in Figures 1 and 2. The classification based on the flow considers the surface flow (also called free-water surface or FWS) and the subsurface flow (vertical or horizontal). Typical plants used in the surface flow systems are emergent, submerged, free-floating and floating-leaved plants, while in the subsurface flow system aquatic vascular plants are typically used. The subsurface systems have been commonly used in Europe while free water surface systems have been more prevalent in North America (Vymazal, 2011). Subsurface flow CWs can degrade xenobiotics that normal sewage treatments, designed to deal with more ordinary contaminants such as nutrients, organic matter, suspended solids and microorganisms, may show limitations (Dordio et al., 2007).

Subsurface flow is divided into horizontal and vertical flow, with vertical being further subdivided into tidal, upflow and downflow. When combining different flows a hybrid system can be denominated (Vymazal and Kröpfelová, 2008). Surface flow systems are closer in similarity to natural wetlands, having their flow at less than 60 cm of depth, over a saturated soil surface. They are capable of fitting into the landscape and provide habitat for a wide range of biota such as macroinvertebrates (Becerra Jurado et al., 2010). Subsurface flow mostly use gravel or expanded clay to support the growth of plants and biofilm development (Saeed and Sun, 2012). In general, if the wastewater percolates through the bed vertically and in an intermittent operation the system is designated as vertical subsurface flow system, being used more frequently due to their higher oxygen transfer and small footprint (Zhou et al., 2018). If the wastewater percolates horizontally and in a continuous mode, the system is designated horizontal subsurface flow system. There are also enhanced CWs, such as the artificial aerated CWs, hybrid towery CWs, circular flow corridor CWs, step feeding CWs and baffled flow CWs, among others, with the purpose of enhancing the performance of systems for wastewater treatment (Wu et al., 2015).

There are a few other types of CWs that do not fit within this flow-based classification, one is Floating Wetlands, where emergent macrophytes grow in a floating platform and directly adsorb nutrients, like nitrogen or phosphorus, from the water. The structure provides shade and refuge for several types of wildlife, from algae to other aquatic species to birds (Karstens et al., 2021).

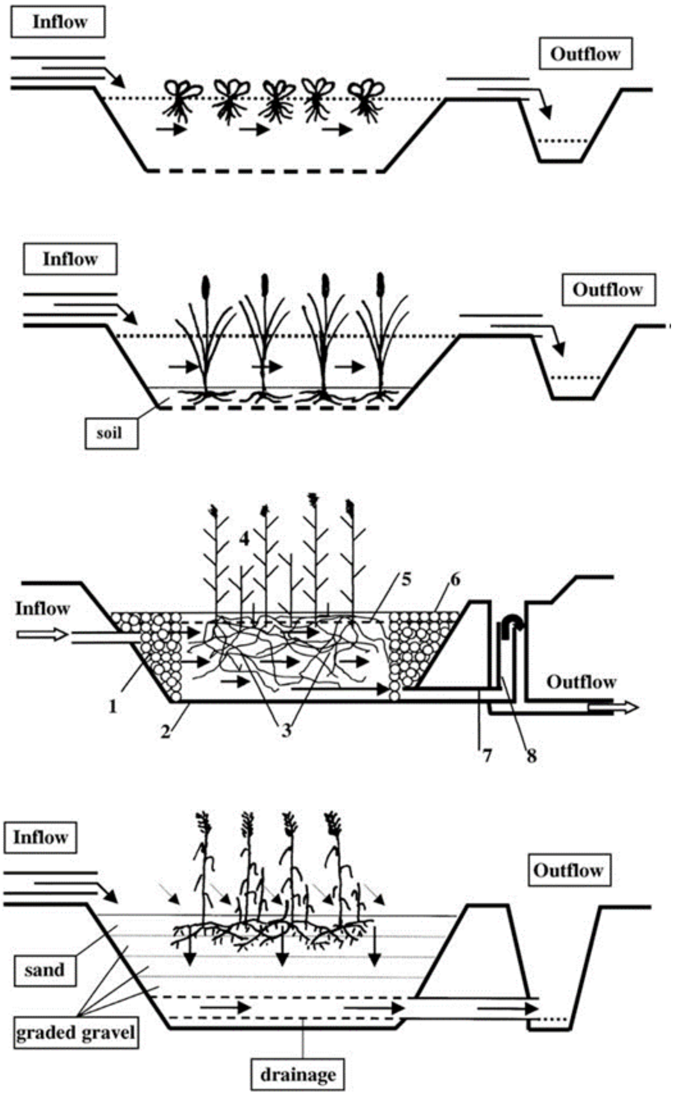

Figure 1- Different types of vegetation arrangement in constructed wetlands (CWs), from up to the bottom, CW with free-floating plants, CW with free-water surface and emergent macrophytes, CW with a horizontal subsurface flow and a CW with a vertical subsurface flow retrieved from (Vymazal, 2001).

1.2. Water treatment by constructed wetlands

Constructed wetlands, in recent decades, have been used to treat various kinds of wastewater, such as domestic sewage, agricultural wastewater, storm water, polluted river water, industrial effluent, landfill leachate, mine drainage and urban runoff (Wu et al., 2015; Varma et al., 2021). Depending on the level of treatment it can be possible the reuse of water in a non-potable state (Rahman et al., 2023).

Traditionally, wastewater treatment methods consist in four main steps. The first is the preliminary treatment, which comprises the physical separation and basic clean-up, reducing in size the large, entrained, suspended or floating solids, mostly by screening and grit removal. The primary treatment that involves physicochemical methods, through sedimentation, mostly to remove solids and lower the organic content. Followed by secondary treatment involving biological methods to biodegrade organic compounds, indicated by the chemical (COD) or biochemical oxygen demand (BOD) among other contaminants. Finally, tertiary treatment is considered for water polishing and finishing treatment, with pH adjustments or ion exchange being common as goals (Aleissa and Bakshi, 2021). Constructed wetlands can be used for secondary or tertiary treatment, depending on the purpose to be considered (Hsu et al., 2011).

Constructed wetlands encompass the treatment of wastewater through filtration, settling, precipitation, volatilization adsorption and complexation, as main physical and chemical processes, and biological processes like bacterial degradation and plant uptake (Kennedy and Mayer, 2002).

1.3. Components of the constructed wetlands

Constructed wetlands comprise several components, such as impermeabilization liners, substrate, vegetation and associated fauna. Each component requires careful selection according to the treatment purpose, the climatic conditions and economic feasibility (Zhao et al., 2010).

1.3.1. Substrate and impermeabilization layers

The CWs media or substrate is an important design component, since it can be used to remove significant contaminants in the wastewater through sedimentation, filtration and adsorption (Zhou et al., 2018). It also provides a suitable growing medium for macrophytes and associated fauna, besides being important to assure efficient hydraulic performance of the system. Substrates are in general selected according to their hydraulic permeability and porosity. If the hydraulic permeability is poor, the system becomes clogged, decreasing its effectiveness. If the substrate sorption is low, long-term removal of the CWs is affected. Substrates can be made of natural materials, industrial by-products or artificial media, or gravel, sand, clay, marble, calcite, limestone, shell, fly ash dolomite, wollastonite, zeolite, vermiculite, bentonite, activated carbon and light weight aggregates (Wu et al., 2015). The substrates are accompanied by impermeabilization layers, such geomembrane and geotextiles membranes (Calheiros et al., 2019a).

1.3.2. Vegetation

Macrophytes is the typical vegetation used in CWs, such as emergent plants, submerged plants, free-floating plants and floating leaved plants that due to their intrinsic properties aid in the functions of the CWs (Wu et al., 2015). These properties are provided by the adaptations of their tissue to an environment where waterlogging and flooding occur. Main characteristic of these plants is the presence of aerenchyma and lacunae, allowing the plant to survive in the anaerobic environment of the wetland (Vymazal, 2013).

Macrophytes have distinct functions in the CW. The aerial tissues help, for example, with insulation during the winter, serves as a storage for nutrients and for aesthetic purposes. The parts of the macrophyte submerged in water, when applicable, can filter out debris, excrete photosynthetic oxygen, promoting aerobic decomposition, uptake of nutrients as well and providing surface for the periphyton (that is, surface floating plants). Rhizomes and roots provide a stabilizing effect on the substrate of the CWs and resisting erosion. These plant structures create channels where water flows, increasing the hydraulic conductivity, which helps the flow of water through the CW, providing the surface for bacterial growth that in turn, oxidizes the usually anoxic substrate, promoting the aerobic decomposition of organic matter and the growth of nitrifying bacteria. The macrophyte, as a whole, or as their component regions, can also provide as a habitat for wildlife (Brix, 1994; Liu et al., 2009; Vymazal, 2013).

To be selected as a macrophyte several criteria must be considered: (1) ecological acceptability, as the plant must not pose a risk of being an invasive species, a “weed”, or of spreading diseases to the neighbouring natural ecosystems, thereby preserving their genetic and ecological integrity, (2) tolerance to local climatic conditions, pests and diseases, maintaining a high survival rate, (3) tolerance of pollutants and hypertrophic waterlogged conditions, (4) ready propagation, (5) the capacity to rapidly establish, spread, grow, with long growing seasons, is another criterion, and (6) have a high pollutant removal capacity, directly, such as with assimilation, or indirectly, through the processes of nitrification or denitrification. Since the functional role of the plants can vary between different CWs needs, the species requirements for each might vary, including, as an example, the need for the macrophytes to be aesthetically pleasing or having high economic value (Tanner, 1996; Lu et al., 2016).

There have been more than 150 species of macrophytes used in CWs, however, only a small number of these is used regularly. The most common species in use are emergent plants, belonging to the genera *Typha spp.*, also known as cattails, *Scirpus* spp., bulrushes, *Phragmites* spp., the common reed, *Juncus* spp. and *Eleocharis* spp., both known as spikerushes, being present in the treatment of a variety of types of wastewater, from agricultural wastewater to sewage to industrial runoff, among others (Vymazal, 2013). Submerged plant species that are commonly used are *Hydrilla verticillata*, *Ceratophyllum demersum*, *Vallisneria natans*, *Myriophyllum verticillatum* and

Potamogeton crispus. For floating plants, the species *Nymphaea tetragona*, *Nymphoides peltata*, *Trapa bispinosa* and *Marsilea quadrifolia* are the most used. Finally, the most common species of free-floating plants in use are *Eichhornia crassipes*, *Salvinia natans*, *Hydrocharis dubia* and *Lemna minor* (Wu et al., 2015).

1.3.3.Fauna

Constructed wetlands can harbour a wide range of organisms, from macrofauna, such as birds, to microorganisms, who play a key role in nutrient transformation and removing pollutants, through the macrophyte's ability to transfer oxygen to the substrate, promoting adequate conditions for the establishment of these microorganisms around the roots (Kataki et al., 2021).

Birds have been used as bioindicators (Schäfer et al., 2004) and in relation to bird biodiversity and abundance, it has been suggested to be related to the area of CW, with larger areas having a higher degree of biodiversity (Hsu et al., 2011).

Soil fauna can be classified as macro, mesofauna and microfauna. Soil macrofauna comprises invertebrates with dimensions between 2 and 20 mm (Zagatto et al., 2020). Macrofauna, in turn can create tunnels in soil, improving the aeration and water infiltration, creating micro-habitats for the microfauna, they can also convert biopolymers into biodegradable substrates (Ouattara et al., 2009). Macroinvertebrates are also seen as excellent bioindicators for aquatic systems due to their life cycles, their species being abundant and diverse with varying life history strategies regulated to habitat, as well as being ubiquitous (Espanol et al., 2015). Mesofauna (also spelled meso-fauna) contains fauna from 0.2 mm up to 2 mm in body size (Zagatto et al., 2020; De La Rosa and Peralta-Videa, 2022). Finally, microfauna comprises organisms with a body width less than 0.2 mm (Zagatto et al., 2020). Communities of this type of fauna have been used as tools for estimating the nutrient and organic loading into the CW (Ouattara et al., 2009).

According to Puigagut et al. (2007), the most represented groups of microfauna in two horizontal subsurface flow CWs were ciliated protozoa and microflagellates, whose ratio changed according to the environmental conditions. Microflagellates were more abundant in poor quality environments, such as those with a high organic content. Ciliated protozoa were shown to be important for wastewater treatment processes, fulfilling several roles, such as, reducing the number of dispersed bacteria, clarifying the effluent, contributing to floc formation, and reducing sludge formation. They serve as well as indicators of the functional conditions of wastewater treatment plants and the presence of toxic compounds.

Microorganisms mainly help in the removal of nitrogen, phosphorus, metals and antibiotics. The main phylum related to functional microorganisms in CWs was noted in a bibliometric analysis of microbial studies in CWs made by Wang et al. (2022), in which included *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes*. Eubacteria dominate the microbial community, with 85% of total viable bacteria measured being of that taxa (Krasnits et al., 2009). A very relevant process is done by these microorganisms in CWs through nitrification, which is a chemoautotrophic process, consisting in the biological aerobic oxidation of ammonia into nitrite, this being called nitritation, or, through nitrate oxidizing bacteria, into nitrate, this designated as nitratation (Albuquerque et al., 2009). This and its opposite reaction, denitrification, are part of the nutrient cycle, thusly, are the main ways carbon and nitrogen can be removed in the CW (Zhou et al., 2018). Biodegradation, biosorption and the supporting of plant growth are some of the other methods these microorganisms use in degrading pollutants (Wang et al., 2022).

Some metals are degraded by biosorption, biomineralization and valence transformation pathways. Bacteria that are effective in the treatment of metal contaminated waters belong in general to the class Deltaproteobacteria and phylum Proteobacteria. The more popular taxa include the genera *Desulfovibrio*, *Desulfobacter*, *Desulfobulbus*, and *Desulfurobacterium* (Chen et al., 2021; Wang et al., 2022).

Phosphorus is mainly removed by phosphorus-accumulating organisms, storing phosphate in their cells. Proteobacteria is the main phylum comprising this kind of bacteria with most of the species belonging to *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* (Shi et al., 2017; Wang et al., 2022).

Finally, CWs have been found highly suitable in removing antibiotics, with removal efficiencies as high as 91.8 to 99.5%, which require complex physical, chemical and biological series of processes like adsorption, microbial degradation and precipitation. The main taxa of microorganisms that are involved in antibiotic removal are *Proteobacteria*, *Actinobacteria* and *Firmicutes*. CW microorganisms have also been shown to use this type of pollutants as their sole source of carbon, and these include mainly microorganisms of the genus *Microbacterium*, belonging to the phylum *Actinobacteria* (Wang et al., 2022).

1.4. Importance of biodiversity in constructed wetlands

Biodiversity, per the definition used by the Parties to the Convention on Biological Diversity is "*the variability among living organisms from all sources including, interalia, terrestrial, marine and other aquatic ecosystems and ecological complexes of which they* *are part, this includes diversity within species, between species and of ecosystems*" (Mace et al., 2012).

Plant biodiversity has also been shown to increase the ability of the CWs to act as a CO_2 sink (Jamion et al., 2023) and a major influence in the general biodiversity of the CW. Water quality and level of contamination are factors that affect the diversity and development of macrophytes (Knapp et al., 2019). Furthermore, plant biodiversity has been shown to increase plant biomass production and nitrate retention in the substrate, with a high degree of diversity being theorized as an important factor in increasing the removal of N in the CWs (Zhu et al., 2010).

Regarding richness in CWs, a study by Hansson et al. (2005) reported that the lack of an observed relationship between nutrient loading and species richness or the biodiversity of macrophytes or their cover and nutrient retention was due to the fact that the CWs studied were of relatively low age. Regarding the biodiversity of microbial communities, both the richness and diversity that they possess, have been shown as key factors for efficient wastewater treatment by the CW (Wang et al., 2022).

CWs can provide a habitat for several species, however this depends in part on the characteristics of the surrounding land. In short-term studies, indicator variables such as insects group activity and richness increased over time or remained similar to the state used as a reference (Wiegleb et al., 2017).

A number of Floating Wetlands were shown to serve as a hunting and nesting ground for grey herons, a bird that hunts fish, frogs, snakes and insects. These CWs also served as shelter for juvenile eels, shrimp and snails (Karstens et al., 2021).

1.4.1.Factors influencing biodiversity

Several factors in the CWs have been noticed to influence biodiversity of macroinvertebrates, including the water quality, trophic conditions, water body size, habitat structure, shifting the abundance and species composition. It has also been reported that some invertebrate taxa have preferences in vegetation and substrate and that can influence the CWs' biodiversity. Community structures in these macroinvertebrates are differentiated, in turn by wetland morphological differentiation and water quality. Finally, in terms of recovering biodiversity in CWs, habitat heterogeneity is considered the most effective strategy (Sartori et al., 2015).

The enhancement of the ecological carrying capacity of the ecosystem, meaning "the level of production that does not impact undesirably on the surrounding ecosystem(s)" (Tett et al., 2015), can be done by CWs, through the sustainable treatment of wastewater (Jamion et al., 2023). Constructed Wetlands' area extension has been shown to correlate to the species richness of several taxa, such as birds, benthic invertebrates and amphibians (Hansson et al., 2005).

Microfauna growth in CWs has been shown to be affected by organic matter content and oxidizing conditions, relating to the design configuration of the system. Microfaunal abundance and bacteria removal are also influenced by vegetation, which promotes favourable environments for meta-zoa diversification, and by flow type (Pedescoll, Rodríguez et al. 2016).

In some short-term studies of CWs, some parameters such as richness and activity of insects increased over time or remained similar to the reference state. However, in some long-term studies, after the span of 10 to 20 years, deterioration was noted in the sites, as they were not following the desired trajectories. It is suspected that the initial enhancement of species richness is not sustainable due to the dynamic nature of ecosystems. Constructed wetlands that were surrounded by agricultural or urbanized sites have been subjected to increased rates of nutrient supplies or pollution, due to anthropogenic disturbances, thusly the pool of potential target species was reduced which caused higher costs in the introduction of species. Being near plant seed sources could also influence succession on a site, while agricultural wetlands would quickly establish perennial communities with their rapid succession (Wiegleb et al., 2017).

1.5. Knowledge gaps

The research on CWs has been in general, focused on their implementation and operation in terms of wastewater treatment efficiency. Nowadays, the potential of CWs to deliver several ecosystem services, such biodiversity promotion, is gaining interest. Constructed wetlands underlying operational processes are associated to biodiversity and these systems can also promote biodiversity through for example creation of habitat. Although the biodiversity dynamics are not fully studied neither understood. Thus more research is needed on CWs and their associated biodiversity (Benyamine et al., 2004; Zhang et al., 2020).

1.6. Aims

The aim of the present thesis is to assess the long-term performance of a CW implemented in a tourism unit and the associated fauna diversity. For that, the CW efficiency at the inlet and outlet was investigated as well as the biodiversity associated to the system, specifically the fauna linked to the CW's substrate surface. The novelty of this work relies on the proposed methodological approach that enables to study the CW's substrate surface biodiversity and relate to the water quality, that to our knowledge is addressed for the first time.

Research questions that this thesis strives to answer are: What is the biodiversity of the fauna associated to a CW with a long-term operation? What are the characteristics of the ecosystem present in and around the CW? What is the efficiency of the CW, after long term operation, to treat the wastewater from the tourism unit?

2. Materials and Methods

2.1. Study Site

The CW under study has been in operation for 12 years (was implemented in 2011) and is located in Paço de Calheiros, in Calheiros, Ponte de Lima in northern Portugal, an agro-tourism unit (Figure 2).

Figure 2 - Constructed wetland implemented at Paço de Calheiros.

The wastewater treatment of the tourism unit comprises a septic tank (acting as preliminary and primary treatment) followed by the CW (acting as secondary treatment). The CW operates with a horizontal subsurface flow, has an area of A = 40.5 m^2 (13.5 m x 3 m), with an effective depth of the substrate, h = 0.4 m. The substrate used as filling of the CW is made of expanded clay (Leca®, with a particle size ranging from 4 to 12.5 mm, supplier: SaintGobain Weber Portugal, S.A.), laid on a layer of geotextile followed by a geomembrane (Figure 3). It is surrounded by an orange tree orchard and grass, with vineyards in relative proximity.

The CW was vegetated with a polyculture of the macrophyte species: *Agapanthus africanus*, *Canna flaccida*, *Canna indica*, *Watsonia borbonica* and *Zantedeschia* *aethiopica*. Detailed description of the CW implementation setup and operation is detailed in Calheiros et al. (2015).

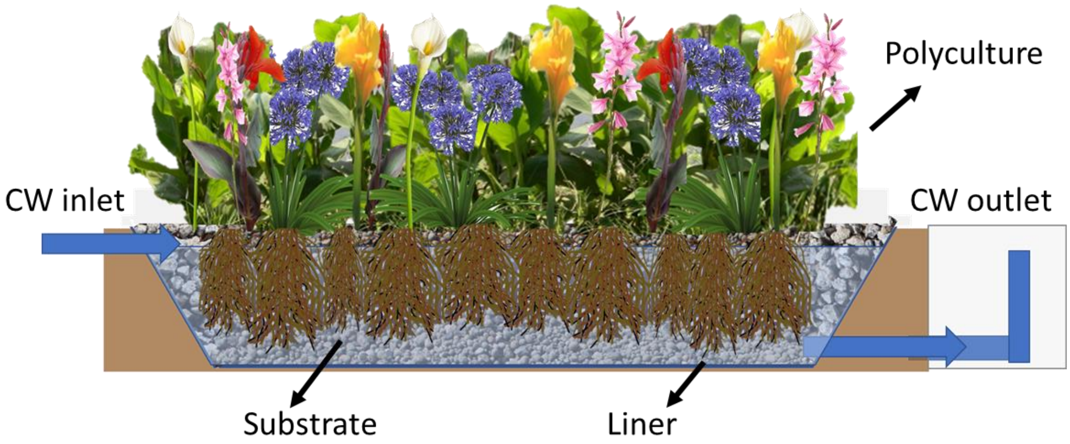

Figure 3 - Schematic representation of the constructed wetland system (Adapted from: Calheiros et al., 2019a).

Climacteric data associated to the CW monitoring site area was provided by Instituto Português do Mar e Atmosfera (IPMA), for the months under study, November (2022) and February (2023), concerning the parameters: air temperature and precipitation. The station from which these data was gathered was the Ponte de Lima/ Escola Agrícola meteorological station.

2.2. Wastewater and substrate sampling and analysis

Wastewater from the inlet and outlet of the CW was analysed for performance proposes, concerning physicochemical and microbiological parameters (Figure 4).

Figure 4 - Sampling boxes at the inlet (a) and outlet (b) of the constructed wetland implemented at Paço de Calheiros.

Wastewater samples at the inlet and outlet were collected monthly from November 2022 to May 2023. It should be noted that wastewater was only sampled at these sites and not inside the CW.

The physicochemical parameters of the wastewater under evaluation were: chemical oxygen demand (COD), nitrogen (NH_4^+-N, NO_2^- -N, NO_3^- -N) and phosphorus (PO_4^{3-} -P), determined with photometric test kits (Spectroquant®, Merck KGaA, Darmstadt, Germany), following approach undertaken previously by (Calheiros et al., 2019a). pH and conductivity were monitored with a WTW handheld multiparameter instrument 340i. Microbiological assessment focused on *Escherichia coli* and total coliforms enumeration by plate counting using ChromoCult1 Coliform Agar (Merck), according to (Calheiros et al., 2015).
Concerning the substrate sampling and analysis, they were retrieved at the same time as the macrofauna assessment described in section 2.3 (November 2022 and February 2023), from the CW. For that, the CW unit was divided into 3 monitoring zones: entrance (A), middle (B) and exit (C), (Figure 5).

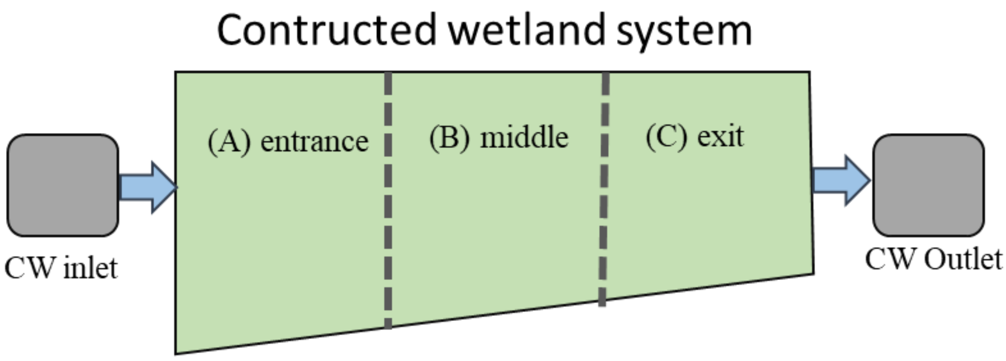

Figure 5 - Schematic representation of the constructed wetland (CW) system, implemented at Paço de Calheiros, with the monitoring zones: CW inlet, (A) entrance, (B) middle, (C) exit, and CW outlet.

A volume of substrate sample of 785 cm^3 was retrieved with a core from each zone, where three subsamples were pooled to form one composite sample, in order to be representative of the correspondent zone. The samples were sent for analysis at A2 Análises Químicas, LDA, following standard methods of analysis, for several parameters: moisture (gravimetry), pH (potentiometry), electric conductivity (conductimetry), organic carbon (catarometry), total nitrogen (catarometry), organic matter (by calculation), carbon: nitrogen ratio (by calculation), phosphate (extraction through water (1 : 5)), potassium oxide (extraction through nitric acid and hydrogen peroxide), calcium (extraction through nitric acid and hydrogen peroxide), magnesium (extraction through nitric acid and hydrogen peroxide), sulphur trioxide (extraction through nitric acid and hydrogen peroxide), boron (extraction through nitric acid and hydrogen peroxide), iron (extraction through nitric acid and hydrogen peroxide), manganese (extraction through nitric acid and hydrogen peroxide), molybdenum (extraction through nitric acid and hydrogen peroxide) and sodium (extraction through nitric acid and hydrogen peroxide).

2.3. Associated fauna assessment

In order to assess the macrofauna biodiversity associated to the CW and its surroundings, it was used the methodology approach based on pitfall traps. Pitfalls, were originally developed by Hertz in 1927, and as mentioned by Woodcock (2005) it is a user-friendly methodology and of common use in varied scientific areas, such as measuring abundance and diversity of springtails (Kouakou et al., 2022).

For that, on autumn and winter seasons it was setup 2 sampling trials, corresponding to November 2022 and February 2023. Following the monitoring scheme presented previously for substrate sampling (Figure 5), the same zone division was considered for the pitfall traps implementation. Briefly, in each zone A (entrance), B (middle) and C (exit), it was setup three pitfall traps. In the surrounding area (outside the CW) it was setup six pitfall traps (Figure 6). Plastic cups of 3.5 cm diameter and 100 ml of volume were used as pitfall traps. Each one was filled to about 1/3 of its volume with 70% ethanol according to Kouakou et al. (2022) and left for 7 days, to be later analysed and identified (Figure 7 and 8).

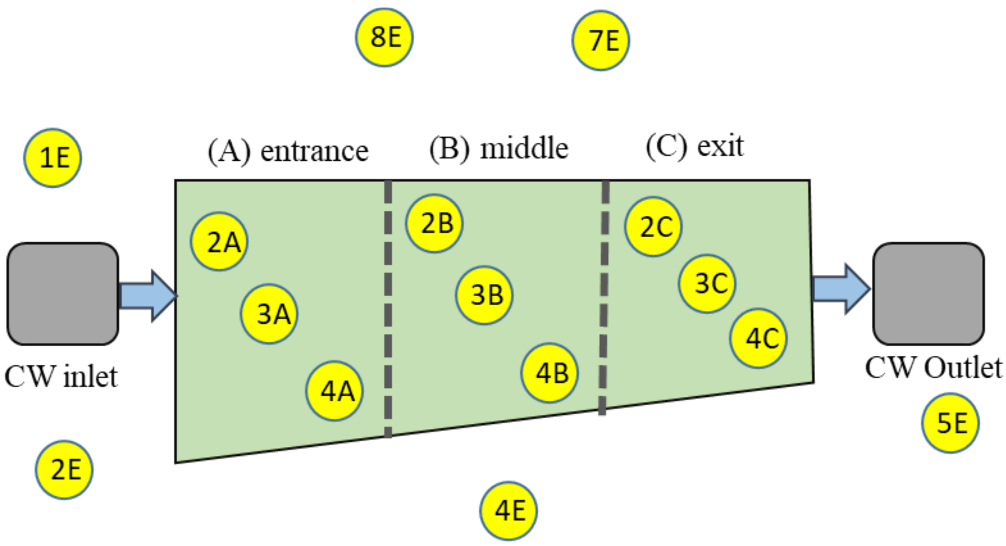

Figure 6 - Schematic representation of the pitfall traps setup at the constructed wetland (yellow circles) (not to scale) implemented at Paço de Calheiros.

Figure 7 - Setting up the pitfall traps at the constructed wetland implemented at Paço de Calheiros.

Figure 8 - Pitfall traps collected at the constructed wetland implemented at Paço de Calheiros after the sampling trials.

The associated fauna that was trapped in the pitfalls was posteriorly separated based on their taxonomical differences. After this all organisms were photographed with an electronic magnifier by using the Leica Application Suite LAS EZ v.3.4.0 software in the case for small fauna and with a photographic camera for macrofauna in order to provide the identification of the associated fauna to the lowest practical taxonomic level, whenever was possible through the partnership with the Environmental Science Program, Faculty of Science and Technology, Beijing Normal University-Hong Kong Baptist University United International College. Based on the fauna identification it was possible to determine the abundance of the associated fauna, and to evaluate these faunae per each selected zone (i.e., surrounding area, entrance, middle and exit).

2.3.1. Associated fauna ecological indexes

Three ecological indexes were calculated using the associated fauna abundance data. The Shannon-Wiener diversity that is a mathematical formula that indicates the diversity of species in a community, considering the number of taxa present and their abundance. The species richness that is the number of different species that are present in an ecological community, region or landscape (Colwell, 2009). And the species evenness that is also known as Pielou's evenness index that shows how evenly the individuals of a community are distributed between the species. It is calculated by taking *H (*the value of the Shannon's diversity index*)* and dividing it with the natural logarithm of species richness, being a value between 0 and 1, with 0 representing no evenness present and 1 complete evenness (Heip et al., 1998).

2.4. Data analysis

ANOVAs were performed to assess the associated fauna in different seasons (i.e., autumn and winter) and zones (i.e., surrounding area, inlet, middle, and outlet).

Spatial differences in abundance of the pitfall traps associated fauna were tested using a one-way ANOVA, with season (one level: autumn, and one level: winter) and zones (four levels: surrounding area, entrance, middle, and exit). ANOVAs were run independently for each season.

The ANOVA was created using the function *aov* from the package car (Fox and Weisberg, 2011) ANOVA's assumptions about the normal distribution and homoscedasticity of the residuals were tested using *shapiro.test* and *leveneTest* functions from the package car (Fox & Weisberg, 2011). When the residuals did not meet these conditions, variables were transformed using the Box-Cox procedure via the *boxcox* function from the Mass package (Ripley, 2002), and in cases where the Box-Cox procedure could not solve the problem, Kruskal-Wallis was used via the *kruskal.test* function from the Stats package (Team, 2010).

The same methodology previously described for the ANOVAs were used to compare the associated fauna Shannon-Wiener diversity, Species richness and Pielou's eveness through the different seasons (i.e., autumn and winter) and zones (i.e., surrounding area, inlet, middle, and outlet).

Indicator species analysis was performed to assess the relevant species of each season (i.e., autumn and winter). The analysis was made with the associated fauna abundance data of each season. The indicator analysis was made using the *multipatt* function from the package indicspecies (Cáceres and Legendre, 2009).

All analyses were performed using R software (version 4.2.1; R Core Team, 2022).

3. Results and Discussion

3.1. Wastewater Sample Analysis

3.1.1. Physicochemical Parameters

Water was characterized for several physicochemical parameters (Table 1). Compared to the results from Calheiros et al. (2015) and Calheiros et al. (2019a; 2019b) about this specific CW, several considerations can be made. Calheiros et al. (2019b) tracked some of the values discussed here over several years, in hot and cold seasons, stating that the water entering in this CW is considered slightly saline and at the outlet is non-saline.

In the present study the pH remained near the value of 7, varying between 7.35 and 7.94 at the CW inlet and between 6.55 and 7.42 at the CW outlet, which were in the same range as the values observed by Calheiros et al. (2019a), being considered neutral. The pH value of the outlet water, seen in Table 1, complies with the "Decree-Law No. 119/2019 establishing the legal regime for producing water to be reused, obtained from wastewater treatment." of the Portuguese Government, which limits the range of pH in reused water for recreative or landscape use from 6.0 to 9.0.

Conductivity, seen in Table 1, registered values of 1420 and 2090 microS/cm as minimum and maximum in the inlet and 161 and 730 microS/cm minimum and maximum at the outlet. Average values for the inlet and outlet were 1727 ± 292 microS/cm and 802 ± 271 microS/cm, respectively. Compared to the data in Calheiros et al. (2019a), in which the inlet had a conductivity between 142 and 2120 microS/cm, and the outlet between 84 and 1060 microS/cm the inlet and outlet conductivity values showed to be within known ranges for this CW.

In a FAO chart found in Drechsel et al. (2023) regarding the parameters with agronomic significance for agricultural irrigation it is possible to ascertain that the levels found in the outlet water, for both samplings, had a pH that causes no harmful effect in its usage in agricultural irrigation, since the values did not stray lower than 6.5 and higher than 8.0.

Conductivity at the outlet was also within acceptable ranges for agricultural irrigation, being lower than 3000 microS/cm (Drechsel et al., 2023).

NO_2^--N, maximum values in the inlet and outlet were of 0.53 mg/L and 0.20 mg/L, respectively, both in December, while the minimum values in this parameter for these two areas were 0.12 mg/L and 0.06 mg/L, in February and April, respectively. The average inlet value was of 0.29 ± 0.16 mg/L. Removal efficiencies were between 80% and 42%, the first corresponding to the sampling made during May and the second to both January and March months. The removal efficiency noted in Calheiros et al. (2015) was 93%, while Calheiros et al. (2019a; 2019b) did not account this parameter. A decrease in the removal efficiency of this pollutant was noted, being more notable in January and March, however Calheiros et al. (2015) had higher concentrations so this might have to be taken into perspective.

NO_3^--N maximum values, in the inlet and outlet, were 5.00 mg/L and 2.50 mg/L, respectively, both in November. The minimum values recorded for the inlet and outlet were 0.90 mg/L and 0.10 mg/L, in April and February. The average inlet value was of 2.93 ± 1.39 mg/L. Efficiencies ranged from 97% to 43%, in February and December, respectively. Most of the efficiencies tended towards the lower end of these extremes. Calheiros et al. (2015) reported a maximum efficiency of 99% and a mean of 85%, as Calheiros et al. (2019a) reported a maximum of 97%. When compared to the previous years' concentrations, these values were higher in the assessment done by Calheiros et al. (2015), with the maximum concentration at the inlet being 37.0 mg/L and 9.4 mg/L and similar by Calheiros et al. (2019a), with 3.70 mg/L and 0.39 mg/L as maximum concentrations in the inlet and outlet. Again, a decrease was noted compared to Calheiros et al. (2015), however due to much higher maximum concentrations, this could not actually be the case, while efficiencies and maximum concentrations remained similar to Calheiros et al. (2019a). Wu et al. (2017), in a study of removal efficiency of several contaminants in an integrated CW in the treatment of mixed sanitary and industrial wastewater, through several years, had 34% removal efficiency, with a concentration of 7.76 mg/L at the inlet and 4.47 mg/L at the outlet, in regards to NO_3^--N; this CW, in contrast, surpassed this removal efficiency value in the samplings done and had lower concentrations at the inlet and outlet, as seen above. Due to the lower inlet concentrations, the relative increase could indeed show a greater efficiency in our CW.

For NH_4^+-N, the maximum concentration levels of this contaminant at the inlet and outlet were 40.00 mg/L and 7.80 mg/L both in May, the minimum values, on the other hand, had concentrations of 0.65 mg/L and 0.05 mg/L in the months of February and December, respectively (Table 3). Maximum and minimum efficiencies were of 98% and 69%, in December and February, respectively. The maximum efficiency was of 84%, reported in Calheiros et al. (2015), preceded by a minimum of 22% and a maximum value of 62% reported by Calheiros et al. (2019b), and Calheiros et al. (2019a) had up to 97%. Previous years had both higher and lower concentration values in this parameter, with values over 60 mg/L (Calheiros et al., 2019b) and lower than 20 mg/L (Calheiros et al., 2019a). Thusly, an increase in the removal of this contaminant was noted, though with the variability that the concentration values had in this year and previous samplings, this observed increase might not be reliable. Wu et al. (2017) had 70% NH_4^+-N removal efficiency, with concentrations of 7.13 and 2.1 mg/L at the inlet in its study integrated on a CW treating mixed sanitary and industrial wastewater, being lower than the most recent results observed for this CW, in relation to efficiency and generally higher, with the exception of May, in inlet concentration.

PO_4^{3-}-P, had its maximum concentration as 24.40 mg/L at the inlet, and 9.00 mg/L at the outlet, both in the month of May (Table 4). Minimum values were of 13.10 mg/L and 0.73 mg/L for the inlet and outlet, both values were observed during the sampling made in November. The concentration of PO_4^{3-}—P generally increased from month to month. An average inlet value of 19.03 ± 3.86 mg/L was noted. Removal efficiencies of PO_4^{3-}-P were between 95% and 63%, with efficiencies generally lowering through the months. Comparing the values observed in the samplings of this CW through the years, Calheiros et al. (2015) reported 73% in removal efficiency. Calheiros et al. (2019b) reported a minimum of 50% and a maximum 82% and Calheiros et al. (2019a) reported a maximum of 91%. As such, levels in concentration in these samplings were similar to those found in the present study. Another increase in the removal efficiency was observed for this parameter in the first months of the present study, with the values observed in the later months being similar to previously recorded ones.

For COD, 320 mg/L and 66 mg/L were the maximum concentration values for the inlet and outlet, both values were observed in the sampling made during May (Table 5). The minimum values were 76 mg/L for the inlet, in January, and 10 mg/L for the outlet, in November. The average inlet value of COD was 178 ± 96 mg /L. Removal efficiencies for COD had their maximum as 89%, in the month of November, and their minimum as 79%, in the month of May. Calheiros et al. (2015) presented an efficiency of 94%, Calheiros et al. (2019b) presented a minimum of 86% and a maximum efficiency of 97%, and Calheiros et al. (2019a) a maximum of 87%. Concentration values were significantly higher in some samplings, as in what was mentioned by Calheiros et al. (2019b). As such, a slight decrease in the removal efficiency was seen, with the note that in previous years the COD concentration was higher in some of the samplings, thus this decrease might not be relevant. Wu et al. (2017) that studied an integrated wetland registered an efficiency of 70% in COD removal, as such this CW had, comparatively, to the several samplings through the years, always superior values in the removal of this parameter throughout the years. Ali et al. (2018) compared hybrid CWs, including their efficiencies for COD, and observed values of 80 and 78%, which were, again lower than the ones detected in the present study.

As a sidenote, it is important to mention that the variations in inlet concentration, in all parameters, also noted here, between samplings, are probably associated to the fluctuations in the number of guests to the tourism unit in Calheiros et al. (2019a).

Drechsel et al. (2023) described the limit values for trace values in drinking water for livestock, and observed that the $NO_2^{—}$N values for the upper limit was 10 mg/L, a value that was not observed in the samples collected in the present study for the outlet zones. The study also specified the upper limit of NO_2^--N + NO_3^- -N, which again, compared to the outlet samples collected in the present study they did not surpass (Drechsel et al., 2023).

The benchmark limit for NH_4^+-N that a high integrity environment surface had was 1000 µg/L, which also wasn't exceeded in the present study (Drechsel et al., 2023).

The PO_4^{3-}-P concentration was within the range for water used in wastewater-fed aquaculture only for the first sampling, during November remaining below 1 mg/L. However, the rest of the samples collected in the present study surpassed this limit (Drechsel et al., 2023).

According to Chapman (1992), the COD values observed for the outflow, from November to January had a concentration found in alignment with non-polluted waters, being lower than 20 mg/L, while the rest of the samples collected, while not on par with non-polluted waters for this parameter, they still weren't considered at the level of industrial wastewater, since had values lower than 100 mg/L.

Thusly, pH and conductivity remained within the same range as in the past (Calheiros et al., 2019a), removal efficiencies generally decreased regarding NO_2^--N, NO_3^- -N and COD, or increased as was the case with NH_4^+-N and $PO_4^{3—}$P. Finally, observing the comparisons done with FAO standards, the outlet wastewater is usable, in certain months for a variety of purposes such as drinking water for livestock, wastewater-fed aquaculture, and that due to NH_4^+-N concentration in the outflow the water present in it could pass as one belonging to a high integrity environment (Drechsel et al., 2023). As such, it is possible to conclude that the depuration process is still effective, after the long-term operation of this CW.

Table 1 - Monthly water samplings values of pH and conductivity in the inlet and outlet zones of the constructed wetland implemented at Paço de Calheiros, Calheiros, collected between November 2022 and May 2023.

Parameter	pH		Conductivity (µS/cm)	
Dates/Sample	Inlet	Outlet	Inlet	Outlet
November/22	7.82	7.12	2090	161
December/22	7.86	7.42	1953	158
January/23	7.94	7.00	1720	297
February/23	7.87	6.55	1824	201
March/23	7.35	6.80	1450	320
April/23	7.43	7.00	1635	802
May/23	7.35	7.29	1420	730

Table 2 - Monthly water sampling concentration values (mg/L) of NO_2^--N, NO_3^--N in the inlet and outlet zones of the constructed wetland implemented at Paço de Calheiros, Calheiros, collected between November 2022 and May 2023, with removal percentages.

Parameter	NO_2^--N (mg/L)			NO_3^--N (mg/L)		
Dates/Sample	Inlet	Outlet	Efficiency	Inlet	Outlet	Efficiency
November/22	0.28	0.12	57%	5.00	2.50	50%
December/22	0.53	0.20	62%	3.70	2.10	43%
January/23	0.26	0.15	42%	3.90	1.30	67%
February/23	0.20	0.09	55%	2.90	0.10	97%
March/23	0.12	0.07	42%	2.30	1.10	52%
April/23	0.15	0.06	60%	0.90	0.50	44%
May/23	0.50	0.10	80%	1.80	0.80	56%

Table 3 - Monthly water sampling concentration values (mg/L) of NH_4^+-N in the inlet and outlet zones of the constructed wetland implemented at Paço de Calheiros, Calheiros collected between November 2022 and May 2023, with removal percentages.

Parameter	NH_4^+-N (mg/L)		
Dates/Sample	Inlet	Outlet	Efficiency
November/22	2.40	0.33	86%
December/22	3.06	0.05	98%
January/23	2.40	0.33	86%
February/23	0.65	0.20	69%
March/23	4.50	0.32	93%
April/23	5.50	0.35	94%
May/23	40.00	7.80	81%

Table 4 - Monthly water sampling concentration values (mg/L) of PO_4^{3-}-P in the inlet and outlet zones of the constructed wetland implemented at Paço de Calheiros, Calheiros, collected between November 2022 and May 2023, with removal percentages.

Parameter	PO_4^{3-}-P (mg/L)		
Dates/Sample	Inlet	Outlet	Efficiency
November/22	13.10	0.73	94%
December/22	21.60	1.50	93%
January/23	20.00	1.04	95%
February/23	21.05	1.16	94%
March/23	15.75	1.25	92%
April/23	17.30	4.35	75%
May/23	24.40	9.00	63%

Table 5 - Monthly water sampling concentration values (mg/L) of COD in the inlet and outlet zones of the constructed wetland implemented at Paço de Calheiros, Calheiros, collected between November 2022 and May 2023, with removal percentages.

Parameter	COD (mg/L)		
Dates/Sample	Inlet	Outlet	Efficiency
November/22	90	10	89%
December/22	87	11	87%
January/23	76	11	86%
February/23	202	31	85%
March/23	220	30	86%
April/23	254	50	80%
May/23	320	66	79%

3.1.2.Microbiological Parameters

In regards to microbiological parameters, *E. coli* in the inlet reached 5.30 x 10^5 CFU/100 mL as a maximum, in the month of May, and a minimum of 4.50 x 10^2 CFU/100 mL in the month of February, while in the outlet *E. coli* were not detected in any of the samplings (Table 6). Total coliforms maximum concentration ranged from 2.10 x 10^6 CFU/100 mL to 2.20 x 10^3 CFU/100 mL in the inlet and outlet, for the months of March and November, while the minimum concentrations were 3.00 x 10^2 CFU/100 mL in the inlet, in January, and not detected in the outlet, in January and February (Table 6).

These parameters were previously evaluated in Calheiros et al. (2015) in which the inlet *E. coli* numbers varied between 1 x 10^3 to 2 x 10^6 CFU 100/mL and between 2 x 10^3 to 3 x 10^8 CFU/100 mL for total coliforms, with both parameters dropping up to 3 logs, showing a consistent removal efficiency throughout the year. Compared to Calheiros et al. (2015) the values of *E. coli* and total coliforms in the inlet are within previously seen ranges. Differences were noted in the removal of *E. coli* in May, which apparently surpassed the removal of 3 logs, by there being a removal of 5 logs. For total coliforms there was not a log difference relating to total coliforms in the outlet in November and just a removal of 1 log in December, thusly a consistent removal efficiency was not noted.

E. coli removal criteria as determined in the European Union in 2020 (Drechsel et al., 2023), demanded to be of 5 log *E. coli*, with the recommended concentration level for unrestricted irrigation of food crops being between 10 *E. coli*/100 mL and 10^3 *E. coli*/100 mL. As we can see, the levels of *E. coli* in the present study for outlet, if correct, allow for unrestricted irrigation. Comparing this presumed efficiency to the results observed by Wu et al. (2017) in CW that had a removal efficiency relating to *E. coli* of 97%, we must assume it is similar to it.

Tunçsiper et al. (2012) observed an average removal of total coliforms of 99% in their CW, whereas Ali et al. (2018) had in its hybrid CWs removal efficiency of 94 and 93% of the bacterial population, with more than 94% of pathogenic microorganisms removed, thus, if we can equate this to the total coliforms removed in the present study for the outlet, during autumn the efficiency of the CW in the present study was much lower than compared to these other CWs previous mentioned, while during winter a comparable efficiency was observed.

In terms of depuration efficiency, *E. coli* removal, as said above, had a removal efficiency reported by Calheiros et al. (2015) of 3 logs, while in the present study, *E. coli* was not detected in the outlet, thusly complying with removal criteria of the European Union and allowing the water to be used for unrestricted irrigation in this parameter, remaining similar to the efficiency noted in the CW of Wu et al. (2017). On the other hand, regarding total coliforms, while some months had the same removal efficiency as *E. coli* in the study of Calheiros et al. (2015), here they varied to a significant degree, with the first two samplings having a very low efficiency, and the rest at least 3 logs, the latter being similar to the removal efficiency seen in a few hybrid CWs (Ali et al., 2018). The reason for the difference in efficiency between years, parameters and samplings might require further samplings to deeply understand.

Table 6 - Monthly water samplings with the values of *Escherichia coli* and total coliforms in the inlet and outlet zones of the constructed wetland implemented at Paço de Calheiros, Calheiros, collected between November 2022 and March 2023, "n/d" stands for "not detected", "Total colif." Stands for "total coliforms".

Colony type	E. coli (CFU/100 mL)		Total colif. (CFU/100 mL)	
Dates/Sample	Inlet	Outlet	Inlet	Outlet
November/22	4.70×10^3	n/d	6.00×10^3	2.20×10^3
December/22	1.70×10^3	n/d	2.37×10^3	2.80×10^2
January/23	n/d	n/d	3.00×10^2	n/d
February/23	4.50×10^2	n/d	7.50×10^3	n/d
March/23	5.30×10^5	n/d	2.10×10^6	1.00×10^3

3.2. Substrate Analysis

Substrate pH ranged from 5.8 to 6.8 in the first sampling and 5.3 to 6.9 in the second, with both showing the same pattern of the highest pH being in the sample from the middle of the CW. According to the United States Department of Agricultural National Resources Conservation Service, the substrate sampled during autumn was moderately acidic to neutral, while during winter it was strongly acidic to neutral (Msimbira and Smith, 2020).

Noting the preferences that the different CW macrophytes possess, *C. flaccida* is adaptable to the soil pH (Society), *C. indica* tolerates a pH range of 4.5 to 8.0 (Pasiecznik, 2008), *W. borbonica* and *A. africanus* (Jamieson, 2004; Notten, 2005) prefer acid soils and *Z. aethiopica* has a preference for mildly acid, neutral and mildly alkaline soils ((L.)Spreng.). Thusly the pH that was observed in the substrate samplings is within the comfortable range of these plants.

Some noteworthy parameters such as the Total N had an 82% decrease in the first sampling and negative efficiency values in the second sampling.

Phosphorous, in the form of phosphorus oxide (P_2O_2), had decreased by 68% in the first sampling and 25% in the second sampling.

Chemical element concentration in the CW like P_2O_2, potassium oxide (K_2O), calcium (Ca), molybdenum(Mo), manganese (Mn), magnesium (Mg), boron (B), sodium trioxide (SO_3), iron (Fe) and sodium (Na) generally decreased along the CW (Table 7), however in certain parameters this pattern showed variation, such as for P_2O_2 during the two samplings, with the concentration in the exit sample site being higher than the middle sample site; or as with the first sampling of K_2O, where the middle saw an increase in concentration relative to the entrance, before having it decreased in the exit; or with the second sampling of the same parameter, the exit concentration being higher than the entrance; or with the case the second sampling of Ca, where the concentrations of the middle and exit sample sites being equal, which, for all of these cases but not limited to them, is contrary to what would be expected to see, as contaminants should be reduced downstream by the phytoremediation processes (Calheiros et al., 2019a). Certain parameters in both samplings showed different patterns such as Mn, where the value of C was higher or equal for the first and second sampling, respectively. Further analyses are required to better understand if these variations in the concentration patterns were consistent through the year or were simply occasional.

The bibliographical search did not yield any examples of substrate analyses done in CWs, which made the comparisons limited relating to this part of the work.

According to the standards of the WHO and FAO (Nunes et al., 2021), several pollutants in the substrate samplings recovered had values below the maximum permissible concentration of potentially toxic elements in the soil, such as molybdenum and boron.

FAO charts in “Harmonized World Soil Database” also indicated that the substrate had low conductivity, being between 2 to 4 dS/m, and that organic carbon content was in the limits for code 3 (medium organic carbon) and 5 (very abundant organic carbon).

It was possible to conclude that, for certain parameters, substrate wise, certain contaminants such as molybdenum and boron were at non-toxic concentrations and that the organic carbon content of this substrate was medium to very abundant, which is promising for the organisms living in it.

Table 7 - Substrate characterization collected during autumn and winter in the different zones, entrance, middle and exit, of the constructed wetland implemented at Paço de Calheiros, Calheiros

Parameters/Season		Autumn			Winter	
Sample	Entrance	Middle	Exit	Entrance	Middle	Exit
Humidity (%)	54.90	50.60	53.40	58.10	46.00	52.10
pH	5.80	6.80	6.20	5.30	6.90	6.00
Conductivity (µS/cm)	20	32	12	28	29	25
Organic C (%m/m)	2.50	2.40	0.80	1.70	2.30	2.10
Organic Matter (%m/m)	4.30	4.10	1.40	2.90	4.00	3.60
Total N (%m/m)	0.22	0.15	0.04	0.12	0.15	0.14
C:N Ratio	11.30	15.70	21.00	14.20	15.30	15.10
P2O2 soluble in H2O (mg/kg)	15.60	3.60	5.00	9.70	3.10	7.30
K2O soluble in H2O (mg/kg)	12.00	14.00	4.60	9.70	14.00	11.90
Ca soluble in H2O (mg/kg)	15.30	18.90	6.00	18.30	13.10	13.10
Mg soluble in H2O (mg/kg)	2.86	3.90	1.40	3.70	3.53	3.42
SO3 soluble in H2O (mg/kg)	19.80	10.80	5.70	19.50	9.80	11.70
B soluble in H2O (mg/kg)	0.03	0.04	0.02	0.02	0.05	0.06
Fe soluble in H2O (mg/kg)	4.00	3.40	1.90	1.30	3.10	4.90
Mn soluble in H2O (mg/kg)	0.03	0.06	0.04	0.06	0.11	0.06
Mo soluble in H2O (mg/kg)	0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Na soluble in H2O (mg/kg)	5.90	5.50	4.30	8.20	10.20	8.70

3.3. Air temperature

Based on the climacteric data provided by IPMA, for the region where the CW was implemented, the average air temperature in the week of the first sampling (November 2022) varied between 10ºC to 16ºC with an average deviation between just below 2ºC and just above 4ºC. There was a slight peak on the 8th, with the average reaching 16ºC (Figure 9). This sampling time corresponded to autumn season.

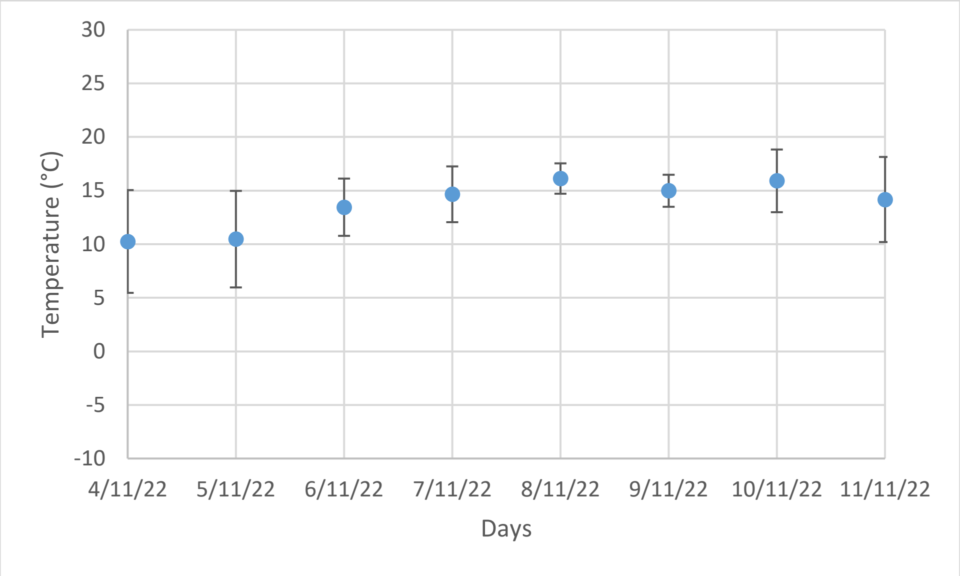

Figure 9 - Average air temperatures in the week of the first biodiversity sampling trial (4-11 November 2022) at the constructed wetland region in Paço de Calheiros, Calheiros.

Compared to the first sampling, average temperatures in the second sampling (February 2023) were lower, however the average deviation was much higher, reaching more than 25ºC in amplitude (Figure 10). This sampling time corresponded to the winter season.

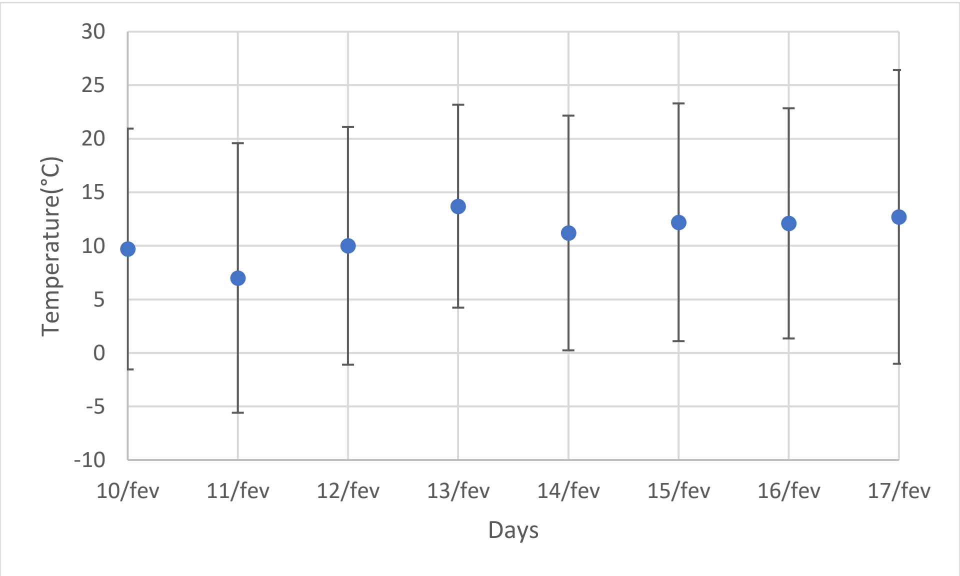

Figure 106 Average air temperatures in the week of the second biodiversity sampling trial (10-17 February 2023) at the constructed wetland region in Paço de Calheiros, Calheiros.

According to Köppen classification, Portuguese climate in Minho region is classified as Csb, i.e., temperate climate with rainy winters and dry summers with mild temperatures (Calheiros et al., 2017; Calheiros et al., 2019b). The air temperature and precipitation, as climacteric conditions, in seasonal contexts, can have impact to different extents on

CW, (Nan et al., 2023). In previous studies (Valkama et al., 2017; Ali et al., 2018; Li et al., 2018; Koskiaho and Puustinen, 2019) it was suggested a positive correlation between the removal efficiency of certain contaminants and temperature.

3.4. Macrofauna

3.4.1. Number of species per group

3.4.1.1. Autumn

It was observed that in the first sampling, made in the autumn, a few taxonomic groups were more prevalent in the samples, such as Acari, Diptera, Hymenoptera (mostly comprising Formicidae), Collembola, Coleoptera and Araneae. Acari was the taxonomic group with the highest number of identified species, with more than 20 species, including two from the family Trombidiidae (Figure 11).

Acari and Collembola, are normally associated to the secondary decomposition pathways in the soil, where key taxa are found in great numbers in all soil layers, with Collembola being capable of adapting to disturbances in a flexible manner (Marx et al., 2012; Zheng et al., 2022). It is possible that this is associated for the high number of Collembola species observed during this sampling.

The presence of larvae in the CW during this season suggest that the CW is probably being used as a nursery area for some species of invertebrates.

The presence of Hemiptera suggests that the macrophyte community present is diversified or/and that other habitats are available, as similar environments, with a diverse macrophyte community are able to host this more specialized taxa (Sartori et al., 2015).

Thysanoptera and Termitidae (Insecta), Scutigeromorpha, Diplopoda, Oligochaeta (Clitellata) and Opiliones (Arachnida), with a further identified individual of the genus Homalenotus, had 1 or 2 individuals representing them.

A dominance of insects, with the prevalence of Diptera, Hymenoptera and Coleoptera were observed in more than 400 soil fauna assemblages, from North America and Europe, related to CW in mild or semi-arid climates (Zheng et al., 2022). Also, in a study by Msaki et al. (2023) on CW in Tanzania it was also observed a prevalence in Diptera, Hymenoptera and Araneae, however unlike this study, Orthoptera (grasshoppers) were also prevalent, which were not found in the samplings of this CW. It is possible that this difference is due to this CW being near an agricultural production site and therefore grasshoppers are not able to proliferate near it, or due to the different climate that each CW are submitted, or even because of the fact that the samplings were made during colder seasons, it is yet not known and difficult to understand from the data collected in the present study. In a study on a CW that studied the macroinvertebrate biodiversity in natural wetlands, artificial ponds and CW by Sartori et al. (2015), insects again dominated all environments studied, with the differences being on the composition of the macroinvertebrate orders.

A soil fauna study done by Leone et al. (2023) in natural wetlands in Eastern Sicily, had several taxa in common with the results observed in the present study. Leone et al. (2023) reported that Acari, Collembola, Diptera and Coleoptera were always present in the samples, patterns also observed in the present study. Vasconcellos et al. (2013), on the other hand, analysed the soil macrofauna in riparian forests in Brazil collected with pitfalls, and observed that Hymenoptera and Isopoda were the main taxa collected, followed by the Coleoptera that was the most numerous during summer, while Hymenoptera that was the most numerous during winter.

Collating these comparisons, we see that the fauna gathered through the course of this thesis shared with other CW soil assemblages, as observed by Zheng et al. (2022) and Sartori et al. (2015), and natural wetlands, as observed by Leone et al. (2023), a dominance of Insecta, with Coleoptera, Diptera and Hymenoptera having dominated the associated fauna, as well as the Araneae, which was also found as a dominant taxon in a CW by Msaki et al. (2023). Thusly, with the exception of Orthoptera and Isopoda that were absent, the taxa encountered in the present study, regarding the order level, were similar to those found in other soils of CW, natural wetlands and riparian forests.

Regarding the trophic levels, a few taxa that were identified can exemplify certain trophic levels found in this CW.

Acari, in general are present at many trophic levels (Manu et al., 2021), with more specifically Trombidiidae, a family of Acari found in this CW, that are called red velvet mites, being parasites of arachnids while in their larval state (Durkin et al., 2021) and active predators in their adult lives (Zhang, 1998).

Araneae (spiders) were also found and, with exception of a single species (Meehan et al., 2009), are carnivorous, being surface active predators (Lehmitz et al., 2020).

Collembola (springtails) , are amongst the most important organisms in the soil food web, feeding on fungal hyphae and decaying plant material, with certain species being carnivores of animals such as rotifers, nematodes and other springtails, serving as regulators and decomposers of the ecosystem (Hopkin, 1997; Marx et al., 2012).

Coleoptera (beetles), can be herbivores, omnivores or predators, in this case it was observed Coleoptera from the familiy Carabidae, a family of Coleoptera known for being predators (Lehmitz et al., 2020), that were found in this CW.

Stylommatophora (land snails and slugs), including an individual of the genus Gyraulus (Figure 12), were also identified in this sampling, being mostly herbivores and detrivores (Barker, 2001).

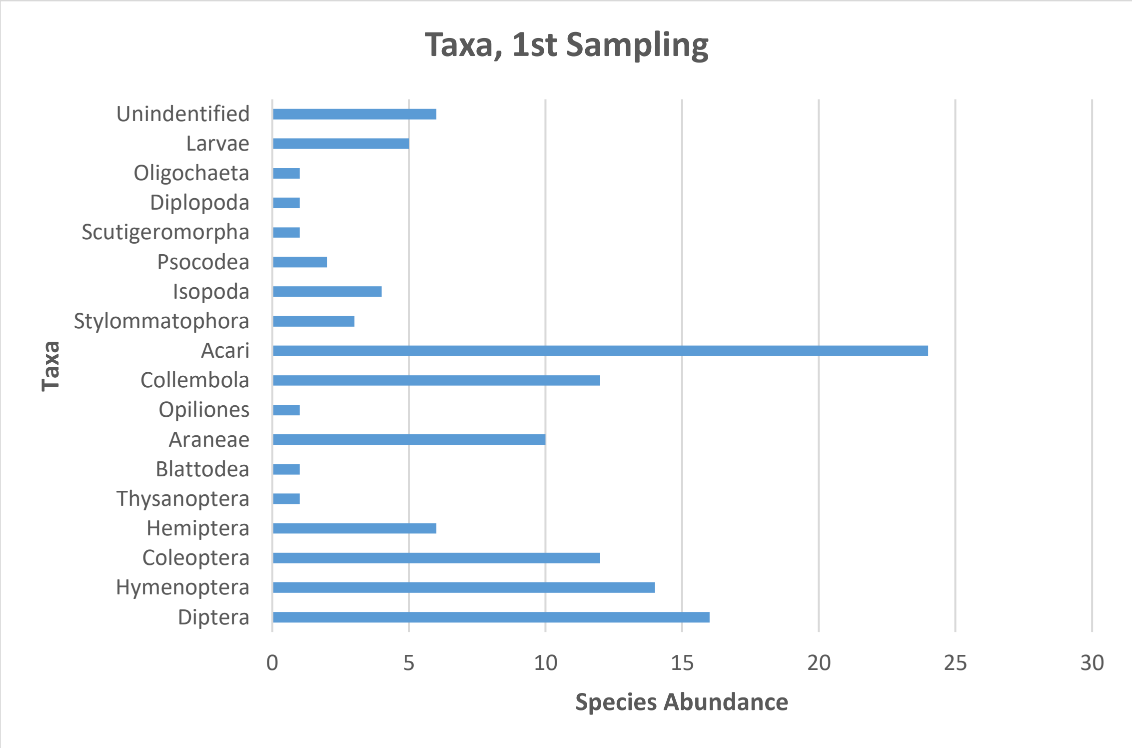

Figure 7 - Abundance per group of the fauna associated to the pitfall traps collected during autumn in the CW located in Paço de Calheiros, Calheiros. With the exception of Oligochaeta (subclass), all groups represent orders. Larvae and Unidentified represent species whose identification was unsuccessful.

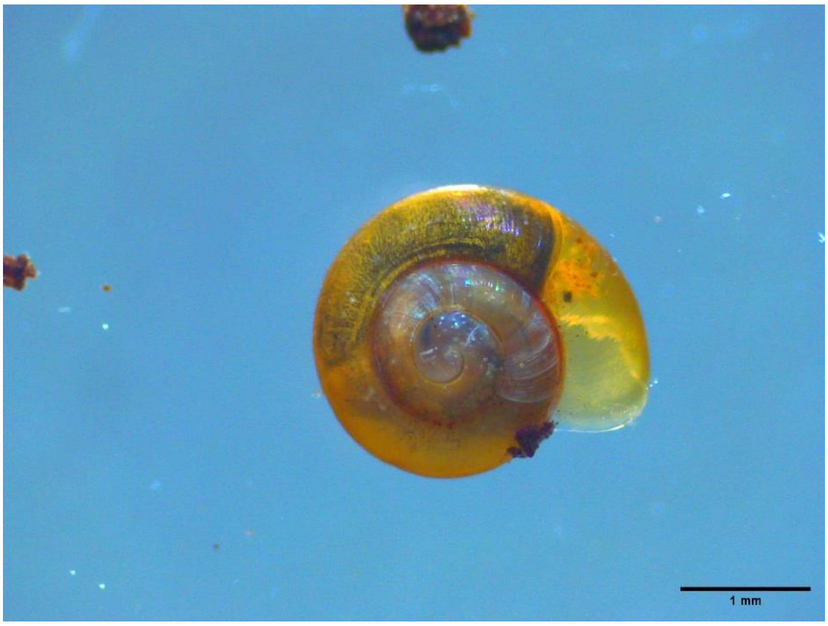

Figure 8 - Gyraulus individual, collected through pitfall traps during the autumn season in the middle section of the CW in Paço de Calheiros, Calheiros.

3.4.1.2. Winter

In the second sampling, done in winter, there were fewer taxonomic groups, with a smaller number of species. Acari, Diptera, Hymenoptera, Collembola, Coleoptera and Araneae were once again the taxonomic groups with the highest number of species, with Acari, once again being the group with the most species (Figure 13). The potential reason for the smaller number of species might be due to the fact that compared to the summer and autumn seasons, which are mating seasons for most species of invertebrates, and thus, with high levels of abundance and richness, the winter season are expected to have lesser abundance and richness of soil fauna when compared to the summer/autumn as it is no longer a mating season. There were new taxa found in this season, such as Cicindelidae (tiger beetles) (Figure 14) and Epuraea (Figure 15), both Coleoptera, but with different trophic levels, with Cicindelidae being fast and exclusive predators (KS, 1999) and Epuraea feeding on rotten citrus (Jang and Kim, 2014), the abundance of prey and of dropped oranges found surrounding the CW might explain the presence of these taxa.

Knowing this, and accounting for the taxa found in the autumn, we observed organisms on a variety of trophic levels, from herbivores such as Stylommatophora (Figure 16) and Epuraea, carnivores such as Araneae, Trombiididae (Figure 17), Cicindelidae and Carabidae (Figure 18), detrivores such as Collembola (Figure 19), and even, with Trombiididae, the possibility of parasites, due to the young of Trombiididae acari being parasites of Araneae, which are also present. But, despite identifying taxa belonging to a variety of trophic levels, there are still species whose identification is only at their order, leaving their trophic level ambiguous, as such, further identification is needed.

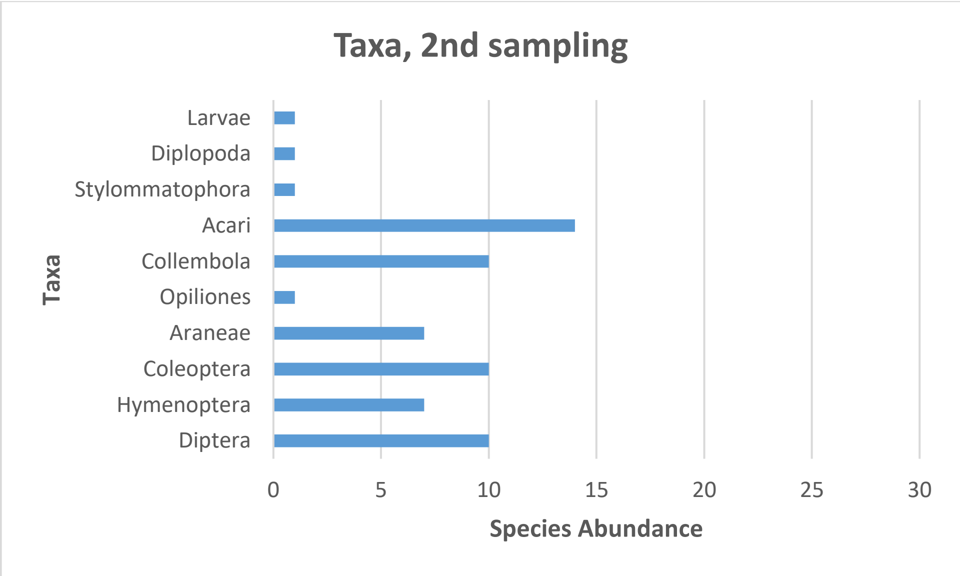

Figure 9 - Abundance per group of the fauna associated to the pitfall traps collected during winter in the CW located in Paço de Calheiros, Calheiros. Larvae represent species whose identification was unsuccessful-

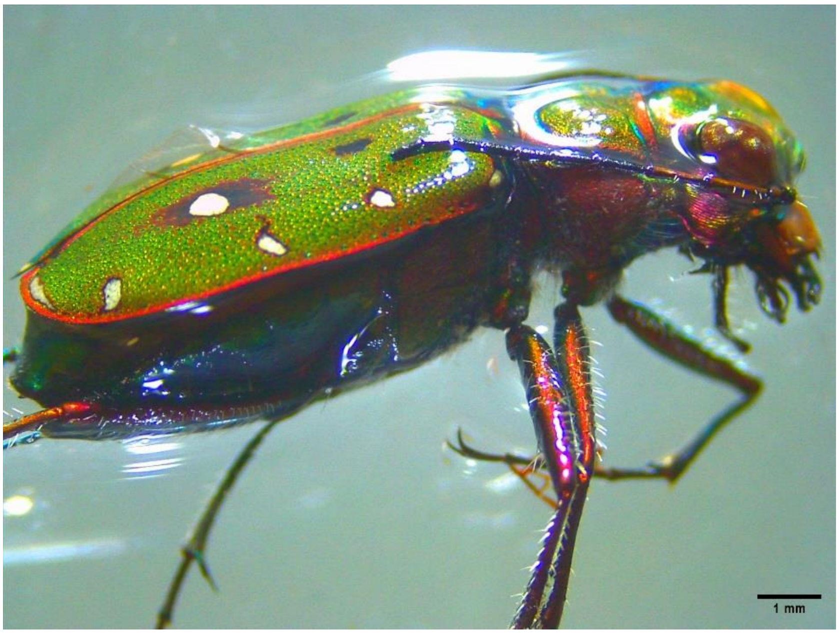

Figure 10 - Cicindelidae individual, collected through pitfall traps during the winter season in the surrounding area of the CW in Paço de Calheiros, Calheiros,

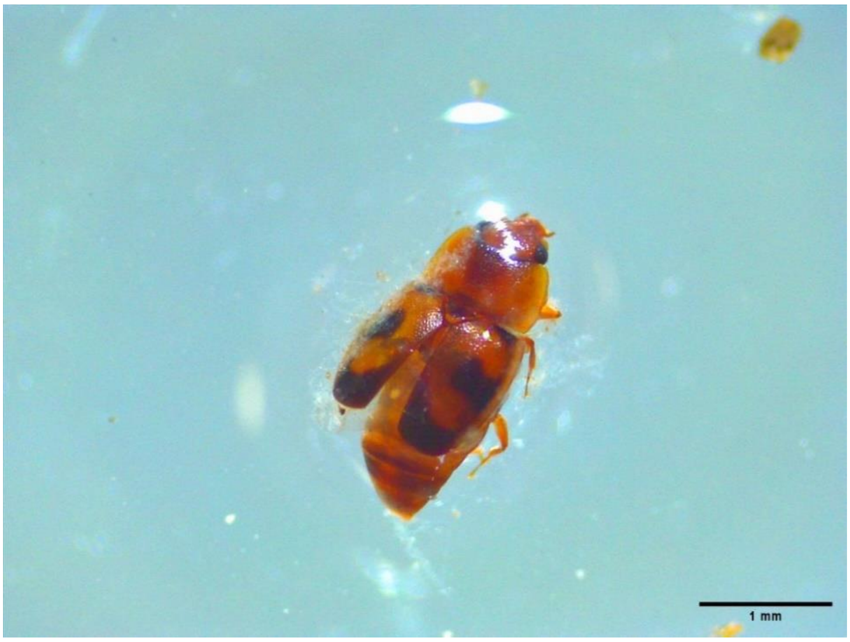

Figure 11 - Epuraea individual, collected through pitfall traps during the winter season in the surrounding area of the CW in Paço de Calheiros, Calheiros

Figure 12 - Stylommatophora individual, collected through pitfall traps during the winter season in the middle section of the CW in Paço de Calheiros, Calheiros,

Figure 13 - Trombidiidae individual collected through pitfall traps during the winter season in the entrance section of the CW in Paço de Calheiros, Calheiros

Figure 14 - Carabidae individual collected through pitfall traps during the winter season in the middle section of the CW in Paço de Calheiros, Calheiros

Figure 15 - Collembola individual collected through pitfall traps during the winter season in the entrance section of the CW in Paço de Calheiros, Calheiros

3.4.2. Ecological Indexes

3.4.2.1. Shannon-Wiener diversity

The Shannon-Wiener diversity values were not significantly different in the comparison per treatment during autumn (F value: 1.17, $P > 0.05$) and winter (F value: 0.342, $P > 0.05$) However, comparing to the values of the Shannon-Wiener diversity from the two seasons it is possible to observe that during autumn, the middle and exit had higher values of diversity (Figure 20), while during winter similar diversity values were observed in the comparison between sampling sites (Figure 21).

According to Sartori et al. (2015) results related to the Shannon-Wiener diversity values, for the associated fauna community of a CWs, it is possible to observe that for the autumn the entrance's sample values were inferior to the values they described in the study (which had a mean value of 2.30 ± 0.62). Equal and higher values were seen in the middle and exit autumn samples. Winter values, compared to the mean values observed by Sartori et al. (2015), showed lower values for the entrance and exit (Figure 21), while the middle included values on par and above it.

In Zhang et al. (2008), a previous study related to rehabilitation of a lakeshore wetland, the level of diversity was studied on plants, they found a diversity value for the associated plant community of 1.619, however they did not elaborate if it was a median or mean. In both samplings, the index values observed for the CW, in their majority, were equal or higher than the results observed by Zhang et al. (2008).

Li et al. (2020) studied the soil fauna at Lake Taihu, in China, at three reed-dominated wetlands, a long-term reed restoration lakeshore, a short-term reed restoration lakeshore and a natural reed lakeshore, measuring several ecological indexes through the year, Shannon-Wiener index included. In the three wetlands seasonality was noted, with Shannon-Wiener value values increasing from spring to summer to autumn, with the autumn having the highest value, and the winter showing a noticeable reduction, except in the natural reed wetland where the winter value was higher than the spring value. Mean values varied approximately between 1.5 and 2, which were slightly below the Shannon Index values observed in the present study.

Looking at the values of a study done by Guariento et al. (2020), which studied the Shannon's diversity in meadows and orchards in extensive or intensive management, it was possible to observe that, in the first sampling (autumn), the reference area's median value was lower than for both meadows and orchards', the values observed for the inlet did not surpass the minimum Shannon value of the meadows and orchards in both types of management, the middle and the outlet medians surpassed the values of diversity in both orchards and meadows, in both treatments. Observing now the second sampling (winter), all medians inside the CW surpassed those of both types of zones, for both treatment types. Thusly, although spring and summer are still needed for a full analysis, the results for the Shannon indicated that the diversity inside the CW is greater than the area surrounding it, and that compared to agricultural orchards and meadows, surpass these areas in diversity.

In conclusion, there were changes in the Shannon-Wiener diversity between samplings, with the middle and outlet showing greater diversity values during autumn, and during winter the Shannon values were very similar in the comparison per sites. Also, when compared with other CWs, natural wetlands and forest soils, the values observed in the present study were slightly above those registered in these contexts.

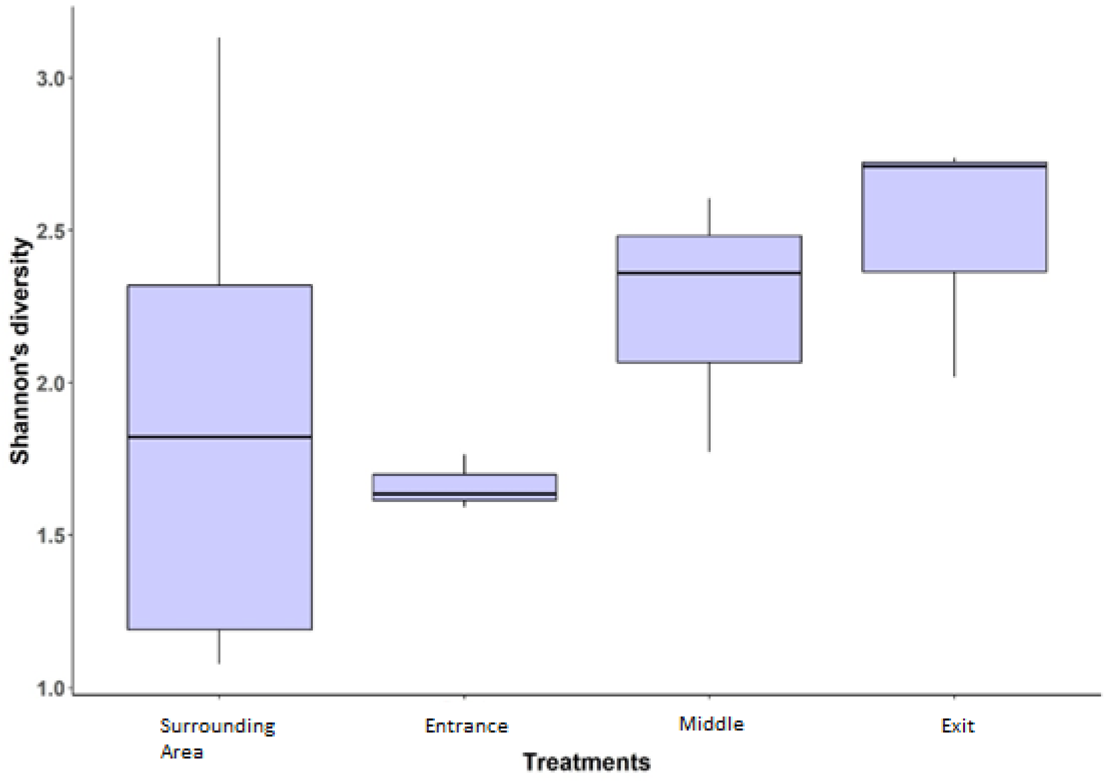

Figure 16 - Median ± SD of the Shannon-Wiener diversity values of the associated fauna collected with the pitfall traps in the Paço de Calheiros CW in Calheiros, during the autumn season.

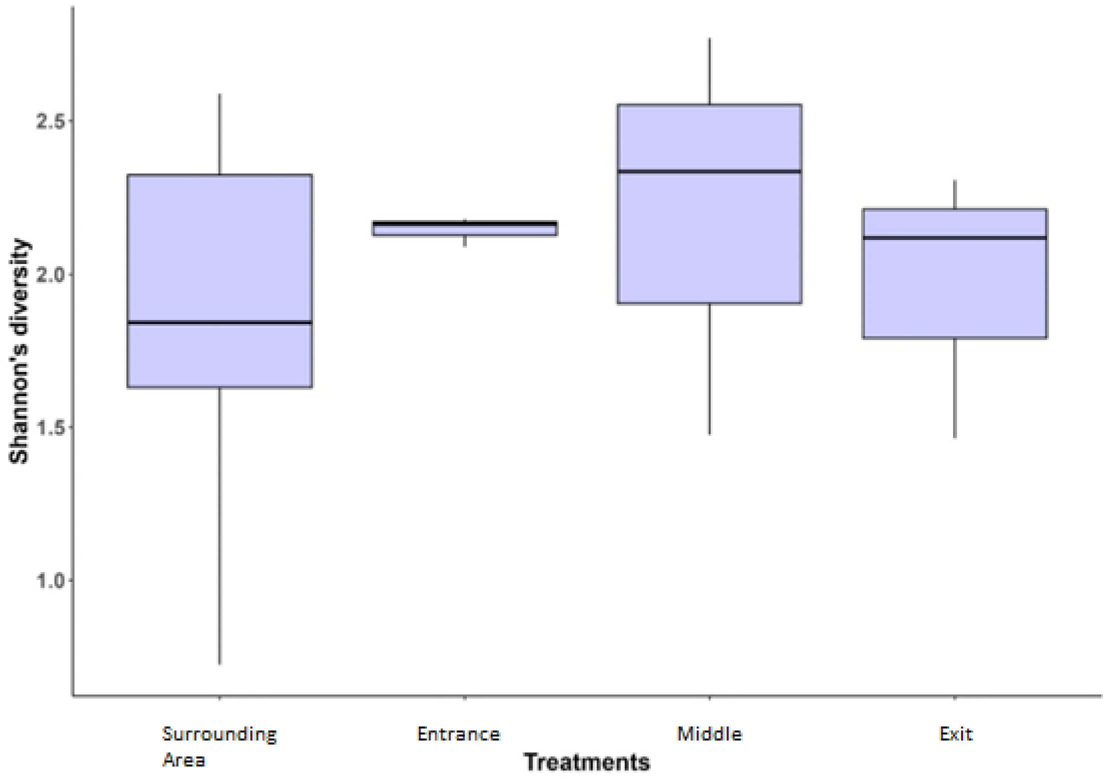

Figure 17 - Median ± SD of the Shannon-Wiener diversity values of the associated fauna collected with the pitfall traps in the Paço de Calheiros CW in Calheiros, during the Winter season.

3.4.2.2. Species richness

The richness of species values was not significantly different in the comparison per treatment during autumn (F value: 3.16, $P > 0.05$) and winter (F value: 0.94, $P > 0.05$). When comparing the richness values during the two seasons, autumn (Figure 22), had much higher richness values than winter (Figure 23), with a possible explanation being that many species have a population explosion in the summer/autumn due to being mating season for several species of macroinvertebrates.

Articles relating to an analysis of the species richness of the whole soil fauna in CWs and wetlands, with relevant information for this work were found to be very sparse, limiting possible comparisons.

When comparing the mean richness values (11.8 ± 2.7) that the CWs studied by Sartori et al. (2015) had, it was possible to observe that regarding the first sampling (autumn) only the surrounding area showed all values higher than in their study, while sites inside the CW had overall most values above it. The second sampling (winter) had the sample sites within the CW with most of their values equal or higher than it.

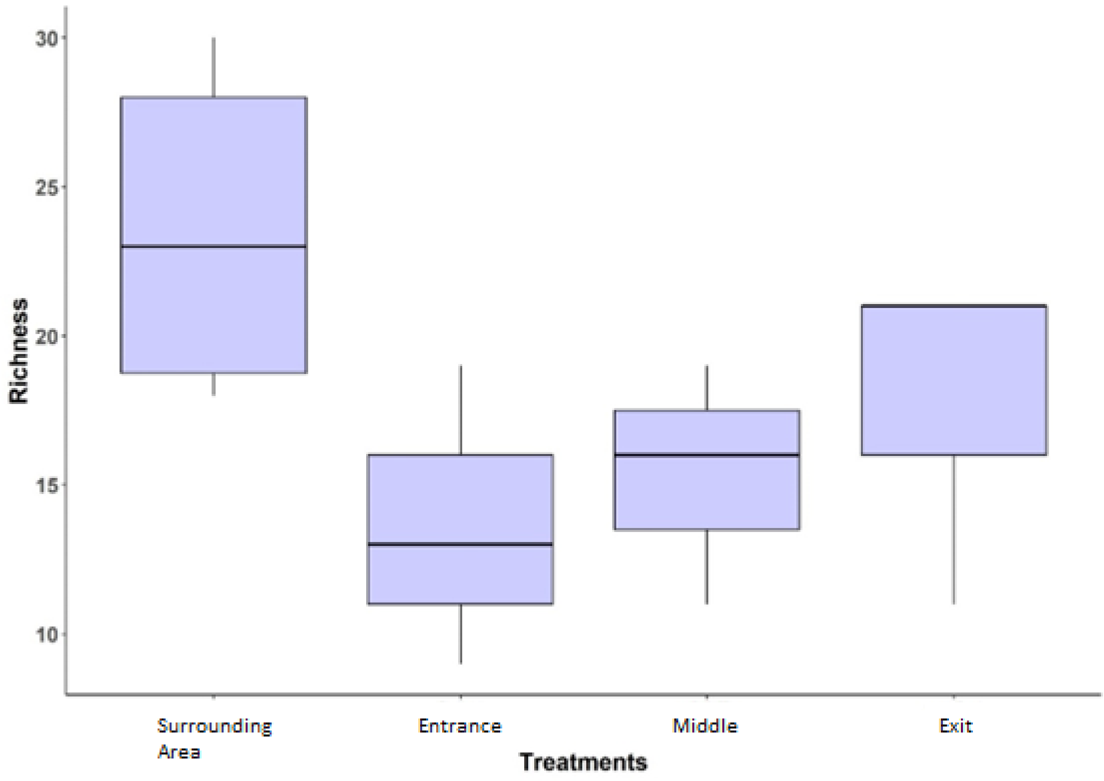

Figure 18 - Median ± SD of the species richness values of the associated fauna collected with the pitfall traps in the Paço de Calheiros CW in Calheiros, during the autumn season.

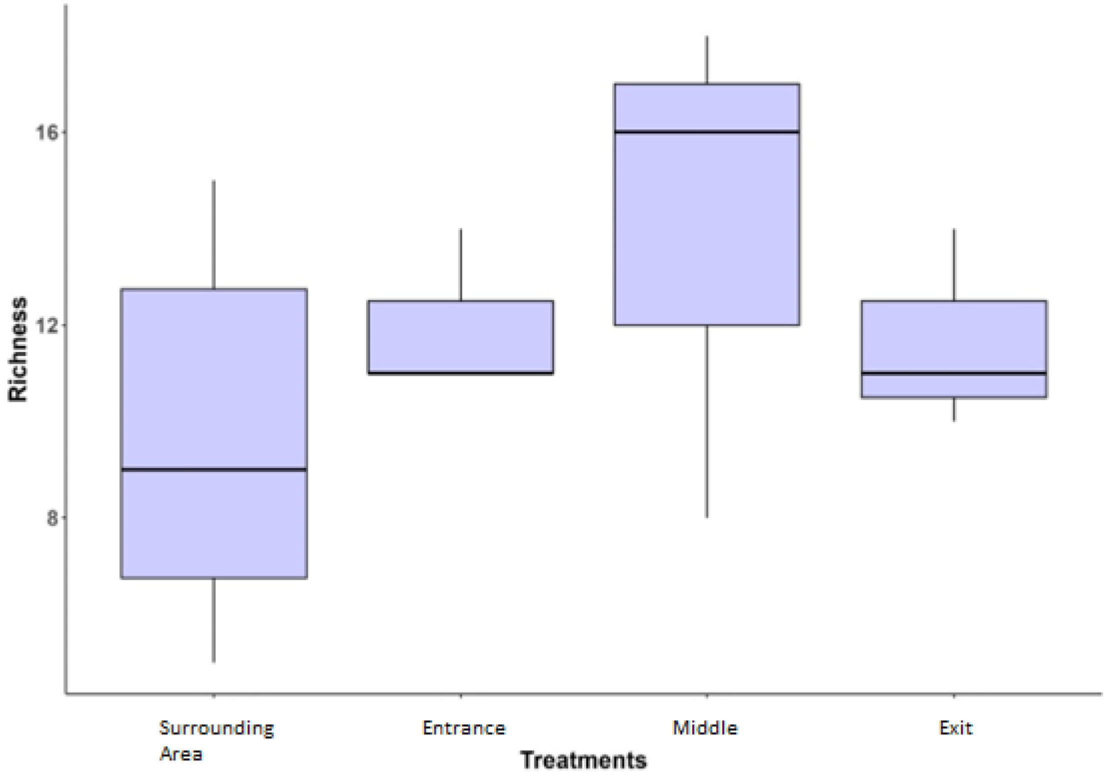

Figure 19 - Median ± SD of the species richness values of the associated fauna collected with the pitfall traps in the Paço de Calheiros CW in Calheiros, during the Winter season.

3.4.2.3. Pielou's evenness

The Pielou's eveness values were not significantly different in the comparison per treatment during autumn (F value: 3.25, $P > 0.05$) and winter (Kruskal-walis chi squared: 0.36, $P > 0.05$). Between seasons, in autumn, the surrounding area and the inlet had a more disproportionate abundance, with lower values of evenness than the middle and outlet (Figure 24), while during winter the values of all sampling sites were very similar (Figure 25).

These values can be contrasted with the mean in Pielou's evenness values (0.65 ± 0.14) seen in Sartori et al. (2015). In autumn, the sites of the surrounding area and inlet had some values below this result, while the middle and outlet were always higher than it, but they were not significant differences, as again, the surrounding area and inlet had many species with an unequal abundance compared to the middle and outlet. During winter, the pattern that was shown between sample sites was one of similarity, as said above, with a few differences in amplitude between sites, perhaps due to this season reducing the species encountered throughout the CW. Seasonality seems to be shown to affect this parameter regarding the soil community in this parameter.

Again, there weren't many articles dealing with Pielou's evenness in the context of the whole soil fauna, in CWs or natural wetlands, stifling comparisons, at this time.

Li et al. (2020) also analysed the Pielou's evenness in three wetlands, with mean values ranging between 0.7 and 0.9, with seasonality effects that differed between long-term reed restoration lakeshore and the rest, with the first having a similar seasonality pattern such as the Shannon Index that showed an increase through spring-summer-autumn and a dip in the winter, while the latter had its dip in the spring and maximum in the summer. Observing the Figures 24 and 25, it is possible to observe that the inlet in the autumn was lower than the results by observed by Li et al. (2020), while the middle and outlet remained in the upper range, whereas during winter the inlet, middle and outlet all stayed in an upper range, compared to Li et al. (2020), and that the seasonality of this parameter might be more similar to short-term reed restoration lakeshore and the natural reed lakeshore.

In short, evenness did not have showed significant changes between the sampling sites in autumn or winter. Autumn however showed a disproportionate abundance in the surrounding area and inlet, while winter had very similar values for all sites. These results, compared to other cases, were similar in terms of the seasonality patterns of a short-term reed restoration lakeshore and a natural reed lakeshore.

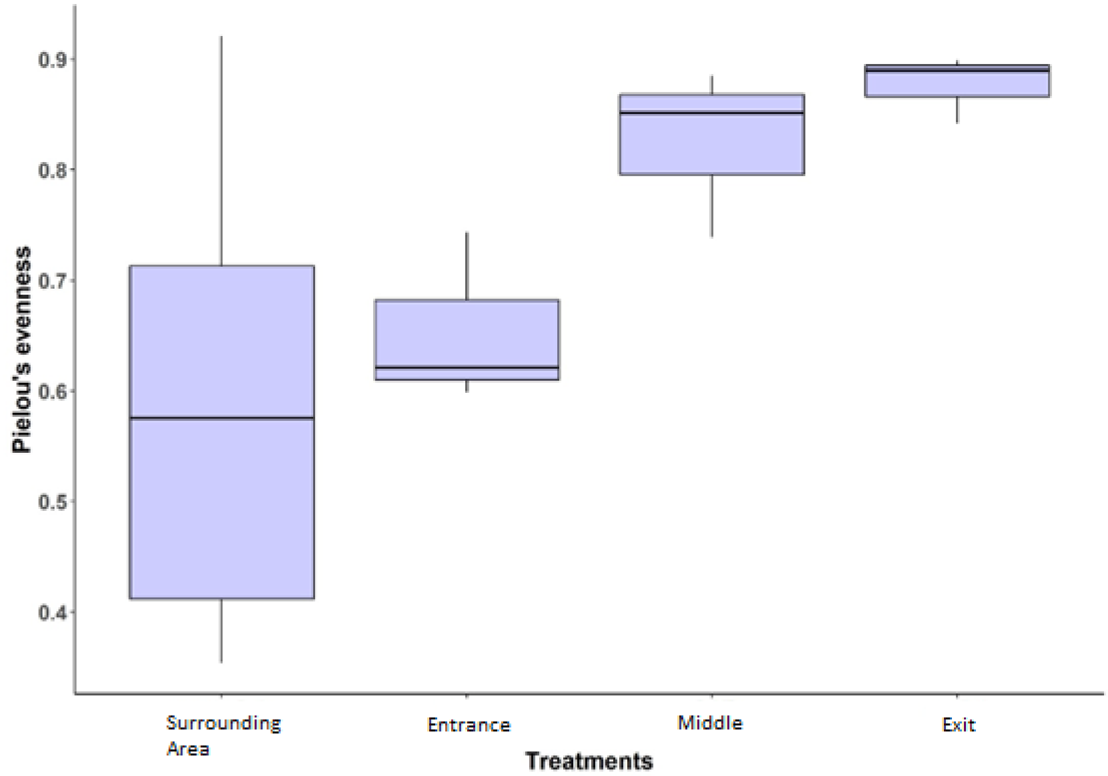

Figure 20 - Median ± SD of the Pielou's evenness values of the associated fauna collected with the pitfall traps in the Paço de Calheiros CW in Calheiros, during the autumn season.

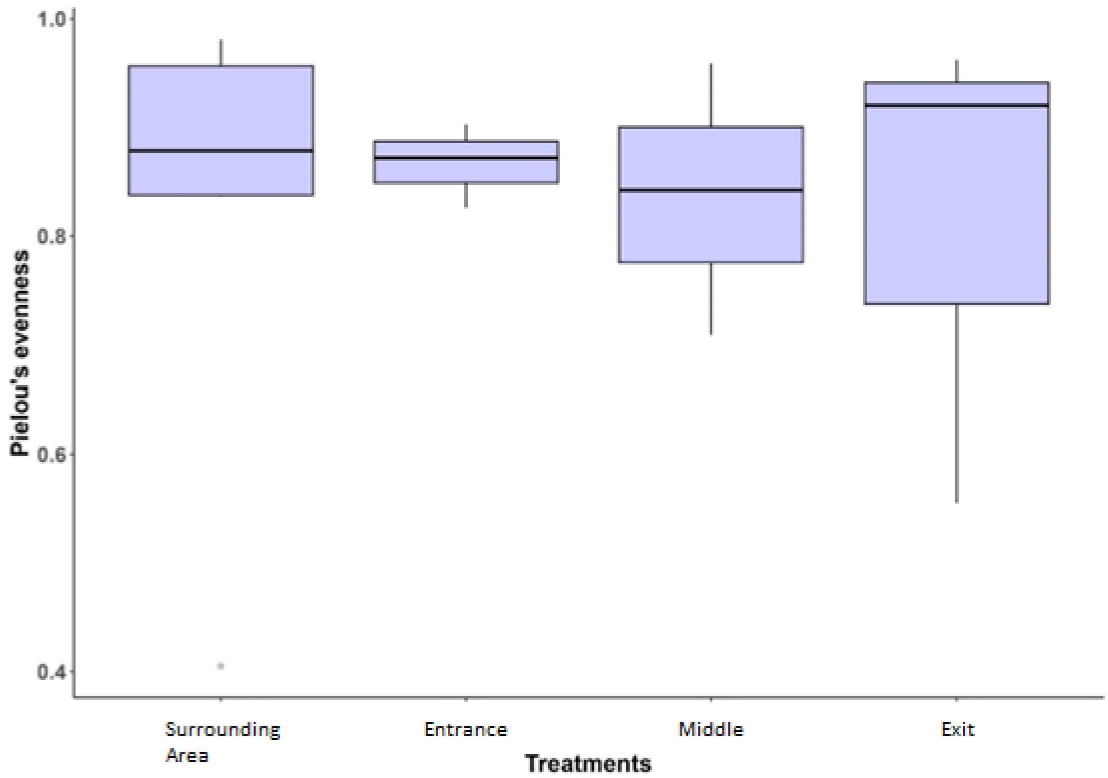

Figure 21 - Median ± SD of the Pielou's evenness values of the associated fauna collected with the pitfall traps in the Paço de Calheiros CW in Calheiros, during the winter season.

3.4.3. Indicator Species

One indicator species, is a species used to monitor environmental changes, providing warning signals for impending ecological shifts and assessing the efficacy of management (Siddig et al., 2016). The indicator species test made for the first sampling, suggested the Acari_13 (Figure 26) as an indicator species for this season for the surrounding area of the CW. Acari_13 occurred mainly in the area surrounding the CW. Acari are used as indicators due to their great sensitivity to disturbances at a micro scale (Gerlach et al., 2013), with a possible reason being that this species was found in great numbers in the surrounding area. This could represent a particular affinity for a parameter found in this area.

For the second sampling, two indicator species were observed, the Araneae_3, in the region corresponding to the entrance, and Araneae_4 for the middle. Araneae have been used before as indicators, reflecting the environmental state, indicating the levels of taxonomic diversity at a site, among other purposes, due to being environmentally sensitive (Gerlach et al., 2013). Araneae_3 was especially numerous in the entrance, while Araneae_4 likewise for the middle. As with Acari_13 in the autumn, this could show a particular sensitivity or predilection for environmental or biological conditions found in these zones, for each species.

However due to these species still being only identified at the Order level, further identification of these species might be needed to understand what specific environmental conditions they indicate.

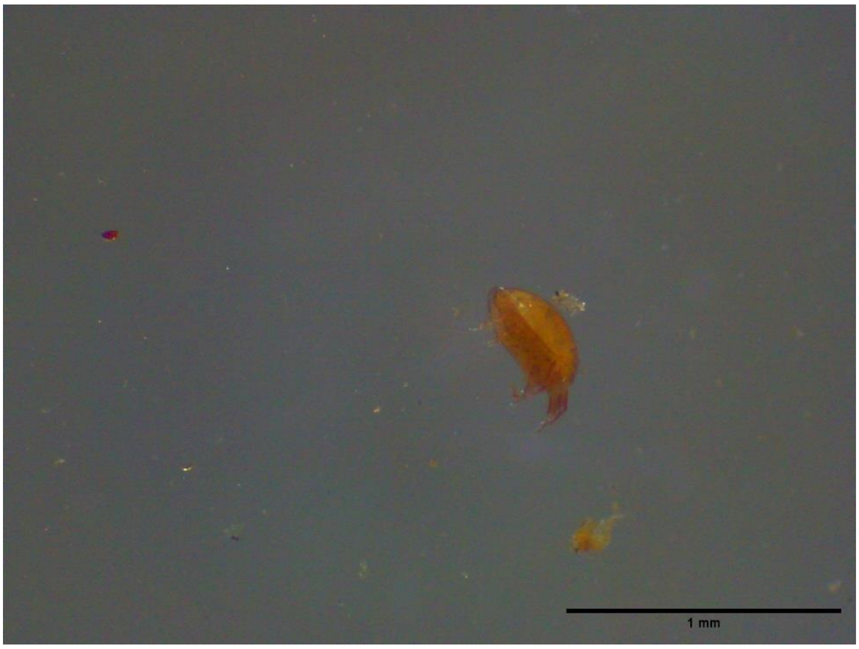

Figure 22 - Acari_13 species' individual, collected through pitfall traps during the autumn season in the surrounding area of the CW in Paço de Calheiros, Calheiros

Figure 23 - Araneae_3 species' individual, collected through pitfall traps during the winter season in the inlet section of the CW in Paço de Calheiros, Calheiros

Figure 24 - Araneae_4 species' individual, collected through pitfall traps during the winter season in surrounding area of the CW in Paço de Calheiros, Calheiros

Conclusion

Interesting results were found in the analysis of the efficiency of this CW, after its long-term operation, and the biodiversity that was associated with it, being among the few studies broaching these topics, in this manner.

The physicochemical water analyses of the samples collected at the inlet and outlet of the CW showed that the phytoremediation process is still effective, in what concerns to the contaminant removal, allowing the water to be reused for a variety of purposes. In terms of the microbial water quality assessment, concerning *E. coli*, it was possible to report the reduction from the inlet to the CW outlet, with a greater removal than those seen in previous studies. Total coliforms had a vast difference in removal efficiency between samplings, being important to continue to monitor its performance. Substrate analysis, though limited by the lack of comparable bibliography on the subject, showed levels of contaminants generally decreasing along the CW.

Taxa wise, the winter sampling had less taxa present as well as a lower number of species belonging to them, compared to the autumn, probably since autumn is a mating season for a range of invertebrate species. The two samplings shared key taxa, such as Diptera, Collembola, Hymenoptera, Araneae and Acari, which were also found in similar studies, with further identified taxa of Trombidiidae, Epuraea, Cicindelidae, Gyraulus showing the presence of organisms in various trophic levels. However, most of the species are still only identified at order level, requiring further identification to get a fuller picture of the trophic levels of the sampled invertebrates. The lack of comparable bibliography limited the comparisons with other studies regarding the ecological indexes (Shannon-Wiener diversity, species richness and Pielou's evenness) values of the associated fauna communities; however, it was noted that for all indexes there were changes between seasons, with richness and Pielou's evenness showing the greatest differences in the comparison per zone of the CW. Other notable difference was that, in the autumn sampling, the middle and outlet of the CW had higher diversities, the surrounding site had higher richness and this surrounding site and the inlet had more disproportionate abundance compared to the other sampling sites. In winter, samplings were quite similar, with exception of species richness being slightly higher in the middle and outlet. Comparing with other works Shannon diversity was generally higher than the compared examples, while richness and Pielou's evenness were generally lower. Due to the differences between seasons, and to further the understanding of the biodiversity of the CW, more samplings, from other seasons are still necessary to better comprehend the patterns observed.

Indicator species were also isolated, being different between autumn and winter, however as these particular species are still only identified at the order level, a more thorough identification will be needed to understand the specific environmental conditions that these species can inform us about.

Finally, this work has shown that after 12 years of operation the CW still performs its function as a wastewater treatment infrastructure, and that it harbours a high amount of invertebrate life.

Future work to be developed would comprise the monitoring, in terms of wastewater and biodiversity analysis, of one year round, in order to deepen the knowledge assorted to different seasons.

References

(L.)Spreng. Zantedeschia aethiopica - (L.)Spreng. *In*, Vol 2023. Plans For A Future https://pfaf.org/User/Plant.aspx?LatinName=Zantedeschia+aethiopica

Albuquerque A, Oliveira J, Semitela S, Amaral L (2009) Influence of bed media characteristics on ammonia and nitrate removal in shallow horizontal subsurface flow constructed wetlands. Bioresource technology **100:** 6269-6277

Aleissa YM, Bakshi BR (2021) Constructed Wetlands as Unit Operations in Chemical Process Design: Benefits and Simulation. Computers & Chemical Engineering **153:** 107454

Ali M, Rousseau DP, Ahmed S (2018) A full-scale comparison of two hybrid constructed wetlands treating domestic wastewater in Pakistan. Journal of Environmental Management **210:** 349-358

Bae S-H, Lee S-D (2018) Construction and Management Plan of Constructed Wetland for Promoting Biodiversity. Journal of People, Plants, and Environment **21:** 185-202

Barker G (2001) Gastropods on land: phylogeny, diversity and adaptive morphology. *In* The biology of terrestrial molluscs. CABI Wallingford UK, pp 1-146

Becerra Jurado G, Johnson J, Feeley H, Harrington R, Kelly-Quinn M (2010) The potential of integrated constructed wetlands (ICWs) to enhance macroinvertebrate diversity in agricultural landscapes. Wetlands **30:** 393-404

Benyamine M, Bäckström M, Sandén P (2004) Multi-objective environmental management in constructed wetlands. Environmental monitoring and assessment **90:** 171-185

Brix H (1994) Functions of macrophytes in constructed wetlands. Water Science and Technology **29:** 71-78

Cáceres MD, Legendre P (2009) Associations between species and groups of sites: indices and statistical inference. Ecology **90:** 3566-3574

Calheiros CS, Barreira E, Ramos NM, Castro PM (2017) Water masses surface temperatures assessment and their effect on surrounding environment. Water Science and Technology **75:** 2916-2925

Calheiros CS, Bessa VS, Mesquita RB, Brix H, Rangel AO, Castro PM (2015) Constructed wetland with a polyculture of ornamental plants for wastewater treatment at a rural tourism facility. Ecological Engineering **79:** 1-7

Calheiros CS, Castro PM, Gavina A, Pereira R (2019a) Toxicity abatement of wastewaters from tourism units by constructed wetlands. Water **11:** 2623

Calheiros CS, Pereira SI, Franco AR, Castro PM (2019b) Diverse arbuscular mycorrhizal fungi (AMF) communities colonize plants inhabiting a constructed wetland for wastewater treatment. Water **11:** 1535

Chapman D (1992) Chapman & Hall on behalf of UNESCO, WHO [and] UNEP. Water quality assessments: a guide to the use of biota, sediments and water in environmental monitoring London

Chen J, Deng S, Jia W, Li X, Chang J (2021) Removal of multiple heavy metals from mining-impacted water by biochar-filled constructed wetlands: adsorption and biotic removal routes. Bioresource Technology **331:** 125061

Choi Y-J, Kim L-H, Zoh K-D (2016) Removal characteristics and mechanism of antibiotics using constructed wetlands. Ecological Engineering **91:** 85-92

Clarkson BR, Ausseil A-GE, Gerbeaux P (2013) Wetland ecosystem services. Ecosystem services in New Zealand: conditions and trends. Manaaki Whenua Press, Lincoln **1:** 192-202

Colwell RK (2009) Biodiversity: concepts, patterns, and measurement. The Princeton guide to ecology **663:** 257-263

Commission E, Research D-Gf, Innovation, Bulkeley H, Naumann S, Vojinovic Z, Calfapietra C, Whiteoak K, Freitas T, Vandewoestijne S, Wild T (2020) Nature-based solutions : state of the art in EU-funded projects. Publications Office of the European Union

De La Rosa G, Peralta-Videa J (2022) Physicochemical Interactions of Engineered Nanoparticles and Plants: A Systemic Approach. Elsevier

Dordio AV, Teimão J, Ramalho I, Carvalho AP, Candeias AE (2007) Selection of a support matrix for the removal of some phenoxyacetic compounds in constructed wetlands systems. Science of the total environment **380:** 237-246

Drechsel P, Marjani Zadeh S, Salcedo F (2023) Water quality in agriculture: risks and risk mitigation.

Durkin ES, Cassidy ST, Gilbert R, Richardson EA, Roth AM, Shablin S, Keiser CN (2021) Parasites of spiders: Their impacts on host behavior and ecology. The Journal of Arachnology **49:** 281-298

Espanol C, Gallardo B, Comín FA, Pino MR (2015) Constructed wetlands increase the taxonomic and functional diversity of a degraded floodplain. Aquatic Sciences **77:** 27-44

Fox J, Weisberg S (2011) An R companion to applied regression. Sage publications

Gerlach J, Samways M, Pryke J (2013) Terrestrial invertebrates as bioindicators: an overview of available taxonomic groups. Journal of insect conservation **17:** 831-850

Guariento E, Colla F, Steinwandter M, Plunger J, Tappeiner U, Seeber J (2020) Management intensification of hay meadows and fruit orchards alters soil macro-invertebrate communities differently. Agronomy **10:** 767

Hansson LA, Brönmark C, Anders Nilsson P, Åbjörnsson K (2005) Conflicting demands on wetland ecosystem services: nutrient retention, biodiversity or both? Freshwater Biology **50:** 705-714

Heip CH, Herman PM, Soetaert K (1998) Indices of diversity and evenness. Oceanis **24:** 61-88

Hopkin SP (1997) Biology of the springtails:(Insecta: Collembola). OUP Oxford

Horne AJ (2020) Phytoremediation by constructed wetlands. *In* Phytoremediation of contaminated soil and water. CRC Press, pp 13-39

Hsu C-B, Hsieh H-L, Yang L, Wu S-H, Chang J-S, Hsiao S-C, Su H-C, Yeh C-H, Ho Y-S, Lin H-J (2011) Biodiversity of constructed wetlands for wastewater treatment. Ecological Engineering **37:** 1533-1545

Jamieson R (2004) Agapanthus africanus. *In*. PlantZAfrica https://pza.sanbi.org/agapanthus-africanus

Jamion NA, Lee KE, Mokhtar M, Goh TL, Simon N, Goh CT, Bhat IUH (2023) The integration of nature values and services in the nature-based solution assessment framework of constructed wetlands for carbon–water nexus in carbon sequestration and water security. Environmental Geochemistry and Health **45:** 1201-1230

Jang YS, Kim D-S (2014) The first report on the winter breeding life history of Epuraea domina (Coleoptera: Nitidulidae) in a citrus grove in Jeju, Korea. Crop Protection **55:** 68-73

Jespersen AN (2020) An Introduction to Constructed Wetlands. Nova Science Publishers

Karstens S, Dorow M, Bochert R, Stybel N, Schernewski G, Mühl M (2022) Stepping stones along urban coastlines—improving habitat connectivity for aquatic fauna with constructed floating wetlands. Wetlands **42:** 76

Karstens S, Langer M, Nyunoya H, Čaraitė I, Stybel N, Razinkovas-Baziukas A, Bochert R (2021) Constructed floating wetlands made of natural materials as habitats in eutrophicated coastal lagoons in the Southern Baltic Sea. Journal of Coastal Conservation **25:** 1-14

Kataki S, Chatterjee S, Vairale MG, Dwivedi SK, Gupta DK (2021) Constructed wetland, an eco-technology for wastewater treatment: A review on types of wastewater treated and components of the technology (macrophyte, biolfilm and substrate). Journal of Environmental Management **283:** 111986

Kennedy G, Mayer T (2002) Natural and constructed wetlands in Canada: An overview. Water Quality Research Journal **37:** 295-325

Knapp S, Schmauck S, Zehnsdorf A (2019) Biodiversity impact of green roofs and constructed wetlands as progressive eco-technologies in urban areas. Sustainability **11:** 5846

Koskiaho J, Puustinen M (2019) Suspended solids and nutrient retention in two constructed wetlands as determined from continuous data recorded with sensors. Ecological Engineering **137:** 65-75

Kouakou AK, Cortet J, Kolo Y, Brauman A (2022) Using Trait-Based Approaches to Assess the Response of Epedaphic Collembola to Organic Matter Management Practices: A Case Study in a Rubber Plantation in South-Eastern Côte d'Ivoire. Insects **13:** 892

Krasnits E, Friedler E, Sabbah I, Beliavski M, Tarre S, Green M (2009) Spatial distribution of major microbial groups in a well established constructed wetland treating municipal wastewater. Ecological Engineering **35:** 1085-1089

KS H (1999) Terrestrial arthropod predators of insect and mite pests. Handbook of biological control. Principles and applications of biological control

Lehmitz R, Haase H, Otte V, Russell D (2020) Bioindication in peatlands by means of multi-taxa indicators (Oribatida, Araneae, Carabidae, Vegetation). Ecological Indicators **109:** 105837

Leone D, Mirabile M, Altieri GM, Zimone A, Torrisi B, Tarasco E, Clausi M (2023) Assessment of soil quality in wetlands in Eastern Sicily. Ecological Indicators **153:** 110428

Li N, Tian X, Li Y, Fu H, Jia X, Jin G, Jiang M (2018) Seasonal and spatial variability of water quality and nutrient removal efficiency of restored wetland: A case study in Fujin National Wetland Park, China. Chinese Geographical Science **28:** 1027-1037

Li W, Dou Z, Cui L, Zhao X, Zhang M, Zhang Y, Gao C, Yang Z, Lei Y, Pan X (2020) Soil fauna diversity at different stages of reed restoration in a lakeshore wetland at Lake Taihu, China. Ecosystem Health and Sustainability **6:** 1722034

Liu D, Ge Y, Chang J, Peng C, Gu B, Chan GY, Wu X (2009) Constructed wetlands in China: recent developments and future challenges. Frontiers in Ecology and the Environment **7:** 261-268

Liu X, Guo X, Liu Y, Lu S, Xi B, Zhang J, Wang Z, Bi B (2019) A review on removing antibiotics and antibiotic resistance genes from wastewater by constructed wetlands: performance and microbial response. Environmental Pollution **254:** 112996

Lu S, Zhang X, Wang J, Pei L (2016) Impacts of different media on constructed wetlands for rural household sewage treatment. Journal of Cleaner Production **127:** 325-330

Mace GM, Norris K, Fitter AH (2012) Biodiversity and ecosystem services: a multilayered relationship. Trends in ecology & evolution **27:** 19-26

Manu M, Băncilă R, Bîrsan C, Mountford O, Onete M (2021) Soil mite communities (Acari: Mesostigmata) as indicators of urban ecosystems in Bucharest, Romania. Scientific reports **11:** 3794

Marx MT, Guhmann P, Decker P (2012) Adaptations and predispositions of different Middle European arthropod taxa (Collembola, Araneae, Chilopoda, Diplopoda) to flooding and drought conditions. Animals **2:** 564-590

Meehan CJ, Olson EJ, Reudink MW, Kyser TK, Curry RL (2009) Herbivory in a spider through exploitation of an ant–plant mutualism. Current biology **19:** R892-R893

Metcalfe CD, Nagabhatla N, Fitzgerald SK (2018) Multifunctional wetlands: pollution abatement by natural and constructed wetlands. Multifunctional Wetlands: Pollution Abatement and Other Ecological Services from Natural and Constructed Wetlands**:** 1-14

Mitsch WJ, Gosselink JG (2015) Wetlands. John Wiley & Sons

Msaki GL, Njau KN, Treydte AC, Lyimo TJ (2023) Characterization of bird, reptile, and insect community diversity in constructed wetlands and waste stabilization ponds across Tanzania. Ecological Engineering **196:** 107082

Msimbira LA, Smith DL (2020) The roles of plant growth promoting microbes in enhancing plant tolerance to acidity and alkalinity stresses. Frontiers in Sustainable Food Systems **4:** 106

Nan X, Lavrnić S, Mancuso G, Toscano A (2023) Effects of Design and Operational Conditions on the Performance of Constructed Wetlands for Agricultural Pollution Control–Critical Review. Water, Air, & Soil Pollution **234:** 434

Notten SNA (2005) Watsonia borbonica. *In*, Vol 2023. PlantZAfrica https://pza.sanbi.org/watsonia-borbonica

Nunes N, Ragonezi C, Gouveia CS, Pinheiro de Carvalho MÂ (2021) Review of sewage sludge as a soil amendment in relation to current international guidelines: A heavy metal perspective. Sustainability **13:** 2317

Ouattara J-MP, Coulibaly L, Tiho S, Gourène G (2009) Comparison of macrofauna communities in sediments of the beds of vertical flow constructed wetlands planted with Panicum maximum (Jacq.) treating domestic wastewater. Ecological Engineering **35:** 1237-1242

Pasiecznik N (2008) Canna indica (canna lilly). *In*, Vol 2023. CABI Digital Library, CABI Compendium https://www.cabidigitallibrary.org/doi/10.1079/cabicompendium.14575

Puigagut J, Salvadó H, García D, Granes F, García J (2007) Comparison of microfauna communities in full scale subsurface flow constructed wetlands used as secondary and tertiary treatment. Water research **41:** 1645-1652

Rahman KZ, Al Saadi S, Al Rawahi M, Knappe J, van Afferden M, Moeller L, Bernhard K, Müller RA (2023) A multi-functional nature-based solution (NBS) for greywater treatment and reuse at the same plot. Ecological Engineering **191:** 106952

Ripley BD (2002) Modern applied statistics with S. springer

Saeed T, Sun G (2012) A review on nitrogen and organics removal mechanisms in subsurface flow constructed wetlands: dependency on environmental parameters, operating conditions and supporting media. Journal of environmental management **112:** 429-448

Sartori L, Canobbio S, Cabrini R, Fornaroli R, Mezzanotte V (2015) Macroinvertebrate assemblages and biodiversity levels: ecological role of constructed wetlands and artificial ponds in a natural park. Journal of Limnology **74**

Schäfer ML, Lundström JO, Pfeffer M, Lundkvist E, Landin J (2004) Biological diversity versus risk for mosquito nuisance and disease transmission in constructed wetlands in southern Sweden. Medical and Veterinary Entomology **18:** 256-267

Shi X, Fan J, Zhang J, Shen Y (2017) Enhanced phosphorus removal in intermittently aerated constructed wetlands filled with various construction wastes. Environmental Science and Pollution Research **24:** 22524-22534

Siddig AA, Ellison AM, Ochs A, Villar-Leeman C, Lau MK (2016) How do ecologists select and use indicator species to monitor ecological change? Insights from 14 years of publication in Ecological Indicators. Ecological Indicators **60:** 223-230

Society FNP *Canna flaccida*. *In*, Vol 2023. Florida Native Plant Society https://www.fnps.org/plant/canna-flaccida

Tanner CC (1996) Plants for constructed wetland treatment systems—a comparison of the growth and nutrient uptake of eight emergent species. Ecological engineering **7:** 59-83

Team RDC (2010) R: A language and environment for statistical computing. (No Title)

Tett P, Black K, Brennan R, Cook E, Davidson K (2015) Sustainable Mariculture at High Latitudes. Coastal Zones: Solutions for the 21st Century**:** 73-81

Tunçsiper B, Ayaz SÇ, Akça L (2012) COLIFORM BACTERIA REMOVAL FROM SEPTIC WASTEWATER IN A PILOT-SCALE COMBINED CONSTRUCTED WETLAND SYSTEM. Environmental Engineering & Management Journal (EEMJ) **11**

Valkama P, Mäkinen E, Ojala A, Vahtera H, Lahti K, Rantakokko K, Vasander H, Nikinmaa E, Wahlroos O (2017) Seasonal variation in nutrient removal efficiency of a boreal wetland detected by high-frequency on-line monitoring. Ecological Engineering **98:** 307-317

Varma M, Gupta AK, Ghosal PS, Majumder A (2021) A review on performance of constructed wetlands in tropical and cold climate: Insights of mechanism, role of influencing factors, and system modification in low temperature. Science of the Total Environment **755:** 142540

Vasconcellos RL, Segat JC, Bonfim JA, Baretta D, Cardoso EJ (2013) Soil macrofauna as an indicator of soil quality in an undisturbed riparian forest and recovering sites of different ages. European Journal of Soil Biology **58:** 105-112

Vymazal J (2011) Constructed wetlands for wastewater treatment: five decades of experience. Environmental science & technology **45:** 61-69

Vymazal J (2013) Emergent plants used in free water surface constructed wetlands: a review. Ecological engineering **61:** 582-592

Vymazal J (2013) Plants in constructed, restored and created wetlands. Ecological engineering **61:** 501-504

Vymazal J, Kröpfelová L (2008) Types of constructed wetlands for wastewater treatment. Wastewater treatment in constructed wetlands with horizontal sub-surface flow**:** 121-202

Wang J, Long Y, Yu G, Wang G, Zhou Z, Li P, Zhang Y, Yang K, Wang S (2022) A review on microorganisms in constructed wetlands for typical pollutant removal: species, function, and diversity. Frontiers in Microbiology **13**

Wiegleb G, Dahms H-U, Byeon W, Choi G (2017) To what extent can constructed wetlands enhance biodiversity. International Journal of Environmental Science and Development **8:** 561-569

Woodcock B (2005) Pitfall trapping in ecological studies. Insect sampling in forest ecosystems **5:** 37-57

Wu H, Zhang J, Ngo HH, Guo W, Hu Z, Liang S, Fan J, Liu H (2015) A review on the sustainability of constructed wetlands for wastewater treatment: design and operation. Bioresource technology **175:** 594-601

Wu P, Zhang H, Cui L, Wickings K, Fu S, Wang C (2017) Impacts of alpine wetland degradation on the composition, diversity and trophic structure of soil nematodes on the Qinghai-Tibetan Plateau. Scientific reports **7:** 837

Wu Y, Han R, Yang X, Zhang Y, Zhang R (2017) Long-term performance of an integrated constructed wetland for advanced treatment of mixed wastewater. Ecological Engineering **99:** 91-98

Zagatto MRG, Oliveira Filho LCI, Pompeo PN, Niva CC, Baretta D, Bran Nogueira Cardoso EJ (2020) Mesofauna and Macrofauna in Soil and Litter of Mixed Plantations. Mixed Plantations of Eucalyptus and Leguminous Trees: Soil, Microbiology and Ecosystem Services**:** 155-172

Zhang C, Wen L, Wang Y, Liu C, Zhou Y, Lei G (2020) Can constructed wetlands be wildlife refuges? A review of their potential biodiversity conservation value. Sustainability **12:** 1442

Zhang J, Meng F, Lu Y, Jing Y, Zhang H, Zhang B, Zhang C (2008) Ecological assessment of lakeshore wetland rehabilitation on eastern route of South-to-North Water Transfer Project. Frontiers of Environmental Science & Engineering in China **2:** 306-310

Zhang Z-Q (1998) Review Biology and ecology of trombidiid mites (Acari: Trombidioidea). Experimental & Applied Acarology **22:** 139-155

Zhao Y, Liu B, Zhang W, Hu C, An S (2010) Effects of plant and influent C: N: P ratio on microbial diversity in pilot-scale constructed wetlands. Ecological Engineering **36:** 441-449

Zheng X, Wang H, Tao Y, Kou X, He C, Wang Z (2022) Community diversity of soil meso-fauna indicates the impacts of oil exploitation on wetlands. Ecological Indicators **144:** 109451

Zhou X, Liang C, Jia L, Feng L, Wang R, Wu H (2018) An innovative biochar-amended substrate vertical flow constructed wetland for low C/N wastewater treatment: impact of influent strengths. Bioresource Technology **247:** 844-850

Zhu S-X, Ge H-L, Ge Y, Cao H-Q, Liu D, Chang J, Zhang C-B, Gu B-J, Chang S-X (2010) Effects of plant diversity on biomass production and substrate nitrogen in a subsurface vertical flow constructed wetland. Ecological Engineering **36:** 1307-1313