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Effects of coagulation pre-treatment on chemical and microbial properties of water-soil-plant systems of constructed wetlands

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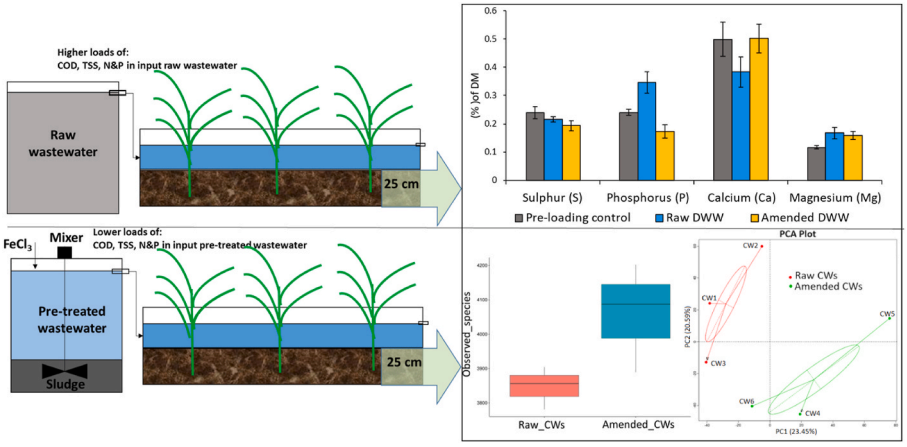
HIGHLIGHTS

- Wastewater pre-treatment using $FeCl_3$ improved the performance of CWs.
- $FeCl_3$ pre-treatment had no negative effects on soil and plant properties.
- $FeCl_3$ pre-treatment did not alter microbial community diversity and composition.

GRAPHICAL ABSTRACT



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ABSTRACT

Chemical coagulation has gained recognition as an effective technique to enhance the removal efficiency of pollutants in wastewater prior to their entry into a constructed wetland (CW) system. However, its potential impact on the chemical and microbial properties of soil and plant systems within CWs requires further research. This study investigated the impact of using ferric chloride ($FeCl_3$) as a pre-treatment stage for dairy wastewater (DWW) on the chemical and microbial properties of water-soil-plant systems of replicated pilot-scale CWs, comparing them to CWs treating untreated DWW. CWs treating amended DWW had better performance than CWs treating raw DWW for all water quality parameters (COD, TSS, TP, and TN), ensuring compliance with the EU wastewater discharge directives. Soil properties remained mostly unaffected except for pH, calcium and phosphorus (P), which were lower in CWs treating amended DWW. As a result of lower nitrogen (N) and P loads, the plants in CWs receiving $FeCl_3$-amended DWW had lower N and P contents than the plants of raw DWW CWs. However, the lower loads of P into amended DWW CWs did not limit the growth of *Phragmites australis,* which

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were able to accumulate trace elements higher than CWs receiving raw DWW. Alpha and Beta-diversity analysis revealed minor differences in community richness and composition between both treatments, with only 3.7% (34 genera) showed significant disparities. Overall, the application of chemical coagulation produced superior effluent quality without affecting the properties of soil and plant of CWs or altering the functioning of the microbial community.

1. Introduction

Constructed wetlands (CWs) have gained significant attention and popularity as a sustainable and cost-effective method for wastewater treatment (Stefanakis, 2019). These systems have emerged as an alternative method for wastewater treatment, particularly in decentralized and rural areas where conventional treatment methods are often impractical or cost prohibitive (Valipour and Ahn, 2016). These engineered systems mimic the natural wetland ecosystem, utilizing a mixture of chemical, physical, and biological processes to remove nutrients, metals, pathogens and organic compounds, while promoting ecological diversity and enhancing aesthetic appeal (Harrington and McInnes, 2009). CWs have proven to be effective in treating various types of wastewaters, including domestic, industrial, agricultural, and stormwater runoff wastewaters (Vymazal, 2009; Kadlec and Wallace, 2008). One crucial aspect of wastewater treatment in CWs is the pre-treatment stage, where various techniques are employed to enhance the efficiency of pollutant removal (Álvarez et al., 2008; Bosak et al., 2016) and optimise the overall performance of the system (Nan et al., 2020). In particular, high-strength wastewater, such as agricultural dairy wastewater (DWW), requires a pre-treatment stage to reduce influent loads since the individual capacity of CWs fails to meet stringent treatment standards. Additionally, the pretreatment stage aids in addressing inherent operational challenges of CWs, such as the potential of CWs clogging or a reduction in soil capacity to adsorb phosphorus (P) with the time (Wang et al., 2021; Mohamed et al., 2022). The pre-treatment stage involves the removal of large, suspended particles, sedimentation of heavy metals, and reduction of organic matter, among other processes (Healy et al., 2007). Various pre-treatment techniques have been employed; among these techniques chemical coagulation has gained recognition as an effective technique to enhance the removal efficiency of pollutants in wastewater prior to their entry into the wetland system (Liang et al., 2019; Xu et al., 2020; Mohamed et al., 2022).

Chemical coagulation involves the dosing of coagulants such as ferric chloride or aluminium sulphate (alum) to wastewater. These coagulants facilitate the aggregation of suspended particles, resulting in the formation of larger flocs that can be more easily removed through sedimentation or filtration processes (Jiang, 2015). Chemical coagulation has been extensively studied and implemented in conventional water treatment processes as well as in wastewater pretreatment and purification. However, the impact of chemical coagulation on CWs has not yet been investigated. The application of chemical coagulation in the pre-treatment of wastewater raises concerns about its potential impact on the chemical and microbial properties of soil and plant systems within CWs. This because the coagulants may introduce chemicals and ions into the wetland environment, altering soil chemistry and potentially affecting plant growth and microbial activity (Liang et al., 2019). These alterations may have cascading effects on the overall functioning of the wetland system, including nutrient cycling, organic matter decomposition, and pollutant removal.

The introduction of chemical coagulants to the soil system may also alter key chemical characteristics, such as cation exchange capacity (CEC), pH, and nutrient availability. Aluminium-based coagulants, for example, may increase soil acidity due to the release of aluminium ions, potentially affecting soil microbial communities and nutrient uptake by plants (Babatunde et al., 2011). Additionally, the increased levels of metals, such as aluminium and iron, arising from the use of the coagulants may influence the availability and mobility of other essential nutrients in the soil (Turner et al., 2019).

Soil microbial communities play a crucial role in organic matter decomposition, nutrient cycling, and overall soil health. The application of chemical coagulants can impact microbial populations and diversity, potentially leading to shifts in community composition and function (Wu et al., 2019). Some studies have suggested that the presence of coagulants may inhibit microbial activity and reduce the abundance of certain microbial groups, such as nitrogen-fixing bacteria, which are essential for nutrient cycling and plant growth (Belkin et al., 2017).

The potential impact of chemical coagulation pre-treatment on plant systems within CWs is another important consideration. Plants play a vital role in pollutant removal, as they take up nutrients and metals from the wastewater through their roots. Changes in soil chemistry resulting from coagulant application may affect plant growth, nutrient uptake, and overall plant health (Babatunde et al., 2011). Furthermore, the direct exposure of plant roots to coagulants may lead to physiological stress and altered plant responses (Liang et al., 2019).

Understanding the effects of chemical coagulation pre-treatment on the chemical and microbial properties of soil and plant systems in CWs is crucial for the successful implementation of these systems. Therefore, further research is needed to investigate the interactions between coagulants and water-soil-plant-microbial systems. The current study examined the effects of $FeCl_3$ pre-treatment of DWW on chemical and microbial properties of soil and plant systems of CWs that were operated for a period of 301 days, compared to CWs receiving raw untreated DWW. This knowledge will contribute to the development of optimized coagulation strategies and the sustainable management of CWs as efficient wastewater treatment systems.

2. Materials and methods

2.1. Experimental set-up

The experimental set up in the current study is described in the study of Mohamed et al. (2022). Briefly, six outdoor pilot-scale free water surface constructed wetlands (FWS CWs) were used to treat raw DWW (n = 3) and pre-treated DWW (n = 3) and operated for a duration of 301 days (Fig. S1, Appendix A). The dimensions of each CW container were 0.5 m (width), 1.75 m (length) and 0.5 m (height) (Fig. S1). Each CW container was filled with loam-textured soil (locally sourced) to a depth of 0.25 m and planted with mature common reed (*Phragmites australis* (Cav.) Trin. Ex Steud.), at density of 200 plants per m^2. In FWS CWs, the water usually flows over the surface of the soil. Therefore, normal soil was used as a substrate instead of granular materials, which are typically used in subsurface horizontal flow CWs (SSHF CWs) or sub-surface vertical flow CWs (SSVF CWs). Locally sourced soil is relatively cheap and easily available on farms. The water level was maintained at a height of 0.15 ± 0.03 m above the soil surface.

Raw DWW was collected weekly from the adjacent dairy farmyard and delivered to a 1000-L storage tank (Fig. S1). The DWW was composed of washings from the holding area, milking parlours, and the cleaning process of the milking equipment. It was a blend of milk, water, urine, cow faces, cleansing agents, and solid particles. Amended DWW was prepared by mixing raw DWW with ferric chloride ($FeCl_3$; Table S1, Appendix A) at a dose of 440 mg Fe per each L of DWW based on the study of Mohamed et al. (2020). The amended DWW was then left to settle for 3 h to separate solids from the clean water (supernatant). Raw

Table 1
Operational conditions and experimental phases applied to raw and amended DWW CWs for a study duration of 301 days (Mohamed et al., 2022).

Operation approach	Phase	Operational days	Wastewater type	OLR (g m^{-2} d^{-1}) Mean ± SD	HLR (L m^{-2} d^{-1}) Mean ± SD	Dosing frequency[a]
Identical OLR	1	49	Raw DWW	7.0 ± 1.5	1.4 ± 0.4	4
			Amended DWW	7.0 ± 1.5	4.7 ± 0.9	
	2	154	Raw DWW	3.5 ± 1.0	0.7 ± 0.2	4
			Amended DWW	3.5 ± 1.0	2.3 ± 0.5	
Identical HLR	3	42	Raw DWW	13.0 ± 2.0	2.3 ± 0.5	4
			Amended DWW	3.5 ± 1.0	2.3 ± 0.5	
	4	56	Raw DWW	25.0 ± 2.5	4.7 ± 0.9	8
			Amended DWW	7.0 ± 1.5	4.7 ± 0.9	

[a] Dosing frequency denotes the number of times which CWs received raw and amended DWW per day.

and amended DWW were prepared every week and were fed/dosed discontinuously (intermittently 4 or 8 times per day, Table 1) onto the replicated (n = 3) CWs using diaphragm waste pumps, which were controlled by electronic timers. The duration of each feeding interval was less than 1 min. The experiment was separated into four phases with different hydraulic and organic loading rates (Table 1). The research spanned an entire milking season, from February to December, comprising 301 days. Spring-calving dairy herds undergo an 8–10-week pre-calving period during which they are not milked, resulting in the absence of DWW production at the study site, typically occurring from early December to mid-February.

2.2. Analysis

2.2.1. Chemical properties of water and soil

Water samples were taken and analysed weekly from the influent raw and amended DWW (after $FeCl_3$ pretreatment) and from the effluent of raw and amended DWW CWs. All water analyses were conducted in accordance with established protocols outlined in the APHA (2005) standard methods. Chemical oxygen demand (COD) was quantified through HACH kits (HACH, USA) using the dichromate-digestion procedure. Total suspended solids (TSS) was assessed through the APHA (2005) gravimetrical method. Total nitrogen (TN) and total P (TP) concentrations were quantified using the Persulphate Oxidative-Digestion procedure carried out with HACH Ganimede instruments (HACH, USA).

Bulk samples of soil, collected from all CWs at the beginning and end of the experiment, were dried and sieved through a 2 mm mesh before chemical analysis. Loss on Ignition (LoI) method was used to assess the amount of organic matter content (OM) through drying soil samples at 105 °C, and then ashing them at temperatures of 450 °C (British Standards Institution, 1990). Total organic carbon (TOC) was quantified through the combustion method of DUMAS (British Standards Institution, 2012; BS EN 15936). Total nitrogen was assessed through the Dumas procedure (AOAC, 1990; Method 949.12). Total elements in soil (Fe, Al, Ca, Mg, P, K) were quantified using both nitric and hydrochloric acid digestion techniques (aqua-regia) (US Environmental Protection Agency, 1996; SW 486 Method 3050 B).

2.2.2. Chemical properties of plant tissue

Bulk samples of plant tissue were also collected from all CWs at the beginning (before loading the systems with DWW; October 2020) and end of the experiment (December 2021). Plant tissue samples comprised both leaves and stems/shoot above the soil and water surfaces (emergent plant). The DUMAS method (AOAC, 1990; Method 949.12) was used to quantify TN after drying and sieving plant samples through a 0.5 mm screen. Elemental content of plant tissue was determined using both nitric and hydrochloric acid digestion solutions (aqua-regia) (MAFF, 1986), after drying and sieving plant samples through a 1-mm screen. Coupled Plasma Optical Emission Spectroscopy (ICP-OES) was then used to determine elements in solution (P, Mg, K, Ca, Mn, S, Fe, B, Zn and Cu). These trace elements are commonly used to study the bioaccumulation capacity of *P. australis* and their higher concentrations in plants be toxic (Vymazal et al., 2009; Bonanno, 2011).

2.2.3. Microbial properties of soil

The microbial community structure within the CWs were analysed by taking wet bulk samples of soil at the end of the experiment. Each sample was thoroughly homogenised and genomic DNA was extracted from a 0.3 g aliquot through the QIAGEN DNeasy PowerSoil Pro Kit (Germantown, Maryland, US), following the provided manufacturer's instructions. The extracted DNA was frozen and transported on ice bags to NovoGene Ltd (Cambridge, UK) for the subsequent steps of amplicon library preparation and sequencing. The amplicon library preparation was carried out using 515 F (GTGCCAGCMGCCGCGG) forward primers and 806 R (GGACTACHVGGGTWTCTAAT) reverse primers, which target the V4 hypervariable region. PCR cycling was performed as follows: an initial denaturation step of DNA strands at 94 ± 1 °C for 5 min, then 30 cycles consisting of denaturation at 94 ± 1 °C for 30 s, followed by annealing at 56 ± 1 °C for 45 s and elongation at 72 ± 1 °C for 2 min; and last elongation step at 72 ± 1 °C for 10 min before cooling to 4 °C. All PCR reactions were carried out with Phusion® PCR Master Mix/High Fidelity (New-England Bio-labs). PCR products were combined in equal volumes with loading buffer containing SYBR green, and electrophoresis on a 2% agarose gel was employed for PCR detection. Only samples displaying shiny main strands within the 400–450 base pair (bp) range were considered qualified for sequencing. PCR products were combined in equi-density ratios and subsequently purified using Gel Extraction Kit (Qiagen, Germany). Then, sequencing libraries were obtained in line with the manufacturer's procedures using NEBNext® Ultra DNA Library Illumina Pre Kit. The quality of the generated library was evaluated using the Qubit@ 2.0 Thermo Scientific Fluorometer. Lastly, amplicon sequencing was carried out using the Illumina NovaSeq PE250 platform (Illumina Inc. USA), targeting a sequencing depth of 100 K raw reads per sample.

2.3. Data analysis

Statistical analysis was conducted using SAS 9.4 (SAS Institute Inc., USA). Chemical properties of soil and plant tissue were analysed using independent sample t-tests to compare raw DWW against the amended DWW for a variety of parameters. The data were checked for normality and homogeneity to satisfy the necessary assumptions of normal distribution. Probability values (p) greater than 0.05 were considered non-significant.

Bioinformatics data analysis was performed by NovoGene Ltd (Cambridge, UK). Paired-end raw reads were retrieved from the sequencing platform and matched to the samples based on their unique barcode. Subsequently, the barcode and primer sequences were trimmed from the reads. These paired-end reads were merged through FLASH algorithm (V1.2.7; Magoč and Salzberg, 2011) and filtered using Cutadapt (Marschner, 2011) to achieve high-quality, clean reads.

The final set of effective reads was obtained after detecting and removing chimera sequences using UCHIME algorithm (Edgar et al.,

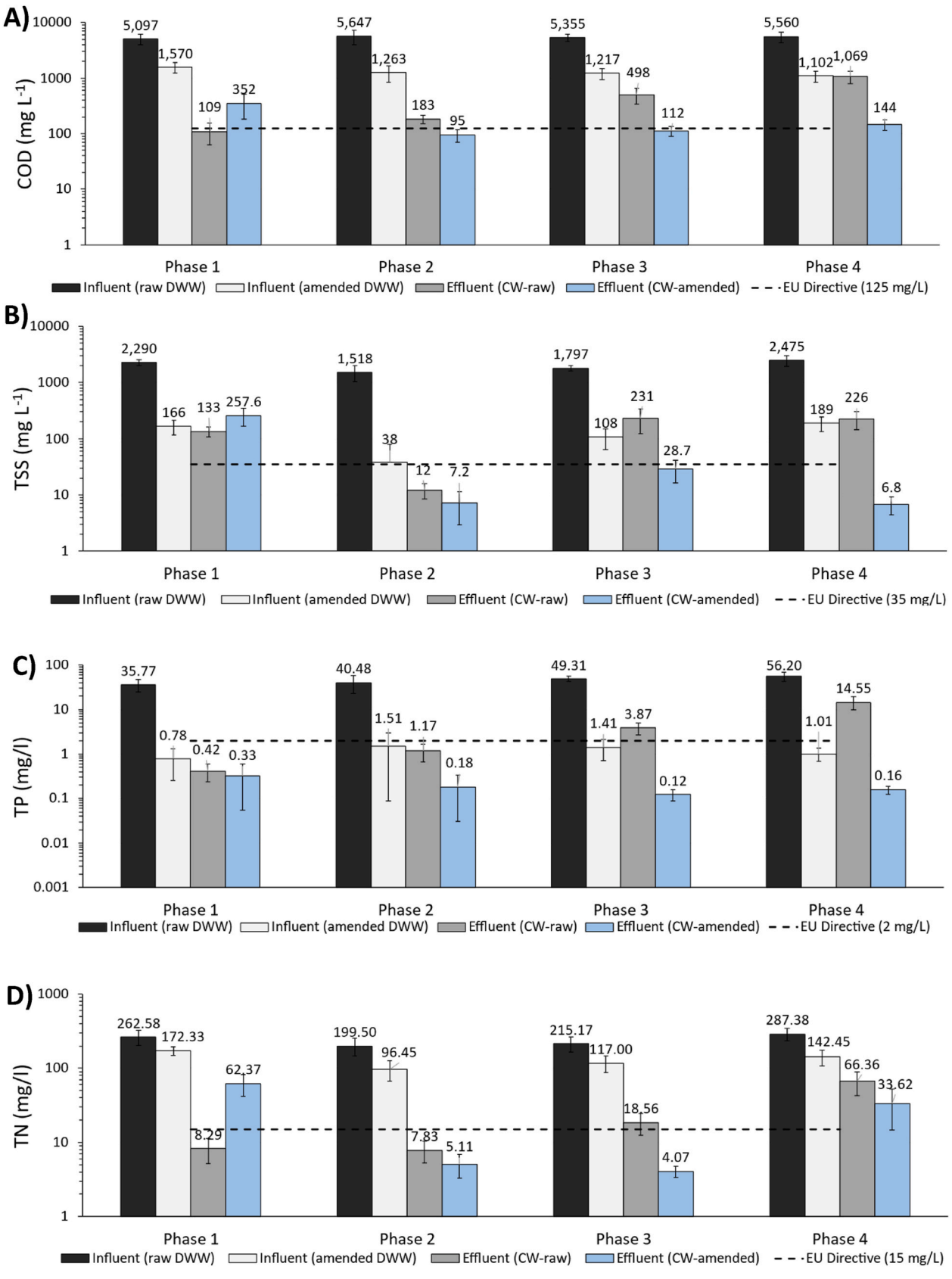


Fig. 1. Influent and effluent concentrations of pollutants and EU regulatory limits: A) chemical oxygen demand (COD); B) total suspended solids (TSS); C) total phosphorus (TP); and D) total nitrogen (TN), for raw and amended DWW constructed wetlands (CWs) for Phase 1 (n = 6), steady state of Phase 2 (n = 8), Phase 3 (n = 6) and Phase 4 (n = 8).

Table 2
Chemical properties of soil of constructed wetlands (Mean ± SD; n = 3) at the beginning and end of experiment for raw and amended DWW units.

Parameter	Unit	Pre-loading Control [a]	Raw DWW	Amended DWW	Statistical analysis [b]		
					Raw × control	Amended × control	Raw × Amended
pH water [1:2.5]		7.0 ± 0.1	8.2 ± 0.1	6.5 ± 0.1	***	*	***
Organic Matter	% w/w	6.6 ± 0.45	8.4 ± 0.2	8.6 ± 0.4	**	*	NS
Total Nitrogen	% w/w	0.32 ± 0.02	0.41 ± 0.01	0.42 ± 0.02	**	**	NS
Total Iron	mg/kg	16,393 ± 538	19,075 ± 673	18,852 ± 522	*	**	NS
Total Aluminium	mg/kg	7889 ± 332	9637 ± 257	9917 ± 422	**	**	NS
Total Calcium	mg/kg	3284 ± 210	5309 ± 521	4053 ± 142	**	*	*
Total Magnesium	mg/kg	2904 ± 94	3455 ± 133	3594 ± 124	**	**	NS
Total Phosphorus	mg/kg	697 ± 44	963 ± 20	833 ± 12	**	*	**
Total Potassium	mg/kg	1106 ± 61	1715 ± 87	1811 ± 63	**	***	NS
Total Carbon	% w/w	3.5 ± 0.2	4.2 ± 0.1	4.2 ± 0.2	**	*	NS
Carbon: Nitrogen Ratio	:1	10.9 ± 0.1	10.2 ± 0.2	10.1 ± 0.2	**	**	NS

[a] Pre-loading control soil is the soil of constructed wetlands at the beginning of the study before loading them with DWW.
[b] Statistical significance levels are denoted as follows: $p < 0.001$ as ***; $p < 0.01$ as **; $p < 0.05$ as * and when there is no significant difference, it is represented as NS.

2011). The analysis of sequences was carried out using UPARSE (v7.0.1001; Edgar, 2013), and sequences sharing ≥97% similarity were grouped into the same operational taxonomic units (OTUs). These OTUs were then annotated via the RDP classifier algorithm (Version 2.2, Wang et al., 2007) with reference to the GreenGenes database (DeSantis et al., 2006).

Alpha and beta diversity analyses were conducted based on normalized OTU data (corresponding to the sample with the least sequences: 96,510). To explore the complexity of species diversity, Alpha diversity indices were estimated using QIIME (Version 1.7.0) and graphed with R (Version 2.15.3). These includes Observed-species, Shannon, Chao1, Simpson, Good-coverage, and ACE. ACE and Chao1 both estimate community richness, while Simpson and Shannon both measure community diversity (Willis, 2019).

Analysis of beta diversity was performed to assess differences in community composition between different samples. Principal component analysis (PCA) was applied as a clustering technique to minimise the dimensionality of the original factors employing the FactoMineR and ggplot2 packages in R (Version 2.15.3). Principal Coordinate Analysis (PCoA) was employed to graph complex-multidimensional data. This involved obtaining a distance matrix of weighted or unweighted unifrac between samples, followed by transformation into a new set of orthogonal axes. The first principal coordinate represented the maximum variation factor, while the second principal coordinate depicted the second maximum variation factor. The PCoA analysis was visualized using the WGCNA, ggplot2, and stat packages in R software (Version 2.15.3). Statistical differences in the microbial community composition between raw and amended DWW CWs were performed by QIIME (Version 1.7.0). PCA is used for dimensionality reduction by finding new axes that capture the maximum variance in the data. It operates on covariance or correlation matrices of continuous variables. PCoA, on the other hand, works directly with dissimilarity matrices, aiming to represent sample relationships in a lower-dimensional space while preserving original distances.

A heatmap graph was generated through the R-package of ampvis2 (Andersen et al., 2018). Biological functions of the genus level taxa next to the heatmap were assigned according to the MiDAS field guide (Dueholm et al., 2022).

3. Results and discussion

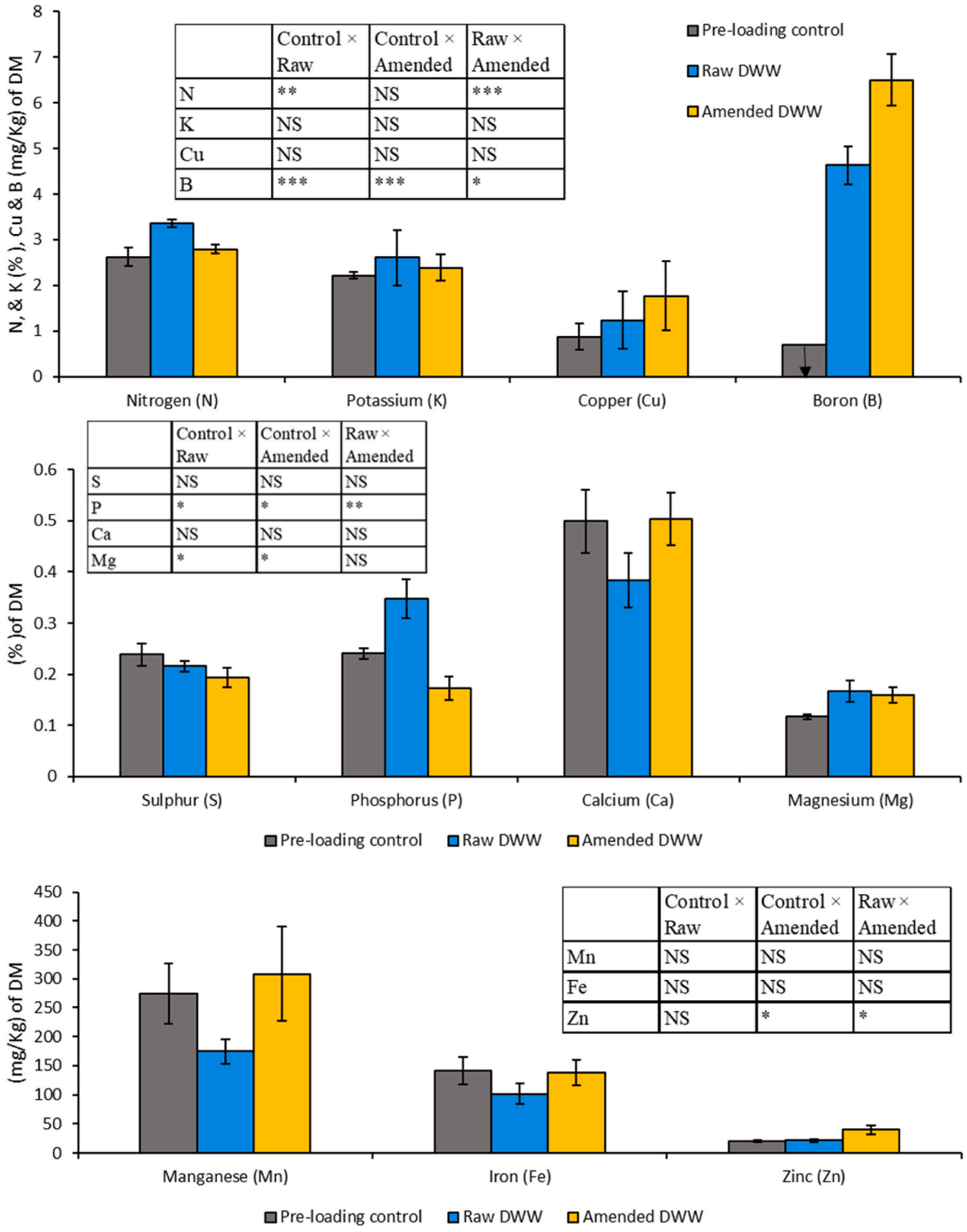
3.1. Water quality parameters and system performance

The introduction of $FeCl_3$ flocculant for the pretreatment of DWW led to a significant reduction in COD, TSS, TP, and TN, with average decreases of 75%, 95%, 96%, and 46%, respectively ($p < 0.001$; Fig. 1). The primary mechanism responsible for the reduction in COD and TN by $FeCl_3$ was the removal of particulate fractions, as reported by Mohamed et al. (2020). Ferric chloride removed these particulate fractions mainly through destabilization and hydrolysis mechanisms (sedimentation of $Fe(OH)_3\downarrow$). What remained in the amended DWW was primarily dissolved COD and ammonium (NH_4–N), as demonstrated by Mohamed et al. (2022). These soluble forms could only be eliminated through alternative processes, such as decomposition and nitrification-denitrification. As for P removal, the principal mechanism appeared to be chemical precipitation of ferric phosphate compound ($FePO_4\downarrow$), as suggested by Bratby et al. (2016).

During the initial start-up phase (Phase 1), CWs treating raw DWW outperformed those treating the amended DWW (Fig. 1) because of the lower HLR of raw DWW CWs (Table 1). However, the CWs achieved steady-state operation in Phase 2 (day 95), when the plants were fully established, and consistent effluent concentrations of COD, TSS, TP, and TN were attained. Throughout all phases, except for Phase 1, the CWs treating amended DWW exhibited superior performance in comparison to CWs treating raw DWW across all parameters and met the effluent quality standards outlined in the EU wastewater discharge directives in Phases 2 and 3 (Fig. 1; 91/271/EEC; EEC, 1991). In Phases 3 and 4, the performance of raw DWW CWs declined when they were receiving HLR similar to amended DWW CWs. An OLR of 3.5 g-COD m^{-2} d^{-1} was found to be optimal for amended DWW CWs, as in Phase 4, when the OLR was increased to 7 g-COD m^{-2} d^{-1}, the systems began to deteriorate and did not meet the EU directive limits for COD and TN.

For raw DWW CWs, the removal of particulate fractions of COD, N, and P primarily occurred through sedimentation mechanisms, as explained by Mohamed et al. (2022). Soluble COD removal in both raw and amended DWW CWs was likely driven by anaerobic and aerobic decomposition mechanisms. Soil adsorption appeared to be the primary mechanism responsible for the removal of soluble forms of P, such as orthophosphate (PO_4–P) and dissolved organic phosphorus (DOP) in the CWs (Vymazal, 2009). In shallow FWS-CWs, nitrification-denitrification typically serves as the primary pathway for NH_4–N removal (Vymazal, 2009).

Overall, the $FeCl_3$-CW combined system consistently produced high-quality effluent that complied with EU directives, a level of performance that would not have been attainable without $FeCl_3$ pretreatment. Additionally, this system could operate at elevated HLRs than traditional CWs, thereby requiring a smaller footprint to treat the same quantity of DWW. It is hypothesised that coagulation pre-treatment can bring extra advantages in SSHF and SSVF CWs because coagulation pre-treatment eliminates all TSS upfront, and therefore prevents clogging, which is a major operational issue in these types of CWs, especially SSVF CWs (De Souza et al., 2021; Wang et al., 2021). This was an observation of Mohamed et al. (2023), who investigated the clogging phenomenon on a combined system of coagulation pretreatment and an intermittent sand



	Control × Raw	Control × Amended	Raw × Amended
N	**	NS	***
K	NS	NS	NS
Cu	NS	NS	NS
B	***	***	*

	Control × Raw	Control × Amended	Raw × Amended
S	NS	NS	NS
P	*	*	**
Ca	NS	NS	NS
Mg	*	*	NS

	Control × Raw	Control × Amended	Raw × Amended
Mn	NS	NS	NS
Fe	NS	NS	NS
Zn	NS	*	*

Fig. 2. Chemical properties of plant tissue of constructed wetlands (Mean ± SD; n = 3) at the beginning (pre-loading control) and end of experiment (December 2021) for raw and amended DWW units. Statistical significance levels are denoted as follows: $p < 0.001$ as ***; $p < 0.01$ as **; $p < 0.05$ as * and when there is no significant difference, it is represented as NS.

filter (ISF) which has properties and characteristics like SSVF CWs. In their experiment, the hybrid ISF system was able to operate at higher OLRs without showing any evidence of clogging as opposed to conventional ISFs which were fully clogged after 280 days of operation. The FWS CWs are usually associated with biological clogging, while clogging in SSHF and SSVF CWs is primarily caused by the build-up of a large amount of TSS and excessive microbial growth, which are physical and biological processes (De Souza et al., 2021; Wang et al., 2021). In general, clogging does not impact the operation of FWS CWs. On the other hand, when the substrate and media of SSHF and SSVF CWs got clogged in the inlet or outlet, it is easy to cause the whole CW system to shut down. This is because wastewater flows over the soil surface in FWS CWs, while it flows mainly through the substrate/media in SSHF and SSVF CWs.

3.2. Chemical properties of soil

There were significant differences in all of the assessed soil properties, of both treatments, between the beginning and the end of the experiments (Table 2). However, there was no significant differences in soil chemical properties between the two treatments at the end of the study ($p > 0.05$), with the exception of pH, total calcium, and total phosphorus ($p < 0.05$; Table 2). The pH was reduced in the case of amended DWW CWs, because of the acidic nature of the chemical coagulant $FeCl_3$ (pH = 1–2, Table S1: Appendix A) used in the pre-treatment step. As a result, the pH of the soil was 6.5 ± 0.1 for amended DWW CWs as opposed to 8.2 ± 0.1 for the soil of raw DWW CWs (Table 2, $p < 0.001$). Several studies have shown that the performance of CWs can be impacted by pH. For example, Sánchez et al. (2021) studied the influence of pH (4 vs 7) on the efficacy of vertical flow constructed wetlands (VF-CWs) treating winery wastewater and found that low pH of 4 can weaken the performance of CWs especially impairing nitrification and partially denitrification. Arienzo et al. (2009) found winery wastewater was toxic to wetland plant species because of the low pH, and recommended pH neutralization. The pH in the current study for amended DWW CWs was closed to neutral pH value of 7, therefore there was no need to add any neutralizer. The performance of the amended DWW CWs was not affected by the chemical pre-treatment, and it was better than raw DWW CWs regarding contaminants removal.

The chemical coagulant significantly reduced the TP by 96% in amended DWW, and hence amended DWW CWs received a lower TP load than raw DWW CWs (Fig. S2). As a result, the soil of amended DWW CWs contained significantly less TP (833 ± 12 mg kg^{-1}, $p < 0.01$) than those in the soil of raw DWW CWs (963 ± 20 mg kg^{-1}; Table 2). Considering the dimensions of CWs (1.75*0.5*0.25 m, effective soil volume in the tank = 70%), and assuming a soil density of 1600 kg/m^3, the increase in soil TP content (due to the input load from DWW) was calculated to be 81 and 3.3 mg kg^{-1} for raw and amended DWW CWs, respectively. These values were lower than the actual increase of soil P content (in relative to the initial status of soil before DWW loading) as shown in Table 2. The soil samples analysed at the beginning of the experiment were taken from the CWs before their planting with *Phragmites australis*. Therefore, the common reeds that were planted mature and densely at the beginning of the experiment may be a reason for this extra P input through the soil-plant interaction/contact, especially through rhizomic system. Considering the high pH of soil from the raw DWW CWs, and the availability of calcium in raw DWW, the high TP load was probably removed through calcium phosphate co-precipitation (Yuan et al., 2005). As a result of calcium phosphate co-precipitation, the total calcium content in the soil of raw DWW CWs was higher than those in amended DWW CWs ($p < 0.05$; Table 2).

The COD load applied to the amended DWW CWs was less than half the load applied to the raw DWW CWs. However, there were no significant differences in organic matter and total carbon contents between the two treatments. Considering the dimensions of CWs, and assuming an influent COD/OM ratio of 1.48, the increased in OM due to DWW input was calculated to be 0.77 and 0.34 % w/w for raw and amended DWW CWs, respectively. These values would be even lower if we incorporate the COD loss by bacterial respiration, and loss of COD in the effluent. Therefore, the significant increase in OM and TC in both types of CWs (relative to the initial status of soil before DWW loading; Table 2) probably originated from the plants that were brought and planted from outside or occurred because of CO_2 sequestration. Many previous studies showed that wetlands are capable of carbon sequestration and hence have a positive role to play with regard to climate change (Adhikari et al., 2009; Mitsch et al., 2013; Villa and Bernal, 2018).

There was no significant difference in TN content of soil between the two treatments, because the cumulative load is similar in both type of CWs (Fig. S2). Overall, there was no significant differences in most of the soil parameters between both types of CWs, which indicated that there is no adverse effect of using chemical coagulation of $FeCl_3$ as a pre-treatment stage on the (assessed) properties of the soil, while allowing for better effluent quality.

3.3. Chemical properties of plant tissue

Plants from raw DWW CWs had higher N and P contents at the end of the study than at the beginning of the experiment (Fig. 2; $p < 0.05$). In contrast, there was no differences in plant N content of amended DWW CWs between the beginning and end of experiment (Fig. 2; $p > 0.05$). Moreover, plants of amended DWW CWs at the end of the study had lower P content than the beginning of the experiment (Fig. 2; $p < 0.05$). The low P inputs in the influent amended DWW (Fig. S2) was expected to be the main reason for this low P content in the amended DWW CWs.

When comparing treatments, the plant N and P contents were higher in the case of raw DWW CWs compared to amended DWW CWs (Fig. 2; $p < 0.05$), likely because of the higher cumulative loads of N and P as shown in Fig. S2. Although the P load into amended DWW CWs was very low, it did not restrict nutrient availability or hinder the growth of *Phragmites australis* plants which were able to uptake other nutrients and trace elements. Similarly, Liang et al. (2019) studied the impacts of ferric sulphate $Fe_2(SO_4)_3$ and poly aluminium chloride (PACL) coagulation-enhanced treatment wetlands on the growth of Typha and found this approach did not restrict the availability of nutrients to Typha growth and did not show any toxicity.

Plants of both raw and amended DWW CWs showed good capability to accumulate boron (B) and magnesium (Mg) compared to the initial status of CWs before loading them with DWW (Fig. 2; $p > 0.05$). In addition, amended DWW CWs had higher zinc (Zn) compared to the initial status of CWs before loading them with DWW (Fig. 2; $p < 0.05$). Seasonal variation and cyclic changes in the nutrients/metals uptake by *Phragmites australis* was expected and is well documented in the literature (Mulkeen et al., 2017).

There were no significant statistical differences in plant contents of K, Cu, S, Ca and Mn between raw and amended DWW CWs at the end of the study ($p > 0.05$, Fig. 2). Excluding N and P, the elemental contents of plants were typically higher in the case of amended DWW CWs than raw DWW CWs. For example, the Mn concentration in the plant of Amended DWW CWs was almost twice as high as that of raw DWW CWs. This was likely because the $FeCl_3$ used in the pre-treatment steps contained multiple trace elements and metals (Table S1: Appendix A). However, this difference was only statistically significant ($p < 0.05$) in the cases of B and Zn. The elevated levels of these elements in the tissue plants of amended DWW CWs (in relation to raw DWW CWs) indicated the potential for *Phragmites australis* to assimilate and uptake these elements without any sign of toxicity. Many previous studies showed and proved the substantial capacity of *Phragmites australis* to accumulate and remove heavy metals (e.g., Weis and Weis, 2004; Bonanno and Giudice, 2010; Vymazal and Březinová, 2016).

The plants of amended DWW CWs had higher Fe content than the plants of raw DWW CWs, but the difference was not statistically significant. This was ascribed to the fact that there was some Fe left in the pre-treated DWW as a residual after $FeCl_3$ addition. Previous studies identified that the critical deficiency Fe concentration in *P. australis* is 40–50 mg kg^{-1} DM, while the critical toxicity Fe concentration in *P. australis* is approximately 150 mg kg^{-1} DM (Ren et al., 2018). In the current study, the plants in all types of CWs were within these limits (Fig. 2), indicating that Fe concentration in influent neither caused deficiency nor toxicity. The Fe toxicity threshold of most wetland plants is about 1100–1600 mg kg^{-1} DM within the leaf tissues (Marschner, 2011). A threshold of Fe concentration of 1 mg L^{-1} in water was identified, above which growth of *P. australis* is notably inhibited (Batty and Younger, 2003; Ren et al., 2018). The selected dose of 440 mg Fe L^{-1} of DWW was based on the study of Mohamed et al. (2020). Their study found this dose was optimum for the removal of pollutants present in DWW, and they did not notice any significant improvements above this dose. This indicates a dosage higher than 440 mg Fe L^{-1} can only incur

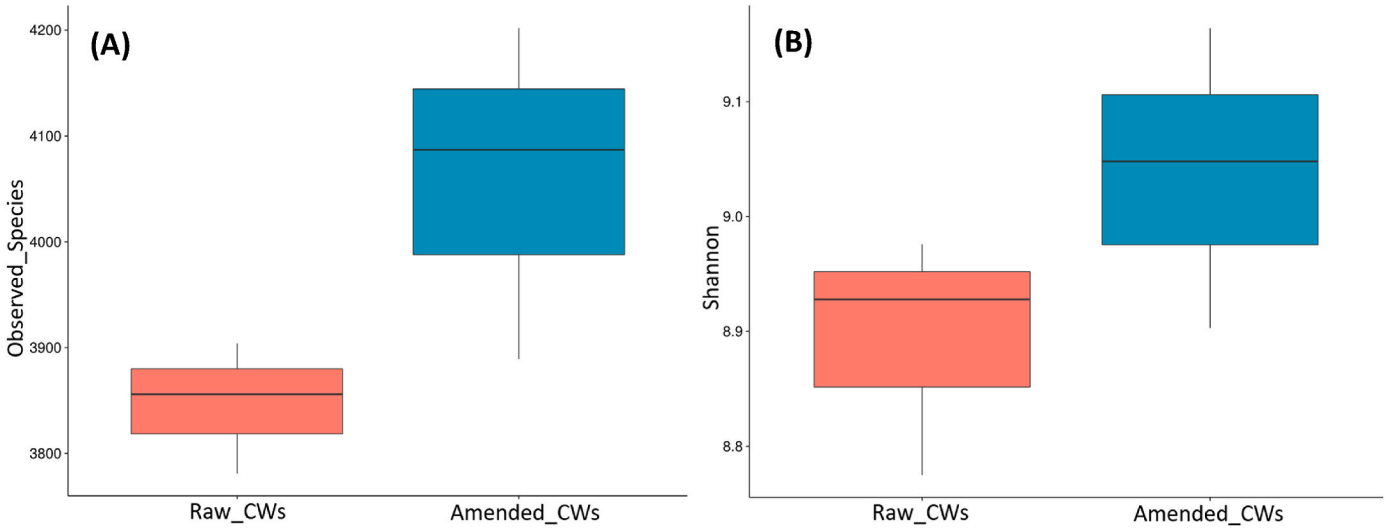


Fig. 3. Box plot of difference of observed species (A) and Shannon indices (B) for raw and amended DWW CWs.

extra cost and probably creates elevated Fe concentrations in the pretreated DWW which can cause toxicity to the plants of downstream CWs. The theoretical Fe required to remove P through chemical precipitation ($FePO_4\downarrow$) was calculated to be 82 mg Fe L^{-1} based on an average P concentration of 45 mg L^{-1} in the raw DWW. The rest of Fe concentration (352 mg Fe L^{-1}) was probably utilized for TSS and turbidity removal through hydrolysis mechanisms (sedimentation of Fe $(OH)_3\downarrow$).

From visual observation, the plants of amended DWW CWs grew at the same rate, density and height as those in the raw DWW CWs during the different stages of the experiment (Fig. S3: Appendix A), indicating that the plants of amended DWW were not impacted by $FeCl_3$ pretreatment. Overall, there was no observed negative effect of using chemical coagulation of $FeCl_3$ as a pretreatment stage on the capacity of plants to remove nutrients and metals from DWW, and no evidence of nutrients (N and P) limitation to the growth of *Phragmites australis*.

3.4. Microbial properties of soil

The microbial community structure within the soil in the CWs was investigated by 16 S rRNA gene sequencing to identify the main genera found in the microbiota and their relative abundance. This approach aimed to investigate the influence of $FeCl_3$ amendments on the microbial community structure within CWs, furthermore, it aimed to provide insights into the microbial mechanisms involved in the removal and/or conversion of carbon, phosphorus, and nitrogen in both raw and amended DWW CWs. A total of 608,182 effective reads (nonchimeric clean tags after quality filtering) were retrieved from 6 samples of high-throughput sequencing, with a minimum and maximum of 96,510 and 104,290 reads, respectively (Table S2).

3.4.1. Species diversity and abundance- alpha diversity

The determination of operational taxonomic units (OTUs) at 97% identity, and the various alpha indices (Simpson, Shannon, ACE, Chao, and good coverage) used to evaluate both the diversity and abundance

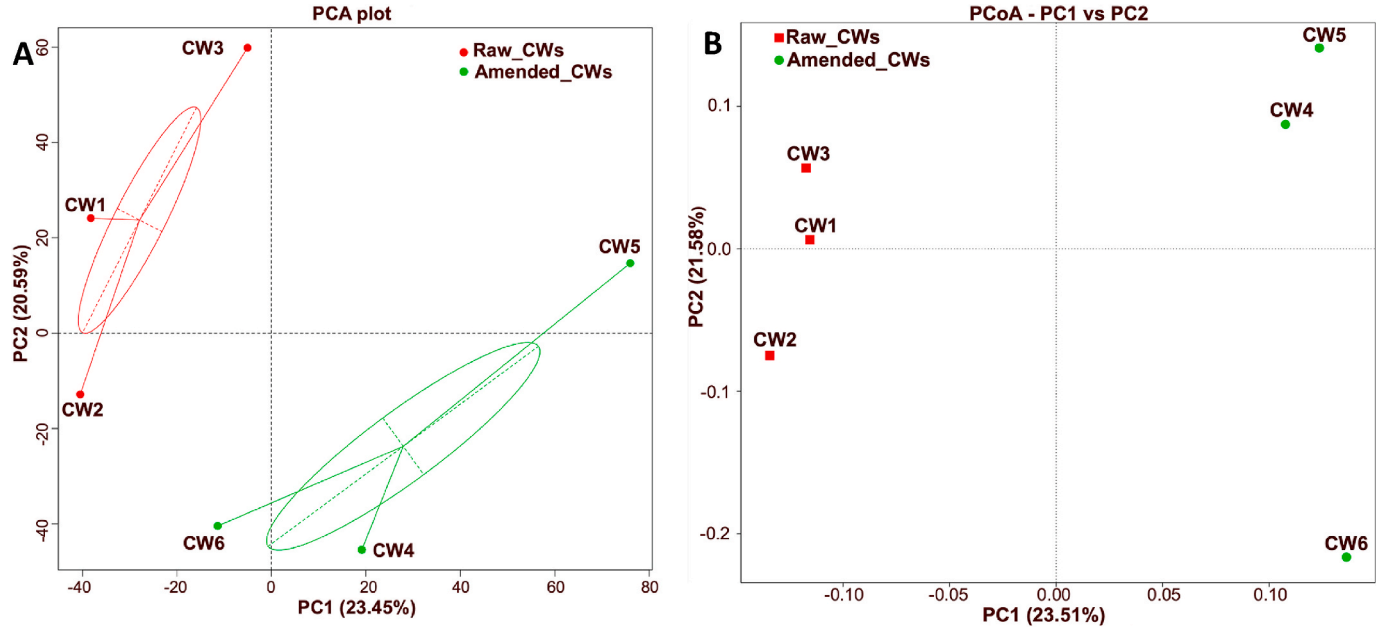


Fig. 4. Microbial community composition (Beta diversity indices) differences between raw and amended DWW CWs: A) Principal component analysis (PCA); and B) principal coordinate analysis (PCoA) based on Unweighted Unifrac distance.

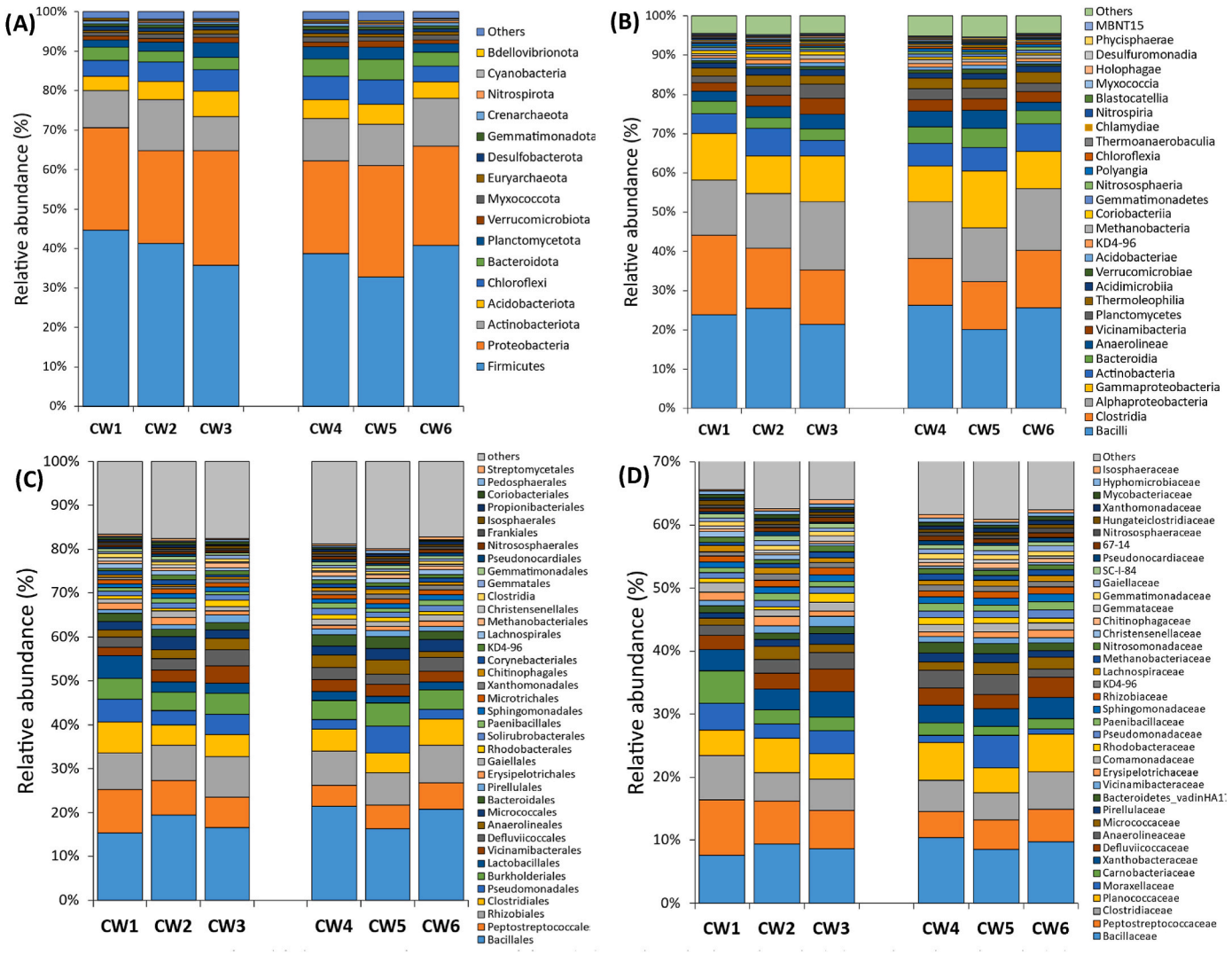


Fig. 5. Microbial community composition (A) at the phylum level; (B) at the class level; (C) at the order level and (D) at the family level for raw DWW CWs (CW1, CW2 and CW3) and amended DWW CWs (CW4, CW5 and CW6).

of the microbial community are displayed in Table S2. The coverage index values for the 6 samples were found to be higher than 0.99 (Table S2), and rarefaction curves were shown to plateau (Fig. S4: Appendix A), signifying that the constructed sequence libraries effectively encompassed the microbial diversity.

The amended DWW CWs contained higher numbers of OTUs than raw DWW CWs (Fig. 3-A), but the difference was statistically insignificant ($p > 0.05$), which indicates that the addition of $FeCl_3$ as an amendment to treat DWW did not reduce the diversity of the microbial community. The Flower diagram (Fig. S5: Appendix A) shows that there was 1941 shared OTUs between all CWs, and there was about 200–300 unique OTUs for each CW. The enhanced abundance in the microbial communities in the amended DWW CWs (Fig. 3A) correlates with the higher diversity (Fig. 3B–Table S2), but with no significant differences ($p > 0.05$) from raw DWW CWs. Other alpha diversity indices (Chao, ACE and Simpson; Table S2), which provide the richness of each species and spreading of the microbial community, also exhibited similar trends and indicated that amended DWW CWs hosts more diverse microbial community than raw DWW CWs, but the difference was statistically insignificant ($p > 0.05$).

3.4.2. *Microbial community composition- beta diversity*

Principal component analysis (PCA) (Fig. 4-A), and principal coordinate analysis (PCoA) based on Unweighted UniFrac distance (Fig. 4-B) revealed some differences in community composition (i.e., beta-diversity) between raw and amended DWW CWs. Raw DWW samples clustered together, while amended DWW samples generally didn't cluster together, but were positioned separately from the Raw DWW samples cluster. The amended DWW samples were scattered and dispersed on the PCoA plot compared to raw DWW CWs (Fig. 4-B). As a result, analysis of community differences highlighted no statistical differences in the overall microbial community diversity between both types of CWs (Table S3). These includes analysis of similarity (Anosim), analysis of molecular variance (AMOVA), Multi-response permutation procedure (MRPP) analysis, Weighted UniFrac (two-Wilcox & *t*-test) and Permutational multivariate analysis of variance (MANOVA/ADONIS), where in all cases *p* value was >0.05 (Table S3).

3.4.3. *Functional flora structure*

The microbial community composition at the phylum level is shown in Fig. 5-A. The most abundant phyla identified in the CWs were Firmicutes (33–45%), Proteobacteria (23–29%), Actinobacteriota (9–13%), with Acidobacteriota, Chloroflexi, Bacteroidota, Planctomycetota, Verrucomicrobia, and Myxococcota each representing >1% of the community. Firmicutes are typical human/animal faecal-microbes and are commonly detected in domestic wastewater (Ali et al., 2019).

Fig. 6. Microbial community composition for the 6 CWs at Genus level for the top 80 genera. Functional information about the Genus-level OTUs were plotted next to the heatmap and were assigned according to the MiDAS field guide (Dueholm et al., 2022). Green dot indicates positive; red indicates negative, yellow indicates variable; grey indicate not assigned.

Firmicutes are probably dominating in the current study because DWW consisted mainly of cow faeces. Members of the Proteobacteria and Actinobacteria have been reported to be predominant in soil-based systems, and usually are involved in the degradation of organic compounds (De Souza et al., 2021; Ajibade et al., 2021). Proteobacteria also plays a vital role in the elimination of biological phosphorus and nitrogen and other contaminants degradation (Niestępski et al., 2020). There were no statistical differences in bacterial community at the phylum level between raw and amended CWs ($p > 0.05$), except for the phyla Spirochaetota which has abundance less than 0.04% in both systems (Appendix B).

The composition of microbial community at the class rank is illustrated in Fig. 5-B. The relative abundance of Bacilli, Clostridia, Alphaproteobacteria, Gammaproteobacteria, Actinobacteria and Bacteroidia, accounted for >70 % of the bacterial communities at class level. Bacilli and Clostridia are both Firmicutes and commonly found in soil, water, and gastrointestinal tracts of animals and humans. Both types of bacteria grow in low-oxygen environments and help in the fermentation of wastewater; however, the Clostridia are strictly anaerobes, while Bacilli can be aerobic or facultative anaerobes (Stiles et al., 2014). Alphaproteobacteria, Gammaproteobacteria and Bacteroidia, which belong to Proteobacteria, play essential roles in denitrification and organic matter decomposition in CWs in the current study (He et al., 2016; Han et al., 2021). The appearance of Methanobacteria in the top 16 class level of bacterial community indicated that both types of CWs involved anaerobic decomposition of organic matter. Nitrososphaeria (Archaea) and Nitrospiria (nitrite-oxidizing bacteria, NOB) were both appeared in the top 25 class level of the microbial community, which indicated that nitrification was also occurring in the CWs. Out of 151 classes identified, only 5 classes showed statistical difference between the two treatments ($p < 0.05$), namely Coriobacteriia, Cyanobacteriia, Spirochaetia, Chitinivibrionia and ABY1 (Appendix C). Members of the class Coriobacteriia commonly prevail in the animal and human gut (Hoyles, 2019), which is probably why their relative abundance was significantly higher in the case of raw DWW CWs, as opposed to amended CWs where the pre-treatment by $FeCl_3$ remove cow faeces by settlement. Members of Cyanobacteriia in amended DWW CWs had abundance of 0.18%, which was significantly higher than the abundance in raw DWW CWs (0.067%, $p < 0.05$). The availability and penetration of the light in amended DWW CWs, due to the clarity of the water (which has less TSS and turbidity, Fig. 1; Fig. S2) might be the reason for Cyanobacteriia to be in higher abundance compared to raw DWW CWs, as they need light for photosynthesis process.

The composition of microbial community at the order and family levels is illustrated in Fig. 5-B and 5-D, respectively. Out of the 324 orders identified, only 2% (7 orders: Xanthomonadales, Coriobacteriales, Polyangiales, Rickettsiales, R7C24, Elsterales and Spirochaetales) showed significant differences between two types CWs (Appendix D). There were 13 families (2.5%: out of 520 identified families) significantly different between raw and amended DWW CWs (Appendix E).

The microbial community composition at the genus rank is represented in Fig. 6. Both type of CWs hosted diverse functional groups of microbes that are involved in carbon, nitrogen, phosphorus, and sulphur conversions/cycles. These includes aerobic heterotrophs that use short chain fatty acids and sugars as substrates (e.g., *Acinetobacter* (1–5%), *Trichococcus* (1–5%), and *Defluviicoccus* (2–3.5 %), chemoautotroph mixotroph (e.g., *Romboutsia* (1.3–3%)), fermentation organisms (e.g., *Proteiniclasticum* (0.5–3%), *Paeniclostridium* (1–2%) and *Turicibacter* (0.7–1.5%)), acetogenesis (e.g., *Romboutsia* (1.3–3%)), methanogenesis (e.g., *Methanobacterium* (0.5–1%)), nitrifiers (e.g., Nitrospira (0.33–0.5%) and *Candidatus(Ca)_Nitrosocosmicus* (0.036–0.25%)), denitrifiers (e.g., *Pseudomonas* (0.8–1.3%), *Bradyrhizobium* (0.8–1.2%), *Anaerolinea* (0.6–1.3%) and *Thauera* (0.1–0.3%)). While there was evidence that glycogen accumulation organisms (GAO) were presents in the current study (e.g., *Defluviicoccus*), there was no evidence that polyphosphate accumulation organisms (PAO) were present in the current study. This indicated that phosphate was mainly removed through soil-adsorption mechanism, and probably slightly through assimilation by microbial biomass for growth. Out of 913 genera identified, only 3.7% (34 genera) showed significant differences between two types CWs (Fig. 7A and Appendix F), including *Paeniclostridium*, *Pseudolabrys*, *Proteocatella*, *Olsenella*, *Ca. Nitrosocosmicus*, *Tissierella* and *Methanobrevibacter*. *Ca. Nitrosocosmicus* is a genus of ammonium oxidizing archaea, and it had an abundance of 0.36% in amended DWW CWs, which is significantly higher than raw DWW CWs (0.25%, $p < 0.05$). Interestingly, other type of nitrifiers did not show any significant differences between the two treatments at both Genus and Species level: e.g., genus of *Nitrospira*/NOB (0.33–0.5%) (species: *Nitrospira_japonica* and *Nitrosospira*_sp), genus of *Nitrosomonas*/AOB (0.025–0.05%) (species: *Nitrosomonas_ureae* and *Nitrosomonas_communis*) (Appendix F and

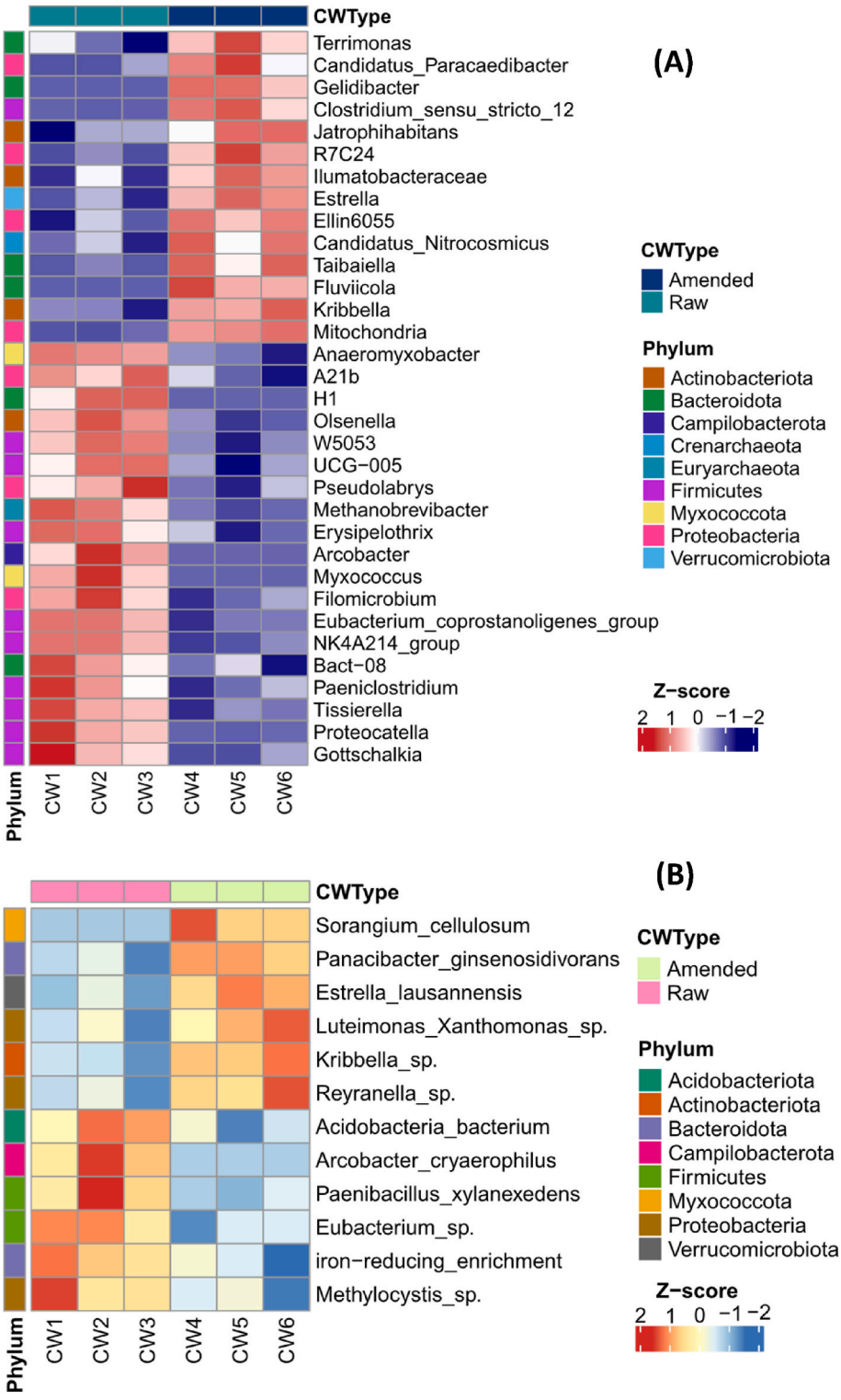


Fig. 7. Significantly different ($p < 0.05$) OTUs between raw and amended DWW CWs (A) at the genus and (B) species levels using T-test differential abundance testing.

Appendix G).

The presence of Mn as a trace element in the solution of $FeCl_3$ coagulant (Table S1) probably led to higher abundance of *Ca. Nitrosocosmicus* in the amended DWW CWs, compared to raw DWW CWs ($p < 0.05$). *Candidatus Nitrosocosmicus* possesses genes encoding Mn catalase (MnKat; Lee et al., 2024), therefore dominated soils of amended DWW CWs, and hence contributed to the development of distinct archaeal communities, which have good nitrification capacity. Genus *Gelidibacter* had also higher abundance ($p < 0.05$) in the amended DWW CWs than in raw DWW CWs (Fig. 7A) potentially due to the availability of trace elements in the $FeCl_3$ solution such as Mercury (Hg) and Mn. Members of genus *Gelidibacter* were found to be effective in Hg removal and bioremediation as they contain genes encoding mercury reductase (*Gbs*MerA; Pardhe et al., 2023). Genera *Ellin6055,* which had higher abundance in

amended DWW CWs than raw DWW CWs ($p < 0.05$; Fig. 7A), is usually associated with arsenic (As) bioavailability and accumulation in the soil (Sun et al., 2023). Other genera that are usually associated with heavy metals (Mn, Zn, Cu and Fe) in wastewater such as *Nakamurella, Micrococcales, Saccharimonadales, Microtrichales, Longilinea and Ferruginibacter* (Zeng et al., 2022) were in higher abundance in amended DWW CWs than raw DWW CWs, although this was not statistically significant ($p > 0.05$; Appendix F). The high penetration of the air into the amended DWW CWs due to the clarity of the water may be a reason for the higher abundance of the obligate aerobic genus of *Terrimonas* (Fig. 7A). On the other hand, raw DWW CWs contained many obligate and facultative anaerobic genera with relatively higher abundance (compared to amended DWW CWs) such as *Olsenella, Methanobrevibacter* and *Anaeromyxobacter*. This probably due to the high organic loading rate of raw DWW CWs as well as the availability of the anaerobic conditions at the base of CWs. In general, most of the genera that had higher abundance in amended DWW CWs than in raw DWW were belonged to phyla Actinobacteriota, Proteobacteria and Bacteroidota, while raw DWW CWs contained many higher abundance genera belonging to phylum Firmicutes as compared to amended DWW CWs (Fig. 7A). Firmicutes are gut derived microbes and probably dominating in the raw DWW CWs, because raw DWW consisted mainly of cow faeces, as opposed to amended CWs where the pre-treatment by $FeCl_3$ remove cow faeces by settlement.

There were only 12 identified species (out of 340 identified species) that showed significant differences between raw and amended DWW CWs (Fig. 7B and Appendix G), corresponding to 190 OTUs out of the 6706 detected. This represents 2.8% disparity in the overall community composition, which is almost consistent across different levels (phylum, class, order, family, genus, and species). *Methylocystis*, which is a strictly aerobic methanotrophic bacteria, had a higher abundance ($p < 0.05$) in the raw DWW CWs than amended DWW CWs (Fig. 7B). This is possibly due to the availability of methane (CH_4) originated from raw DWW or CH_4 generated from the anaerobic treatment occurring in the bed of the raw DWW CWs. Although Fe levels are expected to be higher in the amended DWW CWs, the iron reducing bacteria that reduces Fe(III) to Fe (II) were more abundant ($p < 0.05$) in the raw DWW CWs than amended DWW CWs (Fig. 7A&B). This was evident at both genus (*Anaeromyxobacter*) and species levels (*iron_reducing_enrichment*). This is potentially because this type of bacteria prefers to grow in anaerobic condition that were more likely to occur in the raw DWW CWs due to the high sludge and TSS accumulation. Phosphate solubilizing bacteria, *Paenibacillus xylanexedena,* had higher abundance in raw DWW CWs than amended DWW CWs, potentially because of the higher TP load applied to raw DWW CWs. This genus can decompose both organic and inorganic P, and convert them into soluble P (Dong et al., 2022).

Microbial structure was similar in all CWs, which have been widely reported in the literature (Wang et al., 2022). However, some functional microbes were different as discussed, especially with different influent, Fe and trace elements content.

4. Conclusion

A $FeCl_3$-CW combined system achieved superior effluent quality which complied with EU directives, and hence is suitable for discharge. This system could operate at elevated HLRs than traditional CWs, thereby requiring a smaller footprint to treat the same quantity of DWW. The use of chemical coagulation did not negatively affect the properties of soil and plant system of CWs or alter the microbial community within the CWs. There were no significant differences in most of the soil parameters between both types of CWs. The lower loads of N and P into amended DWW CWs led to lower N and P plant contents compared to raw DWW CWs, but did not limit the growth of *Phragmites australis*, which in most of the cases were able to accumulate higher trace elements than raw DWW CWs. Alpha diversity indices indicated that amended DWW CWs hosts more a diverse microbial community than raw DWW CWs, but the difference was not statistically significant. Similarly, Beta-diversity analysis revealed some differences in community composition (i.e., PCA and PCoA) between raw and amended DWW CWs, but these were not statistically significant. Both type of CWs hosted diverse functional groups of microbes that are involved in carbon, nitrogen, phosphorus, and sulphur conversions/cycles. Only 3.7% (34 genera) showed significant differences between the two types of CWs, which indicated the functionality of microbial community within CW was not significantly affected by the use of the chemical coagulation. *Ca_Nitrosocosmicus,* which is usually involved in the nitrification process, was the key genus that showed differences between the types of CWs. Overall, the use of chemical coagulation in combination with CWs showed a better performance (for treating wastewater) than CWs receiving raw untreated DWW without compromising the quality of chemical and microbial properties of soil and plant systems. Future studies should investigate the specific mechanisms underlying the interactions between coagulants and soil-microbial-plant systems, as well as the long-term effects on pollutant removal efficiency.

CRediT authorship contribution statement

A.Y.A. Mohamed: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **P. Tuohy:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **M.G. Healy:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **D. Ó hUallacháin:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **O. Fenton:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **A. Siggins:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ahmed Mohammad reports financial support was provided by Teagasc. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142745.

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