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Mesocosm-Scale Constructed Wetland of *Canna indica* for Wastewater Treatment

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Micropollutants (MPs) such as caffeine in untreated wastewater pose ecological and human health risks, and conventional treatments often fail to remove them effectively. This study evaluated mesocosm-scale horizontal subsurface flow constructed wetlands (CWs) planted with *Canna indica* under monoculture and polyculture conditions for caffeine and nutrient removal. The CWs contained layered substrates (pebble, sand, gravel, cocopeat, activated carbon, and soil) to enhance contaminant retention. Wastewater spiked with 200 mg L^{-1} caffeine was introduced, and parameters including ammoniacal nitrogen (AN), biochemical oxygen demand (BOD), total nitrogen (TN), caffeine concentration and plant physiological indicators were analyzed. Monoculture *Canna indica* reduced caffeine concentration to 46.20 ± 0.09 mg L^{-1}, AN to 5.80 ± 1.98 mg L^{-1}, BOD to 36.50 ± 2.12 mg L^{-1}, and TN to 12.00 ± 1.41 mg L^{-1}. Polyculture CWs showed slightly better performance, lowering caffeine concentration to 45.83 ± 0.51 mg L^{-1} and achieving greater BOD and TN removal, likely due to synergistic plant microbe interactions enhancing biodegradation. The activated carbon and cocopeat layers contributed to adsorption followed by microbial degradation. Lipid peroxidation indicated the highest malondialdehyde (MDA) concentration in *Heliconia* spp. (0.0155 mM g^{-1}), whereas in *Canna indica* grown in polyculture exhibited the lowest (0.0070 mM g^{-1}), indicating reduced physiological stress, likely associated with improved nutrient cycling. Overall, *Canna indica,* particularly when cultivated in polyculture systems, demonstrates strong potential for decentralised wastewater treatment, supporting Sustainable Development Goals (SDGs) 6 and 14.

Keywords: Constructed wetlands, caffeine, *Canna indica*, micropollutants, ornamental plants

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Micropollutants (MPs) are an increasing environmental concern due to their persistence and bioactivity, even at trace concentrations. These compounds, which include pharmaceuticals, personal care products, and stimulants, can pose serious risks to aquatic ecosystems and human health [1, 2]. Among these contaminants, caffeine, a naturally occurring stimulant commonly found in beverages, medications, and personal care products, has gained significant attention due to its ubiquity and potential ecological impacts. Caffeine, chemically known as 1,3,7-trimethylxanthine, is introduced into aquatic environments primarily through human excretion, untreated wastewater, and the improper disposal of caffeine-containing products [3, 4]. In Malaysia, caffeine contamination has been detected in rivers and lakes, particularly in urbanised regions, with concentrations reaching up to 20,620 ng L^{-1} in surface waters.

The presence of caffeine in aquatic environments poses substantial ecological risks. Even at low concentrations, it can adversely affect aquatic organisms by disrupting behaviour, physiology, and development. For instance, in fish, caffeine exposure has been linked to increased stress, aggression, and reduced liver size. In zebrafish embryos, caffeine disrupts vascular development and immune responses, leading to inflammation and developmental abnormalities [5, 6]. Furthermore, caffeine has been shown to induce oxidative stress and lipid peroxidation in marine invertebrates, impair photosynthesis in macroalgae, and hinder plant growth, highlighting its broad ecological impact [7, 8].

Various treatment approaches have been implemented to reduce MPs in surface waters. Malaysia, like many developing nations, faces significant challenges in managing emerging MPs due to the limitations of conventional wastewater treatment technologies. Treatment systems such as activated sludge, membrane bioreactor (MBR), and moving-bed biofilm reactor (MBBR) are widely used for nutrient and organic matter removal [9].

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However, these systems often fail to effectively remove MPs, such as caffeine, due to its persistence in conventional treatment processes [9, 10]. Advanced technologies such as activated carbon adsorption and membrane filtration can achieve higher removal efficiencies but are costly, energy-intensive, and may generate harmful byproducts such as nitrosamines, trihalomethanes, and haloacetic acids [11]. Consequently, caffeine residues persist in treated effluents, contributing to their frequent detection in Malaysia's water systems, particularly in densely populated and urbanised areas [12, 13].

Constructed wetlands (CWs), therefore, offer an eco-friendly, cost-effective solution for mitigating MPs, such as caffeine, in wastewater. CWs are engineered ecosystems that replicate natural wetland processes, utilising a combination of physical, chemical, and biological mechanisms to remove pollutants. Recent research has highlighted the potential of incorporating ornamental plants into CWs to enhance pollutant removal efficiency while providing additional aesthetic and economic benefits [14]. *Canna indica*, in particular, has demonstrated promising capabilities in removing various pollutants, including chemical oxygen demand (COD), nitrogen compounds, and pharmaceuticals. These capabilities are attributed to its robust root system, high biomass production, and adaptability to diverse environmental conditions [15].

MPs such as caffeine are increasingly detected in aquatic systems, yet their impacts in countries such as Malaysia remain understudied [16]. Although CWs offer sustainable treatment options for MPs, existing research has largely focused on conventional wetland plants. The potential of ornamental species such as *Canna indica* for pollutant removal in Malaysian CWs, therefore, remains largely unexplored [17, 18]. Accordingly, this study aimed to design and construct a mesocosm-scale wetland system for removing caffeine from residential wastewater. In addition, the study evaluated the physicochemical properties and caffeine concentrations of wastewater in the CWs before and after treatment. The study also compared caffeine removal efficiency between monoculture and polyculture systems of *Canna indica*, using traditional wetland plants as a positive control.

EXPERIMENTAL

Setup of Mesocosm-Scale Constructed Wetlands

Eight mesocosm-scale CWs were set up at Universiti Malaysia Pahang Sultan Al-Abdullah, Gambang, Kuantan, Malaysia, at the wastewater treatment facility Loji Rawatan (Loji A, 4360 P.E.). Each CW was constructed using an 80 L plastic container (47.0 cm × 63.0 cm × 39.0 cm). The CWs consisted of two monoculture systems planted with *Canna indica*, two polyculture systems planted with *Canna indica* and *Heliconia* spp., two positive control systems planted with *Typha latifolia*, and two negative control systems without plants. Selected plants (*Canna indica* and *Heliconia* spp.) were acclimatised before planting. Each CW contained four to six plants arranged in two rows to allow horizontal subsurface flow.

The substrate layers were added sequentially and consisted of 7 cm of pebbles for drainage, 3 cm of gravel for physical filtration, 2 cm of sand for sediment filtration, 6 cm of cocopeat and activated carbon for moisture retention, microbial support, and pollutant adsorption, and 10 cm of soil to support plant growth and phytoremediation. A stagnant water level of 5 cm was maintained to facilitate the removal of caffeine, a representative MP. Figure 1 shows the arrangement of substrates with specified diameters within the mesocosm-scale CW, while Figure 2 illustrates the overall design of the mesocosm-scale CW.

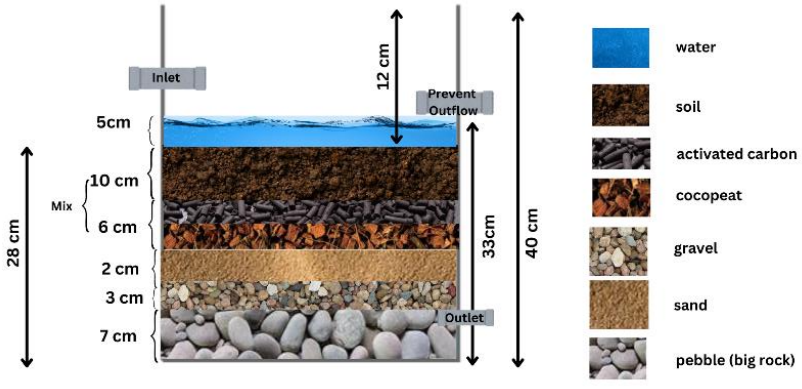

Figure 1. Arrangement of substrates with specified diameters in the mesocosm-scale CW.

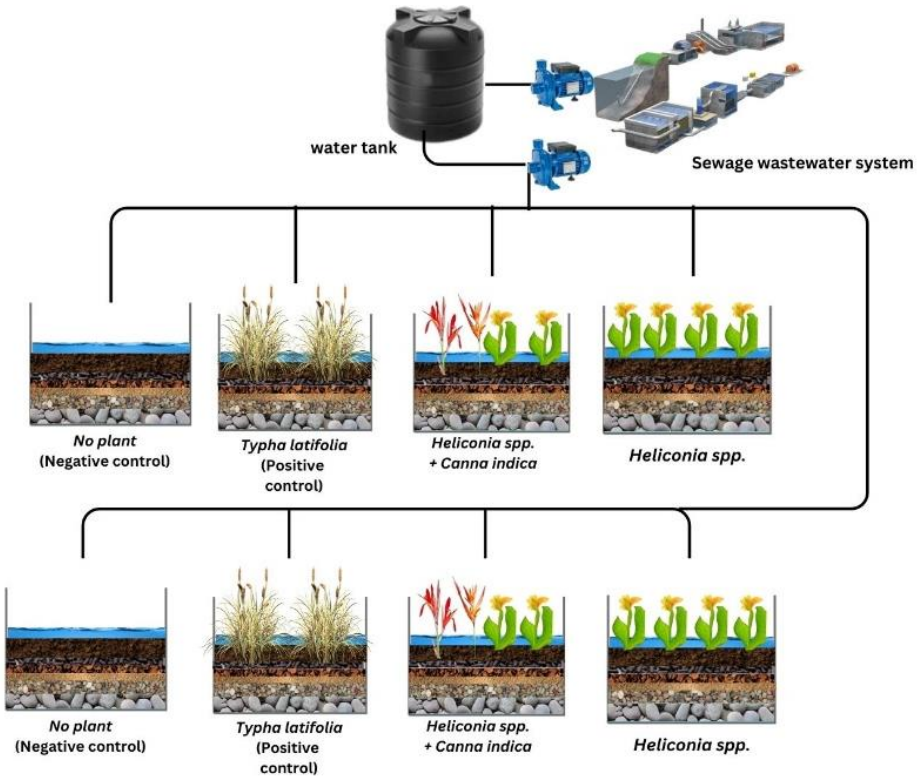

Figure 2. Experimental design of the mesocosm-scale CW.

Physicochemical Analysis of Wastewater

The physicochemical characteristics of wastewater were assessed by analysing total suspended solids (TSS), chemical oxygen demand (COD), biological oxygen demand (BOD), pH, total phosphorus (TP), total nitrogen (TN), total ammonia nitrogen (NH_3-N), and dissolved oxygen (DO). A total of 11 samples, including replicates, were collected before treatment (influent) and after treatment (effluent). The Water Quality Index (WQI) and the National Sanitation Foundation Water Quality Index (NSF-WQI) were calculated using Equations (1) and (2), respectively.

$$\boldsymbol{WQI} = \sum (S_i \bullet W_i) \quad (1)$$

where Si represents the sub-index value of each parameter, and Wi denotes the weight assigned to the corresponding parameter.

$$\boldsymbol{NSF-WQI} = 0.22\, S_{DO} + 0.19\, S_{BOD} + 0.16\, S_{COD} + 0.15\, S_{AN} + 0.16\, S_{TSS} + 0.12\, S_{pH} \quad (2)$$

where S_{DO}, S_{BOD}, S_{COD}, S_{AN}, S_{TSS} and S_{pH} represent the sub-index values for dissolved oxygen, biological oxygen demand, chemical oxygen demand, ammonia nitrogen, total suspended solids, and pH, respectively.

Caffeine Extraction and Quantification

Caffeine standards (50, 100, 125, and 200 mL^{-1}) were prepared by serial dilution from a 500 mgL^{-1} stock solution. Caffeine detection was performed using Solid-Phase Microextraction (SPME) with Gas Chromatography-Flame Ionisation Detection (GC-FID). A 3 mL sample aliquot was placed in a 20 mL vial, sealed, preconditioned at 100°C, and exposed to a PDMS/DVB fibre for 45 minutes. GC-FID analysis was performed using an Agilent 19091J-436 column with helium as the carrier gas, at a flow rate of 1.2 mL min^{-1} and a temperature programme from 50°C to 280°C over 9.75 minutes. Wastewater was spiked with caffeine at a concentration of 200 mL^{-1} caffeine by dissolving 195.5 g in 977 L of wastewater. Homogeneity was ensured, and the concentration was verified using GC-FID, with adjustments made as required.

Plant Biomass

After one week of treatment, one plant from each CW unit was harvested and separated into leaves, stems, and roots. Wet weights were recorded after gently blotting surface moisture. Samples were dried at 50°C for 2 days until a constant weight was achieved. Biomass was calculated using Equation (3).

$$\boldsymbol{Biomass}\ (\boldsymbol{g}) = Wetweight\ (g) - Dry\ weight\ (g) \quad (3)$$

Plant Total Nitrogen Uptake Analysis

TN content in plant samples was determined using a modified Kjeldahl method [19]. Fresh plant material was oven dried at 50 °C for 48 hours until a constant weight was achieved, ground to a fine powder (≤1 mm), and re-dried. Accurately weighed samples (≤2 mg) were digested with 1 ml of concentrated H_2SO_4 and 0.2 ml of copper sulphate solution (10 g $CuSO_4 \cdot 5H_2O$ in 100 ml deionised water) until complete mineralisation was observed. The digested samples were neutralised with NaOH, adjusted to pH 5-8, diluted to 100 ml, and filtered. Nitrogen quantification was performed spectrophotometrically at 630 nm after reacting the samples with phenol reagent (50 g phenol and 0.25 g sodium nitroprusside in 1000 ml deionised water) and sodium hypochlorite reagent (25 g NaOH and 20 ml 15% NaOCl in 1000 ml deionised water). A standard curve (50-800 µg N L^{-1}) was used for calibration. Results were expressed as mg kg^{-1} dry weight and adjusted for moisture content.

Chlorophyll Content Determination

Chlorophyll extraction was performed according to the method described by Arathi Kizhedath [20]. One gram of fresh leaves was ground with 20–40 mL of 80% (v/v) acetone. The mixture was centrifuged at 5,000–10,000 rpm for 5 minutes, and the supernatant was collected. This step was repeated until the residue became colourless. Absorbance was measured at 645 nm and 663 nm, using 80% acetone as the blank. The chlorophyll content was calculated using Equations (4), (5), and (6), as outlined below.

Here, A_{645} and A_{663} represent the absorbance values at 645 nm and 663 nm, respectively. These equations are widely used for chlorophyll quantification in plant extracts due to their accuracy in estimating specific chlorophyll forms.

Setup of Mesocosm-Scale Constructed Wetlands Lipid Peroxidation Estimation

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) content using the thiobarbituric acid (TBA) assay. Frozen plant tissues (100–150 mg) were homogenised with 2 mL of a reaction mixture containing 10% (w/v) trichloroacetic acid (TCA) and 0.25% (w/v) TBA. The homogenate was heated at 95°C for 30 minutes, cooled for 2 hours, and centrifuged for 15 minutes. The supernatant was collected, and absorbance was measured at 532 nm and 600 nm using a UV-Vis spectrophotometer. MDA concentration was calculated using Equation (7).

$$\boldsymbol{TBA\ RP\ (mMg^{-1})} = \frac{(OD_{532} - OD_{600})}{(155 \times m)} \quad (7)$$

where OD_{532} 2 and OD_{600} represent the optical density readings at 532 nm and 600 nm, respectively. The value 155 represents the extinction coefficient for MDA-TBA adducts (mM^{-1} cm^{-1}), and m represents the sample mass in grams.

Biomass Allocation and Water Content Calculation

Biomass allocation and water content were analysed to assess MP accumulation and plant physiological responses. Calculations were conducted using Equations (8) and (9), respectively.

The dry weight of plant organs, including roots, shoots, and leaves, provided insight into biomass distribution under stress conditions. Water content reflected plant hydration status and physiological adaptation to environmental stressors. These parameters are essential for evaluating plant efficiency in MP uptake and their potential application in wastewater treatment systems.

Characterisation Methods Removal Efficiency Calculation

The removal efficiency of MPs during the treatment process was quantified using Equation (10).

$$\boldsymbol{Removal\ efficiency}\ (\%) = \frac{C_i - C_f}{C_i} \times 100 \quad (10)$$

$$\boldsymbol{Total\ chlorophyll} = 20.2 \times A_{645} + 8.02 \times A_{663} \quad (4)$$

$$\boldsymbol{Chlorophyll\ a} = 12.7 \times A_{663} - 2.69 \times A_{645} \quad (5)$$

$$\boldsymbol{Chlorophyll\ b} = 22.9 \times A_{645} - 4.68 \times A_{663} \quad (6)$$

$$\boldsymbol{Biomass\ Allocation}\ (\%) = \frac{Dry\ weight\ of\ organ}{Total\ dry\ weight} \times 100 \quad (8)$$

$$\boldsymbol{Water\ Content}\ (\%) = (Wet\ weight - Dry\ weight)\ /\ Wet\ weight \times 100 \quad (9)$$

where C_i represents the influent concentration (before treatment) and C_f represents the effluent concentration (after treatment). Both concentrations were expressed in the appropriate units for the MP analysed. This calculation provided a clear indication of treatment system performance in reducing MP concentrations in wastewater.

Characterisation Methods Statistical Analysis

Data variability and validation were assessed using one-way or two-way ANOVA (SPSS Version 23). When significant differences were detected ($\alpha = 0.05$), post-hoc tests such as Tukey's Honest Significant Difference (HSD) test (for equal variances) or the Games-Howell test (for unequal variances) were conducted to determine pairwise differences. Means, variances, and standard deviations were calculated, and the results were interpreted to identify the most effective wetland unit.

RESULTS AND DISCUSSION

Setup of The Mesocosm-Scale Constructed Wetland

The mesocosm-scale CWs were successfully established and operated as horizontal subsurface-flow systems, maintaining consistent water levels and providing optimal conditions for *Canna indica* and *Heliconia* spp. The plants exhibited no signs of waterlogging stress, including wilting, oedema, epinasty, root rot, or fungal growth, demonstrating the system's capacity to sustain healthy vegetation. Figure 3 shows the design of the mesocosm-scale CW, illustrating the layout and operational structure.

Physicochemical Properties of Wastewater

Figure 4 compares the physicochemical properties among the different CW systems. Analysis of the influent revealed poor wastewater quality, with TN, TP, BOD, and ammoniacal nitrogen (AN) classified as Class III or Class V. The WQI score was 47.7652, classified as Class III (Satisfactory), while the NSF WQI score was 39.5174, classified as Class II (Bad). Both indices indicate significant pollution levels. BOD and AN were classified as Class V, while TN and TP were classified as Class III or Class IV. The COD, pH, TSS, and DO were within acceptable levels and classified as Class I or Class II. These findings indicate that the existing wastewater treatment facility is insufficient, particularly for removing organic and nitrogenous pollutants.

In the effluent, the CWs exhibited varying levels of treatment effectiveness. The *Canna indica* monoculture system reduced AN to 5.80 mg L^{-1}, while the polyculture system reduced it to 10.40 mg L^{-1}. The negative control achieved the lowest AN concentration at 3.65 mg L^{-1}. The polyculture system also achieved the greatest reduction in BOD (22.50 mg L^{-1}), representing a 20.59% improvement compared with the monoculture system. *Canna indica* monoculture and the positive control reduced TN concentrations to 12.00 mg L^{-1} and 12.50 mg L^{-1}, respectively, while the negative control exhibited the lowest TN concentration at 10.50 mgL^{-1}. Statistical analysis showed significant effects of wetland setup and pollutant type, with all CW systems significantly improving water quality relative to influent conditions. However, differences in performance among the systems were minimal, suggesting that substrate-mediated processes and plant-associated mechanisms play critical roles in nutrient and organic matter removal.

Figure 3. Design layout of the mesocosm constructed wetland setup.

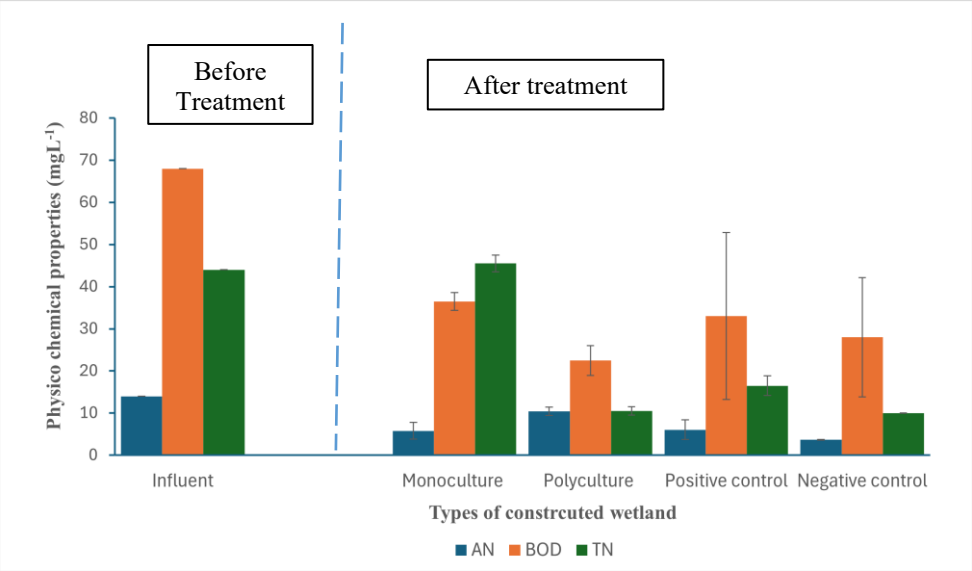

Figure 4. Physicochemical properties of influent and effluent across different types of constructed wetlands.

MP Concentration and Removal Efficiency before and after Treatment

Figure 5 shows the caffeine concentration in wastewater before and after treatment in different types of CWs. The monoculture *Canna indica* wetland exhibited a caffeine concentration of 46.20 mg L^{-1}, while the polyculture wetland showed a slightly lower concentration of 45.83 mg L^{-1}, suggesting marginally higher efficiency in the polyculture system, potentially due to synergistic plant interactions [21]. MP removal efficiencies were similar across all setups, with the monoculture system at 76.90%, the polyculture at77.09%, the positive control at 77.04%, and the negative control at 76.79%. These results suggest that the primary mechanisms for MP removal were physical and chemical processes, including adsorption, filtration, and sedimentation, with minimal contributions from plants [22]. Although the differences were marginal, the polyculture system exhibited slightly higher removal efficiency, which may be attributed to its diverse microbial communities and complementary removal pathways. Statistical analysis revealed significant differences in caffeine concentrations before and after treatment, confirming variability in treatment effectiveness, with all systems demonstrating the ability to reduce caffeine levels.

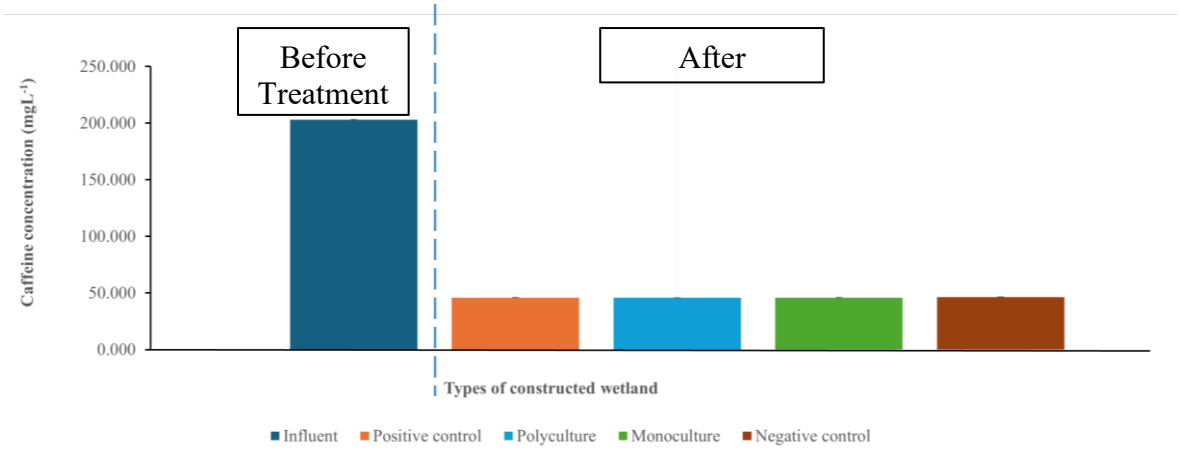

Figure 5. Caffeine concentration in influent and effluent across different types of constructed wetlands.

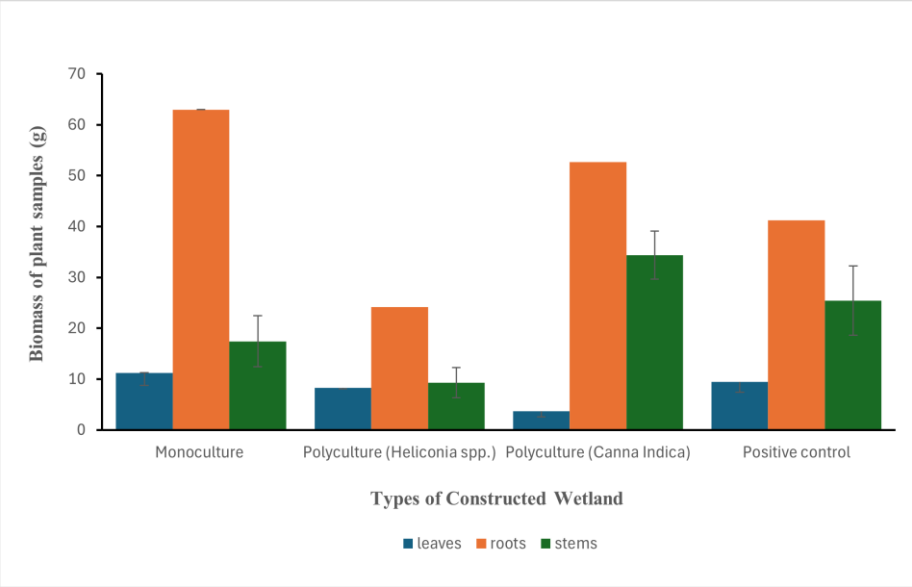

Figure 6. Biomass of plant samples in different types of constructed wetlands.

Biomass of Plants

Figure 6 presents the biomass of plant samples across different types of CWs. In the monoculture *Canna indica* setup, the highest biomass was observed in roots (62.99 ± 33.83 g), reflecting the species' efficient nutrient uptake and water absorption [23]. The polyculture setup showed lower root biomass (24.16 ± 1.63 g), attributed to *Heliconia* spp. having rhizomatous horizontal growth [24]. In the polyculture *Canna indica* setup, root biomass (52.69 ± 1.32 g) and stem biomass (34.36 ± 4.74 g) were higher, while leaf biomass was lower (3.67 ± 1.11 g). This indicates a shift in resource allocation to compete for light and space with neighbouring plants [25]. The positive control showed balanced growth with intermediate biomass values, suggesting sufficient nutrient and water availability. High standard deviations, particularly in monoculture root biomass, were likely due to sampling limitations and environmental variability.

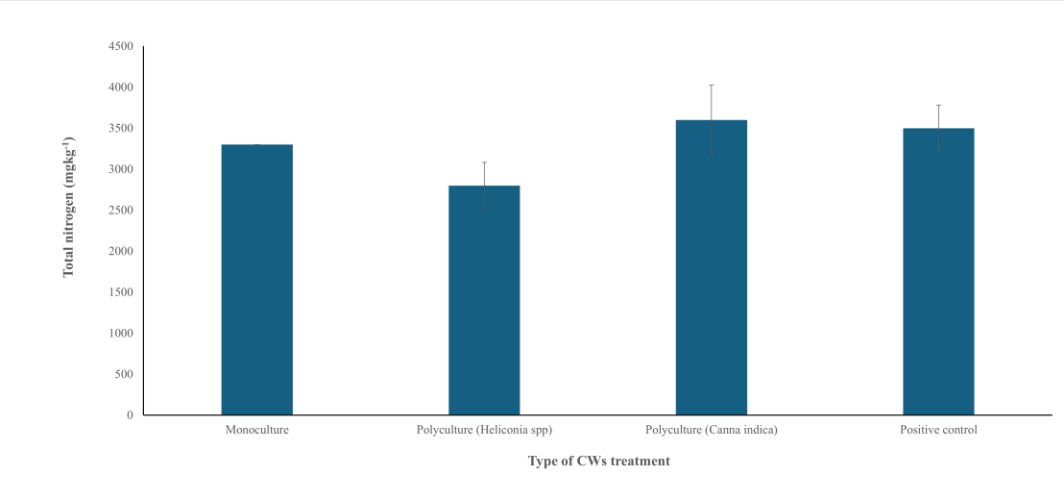

Figure 7. Total nitrogen content of plant samples across different types of constructed wetlands.

Total Nitrogen Uptake in Plants

Figure 7 shows the TN content of plant samples across different types of CWs. The TN content varied among systems, with polyculture *Canna indica* exhibiting the highest concentration (3600.00 ± 424.26 mg kg^{-1}), followed by the positive control (3500.00 ± 282.84 mg kg^{-1}), monoculture *Canna indica* (3400.00 ± 141.42 mg kg^{-1}), and polyculture *Heliconia* spp. (2800.00 ± 282.84 mg kg^{-1}). The polyculture *Canna indica* system exhibited 5.88% higher nitrogen uptake than monoculture systems. Polyculture systems likely benefited from enhanced nutrient cycling and microbial activity. *Canna indica* has a well-developed root system that facilitates nitrogen assimilation [25], while *Heliconia* spp. exhibited lower TN content, possibly due to interspecific competition or less efficient nitrogen uptake [24].

Chlorophyll Content in Plants

Figure 8 shows the chlorophyll content of plant samples across different types of CWs. Polyculture *Heliconia* spp. exhibited the highest total chlorophyll content (44.90 ± 8.11 µg mL^{-1}), primarily due to high chlorophyll b content (40.14 ± 8.90 µg mL^{-1}). The higher proportion of chlorophyll b indicates adaptation to shaded conditions in polyculture, where the species may minimise photoinhibition by adjusting leaf orientation to reduce light exposure [26, 27]. Monoculture *Canna indica* showed moderate chlorophyll content (36.50 ± 0.78 µg mL^{-1}), with a balanced chlorophyll a/b ratio (0.96), indicating adaptability to varying light conditions. The positive control (25.07 ± 9.98 µg mL^{-1}) showed the lowest chlorophyll content but the highest chlorophyll a/b ratio (1.12), reflecting higher efficiency under high-light conditions [28]. Polyculture *Canna indica*, exhibited lower chlorophyll content (28.28 ± 0.82 µg mL^{-1}), indicating reduced photosynthetic capacity due to interspecies competition for light. Vertical leaf orientation likely led to greater exposure to direct sunlight, resulting in leaf damage, reduced chlorophyll content, and impaired photosynthesis.

Lipid Peroxidation (MDA Analysis)

Figure 9 presents lipid peroxidation (MDA concentration) across different CW systems. Polyculture *Heliconia* spp. exhibited the highest MDA concentration (0.0155 mM g^{-1}), indicating greater physiological stress likely due to resource competition [29]. Polyculture *Canna indica* showed the lowest MDA concentration (0.0070 mM g^{-1}), representing a 43.55% reduction compared with monoculture systems. This indicates reduced oxidative stress and suggests beneficial synergistic interactions between plant species. Monoculture *Canna indica* exhibited higher stress levels (0.0124 mM g^{-1}), potentially due to competition for nutrients and light. The positive control showed moderate MDA concentration (0.0107 mM g^{-1}), reflecting adaptation to wetland conditions. Statistical analysis (ANOVA: $p = 0.408$) indicated no significant differences among treatments, suggesting comparable oxidative stress levels across systems.

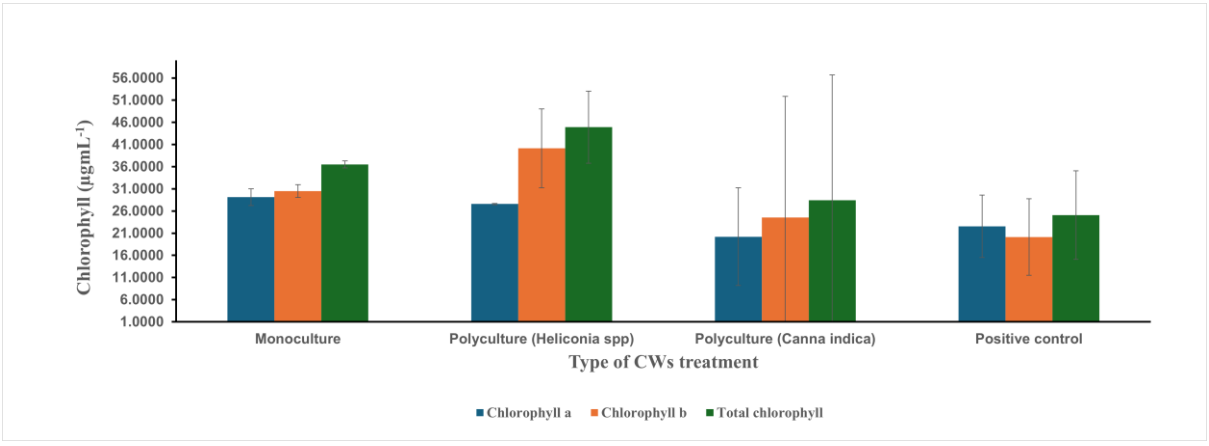

Figure 8. Chlorophyll content of plant samples across different types of constructed wetlands.

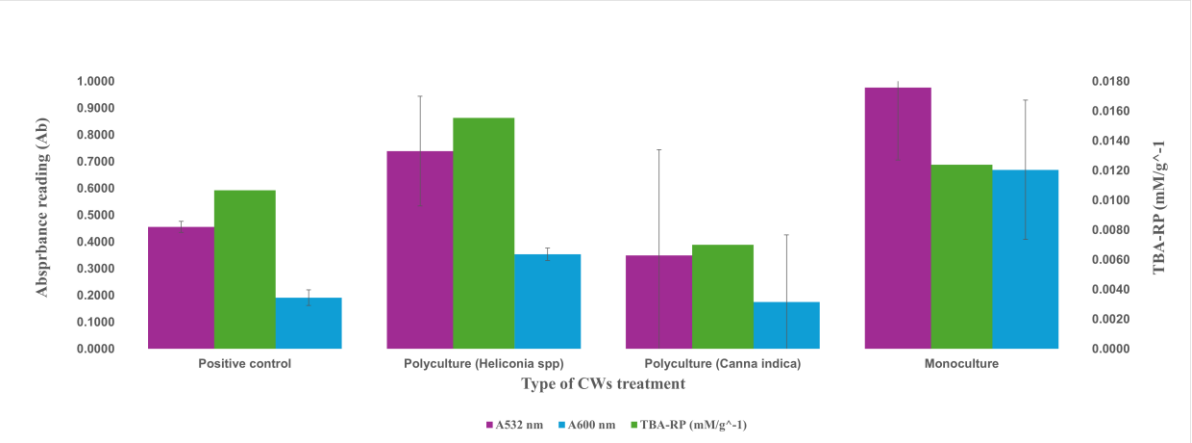

Figure 9. Lipid peroxidation in different types of CWs treatment.

Biomass Allocation and Water Content

Table 1 shows biomass allocation and water content of plant samples across different types of CWs. In monoculture systems, plants allocated more biomass to roots and shoots at 46.10% and 46.20%, respectively, to maximise resource acquisition. Polyculture *Heliconia* spp. allocated 62.91% of biomass to roots, enhancing nutrient uptake. Polyculture *Canna indica* exhibited balanced biomass allocation, with 61.39% allocated to roots. *Typha latifolia* in the positive control allocated 60.10% of biomass to roots, reflecting adaptation to wetland conditions. Water content in monoculture plants was highest in leaves at 86.54%. In polyculture systems, *Heliconia* spp. stored more water in shoots at 87.50%, while *Canna indica* exhibited balanced water retention [24, 30]. *Typha latifolia* showed the highest water content in shoots at 89.28% and roots at 86.37%, consistent with wetland adaptation [31]. Polyculture systems promoted balanced growth and more efficient resource utilisation.

Table 1. Biomass allocation and water content of plant samples across different types of wetlands.

Types of constructed wetland		Biomass allocation (%)			Water content (%)		
	Types of plant samples	leaf	root	shoot	leaf	root	shoot
Monoculture		7.70	46.10	46.20	86.54	82.46	74.75
Polyculture	*Heliconia spp.*	10.60	62.91	26.49	74.15	56.83	87.50
	Canna Indica	11.14	61.39	27.47	82.33	79.08	75.19
Positive control		10.44	60.10	29.45	71.72	86.37	89.28

CONCLUSION

This study successfully designed and evaluated mesocosm-scale CWs for wastewater treatment, achieving significant reductions in AN, BOD, and TN. In some setups, TN levels improved to Class III. Polyculture systems showed a 20.59% increase in BOD removal, a 0.99% improvement in overall removal efficiency, 5.88% higher nitrogen uptake, and a 43.55% reduction in MDA concentration compared with monoculture systems, highlighting the benefits of plant diversity. The substrate-only control outperformed planted systems in AN and TN removal, emphasising the importance of abiotic processes such as adsorption and microbial activity. MP removal averaged approximately 76% across all systems, and was driven primarily by filtration and adsorption processes, with plants playing a secondary role. These findings demonstrate the potential of CWs as eco-friendly, cost-effective systems for decentralised wastewater treatment. Future studies should focus on optimising substrate composition, refining plant selection, and mitigating environmental factors to further enhance system performance.

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