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Marwa Ben Saad

Tunis El Manar University

Eya Ben Othmen

Tunis El Manar University

Syrine Boukhili

Tunis El Manar University

Hajer Ennouri

Tunis El Manar University

Khadija Kraiem

Tunis El Manar University

Wided Ben Ammar

Tunis El Manar University

Maher Gtari

Université Tunis Carthage

Karim Ben Slama

Tunis El Manar University

Atef Jaouani

`atef.jaouani@issbat.utm.tn`

Tunis El Manar University

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Marwa Ben Saad¹, Eya Ben Othmen¹, Sirine Boukhili¹, Hajer Ennouri¹, Khadija kraiem¹, Wided Ben Ammar², Maher Gtari³, Karim Ben Slama¹, and Atef Jaouani^{1*}

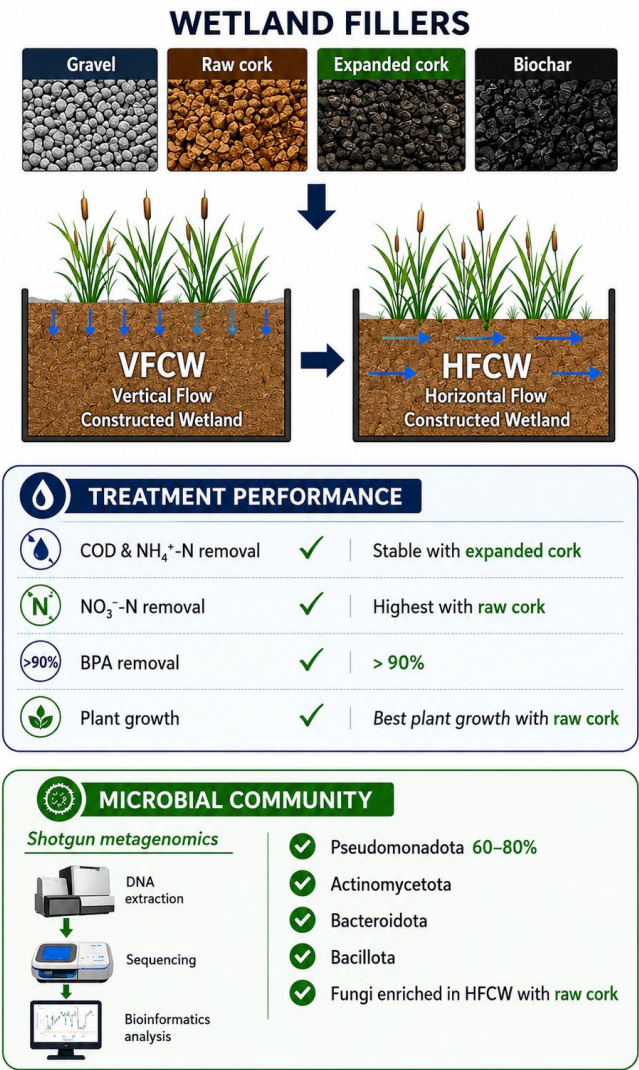
¹ Laboratory of Bioressources, Environment and Biotechnology (LR22ES04), Higher Institute of Applied Biological Sciences of Tunis (ISSBAT), University of Tunis El Manar, 9 Rue Zouheir Essafi, CP 1006, Tunis, Tunisia

² Laboratory of Plant Productivity and Environmental Constraints (LR18ES04), Department of Biology, Faculty of Sciences of Tunis, University of Tunis El Manar, 1060 Tunis, Tunisia

³ Department of Biological and Chemical Engineering, USCR Molecular Bacteriology and Genomics, University of Carthage, National Institute of Applied Sciences and Technology, Tunis, 2080, Tunisia

*Corresponding author, E-mail: atef.jaouani@issbat.utm.tn

Graphical abstract



Abstract

This study aimed to evaluate the treatment performance of constructed wetland (CW) systems using alternative sustainable substrates derived from cork as filter media. Four lab-scale CW units were operated using raw cork, expanded cork, gravel enriched with biochar-based cork, and gravel as substrate materials. The effects of substrate type on organic matter removal, nitrogen transformation, micropollutant attenuation, plant development, and microbial community composition were investigated. Results showed significant differences among substrates for chemical oxygen demand (COD) and nitrate nitrogen (NO_3^- -N) removal (ANOVA test, $p < 0.05$). Expanded cork showed more consistent COD removal performance, with lower variability, while cork-based substrates showed enhanced nitrate removal compared with conventional gravel-based systems. These results suggest that cork substrates may provide favorable physicochemical conditions for microbial processes involved in nitrogen transformation. For bisphenol A (BPA), raw and expanded cork achieved high removal rates $>90\%$ under the applied experimental loading conditions. Cork based-substrates also provided favorable conditions for plant growth, especially in horizontal flow constructed wetlands (HFCWs). Shotgun metagenomic sequencing revealed that microbial communities were dominated by bacterial taxa across all configurations, with Pseudomonadota being the most abundant (60–80%), followed by Actinomycetota, Bacteroidota, and Bacillota. Fungal taxa were detected only in the HFCW with raw cork (HFCW2). Overall, the findings highlight the potential of cork-based substrates as sustainable filter media for CW applications.

Keywords: Bisphenol A; constructed wetlands; cork substrate; metagenomics; plant growth; wastewater treatment.

Highlights

- Cork-based substrates improved pollutant removal in constructed wetlands.
- Expanded cork showed consistent COD removal, while cork favored NO_3^- -N removal.
- BPA removal exceeded 90% in cork-based constructed wetlands.
- Raw cork supported optimal plant growth in horizontal flow CWs.
- Cork substrates influenced microbial community composition in HFCW systems.

Introduction

Constructed wetland systems (CWS), as engineered ecosystems, represent a relevant example of nature-based solutions and provide a sustainable and effective method for water treatment. They can be applied to various types of wastewater and integrated into different stages of the treatment process. This promising approach offers a low-cost, energy-efficient, and ecological solution alternative to conventional water treatment systems [1]. CWs have been developed since the 1960s. Their fundamental principle consists of natural self-purification, in which wastewater is filtered through a basin filled with a substrate, usually gravel, and planted with macrophytes, under controlled conditions [2]. They are generally divided into surface flow and subsurface flow CWs, with the subsurface flow further divided into horizontal and vertical based on the flow direction of the influent. Three key components are present in CW system: substrate, vegetation, and microorganisms. The removal of contaminants from wastewater occurs through the interaction among these three compounds [3]. Various mechanisms are involved in the removal of contaminants such as filtration, sedimentation, adsorption, microbial degradation, and plant uptake [4]. Despite their apparent simplicity, CW systems are highly complex. Many researchers have referred to them as a “black box” because of the complicated processes occurring within them [5–8]. To ensure a sustainable system and improve its efficiency, many scientists have focused on enhancing CW performance. Studies have revealed that adjusting one or more parameters can affect the functioning of CWs. The type of CW, its dimensions, the selection of plants, the substrate and the characteristics of the influent to be treated are all key parameters for assessing the CW efficiency [9, 10].

The substrate plays a crucial role in CWs. It is a fundamental component that supports plant growth and serves as a habitat for the microbial communities. In addition, all mechanisms involved in water treatment occur within the substrate media. Since the inception of CWs, three main types of substrates have been tested as filter media: natural, by-products and synthetic substrates [3]. Gravel is the most common substrate, followed by soil and sand. Various alternative substrates have been developed, such as anthracite, apatite, calcite, ceramsite, construction wastes, biochar, bauxite, oyster shell, peat, wood mulch, rice straw, zeolite, alum sludge, and volcanic rock [11, 12]. According to Yang et al. (2018), two principal factors determine substrate selection: cost and local availability. The physicochemical characteristics of the substrate must be considered, including optimal hydraulic conductivity, high porosity, and suitable chemical properties to support plant growth, microbial activity, and efficient contaminant removal [3]. Therefore, selecting the most suitable substrate is a critical factor in determining the overall performance and sustainability of a CW. Several studies have suggested using a natural carbon-rich substrate as filter medium, such as biochar, agricultural wastes, and recycled materials. These materials offer potential advantages, including improved treatment efficiency, reduced environmental impact, and enhanced resource valorisation [13]. In recent years, biochar filler has been widely explored to enhance CW performance. Biochar is obtained via the pyrolysis of biomass such as reeds, agricultural waste and sludge. The addition of biochar in CW enhance significantly the removal of organic matter, total nitrogen, nitrates and ammonia from wastewater [14, 15]. Cakin et al. (2024) evaluated different CW substrates (pea gravel, LECA and Alfagrog), with and without the addition of 20% biochar. Obtained results showed that LECA + biochar and gravel + biochar revealed promising results for COD, ammonium and phosphorus removal [16]. Aguilar et al. (2019) applied cork as a filter medium in CWs used for treating agricultural runoff contaminated with nitrates. The results showed significantly higher nitrate removal in CWs using cork, compared to the gravel system, reaching removal rates from 80 to 99% compared to 5–46% [17]. In their study, Guerrero-Brotons et al. (2024) evaluated the effects of

adding natural wetland soil and biochar to gravel on nutrient imbalance mitigation in agricultural drainage water, as well as on the growth of plants and microorganisms. Plant growth rates considerably differed among CW substrates. The results demonstrated that *Phragmites australis* exhibited the highest growth rate in the gravel + soil treatment wetland (9.88 ± 0.57 cm per month), followed by gravel + biochar (6.21 ± 0.42 cm per month), and finally gravel alone (2.45 ± 0.42 cm per month) [18]. In addition to natural and conventional substrates, synthetic and modified materials have recently emerged as promising alternatives for enhancing constructed wetland performance. In particular, iron-based substrates, such as ferrous sulfide (FeS)-based materials, have been shown to significantly improve nitrogen removal efficiency by enhancing electron transfer activity, stimulating denitrifying enzyme activity, and enriching functional microbial communities, especially under low carbon-to-nitrogen conditions [19]. Substrate not only has an impact on pollutant removal but also provides physical support for the plants and microorganisms [20].

In CWs, microorganisms play a crucial role in contaminant removal through processes such as organic matter degradation, nitrification–denitrification, and the biodegradation of emerging contaminants [21]. Recent studies have increasingly focused on the influence of substrates on microbial communities. For instance, Gao et al. (2025) investigated the effect of functional substrates (vermiculite–tourmaline modified polyurethane) on microbial populations involved in nitrogen cycling. Their results highlighted the important role of substrate properties in shaping microbial activity and enhancing nitrogen removal in CW systems [22].

Although numerous researches have studied CW substrates and their effects on contaminants removal, there remains a significant gap in understanding the interactions between substrates, wetland plants, and microbial communities in CW [23]. Therefore, it is crucial to analyse the structure and diversity of microbial communities for evaluating the effect of substrate on these communities and understanding the mechanisms involved in water purification [24].

This study aimed to enhance the performance of CW systems by exploring innovative substrate materials. Among the substrates investigated, cork emerges as a promising biomaterial, widely used in various applications due to its exceptional physicochemical characteristics, including compressibility, resilience, and natural durability [25, 26]. Preliminary investigations have demonstrated that cork has a promising potential in nitrate removal from agricultural drainage and in reducing some pharmaceutical contaminants. Cork may also be a source of carbon, which is necessary for the growth of heterotrophic bacteria [17, 27]. In this study, different forms of cork obtained through various treatment processes were evaluated to assess their effects on contaminant removal, plant growth, and microbial community structure in CWs.

Material and methods

Experimental setup

The lab-scale system was implemented at the Higher Institute of Applied Biological Sciences of Tunis. It consisted of four parallel lines, each featuring a hybrid CW system (horizontal and vertical CW) (Fig. 1). Each CW unit was built from polyethylene plastic and had dimensions of $0.39 \times 0.3 \times 0.31$ m (length $\times$ width $\times$ height) corresponding to a total bed volume of approximately 36.3 L and a surface area of 0.117 m².

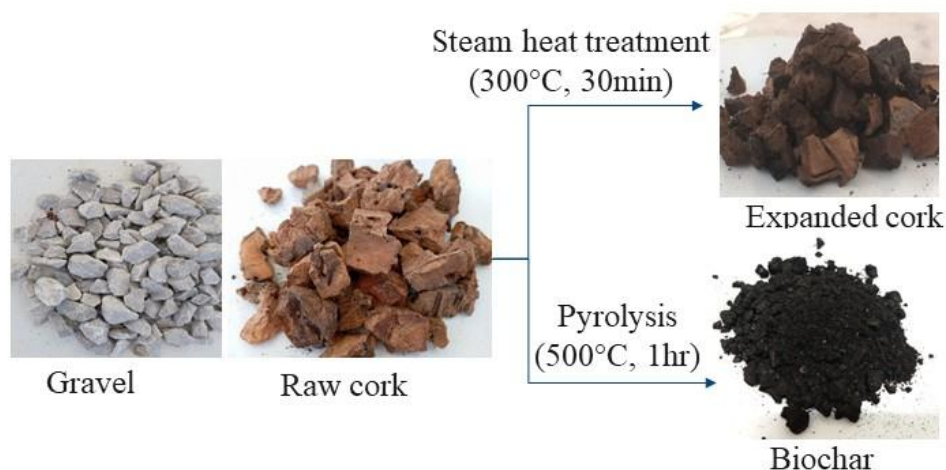
The difference among the lines is based on the substrate used as filter media. Two fundamental substrates were examined: gravel (coarse gravel 15–28 mm Ø, fine gravel 6–12 mm Ø) and cork (5–20 mm Ø). To reduce clogging

problems and improve effluent circulation, coarse gravel was used at the inlet and outlet of the horizontal CWs and at the bottom of the vertical CWs.

Varied treatment approaches were applied to raw cork, resulting in diverse cork forms. Raw cork was subjected to two specific thermal treatments. Initially, it underwent steam heating at 300°C for 30 minutes, causing cork granule expansion and darkening of color, resulting in expanded cork (or black cork). Subsequently, raw cork underwent pyrolysis at 500°C, producing biochar. Raw cork, expanded cork, and biochar were characterized through Fourier Transform Infrared Spectroscopy (Perkin Elmer-FTIR 2000) and X-ray Diffraction (X'Pert PRO MPD) analysis.

The CWs in the first line, L1, were filled with expanded cork (VFCW1 and HFCW1). In the second line, L2, natural raw cork was used (VFCW2 and HFCW2). The third line, L3, was filled with gravel amended with 10% biochar (v/v) and included VFCW3 and HFCW3, while the fourth line, L4, contained only gravel and served as a control (VFCW4 and HFCW4) (Fig. 2). All CW basins were planted with *Phragmites australis*. The filter media depth was 0.26 m and the system was operating at ambient temperature (Fig. 1).

(A) Wetland fillers



(B) Lab-scale system

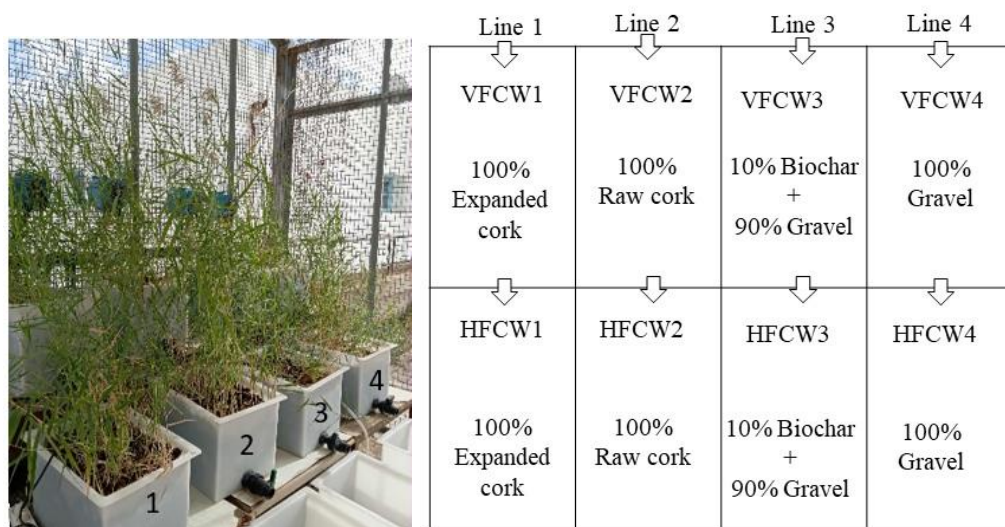


Fig. 1 (A) Wetland fillers and (B) lab-scale system: Gravel, raw cork, expanded cork, and biochar in Vertical Flow (VF) and Horizontal (HF) Flow Constructed Wetlands (CW)

Operation and physicochemical analysis

The experimentation started in April 2024 and continued until November 2024. During the acclimatization period, which lasted approximately two months, the experimental units were supplied with tap water. During the experimental phase, the lab-scale CW system was operated under controlled conditions, with a flow rate of 10 L/d under batch feeding mode, in which wastewater was introduced at a defined feeding frequency, while the outlet remained configured to allow drainage after each feeding event. The hydraulic retention time (HRT) was estimated based on the effective water-filled volume of the filter beds and the applied flow rate, resulting in a nominal HRT of approximately 24 h. This corresponded to a hydraulic loading rate (HLR) of 0.085 m d⁻¹ (85.5 L m⁻² d⁻¹). The applied organic loading rate (OLR) was calculated based on the influent COD concentration (410 ± 55 mg L⁻¹), the applied flow rate, and the surface area of the CW units. The resulting OLR was approximately 34.9 ± 4.7 g COD m⁻² d⁻¹. The applied nitrogen loading rate (NLR), calculated from the influent inorganic nitrogen concentration (NH₄⁺-N + NO₃⁻-N + NO₂⁻-N = 2.74 ± 0.71 mg L⁻¹), flow rate, and surface area of the CW units, was approximately 0.23 ± 0.06 g N m⁻² d⁻¹. During the experimental period, the temperature was monitored and ranged from 24.3 to 25.5 °C. A synthetic wastewater simulating treated wastewater was used to assess the potential of the lab-scale system as a complementary treatment method. A concentrate solution was prepared including sodium acetate (CH₃COONa), ammonium chloride (NH₄Cl), potassium nitrate (KNO₃), monopotassium phosphate (KH₂PO₄) and yeast extract [28]. This solution was then diluted with tap water in order to prepare the influent. Fine sand particles (0.1–0.2 mm Ø) and *E. coli* cells were added to simulate TSS and microbial contamination respectively (Table 1).

Table1 Statistical data of the inflow values of each parameter during the study period

Parameter	Average ± standard deviation	Minimum	Maximum
pH	7.05± 0.02	6.87	7.28
DO (mg/L)	4.78±0.11	3.96	5.80
EC (ms/cm)	4.41±0.10	3.69	5.30
TSS (mg/L)	153±1	140	168
COD (mg/L)	410±55	315	525
NH ₄ ⁺ -N (mg/L)	0.33±0.10	0.02	0.80
NO ₃ ⁻ -N (mg/L)	2.36±0.70	0.72	3.50
NO ₂ ⁻ -N (mg/L)	0.05±0.02	0.01	0.13
PO ₄ ³⁻ -P (mg/L)	3.84±1.60	0.50	5.18
Total coliform	5.3x10 ⁵ ±10 ⁵	4.9x10 ⁵	6.1x10 ⁵
<i>E.coli</i>	6.7x10 ⁴ ±10 ⁴	5.9x10 ⁴	8.2x10 ⁴

For water quality monitoring, samples were collected from the outlet of each line for analysis. Physicochemical analyses were conducted. Dissolved oxygen (DO), pH, and electrical conductivity (EC) were measured using a multi-parameter water quality meter (SensoDirect 150, Loviband, Dortmund, Germany). Chemical oxygen demand (COD), ammonium nitrogen (NH₄⁺-N), phosphate phosphorus (PO₄³⁻-P), nitrate nitrogen (NO₃⁻-N), and nitrite nitrogen (NO₂⁻-N) were measured with Hach Lange test cells (LCI 400, 304, and 349, respectively, Düsseldorf, Germany) on a spectrophotometer (DR 1900, Hach Lange, Dortmund, Germany). Total suspended solids (TSS) were determined by standard method 2540.

Micropollutant removal analysis

Bisphenol A (BPA) was analysed using U-HPLC (Ultimate 3000), consisting of an automatic injection system, high pressure pump, C18 column and UV detector. The HPLC-UV method for detecting BPA was performed according to Campos et al., 2019 [29]. A stock solution of BPA was prepared and a series of standard solutions were used to generate a calibration curve. To assess the performance of lab-scale system in BPA removal, 50 µg/L of BPA was added to the synthetic wastewater and injected into the different units of CWs. After feeding the CW units, 500 mL effluent samples were collected from the outlet of each HFCW. Prior to analysis, samples underwent solid-phase extraction using CHROMABOND HLB (60 µm).

Plant development

Plant development was monitored through metrics such as plant length, photosynthetic pigmentation levels (Chlorophyll a, chlorophyll b and carotenoids), and malondialdehyde (MDA) concentration. For the extraction of the photosynthetic pigments, fresh leaves of *Phragmites australis* (0.1 g), from each basin, was homogenized using a mortar. 5 mL of acetone was added at 80% and centrifuged at room temperature for 10 min at 4400 rpm. The supernatant was analysed using spectrophotometer (Specord 210 Plus, Analytik Jena, Germany) at 470, 646 and 663 nm. Chlorophyll a, chlorophyll b and carotenoids concentrations were determined according to the equation proposed by Wellburn 1994 [30, 31].

Lipid peroxidation in *Phragmites* leaves was evaluated by determining the malondialdehyde (MDA) content using the method described by Heath and Packer (1968). Fresh leaves (0.2 g) from each unit were ground with 1 mL of 0.1% trichloroacetic acid (TCA). After centrifugation at 15,000 rpm for 10 min, 250 µL of the supernatant was mixed with 1 mL of 0.5% thiobarbituric acid (TBA) solution prepared in 20% trichloroacetic acid (TCA). The mixture was incubated at 95°C for 25 minutes and then cooled on ice for 10 min to stop the reaction. The absorbance of the supernatant was measured at both 532 nm and 600 nm. The MDA content was determined by calculating the difference in absorbance readings and applying the extinction coefficient $\epsilon=155 \text{ mM}^{-1} \text{ cm}^{-1}$ [32, 33].

Metagenomics analysis

Substrate samples (~100g) were collected from each unit and suspended in 200 mL PBS 1× (130 mM NaCl, 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.2). After sonication for 3 min in an ultrasonic bath (S40H Elmasonic), the supernatant was centrifuged at 4000 rpm for 10 min (UNIVERSAL 320R Hettich). DNA was extracted using the Genomic DNA Kit Soil (GRISP GK30.0050) according to the manufacturer's instructions. DNA quality and quantity were assessed by spectrophotometry (NanoDrop) and fluorometry (Qubit).

For each sample, 50 ng of genomic DNA was used for library preparation with the Rapid Sequencing DNA V14 – Barcoding kit (SQK-RBK114.96, Oxford Nanopore Technologies, UK). Libraries were loaded onto a MinION MK1B sequencer equipped with a Flow_Min 106 flow cell (1500 active nanopores). Sequencing was performed using MinKNOW (v24.1.18) on a Windows 10 system. Eight DNA samples, corresponding to the eight individual CW units, were multiplexed and sequenced in a single run. After sequencing, the flow cell was washed using the EXP-WSH004 kit according to the manufacturer's protocol. Basecalling and demultiplexing were performed with Guppy (v6.4.6) using the super-accurate model. Taxonomic classification was carried out with the EPI2ME platform (Oxford Nanopore Technologies) using the *What's In My Pot (WIMP)* workflow, which aligns reads against the NCBI nt database. Reads classified as “unclassified” were those with no confident taxonomic

assignment (alignment identity <80% or coverage <50%). Downstream analysis was performed in R (v4.2) using the *vegan* and *ggplot2* packages. Alpha diversity was estimated using the Shannon index, and beta diversity was assessed by Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarity. Sequencing output was assessed based on the number of high-quality reads obtained after filtering. A total of 382,751 high-quality reads were generated across the eight multiplexed samples.

Statistical analysis

Statistical analysis was performed using Microsoft Excel and IBM SPSS Statistics (version 27.0.1) for water quality monitoring parameters. The distribution normality was verified using Kolmogorov-Smirnov test. Differences in performances among substrates were evaluated using one-way ANOVA and pairwise differences were tested using Tukey’s post-hoc test ($P \leq 0.05$).

Pollutant removal efficiency was calculated as follows [34]:

$$\text{Removal efficiency (\%)} = \left[\frac{C_0 - C_e}{C_0} \right] \times 100$$

Where:

C_0 : Influent concentration

C_e : Effluent concentration

Metagenomic datasets were analysed through Principal Coordinates Analysis (PCoA) based on the Bray-Curtis distance matrix performed in Colab Python (v3.10).

Results

Substrate characterization

FTIR spectra of the tested cork-based materials revealed that raw cork showed characteristic peaks of lignocellulose, including a broad absorbance band of O–H between 3200 and 3500 cm^{-1} , C–H stretching at 2800–3000 cm^{-1} , and functional groups such as C=O and C–O in the ranges of 1500–1700 cm^{-1} and 1000–1300 cm^{-1} , respectively. The obtained profile is in agreement with a previous study on natural cork, considering its three main components: suberin, lignin, and polysaccharides [25]. For the expanded cork the intensity of O–H peaks was reduced, carbonyl group signals near 1600–1700 cm^{-1} were increased and the polysaccharide fingerprint was altered, indicating partial degradation of lignin and cellulose. The FTIR spectrum of biochar indicated diminished O–H and polysaccharide-related peaks alongside a dominant aromatic C=C stretch around 1600 cm^{-1} , reflecting enhanced aromaticity and carbonization.

XRD analysis revealed a clear progression in structural complexity of cork materials. The raw cork spectrum showed a broad peak, centered between 15° and 25° (2 θ), characteristic of its amorphous structure [35]. For expanded cork, the broad peak was retained, but additional features appeared, revealing a partial reorganization of the amorphous structure. The biochar spectrum indicated a mix of amorphous and crystalline regions, with new phases formed during pyrolysis. The various profiles observed for the cork materials highlight the significant impact of thermal treatment on raw cork, which aligns well with findings from previous studies. Indeed, the literature indicates that exposing cork to elevated temperatures ($T > 200^\circ\text{C}$) can lead to the decomposition of its chemical constituents, resulting in its transformation. At 300°C, the color of cork changes to dark brown and then black [36] (Fig. 2).

(A) FTIR spectra

(B) XRD patterns

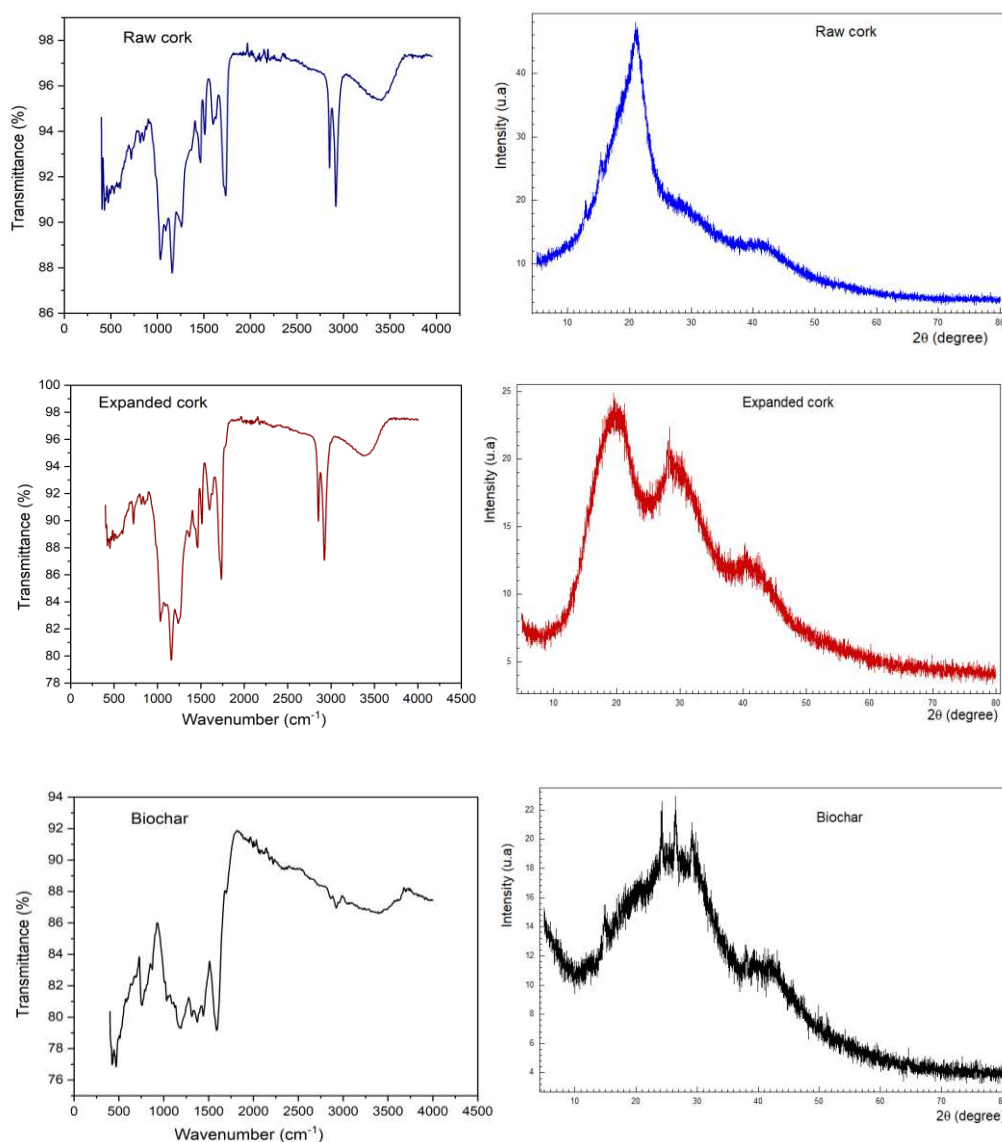


Fig. 2 FTIR spectra (A) and XRD patterns (B) of raw cork, expanded cork, and biochar

Physico-chemical performance assessment

The results of physicochemical parameter monitoring (Table 2) indicate that pH values remained stable (around 6.8–7.2) in all CW units, revealing a near-neutral environment suitable for microbial activity and biodegradation processes [37]. Electrical conductivity (EC) consistently decreased after treatment compared to the influent, indicating a reduction in dissolved salts and ion concentrations, which reflects improved water quality [38]. Dissolved oxygen (DO) levels, while initially lower in the treated effluents than in the influent, tended to gradually increase over time, particularly in gravel-based CW units. This finding can be explained by the ability of *Phragmites australis* to transport oxygen from the aerial parts down into the root zone, a process known as radial oxygen loss (ROL)[39].

Table 2 Physico-chemical performance of CW units, Values are expressed as mean ± standard deviation (SD) (n=22)

Parameter	Expanded cork	Raw cork	Gravel + biochar	Gravel
pH	7.08 ± 0.01	7.05 ± 0.01	7.01 ± 0.02	7.04 ± 0.01
DO (mg/L)	3.93 ± 0.17	3.78 ± 0.21	4.02 ± 0.14	3.71 ± 0.22
EC (mS/cm)	2.90 ± 0.09	2.86 ± 0.07	3.63 ± 0.11	3.83 ± 0.08
TSS (mg/L)	74.7 ± 8.50	89.0 ± 10.20	42.0 ± 7.10	46.0 ± 5.90
COD (mg/L)	105.5 ± 28.10	174.0 ± 36.90	118.0 ± 32.60	167.5 ± 35.10

In terms of total suspended solids (TSS) removal, the best efficiency was registered in gravel with biochar, with a rate of 72.28%, followed by gravel alone at 69.25%. Expanded cork had a removal efficiency of 51.31%, and raw cork had the lowest at 42.13%. Gravel showed the least variation in performance. Expanded cork had moderate TSS removal with lower variability. Raw cork had the highest variability. Gravel with biochar had slightly better TSS removal than gravel alone but was more variable. The higher TSS removal observed in gravel-based systems may be attributed to their more stable granular structure, which provides more efficient physical filtration and minimizes the release of fine particles. In contrast, cork-based substrates may be more susceptible to particle detachment or fragmentation, potentially contributing to lower apparent TSS removal. Differences in surface properties and clogging behavior among substrates may also influence particle retention. ANOVA results showed a statistically significant difference in TSS removal efficiency across the different substrates tested ($F = 237.769$, $p < 0.001$). The very low p -value (< 0.001) indicated that the variations in mean removal rates between the substrate groups were not due to random chance. Tukey's HSD post-hoc test for TSS removal revealed significant differences in removal efficiency among the substrates ($\alpha = 0.05$). Raw cork exhibited the lowest performance, while expanded cork showed intermediate removal. In contrast, gravel and gravel amended with biochar achieved the highest efficiencies. No significant difference was observed between raw cork and expanded cork ($p = 1.000$), nor between gravel and gravel + biochar ($p = 0.572$). Tukey's HSD post-hoc test revealed three distinct homogeneous subsets for TSS removal. Raw cork showed the lowest removal efficiency (group a), expanded cork exhibited intermediate performance (group b), while gravel and gravel amended with biochar achieved the highest efficiencies and belonged to the same homogeneous subset (group c).

COD removal efficiency across different substrates varied, with expanded cork registering the highest mean (73.94%) and the least variability. Raw cork had a lower mean (57.51%) and greater variability, while gravel + biochar and gravel had means of 70.51% and 60.01%, respectively, with moderate variability (standard deviations of 8.58 and 8.29). ANOVA results showed a statistically significant difference in removal efficiency across substrates ($F = 22.025$, $p < 0.05$). According to Tukey's HSD post-hoc test, significant differences in COD removal efficiency across substrates were revealed. Expanded cork had significantly higher removal efficiency than raw cork (16.43%, $p < 0.001$) and gravel (13.94%, $p < 0.001$). Raw cork, in contrast, showed significantly lower efficiency than gravel + biochar (mean difference of -13.00%, $p < 0.001$), while it had no significant difference from gravel in terms of performance (mean difference of -2.49%, $p = 0.728$). Gravel + biochar performed better than gravel alone, with a significant mean difference of 10.51% ($p < 0.001$). Raw cork and gravel exhibited lower removal efficiencies, while gravel + biochar and expanded cork achieved the highest efficiencies.

The lower and more variable COD removal observed in raw cork systems may be partly associated with the potential release of soluble organic compounds naturally present in cork material, which could contribute to effluent COD measurements. Further characterization of dissolved organic carbon (DOC), total organic carbon (TOC), and cork-derived leachates would be required to better elucidate the contribution of substrate-released organic compounds to the observed COD variations. The thermal treatment of raw cork to produce expanded cork

improved its structural stability and enhanced its capacity to remove COD. The expansion process increases the surface area and promotes greater contact between the filter medium and waterborne contaminants, which in turn improves removal efficiency. Similarly, biochar is well recognized for its highly porous and chemically reactive surface, offering multiple adsorption sites for both organic and inorganic pollutants. When combined with gravel, the adsorption capacity of biochar is further complemented by the high surface area and porosity of the gravel matrix, creating favorable conditions for both adsorption and microbial colonization, thus improving contaminant removal performance [18].

Nutrients removal

The efficiency of $\text{NH}_4^+\text{-N}$ removal varied across the four substrate types tested in CW units. Expanded cork achieved a mean removal efficiency of 63.31% with a higher median of 69.38% and the lowest standard deviation of 13.65, reflecting both effective and consistent performance. Similarly, the gravel–biochar mixture exhibited a comparable mean efficiency of 63.80% and a high median of 67.48%, though with a slightly higher standard deviation of 13.96, indicating reliable removal with moderate variability. In contrast, raw cork and gravel alone were less effective, with mean removal efficiencies of 59.96% and 59.10%, respectively; notably, gravel alone showed the highest standard deviation (16.74) and the widest range of removal efficiency (58.30 percentage points), suggesting more erratic performance. Based on the one-way ANOVA results ($F(3,84) = 0.537, p = 0.658$), no statistically significant differences existed in $\text{NH}_4^+\text{-N}$ removal efficiency among the tested substrates. The absence of significant differences among substrates may also be related to the relatively low influent $\text{NH}_4^+\text{-N}$ concentration (0.33 ± 0.10 mg/L), which limited the nitrogen loading available for biological transformation processes. Under such low ammonium loading conditions, the removal performance may be less dependent on substrate-specific effects and may approach a similar range across different media. However, it should be noted that the influent wastewater contained yeast extract, which provides organic nitrogen that can be mineralized into $\text{NH}_4^+\text{-N}$ during treatment. Since only inorganic nitrogen species ($\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$) were monitored, and total nitrogen (TN) or total Kjeldahl nitrogen (TKN) were not measured.

For $\text{NO}_3^-\text{-N}$ removal, raw cork exhibited the highest mean removal efficiency (73.73%), which was significantly higher than both gravel (61.84%, $p = 0.002$) and gravel + biochar (60.08%, $p < 0.001$). Expanded cork (68.35%) performed similarly to raw cork ($p = 0.346$) but was not significantly better than gravel or gravel + biochar ($p = 0.188\text{--}0.057$). Gravel-based substrates showed the lowest removal efficiencies and did not differ significantly from each other. These results indicate that raw cork and expanded cork are the most effective substrates for $\text{NO}_3^-\text{-N}$ removal.

The enhanced nitrate removal observed in cork-containing systems may be associated with the specific physicochemical characteristics of cork, including its porous structure, organic composition, and potential ability to support microbial colonization. In porous substrate-based CWs, nitrate removal can result from multiple simultaneous mechanisms, including biological transformation (e.g., heterotrophic denitrification), adsorption, and ion exchange processes. These findings are consistent with previous studies reporting the potential of cork-based materials for nitrate removal in water treatment systems [17, 40, 41].

In terms of $\text{NO}_2^-\text{-N}$ removal, the descriptive statistics highlighted variability in performance across substrates. Raw cork showed the highest mean removal (50.27%). Gravel followed with a mean removal of 47.79%. Gravel + biochar achieved a mean removal of 46.24%. Expanded cork had the lowest mean removal (41.12%) and the

highest variability. The one-way ANOVA for NO_2^- -N removal showed no statistically significant differences among the substrates.

The analysis for PO_4^{3-} -P removal showed that expanded cork had the highest mean removal efficiency (52.15%) with relatively high variability. Raw cork had a lower mean removal (39.88%) with similar variability. Gravel + biochar registered a mean removal of 42.73% with moderate variability. Gravel had a mean removal of 43.87%, the lowest variability among the substrates. ANOVA results for PO_4^{3-} -P removal indicated a significant difference between the substrates ($F = 2.823$, $p < 0.05$). Tukey's HSD test further showed that the only significant difference in PO_4^{3-} -P removal was between expanded cork and raw cork (12.26%), with expanded cork achieving significantly higher removal efficiency. Gravel and gravel + biochar showed intermediate efficiencies and did not differ significantly from either expanded cork or raw cork. These results indicate that expanded cork could be the most effective substrate for phosphate removal (Fig. 3A).

Pathogen reduction

The removal performance of the tested substrates was also evaluated in terms of total coliforms and *E. coli* reduction. Obtained results revealed that expanded cork had the highest mean log reduction of total coliforms, followed by raw cork, gravel + biochar, and gravel. However, expanded cork also showed greater variability, whereas gravel had more stable performance (Fig. 3B). ANOVA analysis indicated a significant difference among substrates ($p < 0.05$), and Tukey's test revealed that expanded cork performed significantly better than gravel, while other pairwise comparisons were not significant. For *E. coli*, all substrates showed comparable removal efficiencies. Although expanded cork presented the highest mean log reduction, followed closely by raw cork, gravel, and gravel + biochar, the differences were small and not statistically significant (ANOVA, $p > 0.05$).

Micropollutants removal

BPA removal efficiency differed significantly among the tested substrates. Expanded cork achieved the highest mean removal efficiency (97.85%) with low variability, followed closely by raw cork (94.15%), whereas gravel + biochar (67.60%) and gravel alone (62.50%) showed lower removal performance (Fig. 3C). One-way ANOVA confirmed that substrate type had a highly significant effect on BPA removal ($F = 179.68$, $p < 0.001$). Tukey's HSD post-hoc comparisons revealed no significant difference between expanded cork and raw cork ($p = 0.220$), indicating comparable high removal efficiencies for these two substrates. However, both cork-based substrates removed BPA significantly more efficiently than gravel + biochar and gravel alone ($p < 0.001$). Additionally, gravel + biochar showed slightly but significantly higher removal than gravel alone ($p = 0.043$). It should be noted that BPA was selected as a representative emerging contaminant commonly detected in treated municipal wastewater. However, the present study did not include sterilized controls or analysis of transformation products; therefore, the relative contributions of adsorption and biodegradation cannot be experimentally distinguished and the discussion of removal mechanisms should be interpreted accordingly.

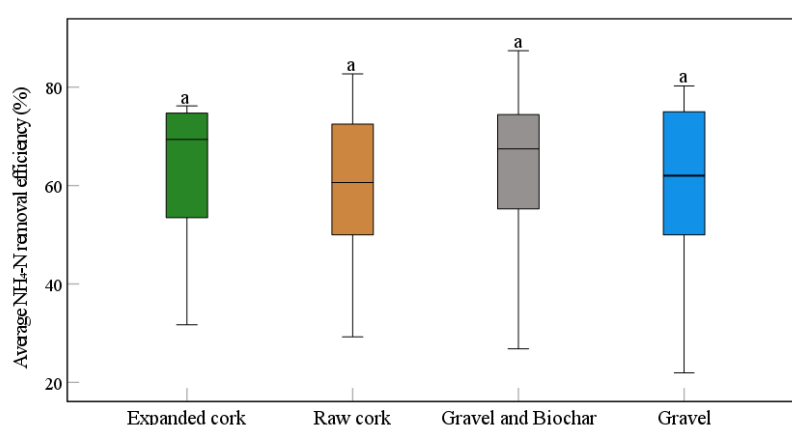
Previous studies have demonstrated the potential of constructed wetlands amended with alternative substrates for BPA removal. Li et al., (2019) demonstrated that an up-flow microbial fuel cell-coupled constructed wetland can effectively treat synthetic wastewater with a high concentration of BPA (10 mg/L) and contribute to typical BPA removal. Amending CWs with biochar or granulated cork significantly increased BPA removal, with some systems achieving >90% removal at influent concentrations up to 200 $\mu\text{g/L}$ and higher [43–45] (Fig. 3C).

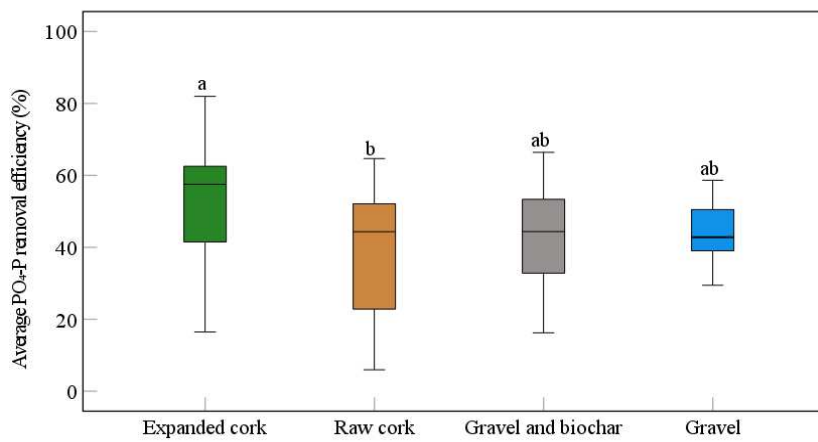
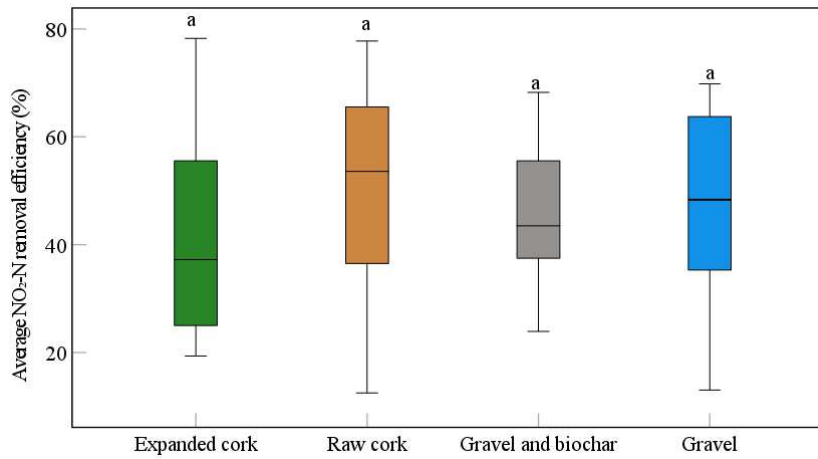
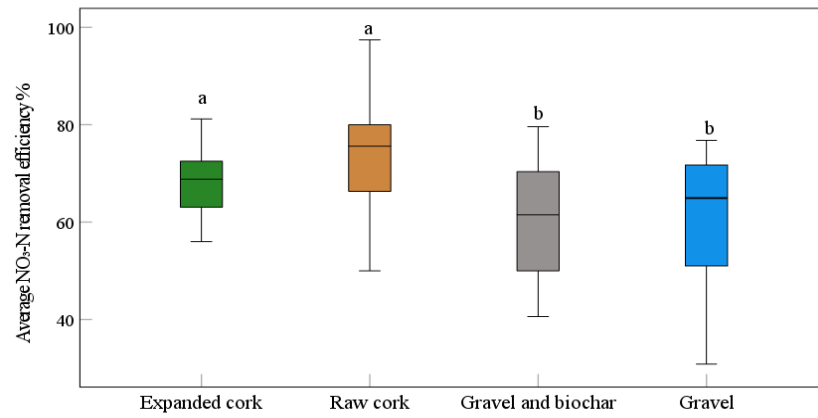
According to Li et al. (2018), BPA removal could be attributed to interconnected physicochemical and biological processes. Adsorption onto the substrate matrix represents a dominant initial step, particularly in systems containing cork and biochar, whose high surface area, porosity, and abundance of functional groups enhance BPA retention through hydrophobic interactions [42]. Subsequently, BPA could be biodegraded by microbial communities leading to its transformation into less harmful metabolites or even complete mineralization. The macrophytes might not only uptake small fractions of BPA but could also promote rhizodegradation through oxygen release and the exudation of organic compounds that stimulate microbial enzyme activities, such as laccases and peroxidases [46].

Plant development, photosynthesis and oxidative stress levels

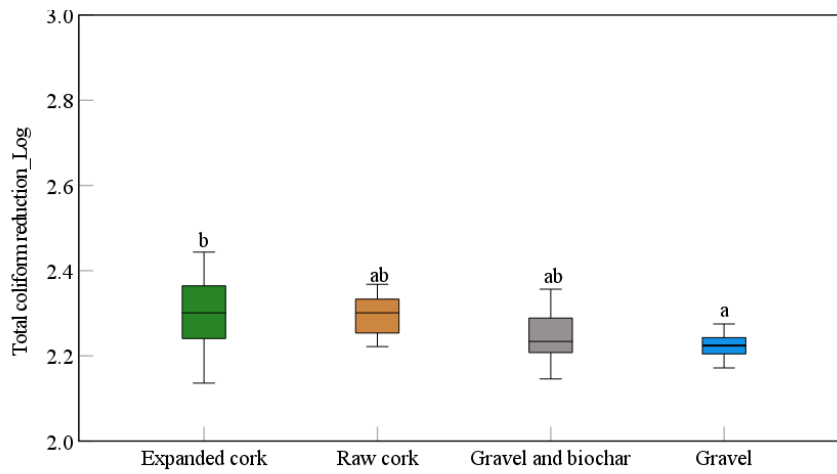
Analysis of plant growth curves revealed significant differences across CW units (one-way ANOVA, $F = 2.56$, $p = 0.025$). HFCW, particularly HFCW2 and HFCW1 with cork substrates, consistently showed the highest plant heights, whereas VFCW3 and HFCW3 (gravel–biochar) showed the lowest values. Photosynthetic pigmentation followed a similar trend: HFCW2 plants had the highest chlorophyll a ($\sim 6.5 \mu\text{g/mL}$) and chlorophyll b ($\sim 5.5 \mu\text{g/mL}$), while VFCW3 and HFCW3 had the lowest pigment concentrations (Fig. 3D). Oxidative stress, assessed via malondialdehyde (MDA) content, also varied with substrate type and flow direction. The highest MDA ($\sim 0.15 \text{ nmol/g}$) was observed in HFCW3, whereas VFCW3 had the lowest ($\sim 0.10 \text{ nmol/g}$). Generally, vertical flow units provided better root-zone oxygenation, supporting antioxidant enzyme activity and mitigating oxidative stress. Other CW units showed intermediate MDA levels ($0.12\text{--}0.14 \text{ nmol/g}$). These findings indicate that substrate type may play a key role in determining plant growth, photosynthetic performance, and oxidative stress levels in CW units. Biomass-based substrates like raw cork or agricultural residues can enhance nutrient availability, water retention, and microbial activity, all of which support robust plant development. Studies show that substrates rich in organic matter like cork provide more carbon and phosphorus, stimulate microbial density, and improve plant tissue quality compared to inert substrates like gravel alone [18, 20, 47].

(A) Nutrients removal

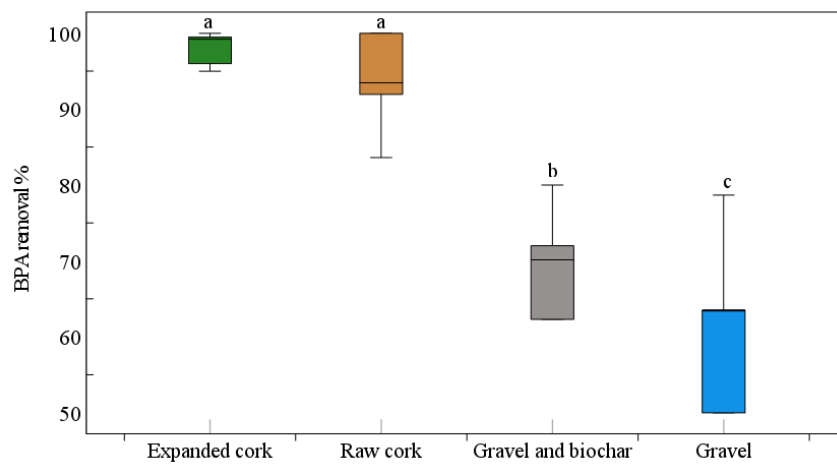




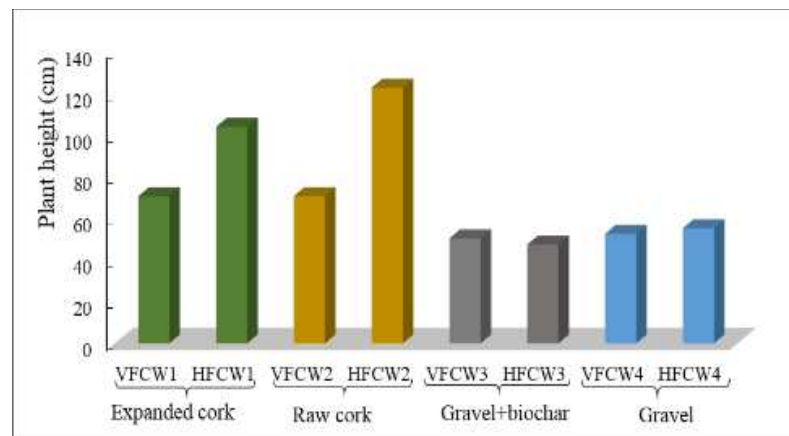
(B) Total coliform reduction



(C) BPA removal



(D) Plant growth and photosynthesis



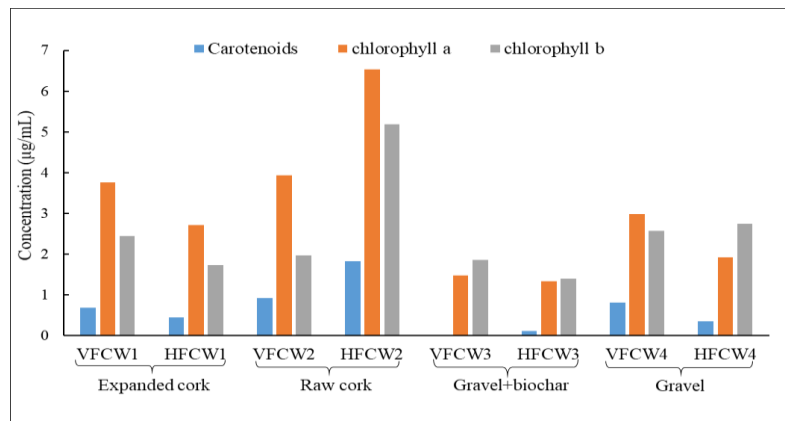


Fig. 3 Nutrient and BPA removal, microbial reduction, and plant development across CW units: (A) Average removal efficiencies of $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{PO}_4\text{-P}$, (B) Total coliform reduction, (C) BPA removal, and (D) plant development in different constructed wetland units. Letters above boxplots (a, b and c) indicate significant differences in removal efficiency among substrates according to Tukey's HSD post-hoc test ($\alpha = 0.05$). Boxes sharing the same letter are not significantly different.

Metagenomics analysis and microbial diversity

Shotgun metagenomics sequencing of the eight CW samples generated 382,751 high-quality reads after filtering, of which 19.1% (73,190 reads) were taxonomically assigned at the phylum level or below. The relatively high proportion of unclassified reads (80.9%) is common in environmental metagenomics and likely reflects the underrepresentation of wetland and soil microorganisms in reference databases; many taxa from such habitats remain uncultured or lack sequenced genomes [48, 49]. Despite this, the obtained taxonomic profiles provided sufficient resolution to compare community composition across substrates.

Taxonomic composition and dominance of bacteria

Across all samples, the microbial communities were overwhelmingly dominated by Bacteria (98.19% of classified reads), with minor contributions from Eukaryota (mainly Fungi, 1.45%), Archaea (0.33%), and Viruses (0.02%). This pattern is consistent with the central role of bacteria in pollutant degradation and nutrient cycling in CWs (Fig. 4A). Among bacterial phyla, Pseudomonadota (Proteobacteria) was the most abundant, accounting for 60–80% of classified reads across configurations. This phylum's metabolic versatility and well-known involvement in organic matter degradation, nitrification, and denitrification make it a key functional group in wastewater treatment systems [50]. The second most abundant phylum was Actinomycetota (Actinobacteria), which is associated with the decomposition of recalcitrant organic compounds and may also play a role in micropollutant degradation [1]. Bacteroidota and Bacillota (Firmicutes) were also detected in variable proportions, particularly in cork-based units.

Fungal taxa (Ascomycota and Basidiomycota) were detected exclusively in the HFCW with raw cork (HFCW2), suggesting that the presence of a lignocellulosic substrate and a more diverse bacterial community may facilitate fungal establishment. Fungi can contribute to the degradation of complex organic matter and may enhance the resilience of the microbial food web [24]. Vertical flow systems generally exhibit higher oxygenation compared to horizontal flow systems, which may limit fungal establishment under more oxidizing conditions. Therefore, the absence of fungi in VFCW2 is more plausibly associated with hydraulic and oxygen-related constraints rather than substrate properties alone [51] (Fig. 4C).

Substrate-driven differences in microbial diversity

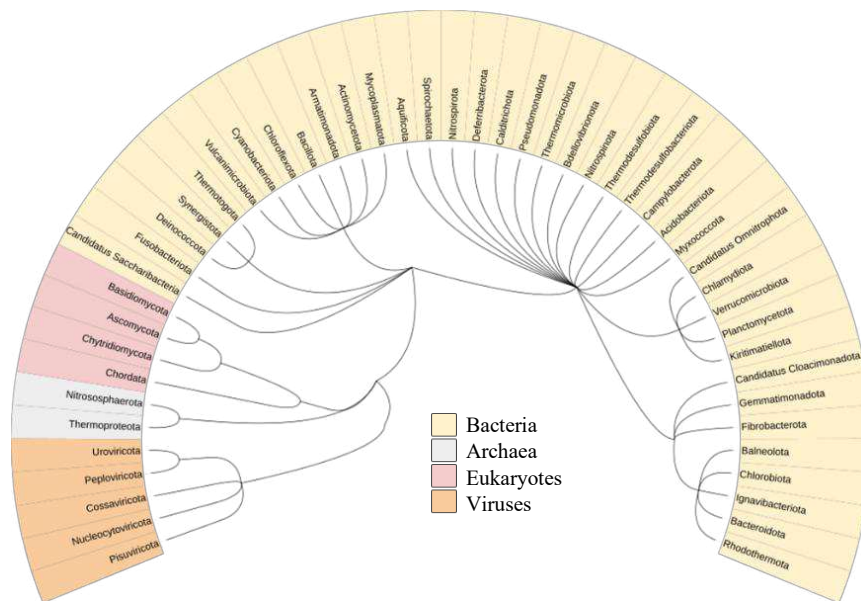
Alpha diversity, estimated by the Shannon index (Fig. 4B), varied markedly among substrates, with the strongest effect attributed to substrate type rather than flow regime. Raw cork exhibited the highest diversity (Shannon index up to 1.8), indicating a heterogeneous and functionally rich microbial assemblage. Expanded cork showed intermediate values (0.8–1.4), while gravel and gravel + biochar displayed the lowest diversity (0.8–1.1). This pattern suggests that thermal expansion alters cork's structural and chemical complexity, potentially reducing niche availability compared to raw cork. However, the effect of biochar amendment on microbial diversity was not pronounced under the present experimental conditions, as the Shannon diversity indices of gravel + biochar and gravel systems showed similar ranges (0.8–1.1). This limited effect may be related to the relatively low biochar proportion (<20% v/v) and the experimental duration, which may not have been sufficient to induce substantial changes in microbial habitat complexity. Therefore, mineral substrates, including gravel with or without biochar, should not be considered inherently less supportive of microbial diversity; rather, their influence depends on substrate characteristics, amendment ratio, and operational conditions. These findings highlight that substrates with greater organic matter availability and structural heterogeneity, such as raw cork, may provide additional ecological niches that favour microbial colonization, while the potential benefits of biochar incorporation may require optimized proportions and longer-term evaluation [52–54]. The addition of biochar to gravel did not substantially increase diversity, possibly due to the low proportion of biochar (estimated <20% v/v) or the short experimental period, which may have limited biofilm colonization.

Beta diversity and community differentiation

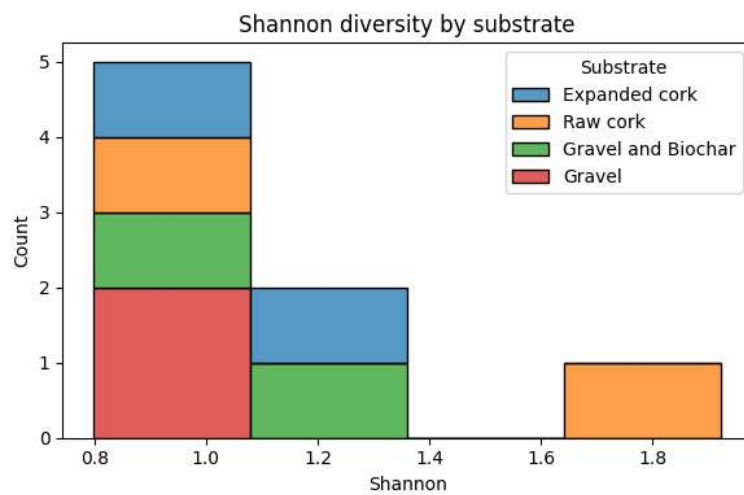
Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarity revealed a clear separation of samples according to substrate type, explaining 92.4% of the total variance (PC1 = 55.1%, PC2 = 37.3%; Fig. 4D). Cork-based units (raw and expanded cork) formed distinct clusters, while mineral-based units (gravel and gravel + biochar) grouped closely together. The high dispersion of raw cork samples between vertical and horizontal flow regimes suggests that, in addition to substrate, hydraulic conditions may interact with substrate properties to further shape microbial communities. In contrast, mineral-based units exhibited low Bray–Curtis dissimilarity values (≤ 0.17), reflecting a more uniform community structure regardless of flow direction.

The distinct clustering of cork-based communities is consistent with previous reports that organic and lignocellulosic materials promote unique microbial consortia enriched in functional groups involved in carbon and nitrogen cycling [17, 55, 56]. The porous architecture and surface chemistry of cork likely create a variety of micro-niches and retain organic carbon, supporting a broader range of metabolic guilds compared to the relatively inert surface of gravel. In contrast, mineral substrates tend to select for more stable but less diverse communities dominated by generalist heterotrophs [16].

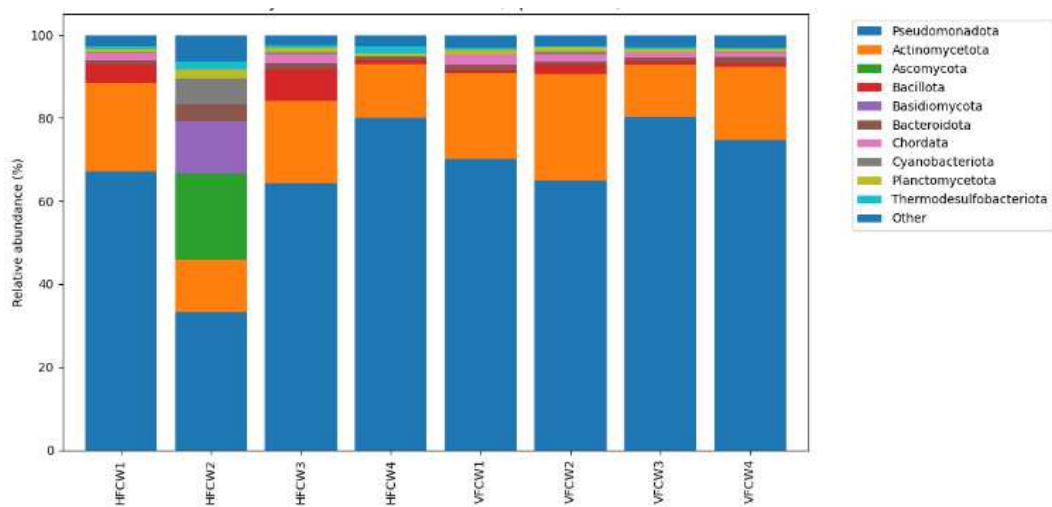
(A)



(B)



(C)



(D)

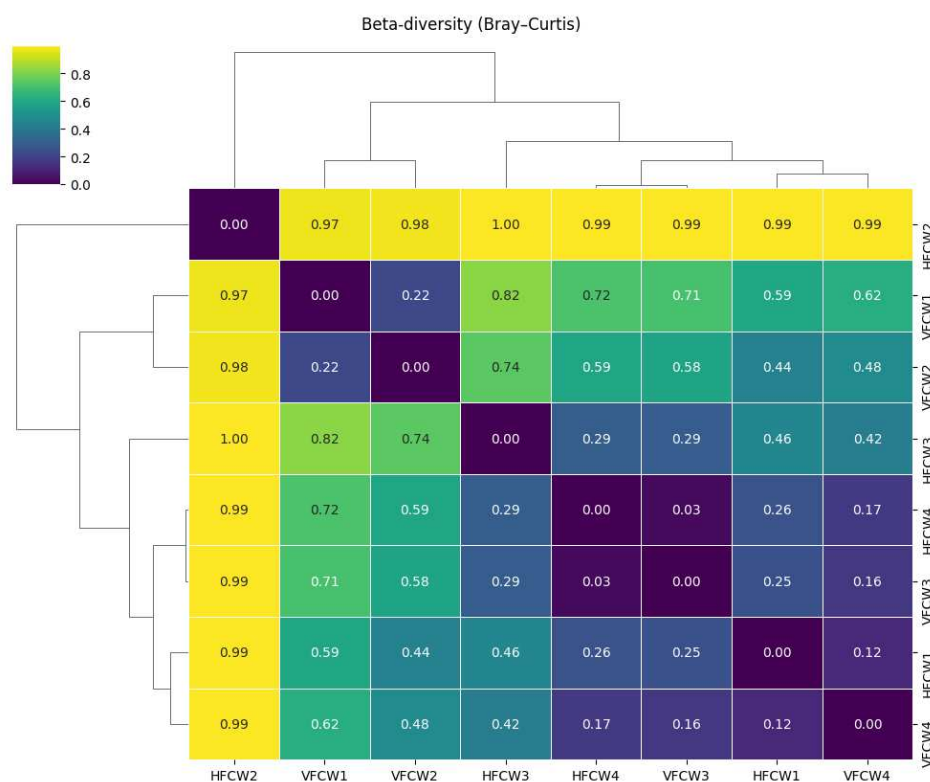
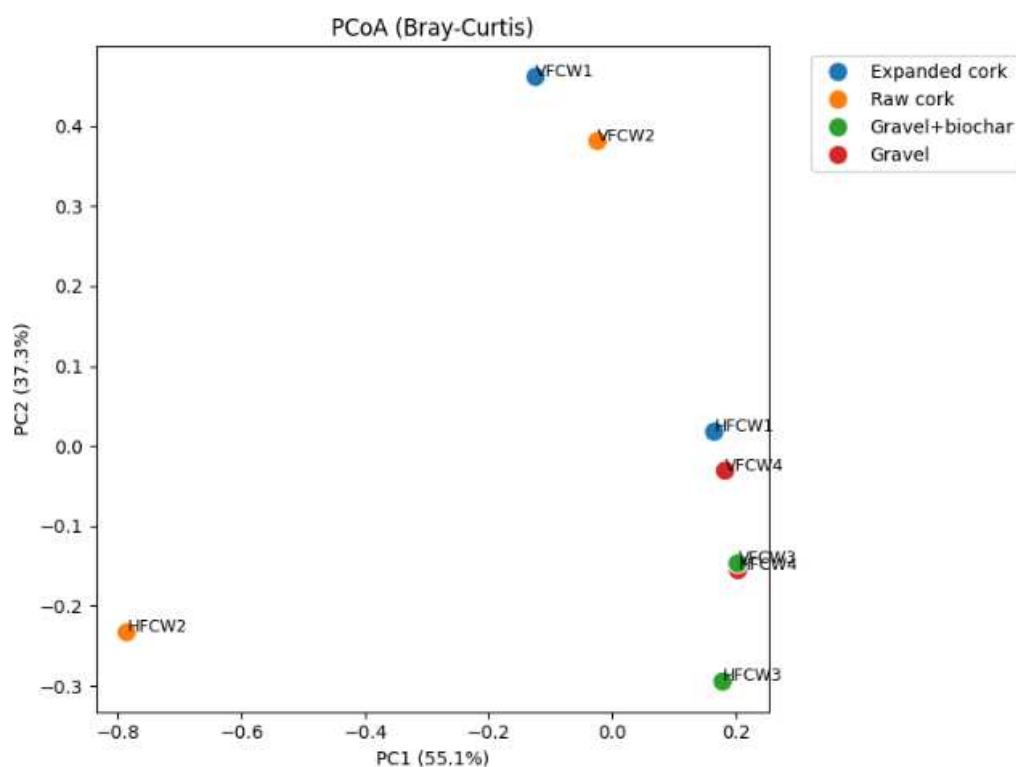


Fig. 4 Microbial community analysis across constructed wetland units: (A) Phylogenetic tree, (B) Shannon diversity, (C) relative abundance of dominant phyla (>1%), and (D) PCoA with Bray-Curtis dissimilarity

Implications for system function

The substrate-driven differences in microbial community composition may contribute to the variations in treatment performance observed among the CW configurations. Cork -based systems showed distinct microbial assemblages compared with conventional gravel-based units, suggesting that substrates characteristics can influence microbial habitat development and community structure. The higher nitrate removal observed in cork containing systems may be associated with favorable physicochemical conditions created by cork matrix [55]. However, the present study did not include functional gene annotation or quantitative analysis of nitrogen-cycling and organic degradation pathways. Beyond its physical role as a support matrix, cork is a chemically complex, carbon-rich substrate composed mainly of suberin, lignin, and polysaccharides. Its capacity to release dissolved organic carbon may provide an internal electron donor, thereby promoting denitrification processes[57]. However, direct measurements of dissolved organic carbon (DOC), total organic carbon (TOC), and substrate-derived leachates were not performed in the present study, and thus the contribution of cork-released carbon to denitrification cannot be quantitatively established. Previous investigations have reported that cork-associated microbiota can include bacterial and fungal groups involved in the transformation of aromatic and lignocellulosic compounds, including genera such as *Streptomyces*, *Burkholderia*, *Caballeronia*, and *Paraburkholderia*. In addition, cork materials have demonstrated potential for sorptive removal of organic micropollutants such as bisphenol A due to their porous structure and surface properties [58] These characteristics may contribute to the enhanced attenuation of contaminants observed in cork-based systems . The honeycomb-like porous architecture and organic composition of cork likely provide favorable conditions for microbial attachment and biofilm development, potentially supporting coupled biological and physicochemical removal mechanisms. Future studies integrating functional metagenomic analyses, including quantification of nitrogen transformation genes (e.g., nitrification and denitrification markers) and organic compound degradation pathways, together with DOC/TOC monitoring, are required to clarify the mechanisms linking cork properties, microbial functions, and pollutant removal performance [57].

Discussion

Influence of substrate type on pollutant removal and microbial communities

The results demonstrate that substrate selection strongly influences CW treatment performance, although the magnitude of this effect varied among pollutants. Substrates serve multiple functions, including particle filtration, adsorption, microbial attachment, and, depending on their composition, provision of carbon or reactive elements that can participate in pollutant transformation [3, 13, 20]. Consequently, substrate performance depends on a combination of physical and chemical properties, including porosity, surface characteristics, organic matter content, and hydraulic behavior, as well as their interactions with microorganisms and plant roots. In the present study, expanded cork showed the highest and most consistent COD removal, whereas raw cork achieved the highest NO_3^- -N removal. The performance of cork-based substrates may be related to their organic and porous structure, which can provide microbial attachment sites and contribute to pollutant retention and transformation. Thermal expansion further modifies the structure and surface properties of cork, potentially affecting water–substrate interactions and microbial colonization. Nevertheless, the observed differences cannot be attributed to substrate composition alone, since hydraulic regime, effective porosity, oxygen availability, and microbial activity may also influence treatment performance. This is particularly relevant for nitrogen removal, which results from

the interaction of nitrification, denitrification, substrate-mediated electron transfer, and oxygen/redox gradients. Recent reviews emphasize that nitrogen removal in CWs can be constrained by insufficient oxygen or electron donors, while substrate modification and appropriate system configuration can enhance these processes [59, 60]. The relatively high NO_3^- -N removal obtained with raw cork may partly reflect the availability of biodegradable carbon associated with the lignocellulosic material, potentially supporting heterotrophic denitrification. This interpretation is consistent with recent research showing that organic or carbon-rich substrates can improve nitrogen removal by providing electron donors, particularly in carbon-limited wastewater [59]. No significant differences in NH_4^+ -N removal were observed among the tested substrates. This result may be partly explained by the low influent NH_4^+ -N concentration and the polishing-treatment conditions of the experiment. Under such low ammonium loading, differences in substrate-specific effects may be less pronounced, while oxygen availability and microbial nitrification activity may become more important determinants of NH_4^+ -N removal [61]. The differences in TSS removal further highlight the importance of substrate structure. Gravel provided the highest TSS removal, whereas raw cork showed the lowest efficiency. The superior performance of gravel may be associated with its stable granular structure, which favors particle interception and filtration while minimizing the release of fine particles. In contrast, cork-based materials may be more susceptible to particle detachment or fragmentation during hydraulic operation. Thus, high porosity alone does not necessarily ensure efficient TSS retention; particle stability, pore structure, and hydraulic conditions also determine filtration performance. For COD, the higher and more stable removal observed with expanded cork may reflect the combined contribution of physical retention, adsorption, and microbial degradation within its porous organic matrix. However, the present data do not allow the relative contribution of these mechanisms to be quantified.

The metagenomic results further indicate that substrate characteristics were associated with differences in microbial community structure. This supports the ecological role of substrates as habitats for microbial colonization rather than merely passive filtration media. Recent reviews emphasize that substrate properties can shape microbial attachment, community assembly, redox conditions, and pollutant transformation in CWs [3, 20]. In the present study, the overlapping Shannon diversity ranges between gravel and gravel + biochar suggest that biochar addition did not substantially modify overall microbial diversity under the tested conditions. This limited effect may be related to the relatively low biochar proportion (<20% v/v), together with the experimental duration. The effect of biochar is known to depend strongly on its feedstock, physicochemical characteristics, amendment ratio, placement within the filter, and operating conditions [61]. Fungal taxa belonging to Ascomycota and Basidiomycota were detected exclusively in HFCW2 containing raw cork. Although the lignocellulosic nature of cork may provide an organic matrix favorable for fungal colonization, this distribution should not be interpreted as a direct substrate-specific effect. Differences between HFCW and VFCW configurations may have generated distinct oxygen and redox conditions, which are important drivers of microbial community assembly. The absence of detectable fungi in VFCW2 may therefore result from the combined influence of substrate characteristics and hydraulic conditions. Overall, these findings reinforce the view that microbial communities in CWs emerge from interactions among substrate properties, hydraulic regime, oxygen availability, and wastewater characteristics rather than from substrate composition alone [24].

Implications for substrate selection and CW optimization

The obtained results indicate that substrate selection should be tailored to the treatment objectives rather than based on a single measure of overall performance. Expanded cork showed advantages for COD removal and stable NH_4^+ -N performance, raw cork favored NO_3^- -N removal, and gravel provided more efficient TSS retention. These contrasting responses demonstrate that no single substrate was optimal for all investigated pollutants. Similar conclusions have been emphasized in recent reviews, which recommend substrate selection according to target contaminants, substrate functionality, and system operating conditions rather than relying on conventional media alone [59]. Recent developments in engineered substrates further support this multifunctional approach. Biochar has attracted considerable interest because of its high surface area, porosity, adsorption capacity, and potential to modify microbial habitats, although its performance depends strongly on material characteristics and application conditions [61]. Similarly, modified iron-based substrates have been investigated to overcome electron-donor limitations and stimulate nitrate reduction through enhanced redox activity and microbial interactions. For example, Guo et al. (2026) reported enhanced nitrate removal in CWs amended with modified FeS-based materials, highlighting the potential of reactive substrates to provide specific functions beyond those of conventional mineral media. Such approaches illustrate how substrate engineering can complement the use of biomass-derived materials such as cork [19].

From a sustainability perspective, cork-based substrates represent an interesting alternative because they combine treatment functionality with the potential valorization of renewable biomass. Their application could therefore contribute to the development of more sustainable CWs, particularly for polishing treated wastewater intended for reuse or managed aquifer recharge. However, substrate selection should be considered together with hydraulic configuration, as differences in porosity and effective water-filled volume may modify the actual residence time and oxygen distribution among systems.

Conclusion

This study investigated the performance of cork-based substrates as alternative filter media in CWs. The results demonstrated that cork-based substrates, particularly raw and expanded cork, represent promising sustainable solutions for enhancing the performance of CWs. Expanded cork exhibited the most stable and efficient removal of COD and NH_4^+ -N, while raw cork achieved the highest NO_3^- -N removal. The consistently high BPA removal (>90%) in cork-based systems under the applied experimental conditions, highlighted a strong retention capacity of cork substrates. However, the specific contribution of adsorption and biodegradation processes could not be distinguished in this study. Metagenomic profiling revealed that microbial communities were dominated by bacterial taxa across all systems, with substrate-dependent variations in community composition and diversity. Cork-based systems, particularly raw cork configurations, supported more diverse microbial assemblages compared with gravel-based systems. However, functional metagenomics profiling was not performed, therefore microbial data reflect community structure rather than direct evidence of metabolic activity. The presence of fungal taxa exclusively in cork-based HFCWs suggested an association between lignocellulosic substrates and distinct microbial habitats, although their functional roles remain to be confirmed. Similarly, observed differences in plant growth and physiological responses may be influenced by multiple interacting factors, including substrate properties and system hydraulics, rather than microbial composition alone.

From a practical perspective, the results suggest that different substrates may offer complementary functions in constructed wetland systems. In particular, gravel-based media may be more suitable for improving physical

609 filtration and reducing suspended solids, while cork-based substrates may contribute to organic matter and
610 micropollutant attenuation and support plant development. Future studies should include long-term monitoring,
611 dissolved organic carbon and total organic carbon (TOC) measurements, and functional gene-based analyses to
612 better elucidate the mechanisms governing pollutant removal in cork-amended CWs.

613 **Author Contributions** M.B. S. Conceptualization, Methodology, Investigation, Data curation, Formal analysis,
614 Visualization, Writing – original draft. E. B. O. Investigation, Data curation, Writing – review & editing. S. B.
615 Investigation, Data curation. H. E. Investigation, Data curation. K. K. Investigation, Resources. W. B. A.
616 Methodology, Validation – review & editing.
617 M. G. Supervision, Validation, Writing – review & editing. K. B. S. Supervision, Validation, Writing – review &
618 editing. A. J. Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review &
619 editing.

620 **Data Availability** No datasets were generated or analysed during the current study.

621 **Declarations**

622 **Competing interest** The authors declare no competing interests.

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