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Dynamics and interrelationships between antibiotic resistance, organic micropollutants and bacterial communities in full-scale rural constructed wetlands

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ABSTRACT

Constructed wetlands systems (CWs) are increasingly regarded as promising alternatives or complements to conventional wastewater treatment processes. However, the fate of chemical and biological contaminants in real-world treatment processes is understudied in this type of systems. This study aimed to fill this gap by evaluating the response of three horizontal subsurface flow CWs, in Northern Portugal, planted with *Phragmites australis*, in operation for >7 years, to reducing the load of fecal contamination, antibiotic resistance genes and organic micropollutants (OMPs).

Influent, effluent and sediments samples (n = 36) were examined for abundance of cultivable *Escherichia coli* and total coliforms, total bacteria (16S rRNA gene), 10 genetic biomarkers associated with anthropogenic contamination (*uidA,* crAssphage, *intI1*, *sul1, ermB, ermF, mefC, qacEΔ1, tetX* and *aph(3″)-Ib*) by quantitative PCR, non-target LC-MS of OMPs and 16S rRNA gene-based bacterial community analysis.

The three CWs showed reduction values (log-units/mL) up to 4.8 of *E. coli* and 3.6 of biomarkers, with the highest values observed in warmer periods. No evidence of for the accumulation microbiological contaminants in the sediments was observed. Among the 59 OMPs detected, reduction rates varied, and the concentration of the most abundant pharmaceutical compounds in the final effluent varied –reaching ng/L concentrations of ~36 000 for fenofibric acid, ~14 000 for acetaminophen, ~3000 for oxazepam and ~2000 for irbesartan, which can be considered high to discharge in the receiving environment. The bacterial community was dominated by members of the class *Gammaproteobacteria*, with treatment contributing to significant reduction of the relative abundance of members of the classes *Clostridia, Bacilli* and *Actinomycetes.* Compared with wastewater, sediments had significantly higher relative abundance of *Alphaproteobacteria.*

The study confirms that CWs are an adequate alternative for the treatment of domestic wastewater in small communities, although it warns of the need for regular monitoring and adjustment of treatment conditions, especially during cooler periods.

1. Introduction

Effective wastewater treatment faces an increasing number of challenges due to the high diversity and abundance of organic micropollutants (OMPs) and hazardous microrganisms, such as the presence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Angeles et al., 2020; Hazra and Durso, 2023). Antibiotic resistance is considered by the World Heath Organization (WHO) among the major public health threats of our century (World Health Organization, 2023) and its spread through untreated wastewater has been considered among the major causes of environmental contamination (Fang et al., 2017). OMPs include a diverse group of chemicals, such as antibiotics and other pharmaceutical compounds and personal care products, pesticides, herbicides, fertilizers, and per- and polyfluoroalkyl

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substances (Halwatura and Aga, 2023). These substances are released to the environment in small concentrations and are of particular concern due to their potential to persist in the environment and bioaccumulate in different trophic networks, with adverse effects on ecosystems and human health (Contreras et al., 2019; Regulation (EU) 2019/1021 of the European Parliament and the council of June 20, 2019 on persistent organic pollutants (recast)). Domestic wastewater or large agglomerations is normally piped to centralized systems, frequently using conventional treatment based on activated sludge, which despite the multiple benefits to treat domestic effluents, has a limited capacity for mitigating contaminants such as ARB, ARGs and OMPs, due to factors that span the pollution load of the raw influents to the climate conditions (Pärnänen et al., 2019; Manaia, 2023). Treatment objectives, established by common regulatory documents (e.g. the European Urban Waste Water Treatment Directive, Directive 91/271/EEC), aims to protect the environment and the public health. The recent vision of the Urban Waste Water Treatment Directive (European Commission, 2022), is an example, with the inclusion of OMPs and antimicrobial resistance among the treatment or monitoring objectives. However, smaller systems, like scattered dwellings, i.e. individual households or agglomerations of less than 1000 population equivalents (p.e.) can be more difficult to control and measure their impact, although in Europe they can represent a median value of 3 % corresponding to about 25 million inhabitants (Vigiak et al., 2018). The implementation of effective treatment systems to served scattered agglomerations has been recognized as an important mode of preventing adverse environmental impacts due to wastewater discharges. Decentralized wastewater treatment systems are essential for environmental protection in areas where large sanitation infrastructures are not available and, for multiple reasons, preferentially should rely on low-cost and low-energy requirement approaches (Vymazal, 2008; Masoud et al., 2022). These decentralized can have a pivotal role on reducing antibiotic resistance and OMPs loads, to ensure the protection of soils and water bodies.

Nature-based solutions, using low-cost and low-energy requisites, are good alternatives to protect, manage, or restore natural ecosystems, as they can respond to challenges associated with climate change, human health protection, and food and water security (Climate Explainer, 2024). Constructed wetlands (CWs) are increasingly implemented as nature-based solutions, and represent promising approaches to mitigate the spread of environmental contaminants throughout the urban water cycle (Wu et al., 2023). In rural areas or in regions with poor sanitation systems, CWs are regarded as good alternatives for conventional wastewater treatment, given the simple infrastructure required and the reduced maintenance and operation demands, with an overall low cost of implementation and integration into the natural landscape with the creation of wildlife habitats (Masoud et al., 2022). CWs are simple structures that include common some key elements, such as an impermeable liner, a basin of permeable substrate, vegetation (wetland plants), and inlet and outlet drainage systems, the specific configuration needs to be customized according to the requirements and insertion area of a region. Most of the treatment success, relies on the biological processes, which in CWs consists on the use of use of plants to facilitate the transfer of ambient oxygen to the roots around which the microbial growth is supported not only by root exsudates, but also by organic compounds, including hazardous substances present in wastewater, that serve as nutrients and promote the outcompetion with fecal and other animal derived bacteria (Vymazal, 2008; Kataki et al., 2021; Bai et al., 2022). In addition, processes like sedimentation and filtration, natural die-off due to UV exposure, adsorption, biodegradation and photodegradation, volatilization and plant uptake are expected to occure (Wu et al., 2016; Waly et al., 2022). The type of substrate (gravel, soil, sand) and macrophytes (*Phragmites australlis* are the most used plants in this type of systems), the option for horizontal or vertical wastewater flow, the inlet and outlet drainage network, or the type of loading (continuous or intermittent) and volume of wastewater to be treated, are decisions that needs to be considered as a function of the treatment process (UN-HABITAT, 2008). To be effective, CWs need not only to be able to reduce organic matter, nutrients (N, P), and pathogens, but also other contaminants such as ARB, ARGs, and OMPs (Hazra and Durso, 2023). An important advantange of nature-based solutions is the capacity to respond to external factors, such as wastewater composition, hydraulic regime and retention time, and seasonal variations (e.g. temperature, solar intensity) (Wu et al., 2016). Sometimes intermittent failures or poor efficacy of CWs systems are reported, showing the accumulation of ARGs and OMPs residues in the final effluents, receiving water bodies or in the sediments (Fang et al., 2017; Xu et al., 2015; Liu et al., 2022). The lack of studies that included the evaluation of all the mentioned parameters in the same analysis and the applicability as decentralized systems, as non-lab environment was the motivation for this study.

The aim of this study was to evaluate real-world municipal CWs for the removal of fecal contamination, ARB, ARGs and OMPs from domestic wastewater in regions where no other treatment systems exist and that have been operating in rural areas for 7–20 years in agglomerates of less than 400 people. The capacity for reducing OMPs, total coliforms and *E. coli* and anthropogenic microbiological contamination assessed based on biomarkers, previously proposed by Teixeira et al. (2023), for fecal contamination (*uidA, crAssphage),* genetic recombination (*intI1)* and antimicrobial resistance (*sul1, ermB, ermF, mefC, qacEΔ1, tetX and aph(3″)-Ib*)), the possible accumulation of these contaminants in the sediments, the variations in the bacterial community composition and the possible effects of seasonal climate variations were examined.

2. Materials and methods

2.1. Constructed wetlands systems (CWs) characterization and sampling

This study investigated the seasonal variations observed in macrophyte-based full-scale (CWs), based on three independent case studies with similar characteristics and operating modes, representing field replicates and with three technical replicates taken at each sampling campaign, for the type of sample collected, which were processed and analysed in triplicate. The three CWA, CWB and CWC, working in continuous operation mode, are in operation in Northern Portugal, within a distance of 20 km, in 2009, 2016 and 2003, respectively, and each has a dimension (m) of 30 length, 16 width and 0.2 depth and serve less than 400 p.e. (Table 1). The influent accesses the macrophytes (*Phragmites australlis*) pond through an inlet zone comprised of a solids removal grid and a septic tank. The treated effluent is emitted through the outlet zone to the receiving river (Fig. 1). The CWs' properties and physical-chemical parameters, pH; COD (chemical oxygen demand); BOD (biochemical oxygen demand); total N (nitrogen); P (phosphorus), and TSS (total suspended solids) were registered throughout the year of 2023 by the water management company (Table S1).

CWA, CWB, and CWC were sampled in March, May, July and October of 2023 in the winter (season 1), spring (season 2), summer (season 3) and autumn (season 4), respectively. Twenty-four hour composite samples of the CWs influent and effluent were collected using autosamplers, while sediments were collected at the surface and approximately in the center of the pond (Fig. 1). Samples were transported to the laboratory and analysed within 24 h after collection. The first sampling occurred after macrophytes' harvesting, at the end of the winter (March), being the growth of the plants measured in the subsequent sampling dates, based on the average height of 10 plants, at each sampling event (Fig. 2b).

2.2. Sample processing and chemical analysis

Liquid samples (influent and effluent) were analysed for the presence of OMPs after solid phase extraction (SPE) of 500 mL of wastewater sample. Extraction was preceded by a membrane filtration with 1.6 μm glass microfiber filters membranes (GF/A, Whatman), followed by 0.45

Table 1
Characteristics of the three Constructed Wetlands (CW) used in this study.

Constructed wetland (CW)	Starting year	Population equivalent (inhabitants)	Dimensions (LxWxD) (m)	Flow rate (m^3/day)	Hydraulic retention time (days)	Maximum discharge flow (m^3/day)
A	2009	360	30 × 16 × 0.2	13.3	7.2	60
B	2016	400		16.7	5.8	
C – 2 ponds	2003	380		19.8	9.7	

L, length; W, width; D, depth.

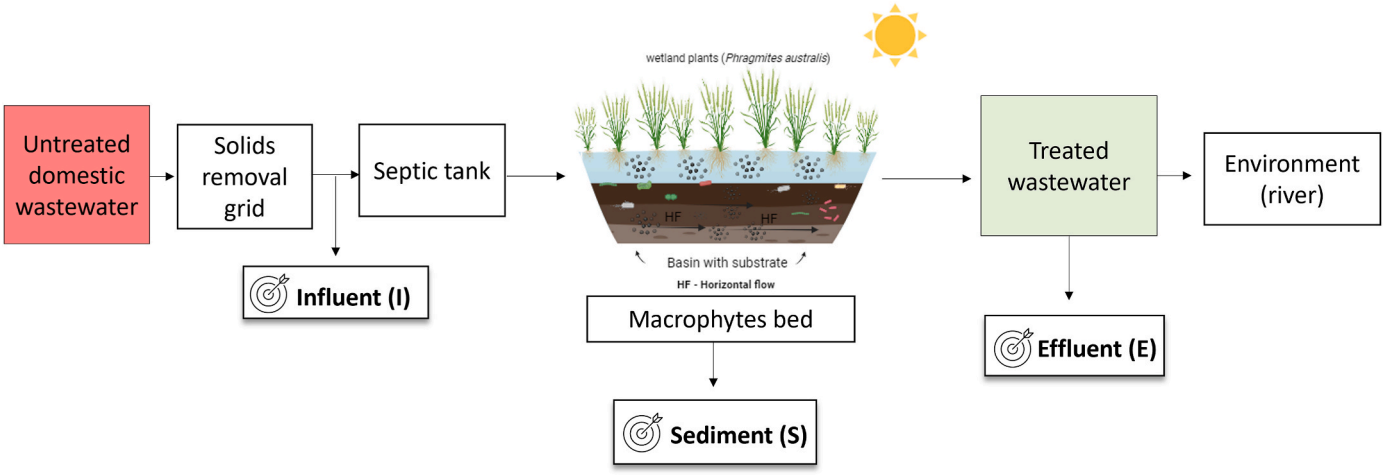


Fig. 1. Flow chart of the CWs (CWA, CWB, CWC) investigated in this study. The untreated domestic wastewater passes through a solids removal grid, a septic tank, and a macrophyte bed. The treated wastewater is returned to the environment (river). The sampling points along the water treatment line are indicated with ◎. CWC includes two macrophyte bed (ponds).

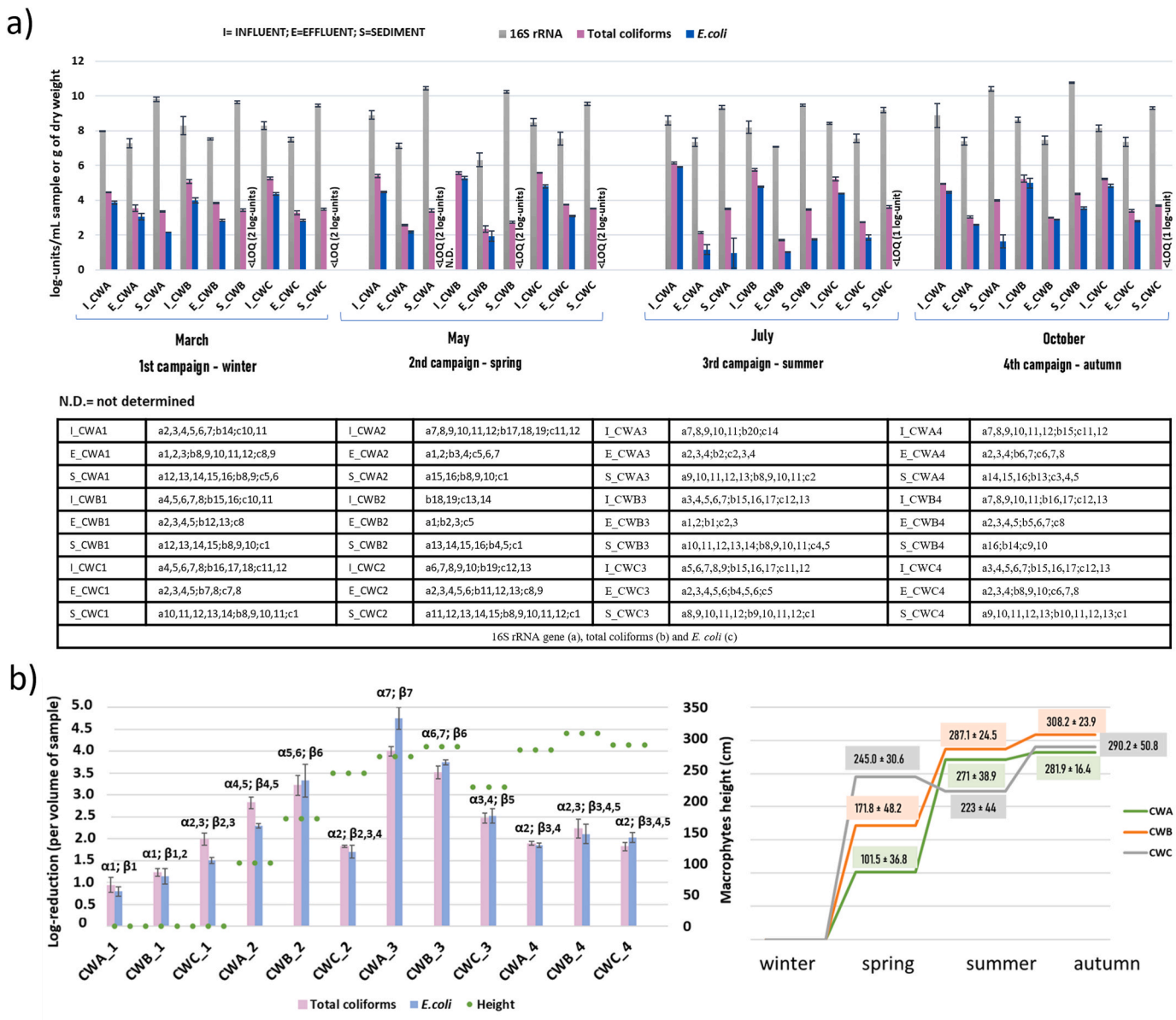
μm Nylon membrane filters (Whatman 7404-004). The filtrate was acidified to pH 2–3 with 37 % (v/v) hydrochloric acid, kept at 4 °C and protected from light until extraction with the Hydrophilic-Lipophilic-Balanced (HLB) and Mixed-Mode Cation-eXchange (CMX) (Oasis™) cartridges, conditioned with 6 mL of methanol, followed by 6 mL of water (LC-MS Grade, LiChrosolv). The samples were loaded onto the SPE cartridges at an approximate flow rate of 3–5 mL/min (60–100 drops/min), after which were left to dry under vacuum. The cartridges were kept at −20 °C until shipment to the University at Buffalo, NY, where the OMPs were analysed using liquid chromatography–mass spectrometry (LC-MS). Unfortunately, the shipment of the cartridges from the 3rd and 4th sampling campaigns were held at the US customs for 2 months, and we considered that the respective results might not be reliable enough to include in this publication. Analytes were eluted from the SPE catridges using the following procedure: the HLB cartridge was eluted consecutively with 3 mL each of (i) methanol, (ii) acetonitrile, and (iii) 1:1 (v:v) acetonitrile: ethyl acetate. The MCX cartridge was eluted into the same collection vessel with 3 mL each of (i) 5 % ammonium hydroxide in methanol, (ii) 5 % ammonium hydroxide in acetonitrile, and (iii) 5 % ammonium hydroxide in 1:1 (v:v) acetonitrile: ethyl acetate. The final extract was evaporated to dryness under nitrogen gas and reconstituted to 1 mL with the starting LC-MS mobile phase. A 50 μL of 1 μg/mL d3-diphenhydramine was added to each vial to serve as instrumental internal standard to correct for any instrument fluctuations. Sample extracts were filtered using 0.45 μm pore size nylon membrane syringe filters prior to LC-MS analysis. Target analysis was carried out in an Agilent 6410 triple quadrupole mass analyzer equipped with a 1200 HPLC system (Palo Alto, CA) under positive mode electrospray ionization (ESI). Non-target analysis was performed in a Thermo Scientific Q-Exactive™ Focus Orbitrap™ LC-MS (Waltham, MA) with Dionex UltiMate™ 3000 ultra-HPLC system with a full-scan data dependent MS2 (ddMS2) acquisition method. Data acquisition and processing were conducted with Xcalibur 2.1 software (Thermo Scientific) (Halwatura and Aga, 2023). The obtained results are presented, in Table S5, (excel file, in supplementary material).

2.3. Enumeration of cultivable fecal bacteria

Fecal bacteria were enumerated by the membrane filtration method on Chromogenic Coliform Agar (CCA – VWR Chemicals, Belgium), immediately upon arrival to the laboratory. Briefly, volumes of 1 mL of wastewater or 1 g of sediment were suspended in 9 mL of sterile saline solution (0.85 % (w/v)) or sodium hexametaphosphate (1 % (w/v)), respectively, and were filtered (or of the respective serial dilutions), through cellulose nitrate membranes (0.22 μm porosity; Sartorius Stedim Biotech, Germany). The CCA cultures were incubated for 18–24 h at 37 °C, according to the manufacter instructions and the standard guidelines (ISO 9308, 2014). Blue colonies were presumptively enumerated as *E. coli* and blue and pink colonies as total coliforms. The limit of quantification (LOQ) was calculated as the lowest number of colony forming units (CFU) observed in the largest volume or weight of original sample analysed. Sediments' dry weight was determined by drying of approximately 1 g of sample (6 replicas) at 70 °C until no weight variation was observed (~3 days).

2.4. Sample processing and DNA extraction

Culture independent analyses used DNA extracts obtained in triplicate for each sample. Liquid samples were concentrated by filtration of accurately measured volumes of 50–100 mL influent and 200–250 mL effluent through polycarbonate membranes (0.22 μm porosity; Whatman, England). The membranes were stored at −80 °C until DNA extraction using the DNeasy PowerWater Kit (QIAGEN, Germany), according to manufacturer's instructions, except the time of lysis that was increased from 15 min to 1 h. Sediment samples were maintained at −80 °C, for no longer than 2 months, until DNA extraction from 0.25 g of



I_CWA1	a2,3,4,5,6,7;b14;c10,11	I_CWA2	a7,8,9,10,11,12;b17,18,19;c11,12	I_CWA3	a7,8,9,10,11;b20;c14	I_CWA4	a7,8,9,10,11,12;b15;c11,12
E_CWA1	a1,2,3;b8,9,10,11,12;c8,9	E_CWA2	a1,2;b3,4;c5,6,7	E_CWA3	a2,3,4;b2;c2,3,4	E_CWA4	a2,3,4;b6,7;c6,7,8
S_CWA1	a12,13,14,15,16;b8,9;c5,6	S_CWA2	a15,16;b8,9,10;c1	S_CWA3	a9,10,11,12,13;b8,9,10,11;c2	S_CWA4	a14,15,16;b13;c3,4,5
I_CWB1	a4,5,6,7,8;b15,16;c10,11	I_CWB2	b18,19;c13,14	I_CWB3	a3,4,5,6,7;b15,16,17;c12,13	I_CWB4	a7,8,9,10,11;b16,17;c12,13
E_CWB1	a2,3,4,5;b12,13;c8	E_CWB2	a1;b2,3;c5	E_CWB3	a1,2;b1;c2,3	E_CWB4	a2,3,4,5;b5,6,7;c8
S_CWB1	a12,13,14,15;b8,9,10;c1	S_CWB2	a13,14,15,16;b4,5;c1	S_CWB3	a10,11,12,13,14;b8,9,10,11;c4,5	S_CWB4	a16;b14;c9,10
I_CWC1	a4,5,6,7,8;b16,17,18;c11,12	I_CWC2	a6,7,8,9,10;b19;c12,13	I_CWC3	a5,6,7,8,9;b15,16,17;c11,12	I_CWC4	a3,4,5,6,7;b15,16,17;c12,13
E_CWC1	a2,3,4,5;b7,8;c7,8	E_CWC2	a2,3,4,5,6;b11,12,13;c8,9	E_CWC3	a2,3,4,5,6;b4,5,6;c5	E_CWC4	a2,3,4;b8,9,10;c6,7,8
S_CWC1	a10,11,12,13,14;b8,9,10,11;c1	S_CWC2	a11,12,13,14,15;b8,9,10,11,12;c1	S_CWC3	a8,9,10,11,12;b9,10,11,12;c1	S_CWC4	a9,10,11,12,13;b10,11,12,13;c1

16S rRNA gene (a), total coliforms (b) and *E. coli* (c)

Fig. 2. a) Total coliforms and *E. coli* colony forming units per volume of sample (log CFU/mL or g of dry weight) and bacterial load based on 16S rRNA gene quantification by qPCR (log gene copy/mL or g of dry weight), in influent (I), effluent (E) and sediment (S) samples of the three CWs (A, B and C). **b)** Enterobacterial reduction values (log CFU/volume) for the three different CWs (A, B and C)) [influent (I) - effluent (E) counts] and macrophytes height (cm) across the four sampling dates (1-winter, 2-spring, 3-summer, and 4-autumn). The different Tukey's groups for 16S rRNA gene, total coliforms and *E. coli* by CW are displayed in the table.

sample with the PowerSoil DNA Isolation Kit (QIAGEN, Germany) according to manufacturer's instructions. DNA extracts were preserved at −20 °C until being analysed and their concentration was determined using the dsDNA HS assay in Qubit fluorometer (Thermo Fisher Scientific, USA), according to the standard kit protocol. In total, 105 DNA extracts (36 samples x triplicates, minus an aborted sample) were available for further analysis.

2.5. DNA-based microbiota analyses: biomarkers and bacterial community

Total bacteria, ARGs and fecal contamination abundance were assessed based on quantitative PCR (qPCR) (n = 105 DNA extracts) targeting the genes 16S rRNA, *intI1, sul1, ermB, ermF, mefC, qacEΔ1, tetX, aph(3″)-Ib,* and *uidA,* crAssphage, respectively (Teixeira et al., 2023). These biomarkers were selected because are associated with resistance, integrate the group of risk determinants, and are not observed to belong to pristine natural environments, but mainly to humans and animals (Teixeira et al., 2023). The primers and conditions are listed in Supplementary Table S2. Briefly, Ct values were interpolated in a calibration curve of Ct values *versus* target gene copy number (Brankatschk et al., 2012), using gene fragments (gBlocks, Integrated DNA Technologies, Inc), or genomic DNA from *E. coli* ATCC 25922 (for the 16S rRNA gene determinations). The four quality criteria for qPCR determination acceptance were those listed by Rocha et al. (2020).

The same DNA extracts were used for the bacterial community analysis, based on the amplicon sequencing analysis targeting the V3/V4 hypervariable region of the 16S rRNA gene (Illumina NovaSeq 250 bp paired-end; Novogene, United Kingdom). Raw data with required quality was filtered, the reads were merged and chimeras were removed. Filtered reads were denoised with DADA2 (Li et al., 2023; Callahan et al., 2016), and defined the amplicon sequence variants (ASVs) (Callahan et al., 2017), using the QIIME2's classify-sklearn algorithm (Bokulich et al., 2018; Bolyen et al., 2019), a pre-trained Naive Bayes

classifier. The representative sequence of each ASV was annotated using QIIME2 against the database Silva 138.1 (Silva, 2024)http://www.arb-silva.de/. Samples were normalized by the total number of reads in the dataset (24 179). The 16S rRNA gene sequences were deposited in the NCBI SRA archive under BioProject number PRJNA1161646.

2.6. Statistical analysis

The results were expressed as the logarithm of the number of colony forming units per volume (log (CFU/mL)) for cultivable fecal bacteria and as the logarithm of gene copy number per volume/mass of sample (log (gene copy/mL) or log (gene copy number/dry weight)) for abundance, and per 16S rRNA gene copy number (log (gene copy/16S rRNA gene copy) or log (gene copy/16S rRNA copy number g dry weight)) for prevalence. The one-way analysis of variance (ANOVA) and Tukey's and Bonferroni post-hoc tests were used to infer statistically significant differences ($p < 0.01$) in the abundance and prevalence of the biomarkers by using the SPSS Statistics for Windows v.28.0 (IBM Corp., Armonk, NY, USA). Values of bacterial or genes abundance reduction were expressed as log-units/mL. Bacterial community analysis data was expressed as relative abundance of the number of reads of a taxon per total reads number. The comparison of the relative abundance of bacterial taxa for two groups was performed using the two-sided Welch's *t*-test (confidence of ≥99 %), using the software STAMP v2.1.3 (Parks et al., 2014). For multivariate ordination analyses, software Canoco for Windows 4.5. version 5 was used. Pearson correlation analysis was performed with GraphPad Prism version 10.2.1. for Windows, with a confidence interval of 99 %.

3. Results

3.1. The influent

The CWs examined in this study serve small populations (~400 people) and are located in rural areas within a distance of 20 km, which could suggest identical organic and nutrient inputs. However, the COD, BOD, N and P influent mesaurements made throughout the year showed differences (Table S1), with the highest organic matter load registered in July for CWA, in March for CWB, and in September for CWC.

In general, the inlet of the CWs (I_CWA, I_CWB and I_CWC) had no significantly different loads of total coliforms and *E. coli*, which values ranged between 4.5 - 6.1 log CFU/mL and 3.9–5.9 log CFU/mL, respectively (Fig. 2a). Over the sampling period (I1, I2, I3 and I4), *E. coli* was significantly least abundant in influent samples collected in March (Fig. 2a). The total bacterial abundance, assessed based on 16S rRNA gene was, on average, 3 log-units above the values determined for cultivable enteric bacteria (Fig. 2a). The abundance of the 16S rRNA gene ranged between 8.2 (March) and 8.7 (May) log-units/mL, with no significant differences among CWs influents (I_CWA, I_CWB and I_CWC) or along the year being observed (Fig. 2a). The abundance (log-units/mL) of the different biomarkers for genetic recombination (*intI1*), fecal contamination (*uidA* and crAssphage) and antibiotic resistance (*sul1, ermB, ermF, mefC, qacEΔ1*, *tetX, aph3('')-ib*) was non-significantly different among the different CWs and ranged between 4.8 ± 0.5 to 6.7 ± 0.7 log-units/mL, being *mefC* the most abundant in CWA and CWC, and *sul1* the most abundant in CWB. The gene *uidA* was the least abundant and prevalent biomarker in all CWs (Fig. S2). In terms of relative abundance (log-units/16S rRNA gene), the gene *qacEΔ1* was significantly more prevalent in CWC than in CWA (Fig. S2). The molecular fecal indicators *uidA* and crAssphage, as well as the resistance genes *sul1*, *ermF, mefC, qacEΔ1*, and *tetX* reached the lowest average log-values (gene copy number/mL) in winter-March, while *intI1, mefC* and *qacEΔ1* peaked in summer-July. The gene *ermB* presented significant variations in the four sampling dates, reaching highest ($p < 0.01$) abundance values in the spring and summer seasons (Fig. S3a). The prevalence values presented the same pattern of variation (Fig. S3b).

OMPs were analysed in samples collected in March and May, with 55 of the 119 target analytes being detected in the influent (Table S5, excel file), four of which (acetaminophen, fenofibric acid, irbesartan and oxazepam) were present in all influent and effluent samples analysed. In general, the semi-quantitative analyses (Fig. S4) suggested that higher concentrations of OMPs reached the CWs in May than in March and that CWA received lower inputs of acetaminophen (433–8150 ng/L) than CWB (77571–227186799 ng/L) and CWC (206418–314435 ng/L). Unfortunately, results from the other two sampling campaigns were not available due to shipping problems that left the SPE cartridges unrefrigerated; these samples were not analysed because OMPs would likely have degraded.

The bacterial community composition of the influent samples was dominated by members of the phylum *Pseudomonadota* (~52 %, mainly classes *Gammaproteobacteria* (34 %) and *Epsilonproteobacteria* (17 %)), followed by *Bacteroidota* (~20 %, class *Bacteroidia* (20 %)), *Bacillota* (~20 %, class *Clostridia* (15 %)) and *Actinomycetota* (~5 %) (Figs. S5 and S6). Over the sampling dates, the bacterial diversity decreased from winter-March to summer-July (Table S4). According to the Principal Component Analysis (PCA) based on the taxonomic profiles at the family level the families that most correlated (VarR - variance of individual response variables) to influent samples were *Arcobacteraceae* (3.7273), *Moraxellaceae* (1.4709), *Comamonadaceae* (0.9010), and *Lachnospiraceae* (0.5796) (Fig. 6). The bacterial community composition at family level was observed to vary over the sampling period (PCA = 73 %), mainly when comparing the periods of winter-March and summer-July (Fig. S7a). Specifically, the relative abundance (average, %) of *Arcobacteraceae* was higher in summer-July than winter-March, in opposition with the relative abundance of *Moraxellaceae* and *Comamonadaceae* that was higher in March than in July (Fig. S7b).

3.2. The effluent

The COD, BOD, N and P effluent mesaurements made throughout the year evidenced marked variations, although presenting COD and BOD values that were below the European Urban Waste Water Treatment Directive threshold of 125 mg/L and 25 mg/L, respectively (Table S1). However, according to the same Directive, both N and P were frequently above the recommended values of 15 and 2 mg/L, respectively (Table S1).

Within each sampling campaign, total coliforms and *E. coli* counts were significantly different among CWs effluents, ranging between 1.7 - 3.8 log CFU/mL and 1.0–3.1 log CFU/mL, respectively (Fig. 2a). For different sampling dates, total coliforms and *E. coli* were significantly less abundant in samples collected in July (more pronounced in CWB) than in other dates (Fig. 2a). The 16S rRNA gene was, on average, 1.5 log-units/mL below influent values (Fig. 2 and S2). Over the sampling period, the abundance of the 16S rRNA gene in the final effluent varied significantly on CWB, being lowest in May. The abundance of the biomarker genes ranged, on average, from 3.0 ± 0.6 to 5.8 ± 0.5 log gene copy/mL (Fig. S1), being *uidA* and *sul1* the least and most abundant and prevalent, respectively. The genes *intI1*, crAssphage, *mefC* and *tetX* abundance showed some significant differences among the three CWs effluents (Fig. S2), being in CWB that these biomarkers had highest reduction. No significant differences of the biomarkers *intI1* and crAssphage were observed between seasons (Fig. S3).

A total of 59 out of 119 OMPs were detected in the effluent samples. Interestingly, four compounds were detected in the effluent but not in the influent: clindamycin and bupropion in CWC, trimethoprim in CWA and acetazolamide in CWB. The four OMPs that were detected in all influent samples were also detected in all effluent samples, in the ranges: acetaminophen: 435–14029 ng/L; fenofibric acid: 623–36091 ng/L; irbersartan: 129–2210 ng/L; and oxazepam: 106–2942 ng/L (Fig. S4), indicating their persistence after the treatment. Without a specific pattern of variation, other compounds such as levetiracetam, losartan, carbamazepine and cetirizine already present in influents, sometimes

increasead their average concentration after the treatment (Table S5). Gabapentin was the only compound which abundance increased after treatment.

The bacterial community composition of the effluent samples had a pattern similar to that of the influent (Fig. S5), dominated by members of the phylum *Pseudomonadota* (~60 %, classes *Gammaproteobacteria* (34 %), and *Epsilonproteobacteria* (23 %)), followed by *Bacteroidota* (21 %, *Bacteroidia* (20 %)), and *Bacillota* (4 %, *Clostridia* (3 %)) (Figs. S5 and S6). However, compared with influent samples, the effluent bacterial community composition presented a more heterogenous distribution (Fig. 6) and significantly lower bacterial diversity indexes (Table S4). Over the sampling period, the diversity indexes decreased from spring-May to summer-July. As observed for the influent samples, a decrease in diversity from winter to summer was observed (Table S4), except for CWB. In the PCA in which influent, effluent and sediment samples were compared, it was observed that the families most correlated (VarR) to effluent were *Rhodocyclaceae* (5.0866), *Sulfurimonadaceae* (1.7436), and *Rikenellaceae* (0.8603) (Fig. 6b). The comparison of all effluent samples at the family level suggested that the bacterial community composition varied throughout the year, with a clear separation over axis 1 (PC1 = 47.3 %) between winter-March and summer-July (Fig. S8a). The relative abundance (average, %) of *Sulfurimonadaceae* and *Rikenellaceae* was as more prevalent in effluents in July than in March (Fig. S8b).

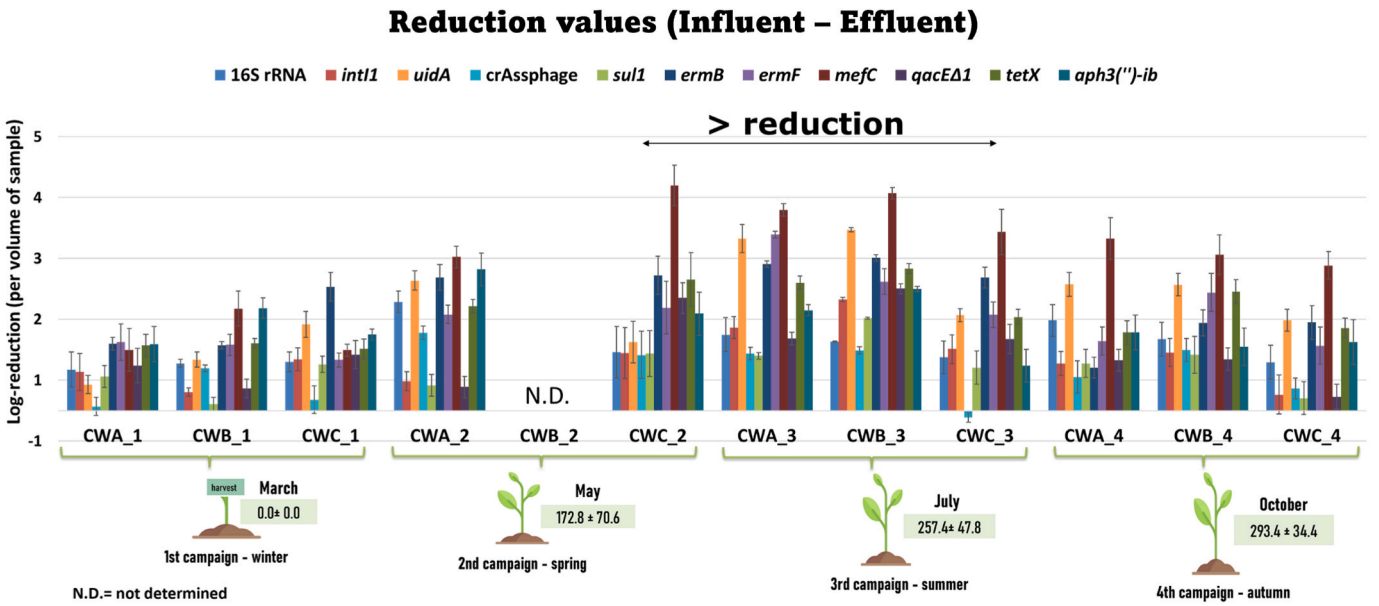
3.3. Performance and possible sediment accumulation of contaminants

This study was motivated by two major questions. One referred to the capacity of the CWs to reduce total coliforms and *E. coli*, total bacteria and genes associated with antibiotic resistance and fecal contamination, and OMPs. The other question referred to the potential accumulation of these contaminants in the sediments.

Regarding the capacity to reduce contaminants, it was observed that the reduction of cultivable bacteria counts was higher than the reduction observed for the total bacteria (16S RNA gene) and the biomarker genes. Total coliforms and *E. coli* reductions ranged from 0.8 to 4.8 log-units/mL, being these decreases significantly higher in summer-July than in winter-March (Fig. 2b). For total bacteria, the average reductions varied between 0.8 (winter-March) to 1.4 (spring-May) log-units/mL (Fig. 3). For the quantified biomarkers, average reductions between 0.9 (winter-March) to 1.8 (summer-July) log-units/mL were observed, being the lowest and highest values of −0.11 and 3.6 log-units/mL for crAssphage and *mefC*, respectively, both in summer (Fig. 3).

Regarding the OMPs, the concentration of acetaminophen was reduced by ~100 % after treatment in CWB and CWC (Fig. 5), others maintained the same range of concentration (e.g. fluoxetine and 1-(3-chlorophenyl) piperazine), while others, specifically gabapentin and fenofibric acid presented increased concentrations after treatment (Table S5, in excel file), which may suggest accumulation in the sediments and subsequent desorption. CWB presented reduction of the concentration of the four OMPs detected in influents and effluents (acetaminophen, fenofibric acid, irbersartan and oxazepam), contrary to the other two CWs (Fig. 5) which presented generally lower values of reduction (Fig. 5).

The bacterial community analysis suggested that treatment might lead to significant decreases of the relative abundance of members of the classes *Clostridia, Bacilli* and *Actinomycetes,* in all CWs and sampling dates, being the highest reductions observed in CWA – May (decrease of 20 %) for *Clostridia*, CWC – March (decrease of 10 %) for *Bacilli* and CWC – May (decrease of 9 %) for *Actinomycetes*, respectively (Fig. S6). The relative abundance of members of the families *Rhodocyclaceae* and *Sulfurimonadaceae* increased significantly after treatment in all CWs, but with more evidence for summer-July samples - CWA_3 (increase of 49 %) and CWC_3 (increase of 34 %), respectively for *Rhodocyclaceae* and *Sulfurimonadaceae*, involved in the carbon, nitrogen and sulfur cycles. In summary, when the performance of each CW was evaluated along the year, it was observed that reductions could be ranked as *E. coli* > total



CWA_1	a1;b1,2;c1;d1,2;e1,2;f1,2;g1,2;h1;i1,2;j1,2;k1,2	CWA_2	a2;b1;c5;d4;e1,2;f4;g1,2,3;h2,3;i1;j2,3,4;k4	CWA_3	a1,2;b2,3;c6;d3,4;e2,3;f4;g4;h3,4;i2,3;j4,5;k2,3,4	CWA_4	a1,2;b1,2;c4,5;d1,2,3;e1,2,3;f1;g,1,2;h3,4;i1,2;j1,2,3;k1,2,3
CWB_1	a1;b1;c1,2;d2,3,4;e1;f1,2;g1,2;h1,2;i1;j1,2;k2,3,4	CWB_2	N.D.	CWB_3	a1,2;b3;c6;d3,4;e3;f4;g3,4;h4;i4;j5;k3,4	CWB_4	a1,2;b1,2;c4,5;c3,4;e2,3;f2,3;g2,3;h2,3;i1,2;j3,4,5;k1,2
CWC_1	a1;b1,2;c2,3,4;d1,2;e1,2,3;f3,4;g1;h1;i1,2;j1;k1,2,3	CWC_2	a1,2;b1,2;c2,3;d3,4;e2,3;f4;g1,2,3;h4;i3,4;j4,5;k1,2,3,4	CWC_3	a1;b1,2,3;c3,4,5;d1;e1,2;f4;g1,2,3;h3,4;i2,3;j1,2,3,4;k1	CWC_4	a1;b1;c2,3,4,5;d1,2,3;e1,2;f2,3;g1;h2,3;i1;j1,2,3;k1,2,3
16S rRNA (a); *intI1* (b); *uidA* (c); crAssphage (d); *sul1* (e); *ermB* (f); *ermF* (g); *mefC* (h); *qacEΔ1* (i); *tetX* (j); *aph3(")-ib* (k).							

Fig. 3. Reduction values (log-units/mL) of 16S rRNA gene and biomarkers for genetic recombination (*intI1*), fecal contamination (*uidA* and crAssphage) and antibiotic resistance (*sul1, ermB, ermF, mefC, qacEΔ1, tetX, aph3(")-ib*) [influent (I) - effluent (E)] and macrophyte height (cm) in the four sampling dates. The different Tukey's groups for the different genes are displayed in the table.

coliforms > biomarkers > total bacteria, by summer-July > spring-May > autumn-October > winter-March. This observation coincided with the highest temperature, plants with larger stem and leaf areas and less rainfall, all associated to higher reductions (Fig. S10).

Regarding the assessment of possible accumulation in sediments, no evidence was found. However, sediments contained total coliforms and *E. coli* in values that ranged between 2.7 - 4.4 and < LOQ – 3.6 log CFU/g dry weight, respectively (Fig. 2), being the highest values reached in October and the lowest in May. Total bacterial abundance ranged between 9.2 and 10.8 log-units (gene copy number/g dry weight), corresponding to the samples with the lowest and highest moisture content (%), respectively (Table S3). The biomarkers abundance ranged between < LOQ or < LOD to 9.0 ± 0.1 log-units/g dry weight of sediment, being *sul1* the gene most frequently detected (50 % of the samples) and *qacEΔ1* the most abundant and prevalent. The gene *uidA* was the least frequently detected, abundant and prevalent biomarker in all CWs (being above the quantification limits only in CWB, in October) (Fig. S1). While absolute quantifications do not permit an evaluation of possible acumulation in sediments, the comparison of prevalence values (biomarker/16S rRNA gene) for each biomarker in wastewater and sediments supports such an analysis. This comparison showed that biomarkers presented lower prevalence in the sediments than in influent or effluent samples (Fig. 4). This may result from the fact that the total number of bacteria is higher in sediments than in water, and suggests that there is no accumulation at least in terms of relative abundance. The fact that we observed distinct bacterial communities in sediments and in influents and effluents suggest that over at least seven years of operation there was no relevant accumulation in the sediments (Fig. 6b).

Sediments were characterized by a bacterial community that could be clearly distinguished from that observed in influent or effluent samples, with both types of community separating over axis 1 of the PCA (Fig. 6). Sediments communities were characterized by a higher relative abundance of members of phyla such as *Pseudomonadota* (43 %), *Acidobacteriota* (16 %), *Thermoproteota* (9 %), *Actinomycetota* (8 %) and *Choroflexota* (6 %) (Fig. S5); and classes *Gammaproteobacteria* (23 %), *Alphaproteobacteria* (17 %), *Acidobacteria* (14 %), *Nitrososphaeria* (9 %), *Gemmatimonadia* (3 %) and *Thermoleophilia* (3 %) (Fig. S6). This observation suggests that sediments maintain an authochnous community, probably actively involved in the treatment process. The families *Rhodanobacteraceae* (1.0645), *Nitrosotaleaceae* (0.9906), *Chitinophagaceae* (0.1803), *Gemmatimonadaceae* (0.1293) *Xanthobacteraceae* (0.0990), *Acidobacteriaceae*_(Subgroup_1) (0.0806), *JG30*-KF-*AS9* (0.0609) and *Solibacteraceae* (0.0547) (Fig. 6) were the most correlated with sediment samples, evidencing the differentiation from wastewater samples. Also, the dynamic variation of the community in each CW sediments, characterized by the significantly higher percentage of members of the family *Moraxellaceae* in winter-March than in summer-July (Fig. S9b), suggest that the sediment communities are active and responding to external variatons. Contrary to what was observed in influent and effluent samples, it was not observed a clear separation of the bacteria community (family level) along the year, suggesting the stability of that ecosystem (Fig. S9a).

4. Discussion

The investigation of three independent CWs operating for at least 7 years to treat raw influents in a rural region confirmed the potential of these systems to remove OMPs, fecal contamination and ARGs, and did not evidence the accumulation anthropogenic biomarkers in sediments. However, possible adverse impacts on downstream environments could not be discarded, as measurable amounts of all these contaminants were observed in the final effluent. This is relevant information, because real-world full-scale systems are under the influence of multiple and non-controlable external factors, such as temperature, rain, wind or the variable inputs of contaminants and nutrients entering the systems, which may trigger unexpected effects. Studies conducted in full-scale systems, in which CWs were used as secondary or tertiary treatment, show these implications (Calheiros et al., 2015; Fang et al., 2017; Sabri, 2020; Pastor-Lopez et al., 2024). Most of the studies that have been published on the capacity of CWs to reduce coliforms, ARGs, ARB or OMPs have been based on laboratory- or pilot-scale experiments, in which most of those external factors are controlled (Wu et al., 2016; Bai et al., 2022). Indeed, pilot-scale studies often provide better efficacy results than real-world systems, possibly due to a size effect and because external factors can be controlled and managed by the operators (Manaia, 2023).

The measurement of standard parameters commonly used to assess the efficacy of wastewater treatment processes, BOD, COD, TSS, N and P, showed the adequacy of these systems to remove organic matter, BOD and COD. However, although the systems examined in this study are not under the scope of the European Urban Waste Water Directive (European Commission, 2022), it was observed that, in most of the effluent samples, N and P concentrations in final effluents did not meet legal recommendations (<15 and < 2 mg/L, respectively) (Table S1). This observation was not totally unexpected, as soil-based systems are generally efficient in removing TSS and BOD, although with limited capacity to remove N and P (Brix et al., 2007). Considering the fecal contamination load, assessed based on the standard indicators total

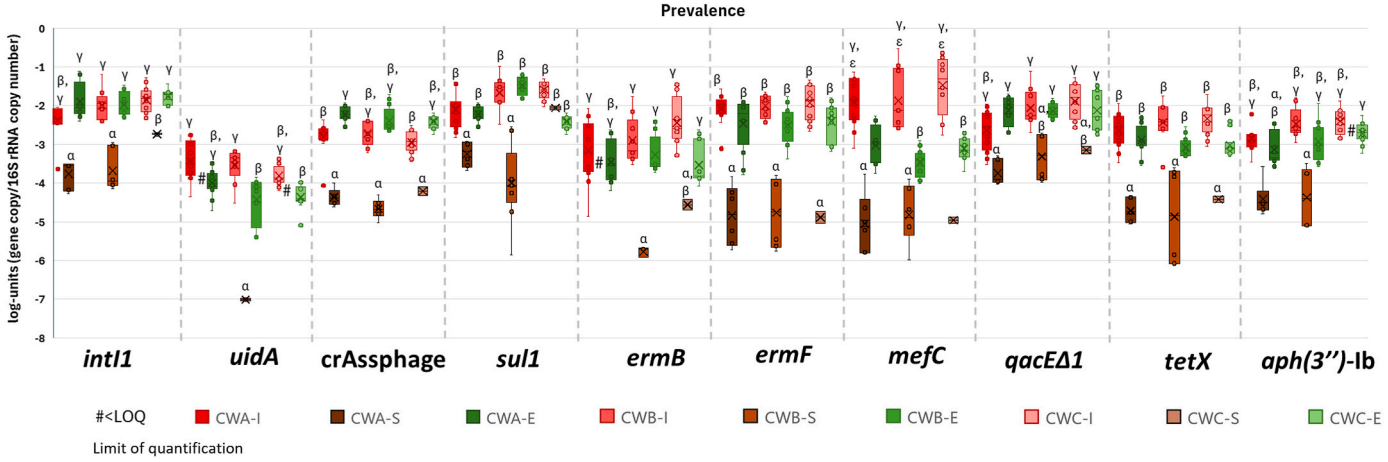


Fig. 4. Prevalence (log-units of the relative abundance by gene copy/16S rRNA copy number) of the biomarkers for genetic recombination (*intI1*), fecal contamination (*uidA* and crAssphage) and antibiotic resistance (*sul1, ermB, ermF, mefC, qacEΔ1, tetX, aph3(''))-ib*) for the influent (I), effluent (E), and sediments (S) samples of the three CWs (A, B and C). The letters (α, β, γ and ε) indicate significantly ($p < 0.01$) different Tukey's groups between different samples for the same gene.

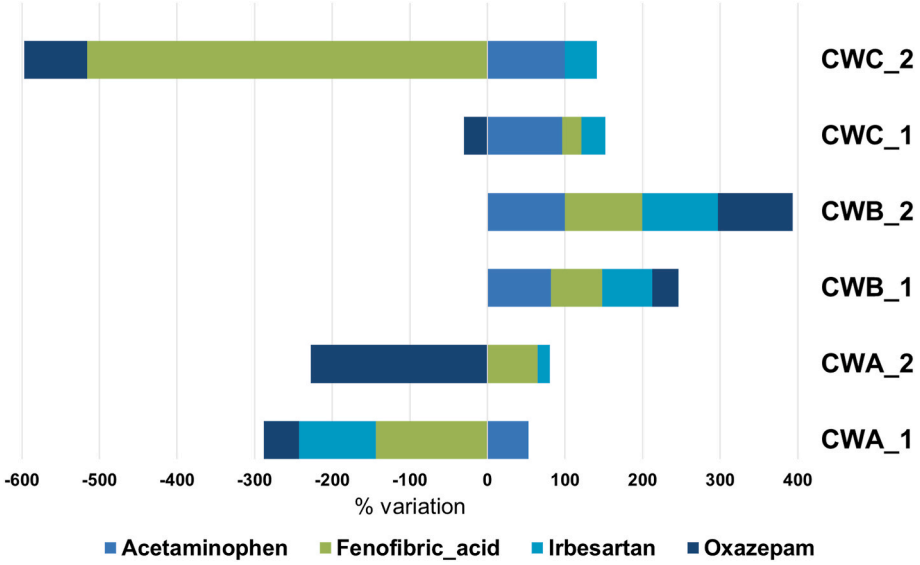


Fig. 5. Variation (%) of the organic micropollutants (OMPs) identified in all the samples collected in the 1st and 2nd campaigns, in March and May, respectively, by (LC-MS), for the three CWs (A, B and C). The variation was determined comparing the concentration in the effluent *vs* the influent [% variation = ($C_{influent}$-$C_{effluent}$)/ $C_{influent}$*100].

coliforms and *E. coli*, the final CWs effluents examined in this study ranged 100 to 10 000 CFU/mL, generally above the lowest quality category (class D) of treated wastewater used for irrigation, which is 10 000 CFU/100 mL (Regulation (EU) 2020/741). Also, the load of OMPs in the range of ng/L and of antibiotic resistance indicators, ranging from 4 to 5 log units gene copy/mL, although not regulated, may be considered excessive if the receiving environment does not have a strong dilution or mitigation capacity. However, these emissions are in the same order of magnitude that have been reported for activated sludge wastewater treatment plants, even using UV disinfection (Narciso-da-Rocha et al., 2018; Lira et al., 2020; Ferreira et al., 2022; Pastor-Lopez et al., 2024).

Currently, there are no regulations concerning the acceptable levels of pharmaceutical residues in treated wastewaters for release into the environment, and the removal of OMPs is widely variable and unpredictable. Some of the OMPs quantified in the wastewater samples in this study are among the therapeutic classes with highest consumption in Portugal, in 2023 (INFARMED, 2023), such as antidyslipidemic, metabolite fenofibric acid, pain killers such as acetaminophen, antihypertensives such as irbesartan, or psychoactive drugs such as oxazepam. Other studies have reported acetaminophen (550–38 000 ng/L), caffeine (13 000–100 000 ng/L), and ciprofloxacin (420–2700 ng/L) as the most abundant chemical compounds in influent samples from seven different municipal WWTPs, with the biological treatment providing a negative to 50 % reduction, while activated carbon and ozonation provided >95 % of reduction of those compounds (Angeles et al., 2020). Studies with four pilot-scale CWs have also reported removal rate for sulfamethazine and tetracycline of 11 %–95 % and 85 %–95 %, respectively (Liu et al., 2014). Statistically significant differences were observed in vertical CWs' with capacity to reduce ofloxacin (~98 %), pipemidic acid (80–99 %), ciprofloxacin (~77 %) and azithromycin (0–53 %) (Ávila et al., 2021). In some cases, it was also observed an increase of the contaminant concentration after treatment, which can be explained by transformation back to original parent compound within the biological treatment system (Angeles et al., 2020).

CWs are interesting ecosystems in which the removal of chemical and biological contaminants is aided by plant-microbe associations, in which the inhibitory or stimulating compounds occurring in wastewater or produced by some organisms may shape the microbial community. Aeration and nutrient availability plays an important role in pattern-driven variations of bacterial community (Zhang et al., 2024). In this study, one of the objectives was to investigate how external factors could be associated with the variations within the CW bacterial ecosystem (Fig. 6). The highest reduction rates for coliforms and the different biomarkers were observed in July, which was also the period in which the concentrations of N and P, as temperature, peaked (Fig. 6a and Fig. S10). In contrast, the period of lowest removal rates, in March, when temperatures were lower, was associated with highest COD and BOD values in the final effluents. Rainfall, probably due to a dilution effect, was negatively correlated with N ($R = -0.92$) and P ($R = -0.79$) as well as biomarkers reduction in the final effluent (Figs. 6a and 7). In this study, as has been discussed by other authors, hydraulic overloading of the treatment system, due to heavy rainfall, can reduce the removal efficiency of microorganisms, possibly due to decreased adsorption to the biofilm or the dilution factor (Wu et al., 2016). It is also described that highest removal efficiencies are obtained when using longest hydraulic retention time (HRT) (Avelar et al., 2014; Wu et al., 2016). However, in this study HRT was not very different or varying among CWs. In CWA (HRT ~7.2 days, Table 1), presented the highest log removals for total coliforms and *E. coli* in summer (Fig. 2), while CWB (lower HRT~5.8 days) demonstrated the highest removal of ARGs also in summer - removal values of 2.2 ± 0.7 and 1.5 ± 0.8 log-units (on average), respectively (Fig. 3). Moreover, in this study, HRT correlated negatively ($r = -0.34$) with the total load of bacterial removal (16S rRNA) and, with total coliforms and *E. coli* removals ($r = -0.25$) and biomarkers removal ($r = -0.22$) (Fig. 7). The temperature was observed to have a positive strong correlation with the macrophytes' height ($r = 0.82$), and with the reduction of biomarkers ($r = 0.81$), *E. coli* ($r = 0.79$), and total coliforms ($r = 0.78$) (Figs. 6a and 7). However, in a system that is mostly driven by plant growth, as is the case of CWs, it is arguable that temperature is associated with plant growth and not directly with treatment efficiency. Indeed, in previous studies, it has been suggested that higher environmental temperatures may support the survival or possible proliferation of human commensals, such as enteric bacteria (Pärnänen et al., 2019).

The microbial community is the major driver of the wastewater treatment process promoted by CWs (Wu et al., 2016). In this study, the predominant phyla in CWs influents and effluents were the same described for urban wastewater treatment plants based on activated sludge processes (Fernandes et al., 2019; Li et al., 2023), with *Pseudomonadota, Bacillota, Bacteroidota* and *Actinomycetota* predominating in influents and *Pseudomonadota* and *Bacteroidota* in the final effluents. The bacterial community in the influent of the three CWs was, irrespective of

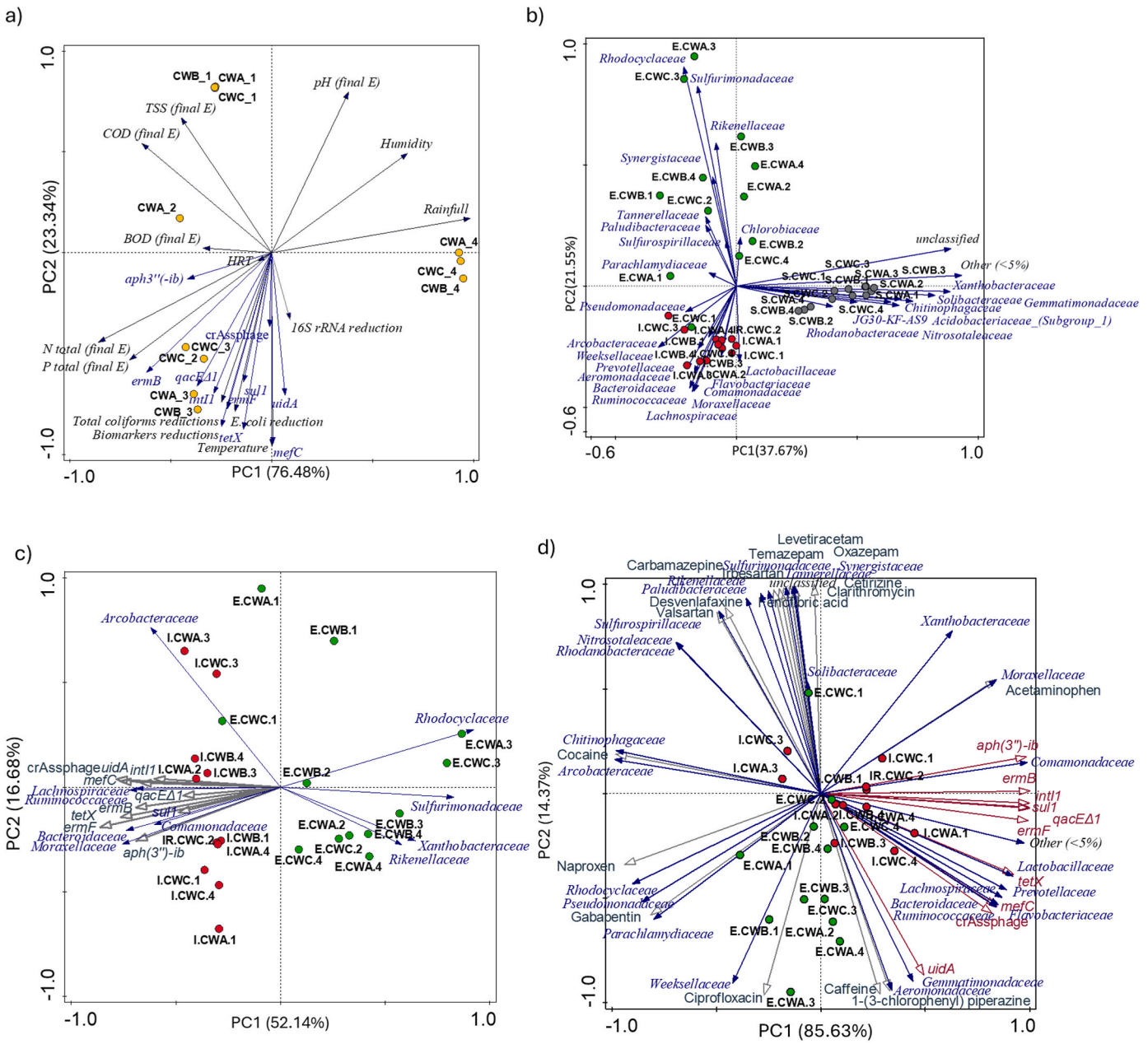


Fig. 6. Unconstrained principal component analysis (PCA) of **a)** biomarkers (*intI1, uidA,* crAssphage, *sul1, ermB, ermF, mefC, qacEΔ1, tetX* and *aph(3″)-Ib*), 16S rRNA gene (log-units gene copy/mL), total coliforms and *E. coli* reductions (log-units CFU/mL), N and P total (final effluent) (mg/L), rainfall (mm), humidity (%), and temperature (°C); **b)** bacterial community composition, expressed as family relative abundance (>5 %) between the influent (I), effluent (E), and sediments (S) samples; **c)** qPCR biomarker quantification (gene copy/mL of sample) and the most 10 most representative bacterial families; and **d)** qPCR biomarkers quantification (gene copy/mL of sample), the most abundant bacterial families and OMPs for the three CWs (A, B, and C) in the different seasons (1-winter, 2-spring, 3-summer, and 4-autumm).

the sampling date, very similar, underlying the concept of the wastewater-based epidemiology, grounded on the principle that the sewage produced by a population contains different markers referring to population health, dietary and consumption habits or prevalence of infectious agents (e.g. virus or antimicrobial resistance determinants) (Aarestrup and Woolhouse, 2020). In contrast, the final effluents, although maintaining the same profile of dominant taxa and showing a reduction of diversity indexes, evidenced a wider distribution of the community composition (Fig. S6), in agreement with previous studies, and which may be attributed to stochastic or at least, yet unknown, events (Fernandes et al., 2019; Lira et al., 2020). In urban wastewater treatment plants with anaerobic-anoxic–oxic treatment process it has been described that treatment is associated to an increase of the relative abundance of *Bacillota* and a decrease of *Pseudomonadota* and *Bacteroidota* (Lira et al., 2020; Li et al., 2023), a shift that was not observed in the current study with CWs.

The abundance of the biomarker genes was positively correlated with the bacterial community composition of influent samples, possibly due to the higher abundance of those genes in raw than in treated wastewater samples (Fig. 6c). Genetic biomarkers, in particular the *mefC* gene (macrolide efflux of *Photobacterium damselae*) and the gene *uidA* (beta-glucuronidase enzyme of *E. coli*) (Fig. S3), had a higher reduction after the treatment than the 16S rRNA gene, suggesting the higher removal of human commensals than of environmental bacteria. This suggestion was also confirmed by the covariation of some human bacterial commensals in the influent, such as *Arcobacteraceae*,

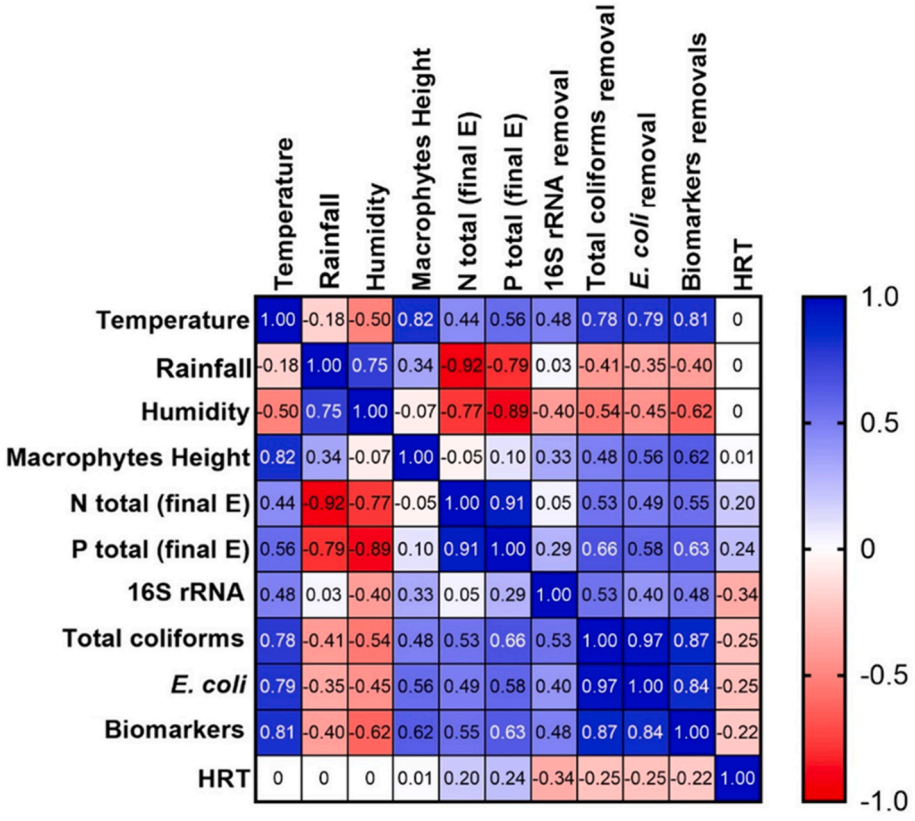


Fig. 7. Pearson's correlation coefficient (R) between the biomarkers for genetic recombination (*intI1*), fecal contamination (*uidA* and crAssphage) and antibiotic resistance (*sul1, ermB, ermF, mefC, qacEΔ1*, *tetX, aph3('')-ib*), 16S rRNA (log-units gene copy/mL), total coliforms and *E. coli* reductions (log-units CFU/mL), N and P total (mg/L), rainfall (mm), humidity (%), temperature (°C), macrophytes height (cm) and hydraulic retention time (HRT - days).

Moraxellaceae, *Comamonadaceae*, *Bacteroidaceae, Ruminococcaceae* and *Lachnospiraceae* (Fig. 6). Some correlations were suggested by the PCA (Fig. 6d), specifically of the families *Comamonadaceae* and *Lactobacillaceae* with the biomarkers examined and the *Moraxallaceae* with the analgesic and antipyretic drug acetaminophen, which probably hint co-occurrence of the different types of contaminant (Fig. 6d).

In general, CWs demonstrated to enhance the quality of treated wastewater and reduce the potential impact on the receptor environment. These systems are sometimes among the only solutions available, given geographic constraints and low population density in the area, and represent clearly a reliable alternative for small populations, rural areas or poor sanitation regions. However, some parameters, such as N and P reductions, need to be improved. Moreover, it may be more suited to warmer climates, like Southern Europe and the rotation of plants that have extensive leaf and steam parts in different times of the year is advisable. The use of alternative substrates, other than soil, may potentially favour oxygenation and drainage and thus nutrient removal (Colares et al., 2020), for example, to produce water with quality for reuse in different activities, as agriculture (Regulation (EU) 2020/741). As real systems operating, in the future, further studies with rotating plants, alternative substrates and contrasting climate conditions may contribute to consolidate these conclusions.

5. Conclusions

The CWs examined in this study showed capability to remove chemical and biological contaminants, although producing effluents in which ARGs, OMPs and fecal contamination were detected in concentrations not adequate for water reuse practices and with some risk for the receptor aquatic environment. Nevertheless, the dynamics of the bacterial community composition and of the biomarkers examined showed a shift of the wastewater microbiota from human commensals to mostly environmental bacteria. The macrophytes growth stage along the year and temperature were associated with the maximal reduction values, observed in the month of July, that corresponds to summer season. The CWs, in operation for more than 7 years, did not evidence the accumulation of biological contaminants in the sediments. The results obtained suggest that the CWs can be a good treatment alternative to treat domestic wastewater in small populations without affecting the natural landscape. However, improvements seem to be possible. For instance, through the optimization of hydraulic conditions to promote an efficient water flow and residence times and optimized aeration, particularly for N and P removal. Studies in full-scale CWs may contribute to gaining confidence on effective decentralized wastewater treatment units to be implemented in regions with low population density. Hybrid systems extended with other technologies in order to improve treatment performance are ideal for achieving higher treatment efficiency, for example, for the reuse of wastewater, in order to promote its correct use and avoid harmful effects on health and the environment.

6. Environmental implication

Constructed wetland systems (CWs) are nature-based solutions seen as promising alternatives for small populations or used as complements to conventional wastewater treatments. However, hazardous microorganisms and micropollutants may persist in the final effluent, raising the risks of water and soil contamination. This study shows that antibiotic resistance or associated genes and some pathogens may persist in the treated wastewater, being pivotal the monitoring of this type of systems. The results suggest that these systems helped to treat wastewater and can be used as decentralized systems in places where no other wastewater treatment systems are available.

CRediT authorship contribution statement

A. Margarida Teixeira: Writing – original draft, Methodology, Formal analysis, Validation, Investigation, Data curation, Writing – review & editing. **Diana Matos:** Resources. **Norberta Coelho:** Resources. **Lahiruni M. Halwatura:** Investigation, Writing – review & editing. **Ivone Vaz-Moreira:** Validation, Writing – review & editing, Data curation. **Paula M.L. Castro:** Supervision, Writing – review & editing. **Diana S. Aga:** Writing – review & editing, Investigation. **Célia M. Manaia:** Project administration, Investigation, Writing – review & editing, Supervision, Methodology, Data curation, Validation, Resources, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2025.146039.

Data availability

Data is available in public databases.

References

Aarestrup, Woolhouse, 2020. Using sewage for surveillance of antimicrobial resistance. Science 6478, 630–632. https://doi.org/10.1126/science.aba3432, 367.

Angeles, L.F., Mullen, R.A., Huang, I.J., Wilson, C., Khunjar, W., Sirotkin, H.I., McElroy, A.E., Aga, D.S., 2020. Assessing pharmaceutical removal and reduction in toxicity provided by advanced wastewater treatment systems. Environ. Sci. Water Res. Technol. 6, 62. https://doi.org/10.1039/c9ew00559e, 2020.

Avelar, F.F., Matos, A.T., Matos, M.P., Borges, A.C., 2014. Coliform bacteria removal from sewage in constructed wetlands planted with *Mentha aquatica*. Environ. Technol. 35, 2095–2103. https://doi.org/10.1080/09593330.2014.893025.

Ávila, C., García-Galán, M.J., Borrego, C.M., Rodríguez-Mozaz, Sara, García, J., Barceló, D., 2021. New insights on the combined removal of antibiotics and ARGs in urban wastewater through the use of two configurations of vertical subsurface flow constructed wetlands. Sci. Total Environ. 755, 142554.

Bai, S., Wang, X., Zhang, Y., Liu, F., Shi, L., Ding, Y., Wang, M., Lyu, T., 2022. Constructed wetlands as nature-based solutions for the removal of antibiotics: performance, microbial response, and emergence of antimicrobial resistance (AMR). Sustainability 14, 14989. https://doi.org/10.3390/su142214989.

Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M.R., Bolyen, E., Knight, R., Huttley, G.A., Caporaso, J.G., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6, 90. https://doi.org/10.1186/s40168-018-0470-z.

Bolyen, E., Rideout, J.R., Dillon, M.R., et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Brankatschk, R., Bodenhausen, N., Zeyer, J., Burgmann, H., 2012. Simple absolute quantification method correcting for quantitative PCR efficiency variations for microbial community samples. Appl. Environ. Microbiol. 78, 4481–4489. https://doi.org/10.1128/AEM.07878-11.

Brix, H., Schierup, H.-H., Arias, C.A., 2007. Twenty years experience with constructed wetland systems in Denmark – what did we learn? Water Sci. Technol. 56, 63–68.

Calheiros, C.S.C., Bessa, V.S., Mesquita, R.B.R., Brix, H., Rangel, A.O.S.S., Castro, P.M.L., 2015. Constructed wetland with a polyculture of ornamental plants for wastewater treatment at a rural tourism facility. Ecol. Eng. 79, 1–7. https://doi.org/10.1016/j.ecoleng.2015.03.001.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., Holmes, S.P., 2016. DADA2: high-Resolution sample inference from illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Callahan, B.J., McMurdie, P.J., Holmes, S.P., 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. ISME J. 11, 2639–2643. https://doi.org/10.1038/ismej.2017.119.

Climate Explainer, 2024. Nature-Based Solutions. worldbank.org.

Colares, G.S., Dell'Osbel, N., Wiesel, P.G., Oliveira, G.A., Lemos, P.H.Z., Silva, F.P., Lutterbeck, C.A., Kist, L.T., Machado, Ê.L., 2020. Floating treatment wetlands: a review and bibliometric analysis. Sci. Total Environ. 714, 136776. https://doi.org/10.1016/j.scitotenv.2020.136776.

Contreras, C.R., López, D., Leiva, A.M., Domínguez, C., Bayona, J.M., Vidal, G., 2019. Removal of organic micropollutants in wastewater treated by activated sludge and constructed wetlands: a comparative study. Water 11, 2515. https://doi.org/10.3390/w11122515, 2019.

(EU) Directive 91/271/EEC: Council Directive of 21 May 1991concerning Urban Waste Water Treatment.

European Commission, 2022. Directive of the European Parliament and of the Council Concerning Urban Wastewater Treatment (Recast), 2022.

Fang, H., Zhang, Q., Nie, X., Chen, B., Xiao, Y., Zhou, Q., Liao, W., Liang, X., 2017. Occurrence and elimination of antibiotic resistance genes in a long-term operation integrated surface flow constructed wetland. Chemosphere 173, 99–106.

Fernandes, T., Vaz-Moreira, I., Manaia, C.M., 2019. Neighbor urban wastewater treatment plants display distinct profiles of bacterial community and antibiotic resistance genes. Environ. Sci. Pollut. Res. Int. 26, 11269–11278.

Ferreira, C., Abreu-Silva, J., Manaia, C.M., 2022. The balance between treatment efficiency and receptor quality determines wastewater impacts on the dissemination of antibiotic resistance. J. Hazard. Mater. 434, 128933. https://doi.org/10.1016/j.jhazmat.2022.128933.

Halwatura, L.M., Aga, D.S., 2023. Broad-range extraction of highly polar to non-polar organic contaminants for inclusive target analysis and suspect screening of environmental samples. Sci. Total Environ. 893, 164707. https://doi.org/10.1016/j.scitotenv.2023.164707.

Hazra, M., Durso, L.M., 2023. Performance efficiency of conventional treatment plants and constructed wetlands towards reduction of antibiotic resistance. Antibiotics 11, 114. https://doi.org/10.3390/antibiotics11010114.

INFARMED, 2023. Análise De Consumo De Medicamentos Em Meio Ambulatório (available online – accessed in March 2024).

ISO 9308, 2014. Water Quality – Enumeration of Escherichia coli and Coliform Bacteria. International Organization for Standardization, Geneva, Switzerland.

Kataki, S., Chatterjee, S., Vairale, M.G., Dwivedi, S.K., Gupta, D.K., 2021. Constructed wetland, an eco-technology for wastewater treatment: a review on types of wastewater treated and components of the technology (macrophyte, biolfilm and substrate). J. Environ. Manag. 283, 111986. https://doi.org/10.1016/j.jenvman.2021.111986.

Li, S., Wang, S., Wong, M.H., Zaynab, M., Wang, K., Zhong, L., Ouyang, L., 2023. Changes in the composition of bacterial communities and pathogen levels during wastewater treatment. Environ. Sci. Pollut. Res. 30, 1232–1243. https://doi.org/10.1007/s11356-022-21947-8.

Lira, F., Vaz-Moreira, I., Tamames, J., Manaia, C.M., Martínez, J.L., 2020. Metagenomic analysis of an urban resistome before and after wastewater treatment. Sci. Rep. 10, 8174. https://doi.org/10.1038/s41598-020-65031-y.

Liu, L., Liu, Y-h., Wang, Z., Liu, C-x., Huang, X., Zhu, G-f., 2014. Behavior of tetracycline and sulfamethazine with corresponding resistance genes from swine wastewater in pilot-scale constructed wetlands. J. Hazard. Mater. 278, 304–310. https://doi.org/10.1016/j.jhazmat.2014.06.015.

Liu, L., Zhang, Y., Chen, H., Teng, Y., 2022. Fate of resistome components and characteristics of microbial communities in constructed wetlands and their receiving river. Sci. Total Environ. 844, 157226. https://doi.org/10.1016/j.scitotenv.2022.157226.

Manaia, C.M., 2023. Framework for establishing regulatory guidelines to control antibiotic resistance in treated effluents. Crit. Rev. Environ. Sci. Technol. 53, 754–779. https://doi.org/10.1080/10643389.2022.2085956.

Masoud, A.M.N., Alfarra, A., Sorlini, S., 2022. Constructed wetlands as a solution for sustainable sanitation: a comprehensive review on integrating climate change resilience and circular economy. Water 14, 3232. https://doi.org/10.3390/w14203232.

Narciso-da-Rocha, C., Rocha, J., Vaz-Moreira, I., Lira, F., Tamames, J., Henriques, I., Martinez, J.L., Manaia, C.M., 2018. Bacterial lineages putatively associated with the dissemination of antibiotic resistance genes in a full-scale urban wastewater treatment plant. Environ. Int. 118, 179–188. https://doi.org/10.1016/j.envint.2018.05.040.

Parks, D.H., Tyson, G.W., Hugenholtz, P., Beiko, R.G., 2014. STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics 30, 3123–3214.

Pärnänen, K.M.M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L.,

Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Della Giustina, S.V., Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5, eaau9124. https://doi.org/10.1126/sciadv.aau9124.

Pastor-Lopez, E.J., Casas, M.E., Hellman, D., Müller, J.A., Matamoros, V., 2024. Nature-based solutions for antibiotics and antimicrobial resistance removal in tertiary wastewater treatment: microbiological composition and risk assessment. Water Res. 261, 122038. https://doi.org/10.1016/j.watres.2024.122038. ISSN 0043-1354.

Regulation (EU) 2019/1021 of the European Parliament and the Council of 20 June 2019 on Persistent Organic Pollutants (Recast).

Regulation (EU) 2020/741 of the european parliament and of the council of 25 may 2020 on minimum requirements for water reuse, available online https://eur-lex.europa.eu/legal-content/PT/TXT/?uri=CELEX%3A32020R0741.

Rocha, J., Cacace, D., Kampouris, I., Guilloteau, H., Jäger, T., Marano, R.B.M., Karaolia, P., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Berendonk, T. U., Schwartz, T., 2020. Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes. J. Environ. Chem. Eng. https://doi.org/10.1016/j.jece.2018.02.022.

Sabri, 2020. Antibiotics and Antibiotic Resistance Genes in Wastewater: Occurrence and Removal Technologies. Phd Thesis. Wageningen University, Wageningen, the Netherlands.

Silva, 2024. Silva 138.1 Database. http://www.arb-silva.de/.

Teixeira, A.M., Vaz-Moreira, I., Calderón-Franco, D., Weissbrodt, D., Purkrtova, S., Gajdos, S., Dottorini, G., Nielsen, P.H., Khalifa, L., Cytryn, E., Bartacek, J., Manaia, C.M., 2023. Candidate biomarkers of antibiotic resistance for the monitoring of wastewater and the downstream environment. Water Res. 247, 120761. https://doi.org/10.1016/j.watres.2023.120761.

UN-HABITAT, 2008. Constructed Wetlands Manual. UN-HABITAT Water for Asian Cities Programme Nepal. Kathmandu.

Vigiak, O., Grizzetti, B., Zanni, M., Dorati, C., Bouraoui, F., Aloe, A., Pistocchi, A., 2018. Estimation of Domestic and Industrial Waste Emissions to European Waters in the 2010s, EUR 29451 EN. Publications Office of the European Union, Luxembourg. https://doi.org/10.2760/08152.JRC113729. ISBN 978-92-79-97297-3.

Vymazal, J., 2008. Constructed wetlands for wastewater treatment: a review. In: Sengupta, M., Dalwani, R. (Eds.), Proceedings of Taal2007: the 12th World Lake Conference, pp. 965–980.

Waly, M.M., Ahmed, T., Abunada, Z., Mickovski, S.B., Thomson, C., 2022. Constructed wetland for sustainable and low-cost wastewater treatment: review article. Land 11, 1388. https://doi.org/10.3390/land11091388.

World Health Organization, 2023. The Selection and Use of Essential Medicines 2023: Executive Summary of the Report of the 24th WHO Expert Committee on the Selection and Use of Essential Medicines, 24 – 28 April 2023. Web Annex C. WHO Access, Watch, Reserve (Aware) Classification of Antibiotics for Evaluation and Monitoring of Use. December 2023.

Wu, S., Carvalho, P.N., Müller, J.A., Manoj, V.R., Dong, R., 2016. Sanitation in constructed wetlands: a review on the removal of human pathogens and fecal indicators. Sci. Total Environ. 541, 8–22. https://doi.org/10.1016/j.scitotenv.2015.09.047.

Wu, H., Wang, R., Yan, P., Wu, S., Chen, Z., Zhao, Y., Cheng, C., Hu, Z., Zhuang, L., Guo, Z., Xie, H., Zhang, J., 2023. Constructed wetlands for pollution control. Nat. Rev. Earth Environ. 4, 218–234. https://doi.org/10.1038/s43017-023-00395-z.

Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., Zhang, Y., Li, X., Meng, W., 2015. Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. Chemosphere 119, 1379–1385. https://doi.org/10.1016/j.chemosphere.2014.02.040.

Zhang, Y., Li, T., Li, G., Yuan, T., Zhang, Y., Jin, L., 2024. Profiling sediment bacterial communities and the response to patterndriven variations of total nitrogen and phosphorus in long-term polyculture ponds. Front. Mar. Sci. 11, 1403909. https://doi.org/10.3389/fmars.2024.1403909.