



Decentralized wastewater management using treatment wetlands: Effective removal of antibiotics, resistance genes and organic micropollutants

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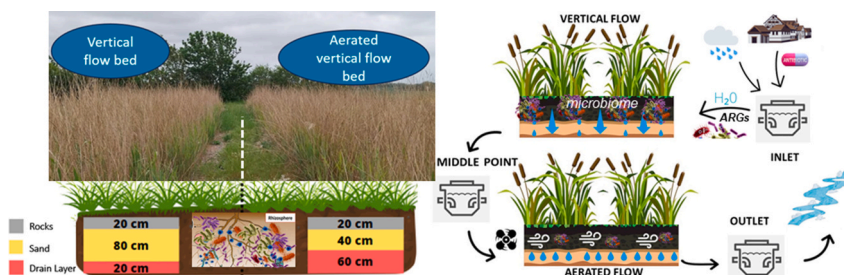
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HIGHLIGHTS

- Treatment wetlands (TWs) effectively remove a range of pollutants from wastewater.
- The TW shows higher micropollutant removal rates than conventional treatment plants.
- Efficiency dipped in winter, likely due to plant dormancy and cooler temperatures.
- Antibiotic resistance genes (*sul1* and *sul2*) and *Int1* gene were significantly reduced in the TW.
- The TW relied on synergic interactions in the rhizosphere reed-microbiome system.

GRAPHICAL ABSTRACT



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ABSTRACT

Treatment wetlands (TW) are a popular choice for decentralized wastewater treatment, with substantial documentation on their capacity to manage conventionally monitored pollutants. However, most insights into their effectiveness against emerging contaminants come from lab and mesocosm studies with a limited number of compounds, highlighting knowledge gaps in their performance at full scale. This study provides a first long-term, full-scale assessment of TW ability to remove a large number of organic micropollutants (OMPs) and manage antibiotic resistance under real-world conditions. The TW, with *Phragmites australis* as the plant species, operational for 10 years with a vertical flow bed followed by an aerated bed, serves a Danish village with a 100 population equivalent. We have monitored its performance over 18 consecutive months (each 3 weeks), analysing organic carbon, nitrogen and OMPs (e.g. pharmaceuticals including antibiotics).

Moreover, the microbial community structure (abundance and biodiversity) and possible presence of Antibiotic resistance genes (ARGs) in wastewater and reed rhizosphere at different points of the TW were analysed in four seasonal samplings. The findings demonstrated robust nutrient and OMP removal, with slight performance reductions in winter, likely due to plant dormancy and lower temperatures. Due to the small population served by the TW, antibiotics entered the system sporadically but at peak concentrations. The *sul1*, *sul2* genes associated

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with sulfonamide resistance and *intI1* gene were detected in all microbiological samples. The vertical flow bed effectively reduced OMPs (including antibiotics) and ARGs thanks to the removal action of the reed-microbiome system. The TW was a biofilter for targeted emerging contaminants, limiting their transfer in the water cycle and this study uniquely demonstrates that full-scale TWs can achieve high removal efficiencies for both antibiotics and ARGs, offering new insights into their practical application and operation for decentralized wastewater management.

1. Introduction

Water scarcity and contamination pose threats to the global environment and well-being and drive a demand for water treatment, storage, and reuse technologies. Since a significant part of water pollution originates from wastewaters not yet connected to treatment plants, this demand could be significantly addressed by decentralized treatment systems. The latter, unlike centralized wastewater treatment plants, are particularly relevant for remote or sparsely populated areas due to their lower infrastructure costs, flexibility in scale and ability to address localized pollution. A growing body of evidence shows that one important cost-effective category of such systems are nature-based solutions (NBS). They can manage and treat wastewater to the required standards, simultaneously offering an array of environmental and societal side benefits (Vymazal, 2010; Langergraber et al., 2019; Waly et al., 2022; Hendy et al., 2023). Among various NBS options, treatment wetlands (TWs) stand out for their proven effectiveness in wastewater treatment due to their ability to replicate and operationalize natural purification processes efficiently (Rosendo et al., 2022; Bakhshoodeh et al., 2020; Masi et al., 2018; Parde et al., 2021). Since 1968, TWs have been increasingly utilized for municipal wastewater treatment (Kadlec and Wallace, 2008) and have evolved to a complex variety of designs that can be tailored for treatment of specific wastewater streams to specific requirements of the effluent quality (Torrijos et al., 2016; Sehar et al., 2016; Liu et al., 2019; Ramírez-Vargas et al., 2019; Sharma and Malaviya, 2022).

The TWs are systems engineered by intentionally selecting porous media (e.g., gravel, sand), plants, and hydraulic conditions with the purpose of maximizing processes that naturally occur in wetlands. The intended processes include sedimentation, adsorption, filtration, microbial degradation and plant assisted bioremediation (Kadlec and Wallace, 2008; Dotro et al., 2017). Effective TWs significantly reduce classical wastewater pollutants, such as suspended solids, organic matter and nutrients. Additionally, some reduction in pathogens and retention of heavy metals is also achievable (Wu et al., 2015; Ladislav et al., 2015; Vymazal and Brezinova, 2016; Aguirre et al., 2005). These systems are therefore applied for primary, secondary and tertiary wastewater treatment (Matamoros et al., 2007; Matamoros et al., 2008; Nan et al., 2020; Patyal et al., 2022; Hassan et al., 2021; Masharqa et al., 2023; Gorito et al., 2017).

Besides classical wastewater pollutants, there is a growing demand for wastewater treatment facilities to reduce the emissions of water-borne contaminants of emerging concern. These contaminants, including pharmaceuticals, pesticides, surfactants and personal care products, pose risks even at low environmental concentrations due to their bioactive properties and potential to accumulate in ecosystems. Because of the relatively low environmental concentrations, the compounds are commonly referred to as organic micropollutants (OMPs). Many of them, such as pharmaceuticals and pesticides, are designed to interact with living cells. Depending on the compound, their environmental concentrations as low as nanograms per litre can disrupt enzymatic and metabolic mechanisms of living organisms, accumulate in food chains and threaten ecosystems, water resources and human health (Daughton and Ternes, 1999; Hoeger et al., 2005; Reemtsma et al., 2016; Schwarz et al., 2017; Saidulu et al., 2021; Oskarsson et al., 2014).

Despite growing environmental awareness, removal of these low-concentration structurally diverse compounds from wastewater

remains challenging and costly due to the need for advanced treatment methods (Aymerich et al., 2016; Oller et al., 2011; Reemtsma et al., 2016; Tran et al., 2018). To ensure the safety of water resources, TWs must also address compounds of emerging concern. The European Commission has recently revised the Urban Wastewater Treatment Directive (European Council, 1991), requiring large wastewater treatment plants to monitor and remove 12 indicative OMPs including some antibiotics, from their effluents (European Parliament and Council, 2024). While not applicable to smaller, decentralized systems like TWs, this Directive highlights the increasing recognition of OMPs as critical targets for water quality improvement. Additionally, TWs can serve as a valuable tertiary treatment step, polishing WWTP outflows to further remove OMPs and provide an environmentally friendly, low-energy option for additional contaminant removal. Limited data exist on the capacity of TWs to remove emerging contaminants at full-scale, particularly at real-world conditions (Sánchez et al., 2022; Ma et al., 2022; Cui et al., 2023). The scarcity of full-scale data particularly affects intensified TWs with controlled aeration, although small-scale studies suggest these systems may enhance attenuation of regulated pollutants compared to classical TWs (Saeed and Sun, 2012; Ávila and García, 2015).

Among OMPs, antibiotics (ABs) pose a significant global environmental and health concern. Beyond their direct biocidal effects on natural microorganisms (including microbiomes of animal and plants), their chronic environmental presence cause antimicrobial resistance (AMR) phenomena. Once acquired, AMR can spread between different species, including from pathogenic to non-pathogenic ones and vice versa and, through ingestion of AB contaminated water or food, between environmental bacteria and human and animal microbiomes (Chen et al., 2023; Grenni et al., 2024). Indeed, microorganisms with AMR genes threaten human and animal healthcare making standard treatments for infections ineffective. Addressing AMR to ensure continued effectiveness of antimicrobial drugs and controlling infections is a critical global health concern.

Pharmaceutical emissions can be significant locally near places of their production, hospitals, agriculture and aquaculture; however, urban wastewater is their dominant global environmental source (deraus et al., 2016; Paut Kusturica et al., 2022). Understanding the role of TWs in mitigating antibiotic resistance is crucial for public health, as TWs foster diverse microbial communities and provide conditions (e.g., oxygen gradients, organic-rich substrates) that enhance microbial degradation, aiding in the breakdown of antibiotics under near-natural environmental conditions. Clinically relevant antibiotic resistant bacteria (ARB) are commonly found in wastewater treatment plants (Chahal et al., 2016; Adelowo et al., 2020; Visca et al., 2022), including in TWs (Song et al., 2018; Zhao et al., 2023). The fate of antibiotic resistant genes in such systems remains largely unknown, with existing literature pointing in different directions (removal and accumulation) (Adelowo et al., 2020; Bai et al., 2022; Ma et al., 2022; Cui et al., 2023). The possibility that effluents of TWs can be a source of ABs and ARB depends on the biotic interactions in the sediment plant-microbiome system which can counteract ARG spread and promote water decontamination. This study addresses a critical knowledge gap regarding the effectiveness of full-scale hybrid TWs in reducing OMPs (including ABs) and ARGs under real-world conditions. Monitoring a system serving ~100 population equivalent (PE) in rural Denmark, we assessed the TW's seasonal and long-term efficiency over 18 months. The hybrid TW,

consisting of a vertical flow (VF) bed followed by an aerated flow (AE) bed, was evaluated for 60 OMPs, including 14 ABs. Wastewater and sediment microbial community structure was analysed in terms of cell abundance (epifluorescence direct counts), biodiversity (amplicon sequencing of the 16S rRNA gene) and quantification (qPCR) of common antibiotic resistance genes (*sul1* and *sul2*, *bla* and *mphE* genes) and the class 1 integron-integrase *int1* gene because of a proxy for anthropogenic pollution.

By providing a long-term, real-world assessment, this study offers critical insights into the practical application of hybrid TW systems for decentralized wastewater management.

2. Materials and methods

2.1. The treatment wetland

The selected hybrid treatment wetland (TW) treats the combined sewage from Tjørnelunde, a typical village in the Danish countryside with ~100 PE. This system (WGS84 coordinates 55.510255, 11.33598) has been operational since 2014. The wastewater from a sedimentation tank is subsequently pulse-loaded in a vertical flow (VF) bed followed by an aerated vertical flow bed (AE), (Fig. 1). While the VF was designed as the main treatment filter, the AE safeguards combined sewage overflow (CSO) events. Each bed has a surface area of 288 m² and approximately 1.2 m depth. Both the VF and AE support aerobic processes like reduction of organic load and nitrification, in compliance with Danish legislation requires. The VF consists of a 20 cm coarse gravel layer on top, followed by an 80 cm sand layer and a 20 cm drain layer. The AE consists of a 20 cm coarse gravel layer, covering a 40 cm sand layer and a 60 cm drain layer. *Phragmites australis* (common reed) was planted in both beds. The hydraulic retention time in the system is estimated around 2–3 days (not experimentally determined). The treated water is discharged into a nearby freshwater stream.

2.2. Samplings

2.2.1. Wastewater collection for analysing organic micropollutants

Wastewater samples were collected from the Inlet and Outlet of the TW approximately once every 3 weeks, covering the period from February 2022 to August 2023. CSO events were avoided, ensuring the

focus of the study remained the treatment of wastewater. Additionally, samples were taken from the effluent of the VF bed (Middle point of the TW system), starting mid-period (from November 2022) until the end of the sampling period. The dates are listed in Table S1 of the Supplementary Material 1 (Word file, SM1). Wastewater samples were collected as grab samples into two types of containers: 250 mL unused plastic bottles for nutrient analysis, 1 L Schott Duran glass bottles (previously heated at 450 °C for 6 h to ensure no contamination) for micropollutant analysis. Physicochemical water parameters (pH, electric conductivity (EC), total dissolved solids (TDS), temperature) were measured on-site using a Hach Lange 5059 multisensor. Subsequently, collected samples were stored at –20 °C for subsequent analysis of nutrients and OMPs.

2.2.2. Wastewater and sediment collection for microbial analysis

Four samplings were conducted to assess the impact of seasonal variations on microbial community structure and antibiotic resistance (one in Autumn: November 2022; one in Winter: January 2023; one in Spring: April 2023; and one in Summer: July 2023, Table S1 of the SM1). Wastewater and sediment from the VF and AE beds were collected in the four seasons.

Wastewater samples (3 replicates) collected at the Inlet, after the exit of the VF bed (Middle point) and at the Outlet were put in sterilized bottles (500 mL in triplicates for each sampling point). Sediment samples (3 replicates) taken at approximately 35 cm depth from the top, 15 cm below the distribution pipes (in both the VF and AE beds) were collected from reed-rhizosphere. All samples were put in sterilized tubes (100 g in triplicates for each sampling point). Analyses for the microbial abundance, antibiotic resistance genes and amplification and sequencing of the 16S rRNA gene (biodiversity) were conducted once for each season.

2.3. Analysis and data treatment of organic pollutants

The Total Nitrogen (TN) and Total Organic Carbon (TOC) were determined by a TOC analyser Shimadzu TOC-L (Kyoto, Japan). OMPs were quantified in wastewater using direct injection into a high-performance liquid chromatography system coupled with tandem mass spectrometry (HPLC-MS/MS). Samples were first centrifuged at 4000g for 7 min, using a Hermle Labortechnik GmbH Z 206 A centrifuge,

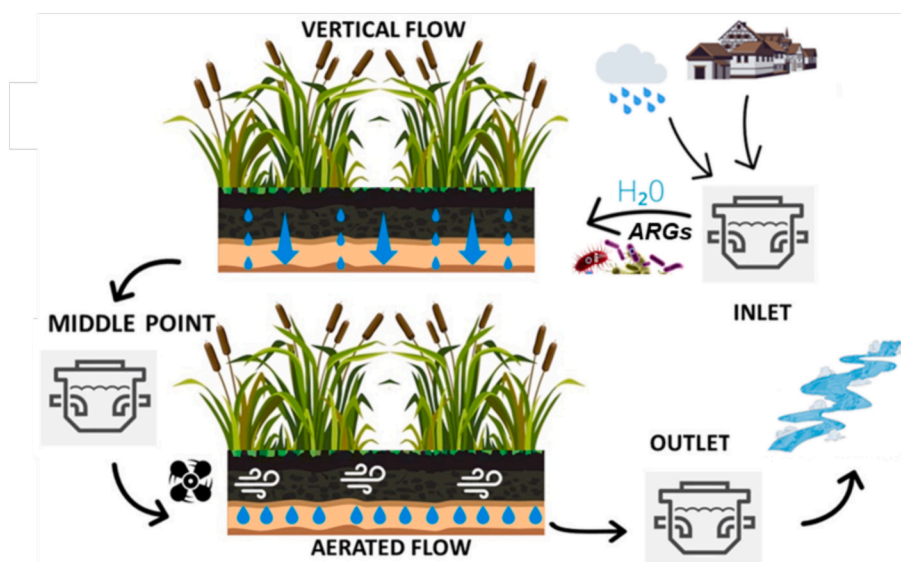


Fig. 1. The hybrid treatment wetland (TW) of this study. It treats the combined sewage from Tjørnelunde (~100 PE). The village is a hamlet in Kalundborg, (Region Zealand, Denmark) in the countryside. The wastewater from a sedimentation tank (INLET) is subsequently pulse-loaded in a vertical flow bed, passing through another tank (MIDDLE POINT), followed by an aerated vertical flow bed and then in the last tank (OUTLET). *Phragmites australis* (common reed) was planted in both beds.

to precipitate particulates. After centrifugation, 900 μL of the resulting supernatant was transferred to a new HPLC vial and mixed with 100 μL of a methanolic internal standard solution. Two injections per sample were analysed, with 20 μL injected each time into an Agilent 1290 HPLC system (Waldbronn, Germany) coupled with a Sciex QTRAP 5500 mass spectrometer (Framingham, MA, USA). Sediment extracts, obtained by a sequential ultrasonic extraction (see SM1) from subs-samples of the sediments collected for microbial analysis, were analysed in the same way of the wastewater samples.

Chromatographic separation was conducted on a Synergi Polar-RP column, 150 $\times$ 2 mm, 4 μm particle size (Phenomenex, Torrance, CA, USA), maintained at a temperature of 20 $^{\circ}\text{C}$. The flow rate of the solvent was set at 250 $\mu\text{L}/\text{min}$. The mobile phase consisted of water (eluent A) and methanol (eluent B), each containing 0.2 % v/v formic acid. A multi-step linear gradient program was employed for the separation: from 0 to 1 min, 0 % B; from 1 to 2.5 min, a gradient from 0 % to 50 % B; from 2.5 to 8.5 min, 50 % to 80 % B; from 8.5 to 9 min, 80 % to 100 % B; maintained at 100 % B from 9 to 14 min; from 14 to 14.5 min, a rapid return to 0 % B; and maintained at 0 % B from 14.5 to 19.5 min.

The mass spectrometer was operated using electrospray ionization (ESI) in positive mode with a capillary voltage set at 4500 V and temperature of 400 $^{\circ}\text{C}$. The concentration of each compound was determined based on a 9-point calibration curve, using certified analytical standards. Compound analytical standard suppliers, chromatographic retention times, precursor and product ions, compound-specific optimized MS parameters, and limits of quantification (LOQ) are provided in Tables S2 and S3 of the SM1.

Occurrences of all antibiotics were documented individually, whereas other OMPs were categorized based on their frequency of detection at the system's Inlet: those detected at least 10 times and those detected less frequently. Remote outliers ($Q = 1$) among the compounds detected at least 10 times were identified and removed using the ROUT function in GraphPad Prism 10. Concentrations within the first and third quartiles were then compared to corresponding concentrations at the Outlet. Outlet concentrations below the limit of quantification (LOQ) were assigned the LOQ value, and the removal efficiency was reported as being greater than or equal to the corresponding percentage ($\geq$). The occurrence and removal of non-antibiotic compounds detected in the Inlet of the TW less than 10 times are summarized in Table S3 of the Supplementary Material 2 (Excel file, SM2).

2.4. Analysis of microbial abundance and antibiotic resistance genes

Microbial abundance (cells/mL) was measured through the epifluorescence direct count method, which relies on the 4',6-diamidino-2-phenylindole (DAPI) fluorescent intercalant to bind adenine-thymine-rich regions of DNA (Barra Caracciolo et al., 2005). Using this method, microorganisms stained with DAPI can be visualized under an epifluorescence microscope, displaying a blue luminescent colour, which contrasts with the yellow appearance of inorganic particles.

Wastewater samples (50 mL each for 3 replicates) were fixed with formalin (2 % final concentration), vortexed and then filtered through a 0.2 μm polycarbonate membrane (Merck Millipore) by a gentle vacuum (0.2 bar). Then different aliquots (50 μL for Inlet and 1000–2000 μL for Middle Point and Outlet) were taken from cleared supernatants and processed with DAPI stain, as described in detail in previous works (Barra Caracciolo et al., 2005). The volume of filtered water was adjusted for each sample to obtain enough cells for a representative enumeration.

Sediment samples (1 g each for 3 replicates) were fixed in a 9 mL fixative solution (PBS: 130 mM NaCl, 7 mM Na_2HPO_4 , 3 mM NaH_2PO_4 , pH 7, 2 % formalin, 0.5 % Tween 20 and sodium pyrophosphate). The fixed samples were agitated at 400 rpm for 15 min and left to settle for 24 h. Subsequently, from cleared supernatants, aliquots (50 μL each) were taken and put in 2 mL of MilliQ and processed as above.

Wastewater and sediment microbial cells were visualized and

enumerated with a fluorescence microscope (Leica DM 4000B, Leica Microsystems GmbH, Wetzlar, Germany). A minimum of 300 cells were counted for each replicate in at least 30 fields selected randomly on each filter (Barra Caracciolo et al., 2010).

Aliquots of wastewater from the Inlet (50 mL), Middle (100 mL) and Outlet (100 mL) points (in 3 replicates) were used for concentrating bacteria on microbiological filters, using a vacuum pump. DNA was then extracted from filters or directly from sediment aliquots (0.25 g for each replicate) using the DNeasy PowerSoil Pro kit (Qiagen, USA), following the manufacturer's instructions and as reported previous works (Visca et al., 2022). A negative control (DNA-free water) was also included. The extraction yield and quality of the DNA were assessed with a spectrophotometer (Multiskan Sky Microplate Spectrophotometer, Thermo Fisher Scientific, USA). The overall DNA extracted was stored at -20°C until use for quantifying the 16S rRNA gene and various antibiotic resistance genes (i.e. *sul1* and *sul2* sulphonamide resistance genes; the beta-lactamase *bla* gene; the macrolide phosphotransferase *mphE* gene) and the class 1 integron-integrase *intI1* gene as a proxy for anthropogenic pollution. An aliquot of DNA was used for 16S rRNA amplicon sequencing.

The qPCR assay was performed with a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) for targeting the 16S rRNA, ARGs and the *intI1* gene, as reported in previous work (Visca et al., 2022). The template was 25 ng of genomic DNA previously extracted from each sample. The list of used primers is in Table S4 of the SM1. All qPCR wastewater and sediment results were reported as relative abundances (normalized by 16S ribosomal RNA abundance).

2.5. Microbial community composition: Sequencing of 16S rRNA gene and bioinformatic analyses

Wastewater and sediment microbial community was investigated over the different seasons using specific primers of 16S V3 and V4 regions. Amplicon library preparation was performed by a two-step PCR, as described by Gobbi et al. (2019). PCR BIO ultramix polymerase (PCR Biosystems, United Kingdom) was used for both amplification and indexing PCRs on a T100 Thermal Cycler (Bio-Rad, United States).

The library was sequenced using the V2 500 cycle reagent kit on the Illumina MiSeq instrument (Illumina, United States) at the Aarhus University (Roskilde, Denmark) Sequencing Center. The raw sequences were imported and demultiplexed using QIIME2 platform v2019.11 (Bolyen et al., 2019) and denoised with the DADA2 plug-in, as previously described by Callahan et al. (2016). The primers were removed using the DADA2 commands: “trim-left-f” for the forward primer and “trim-left-r” for the reverse one. These commands remove the sequences from their beginning to a specific position. The exact length of the primers was 17 nucleotides for the forward and 21 nucleotides for the reverse one, as reported in Mazzurco et al. (2020).

The amplicon sequencing variants (ASV) obtained with denoising process were sorted using the Silva 138 database ([https:// www.arb-silva.de](https://www.arb-silva.de)) with a naive Bayes classifier trained on the amplified regions with 80 % confidence (Bokulich et al., 2018). Finally, the R packages phyloseq and microbiome were used to estimate the ASV abundance in samples (McMurdie and Holmes, 2013).

2.6. Diversity indices and statistical analyses

The Shannon Diversity, Chao1 and Evenness Indexes were estimated with QIIME2 using the command line diversity alpha. The diversity indices, microbial abundance and ARG data from different sampling points and seasons (Autumn, Winter, Spring and Summer) were compared by a One-way and Two-way analysis of variance (ANOVA), using the 4.3.0 R version (Team, 2020). The post-hoc test (Tukey's Honestly Significant Difference) was performed to show significant differences (significance level ≤ 0.05) among conditions.

The differences in microbial community composition from different

sampling points and seasons were evaluated with a principal-coordinate analysis (PCoA) of the ASVs, based on Bray-Curtis distances. A multivariate ANOVA with permutations (PERMANOVA) was applied in order to assess significantly differences between the seasons and the sampling points. The Pairwise PERMANOVA (Anderson and Willis, 2003) was performed using the function pairwise.perm.manova from the package RVAideMemoire (Herve, 2023).

The ASVs were grouped according to bacteria genera, normalized by z-scores and the 40 most abundant genera from wastewater and sediments were selected to create a heatmap. In the heatmap, bacterial genera and sampling points were grouped in accordance with hierarchical clustering dendrograms, which are shown at the top and on the left side of the graphs. The Heatmap was generated to visualize differences in the relative abundance of bacterial genera between wastewater and sediment samples, with hierarchical clustering used to group similar microbial communities, helping to identify patterns of microbial distribution and community composition across different conditions. Differences in microbial class and genera relative abundances within and among the samples were assessed using an ANOVA-like differential expression tool (ALDEx2 package) (Fernandes et al., 2013).

3. Results and discussion

3.1. Main physicochemical wastewater parameters

The main physico-chemical wastewater parameters are reported in Table S4 of the SM2. Wastewater temperatures in the TW ranged from a minimum of 5.3 °C (December) to a maximum of 21.3 °C (July) and were relatively stable throughout the system at a given sampling point. The average measured pH values were slightly alkaline and remained relatively stable at both Inlet and the Outlet (7.5 ± 0.38 and 7.6 ± 0.25 , respectively). TOC was on average lower at the Outlet than the Inlet point, with two peaks in the latter of 164 and 207 mg/L observed in November 2022 and June 2023, respectively. However, these concentrations reduced significantly (7.2 in November and 15.2 mg/L in June) at the Outlet, with values similar to the average all-time-measured TOC outlet concentration of 11.1 ± 4.9 mg/L. The TN was removed only from average concentrations of 31.6 ± 26.8 in the Inlet to 27.9 ± 20.3 mg/L in the Outlet. The observed trends in low nitrogen reduction is in line with other VF and aerated flow beds (Vymazal, 2013). Indeed, the studied TW system was designed to perform nitrification and to perform the reduction of organic loads and this was ensured by the two stages of treatment which favoured aerobic conditions. In any case, in most cases TN decreased from Inlet to Outlet and this was an added value of the studied system. The TW has been operating in full compliance with its permit (pH, total suspended solids, ammonium, oxygen saturation, and biological oxygen demand) since its construction.

Table 1
Occurrence and removal percentages of antibiotics. LOQ: limit of quantification.

Antibiotic	LOQ (µg/L)	Inlet		Outlet		Average total removal (%)	Freshwater PNEC or Surface water quality standard* (µg/L)
		Number positive measurements	Average Concentration (µg/L) ± SD	Number positive measurements	Average Concentration (µg/L) ± SD		
Amoxicillin	0.05	1/27	23.5	0/27		≥ 99.8	0.078
Clarithromycin	0.1	2/27	2.58 ± 3.28	0/27		≥ 96.1	0.06
Clindamycin	0.02	1/27	0.20	0/27		≥ 90.0	0.014
Erythromycin	0.2	2/27	0.80 ± 0.06	0/27		≥ 75.0	0.04
Roxithromycin	0.2	1/27	10.5	0/27		≥ 98.1	0.15
Sulfadiazine	0.01	2/27	0.20 ± 0.15	1/27	0.11	≥ 69.6	4.6
Sulfamethizole	0.01	4/27	3.5 ± 6.7	4/27	0.10 ± 0.03	≥ 98.4	2.54
Trimethoprim	0.01	4/27	0.34 ± 0.33	0/27		≥ 97.1	10

Not detected: Ampicillin, azithromycin, ceftazidime, ciprofloxacin, sulfamethoxazole, sulfapyridine * Kisielius et al., 2023.

3.2. Micropollutant concentrations

Table 1 shows that 8 out of the 14 monitored antibiotics were detected at least once in the Inlet of the system. The highest recorded concentration was 23.5 µg/L for amoxicillin, followed by sulfamethizole (13.6 µg/L) and roxithromycin (10.5 µg/L). Interestingly, sulphonamides were the only ones detected at the Outlet and the sulfamethizole was found four times, suggesting that it was the most used among the household population. Sulphonamides were found in correspondence or close to the microbiological samplings (see Supplementary Materials 1, Table S6).

Concentrations of six antibiotics at the Inlet exceeded the freshwater predicted no-effect concentration (PNEC) or the EU surface water quality standard (Kisielius et al., 2023). While dilution may reduce antibiotic concentrations in receiving waters, the extent of dilution varies considerably across sites and seasons. Therefore, comparing effluent concentrations directly to PNECs provides a conservative and standardized benchmark for evaluating ecological risks, especially in small or intermittently flowing brooks where dilution may be minimal. Notably, high removal efficiencies were observed for antibiotics, frequently exceeding 90 % and leading to Outlet concentrations below the established safety limits (PNECs) or below LOQ (limit of quantification). Only sulfadiazine and sulfamethizole were quantifiable at the Outlet, each with an average concentration of 0.11 µg/L, below the safety limits for the receiving freshwater body.

Given the availability of comprehensive data on human excretion rates for antibiotics, it is possible to demonstrate that each peak detection event could be attributed to a single individual consuming the medication. Thus, it is also possible to verify that no detects were simply due to low consumption of antibiotics and not a limitation of the analytical method. For instance, using the daily recommended dose of roxithromycin (300,000 µg), a 50 % excretion factor (Kisielius et al., 2024), assuming a water usage of 150 L per person per day in a system designed for 100 person equivalents (PE), the expected concentration of roxithromycin attributable to a single individual matches the measured concentration at the Inlet (Table 1), as it would be calculated as follows:

$$\begin{aligned} \text{Concentration (}\mu\text{g/L)} &= \frac{300,000 \mu\text{g (daily dose)} \times 0.5 \text{ (excretion factor)}}{150 \text{ L (daily water usage per capita)} \times 100 \text{ (PE)}} \\ &= 10 \mu\text{g/L} \end{aligned}$$

Equation 1. Predicted sewer concentration of roxithromycin attributable to a single individual's daily dosage.

Table 2 illustrates the normalized occurrence and average removal rates of non-antibiotic household-generated OMPs that were quantified at least 10 times at the system's Inlet. Significant removal efficiencies (> 87.5 %) were observed for different compounds such as benzotriazole, caffeine, diclofenac, fexofenadine, furosemide, gemfibrozil and losartan. Gabapentin exhibited lower removal rates (34.2 %) than the previous

Table 2

Occurrence and removal of organic micropollutants, quantified in the system 10 or more times. LOQ: limit of quantification.

Compound	LOQ (µg/L)	Positive/measured events	Interquartile range of the positive events* (µg/L)	Average removal within each range (%)
Benzotriazole	0.1	23/27	0.80–1.62	≥87.5
Caffeine	0.1	19/20	23.4–111	≥99.4
Diclofenac	0.05	19/27	0.58–1.63	≥92.0
Fexofenadine	0.1	18/20	1.87–9.49	≥96.4
Fluopyram	0.2	16/20	0.22–0.53	–57.0
Furosemide	0.2	10/27	1.08–2.16	≥88.4
Gabapentin	0.02	13/27	1.01–37.1	34.2
Gemfibrozil	0.2	25/27	3.55–15.3	≥96.0
Losartan	0.03	23/27	0.83–2.42	≥97.2

* The range between the 1st and the 3rd quartile, discarding outliers.

ones. Notably, a negative removal rate was recorded for the fluorinated nematicide fluopyram, reflecting the tendency of fluorinated compounds to show low biodegradability.

Sporadic occurrence of other compounds was, as for the antibiotics, presumably due to the low number of population using the system. None of the compounds was measured in the influent 100 % of the time. The highest average concentrations of non-antibiotic OMPs in the inflow were recorded for caffeine, gabapentin, and gemfibrozil. The irregular occurrence of these compounds in the Inlet complicates the collection of comprehensive removal data. While assessing performance under such variable conditions is challenging (in contrast to lab studies under spiking conditions), current results represent true real life operation conditions for decentralized treatment solutions. Interestingly it shows that the smaller the PE served by a TW, the less frequent but higher concentrations the system will be loaded with in contrast to centralized solutions where concentrations tend to be more stable for a larger variety of compounds. Nevertheless, present results also show that despite not being continuously loaded with antibiotics and other OMPs, significant reductions can be achieved by such systems. Presently, average removal rates above 95 % were achieved for caffeine, diethyltoluamide (DEET), fexofenadine, gabapentin, gemfibrozil, losartan, sertraline, and simvastatin (Table 2 and Table S2 of the SM2). Except for fluopyram, no other compound exhibited a higher average concentration in the Outlet compared to the Inlet. The only compounds with an average removal rate below 40 % were gabapentin and lamotrigine, probably not fully due to lower removal, but in the case of lamotrigine attributed to the cleavage of lamotrigine-N2-glucuronide (lamotrigine human metabolite) during the treatment process that results back in lamotrigine (Bollmann et al., 2016).

Regarding the analysis of micropollutants in the sediments, results show limited accumulation during the 10 years of operation. Only a few compounds were consistently quantified in the sediment, indicating limited accumulation for most substances (Table S6 of the SM2). Notably, none of the sulfonamides – despite their frequent detection in the wastewater – were found above the limit of quantification in the sediments. In contrast, macrolide antibiotics such as clarithromycin and roxithromycin, which were rarely measured in the water phase, were repeatedly detected in the sediment, often at elevated concentrations. This pattern suggests a strong tendency of these compounds to sorb to solid phases or persist under sediment conditions.

Removal percentages of both ABs and other OMPs in the TW were significantly higher than those typically observed in conventional activated sludge (CAS) wastewater treatment plants (WWTPs). For example, in two conventional CAS WWTPs located in the same Danish island of the studied TW, the average removal rates for sulfadiazine, sulfamethazole and trimethoprim were 45.2–50.9 %, 47.1–67.6 % and 35.3–46.2 %, respectively (Kisieliu et al., 2024). In contrast, the TW achieved considerably higher removal efficiencies of ≥69.6 %, ≥98.4 %, and ≥ 97.1 % for these compounds, as shown in Table 1. Notably,

additional monitoring of the system's midpoint over the last nine months, encompassing 12 campaigns, indicated that majority of the removal of OMPs occurred in the vertical flow bed of the TW, as outlined in Tables S1–S3 of the SM2.

3.3. Microbial abundance and antibiotic resistance genes in wastewater and sediment samples

The microbial abundance (N. cells/mL) of wastewater samples measured in Autumn (Nov), Winter (Jan), Spring (May) and Summer (Jul) samplings are reported in Fig. 2A. A significant decrease ($p < 0.05$) was found from the Inlet to the Outlet of the TW, in each season. The highest value (9.55×10^6) was detected in Summer, presumably due to high temperatures promoting the proliferation of microbial cells. However, the treatment system consistently reduced the overall microbial abundance, obtaining similar values at the Outlet throughout the year (Autumn: 5.51×10^5 ; Winter: 1.42×10^5 ; Spring: 1.56×10^5 and Summer: 4.97×10^5).

The microbial abundances (N. cells/g) of the sediment samples, collected at approximately 15 cm below the distribution pipes (wastewater inflow) are reported in Fig. 2B. At these points plant roots were well developed, indicating that these samples represented the rhizosphere. A significant ($p < 0.05$) decrease in microbial abundance from the VF to the AE was observed, and this result suggests that the VF and AE beds acted as active filters for wastewater, counteracting bacteria from wastewater.

The relative abundance of ARGs (N. copies/16S rRNA gene) for wastewater and sediment samples are reported in Fig. 3 and Fig. 4. Although ARGs for various antibiotic classes were initially searched for (e.g. the Beta-lactamase gene *bla* and the macrolide phosphotransferase gene *mphE*) only those involved in sulphonamide resistance (*sul1* and *sul2*), together with the class 1 integron-integrase *int1* gene, were detected in the four microbiological samplings. The ARG values were consistent with other works on constructed wetlands (Abou-Kandil et al., 2021; Ávila et al., 2021; Sabri et al., 2021). These findings do not exclude possible presence of other ARGs at different times over the 18-month study, if other antibiotic class would be used among the household population.

A general decrease in ARGs from the Inlet to Outlet was found, in accordance with the reduction in the total microbial abundance. In fact, *sul1*, *sul2* and *int1* genes at the Inlet were significantly higher than those at the Middle ($p < 0.02$) and Outlet ($p < 0.01$) points, except for *sul2* in Winter.

Indeed, at the Middle Point, the highest ARG abundances in wastewater ($p < 0.01$, Fig. 3) were in Winter and *sul2* did not decrease from the Inlet to the Outlet. These results show how wastewater flowing in the VF and AE beds were depurated by the rhizosphere reed-microbiome system. In accordance with these results, significant decrease in sulphonamides and ARGs due to microbiome removal in rhizosphere has been observed in previous soil microcosm studies (Barra Caracciolo et al., 2022). The same work demonstrated that sulphonamides did not bioaccumulate in leaves.

Consistent with the results described above for wastewater, *sul1*, *sul2* and *int1* abundance in rhizosphere sediment samples reduced significantly from the VF ($p < 0.01$) to the AE bed and this phenomenon was less pronounced during Winter (Fig. 4). The abundance of the *sul1* gene did not vary significantly across the four seasons. The abundance of the *int1* gene was significantly higher during Summer ($p < 0.02$) compared to other seasons. Overall, ARGs abundance was higher in Summer, followed by Spring, Winter and Autumn.

Although antibiotic residues were generally not detected at the time of the microbiological samplings (Autumn: November 1st; Winter: January 30th; Spring: May 16th; Summer: July 11th), (see Table S1 of the SM2), *sul1*, *sul2* and *int1* were consistently found. This suggests that sulphonamide ARGs can persist even in antibiotic absence and that other stressors or chemicals may contribute to their presence. The fact that

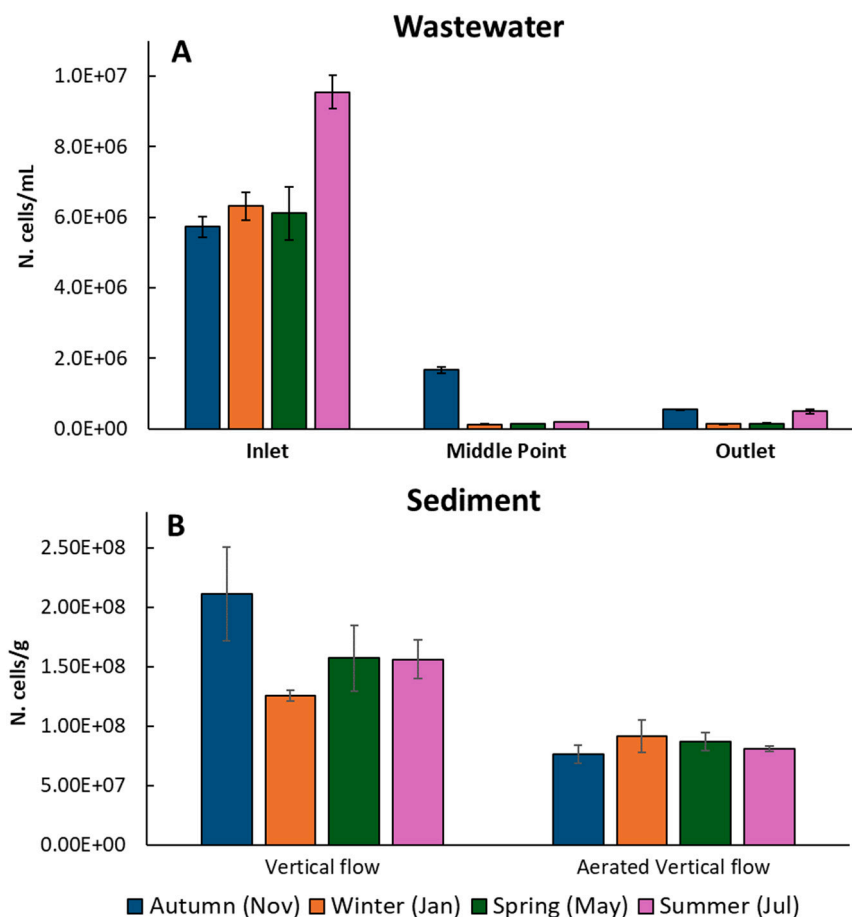


Fig. 2. Microbial abundance (number of cells) in (A) wastewater and (B) sediment samples.

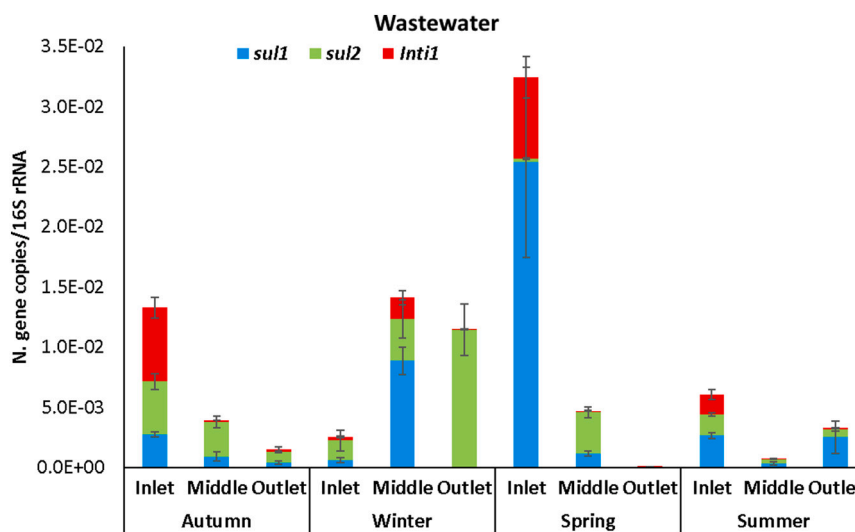


Fig. 3. Relative abundance (N. gene copies/16S rRNA gene) of *sul1*, *sul2* and *int1* in water samples collected from the Inlet, Middle and Outlet points during Autumn (November), Winter (January), Spring (May), and Summer (July) samplings.

some ARGs can be more persistent in environment than their antibiotics has been also found in other studies (Deekshit and Srikumar, 2022). Indeed, environmental factors (e.g. temperatures, other contaminants such as biocides or stressors) can contribute to this phenomenon (Visca et al., 2021).

3.4. Wastewater and sediment microbial community composition

A total of 5,851,991 sequences were obtained, with an average sequencing depth of 57,941 reads per sample. After filtering out singletons and doubletons, 17,471 distinct ASVs were retained, representing 1099 unique genera across the different sample types and seasons. Thus, representing an overall biodiverse community.

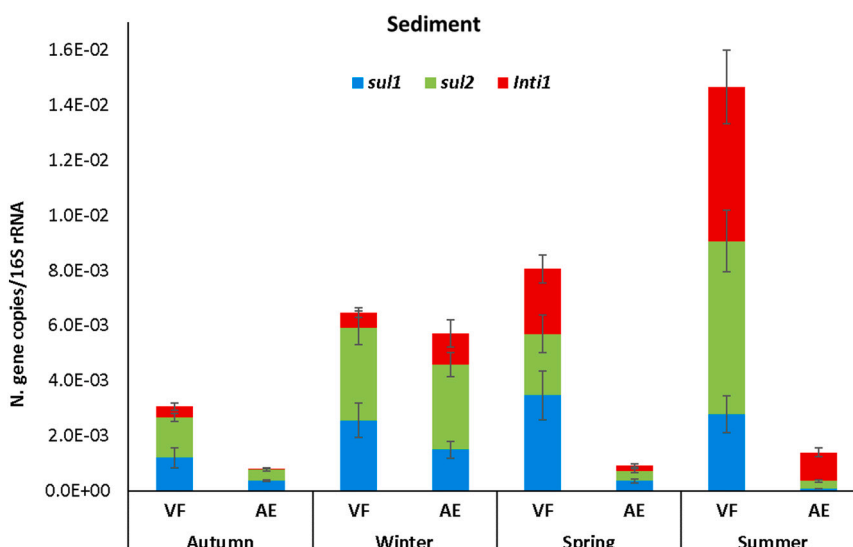


Fig. 4. Relative abundance (N. gene copies/16S rRNA gene) of *sul1*, *sul2* and *int1* in sediment samples from the Vertical Flow (VF) and Aerated Flow (AF) beds during Autumn (A), Winter (B), Spring (C) and Summer (D) samplings.

Table 3 reports the Shannon, Evenness and Chao1 indices. The microbial biodiversity was generally lower in water than in sediment samples, showing a more diverse microbial community in the TW rhizosphere. Moreover, the biodiversity tended to decrease in water between Inlet and Outlet points.

The Principal Coordinates Analysis (PCoA) (Fig. 5) used to graphically represent the dissimilarity matrix of ASV distribution within the samples, explained 22 % of the total variance. The sampling points (VF and AE bed sediments, Inlet, Middle and Outlet points) and the seasons were used as variables. The first axis (Axis 1) accounting for 14 % of the total variance, is associated with the environmental matrix (wastewater or sediment). A significant difference ($p < 0.01$) was observed between the microbial communities in the sediment (right side) and in the water (left side). The axis 2 (which accounts for 8 % of the variation) shows different distribution of the ASVs among seasonal samples. In particular the summer community tend to be different from the autumn and winter ones. These differences may be ascribed to temperature effects on microbial activity, particularly in the rhizosphere. Additionally, variations in precipitation frequency and local human activities can influence both the quantity and the quality of incoming water. For instance, a

substantial nitrogen input observed in autumn was associated with an increase in ammonifying bacteria.

In wastewater (Fig. 6) the predominant bacterial class was *Gammaproteobacteria* (except in one case), ranging from 10.5 % (Spring, May) to 79.5 % at the Inlet point, and from 37.7 % (Winter, January) to 79.1 % (Summer, July) at the Outlet one. The relative abundance of *Bacteroidia* was similar across the four sampling periods, with mean values ranging from 11.97 % to 15.91 %, except for the Spring Inlet, where it was only 2.86 %. In Spring and Summer samplings higher percentages of *Saccharimonadia*, *Clostridia*, *Bacilli* and *Campylobacteria* classes were found at the Inlet compared to Autumn and Winter.

In Autumn (November) wastewater samples, the second most abundant bacterial class was *Bacteroidia* (12.3 % at the Inlet, 13.5 % at the Middle Point and 21.9 % at the Outlet). The percentage of *Actinobacteria* remained relatively stable between the Inlet and Outlet (0.9 % and 0.7 %, respectively, while *Bacilli* relative abundance decreased from Inlet to Middle and Outlet (3.2 %, 1.0 % and 0.2 % respectively) points. On the other hand, *Alphaproteobacteria* increased from the Inlet to the Outlet (1.7 % in the Inlet to 4.2 % in the Outlet).

In Winter (January) microbial community was not substantially

Table 3

Chao1, Shannon and Evenness diversity with corresponding standard errors (s.e.).

Sampling	Sample		Shannon	±s.e.	Evenness	±s.e.	Chao1	±s.e.
Autumn (Nov)	Wastewater	Inlet	4.16	0.03	0.79	0.01	208	0.82
	Sediment	Vertical	5.26	0.04	0.85	0.01	511	1.69
	Wastewater	Middle Point	4.03	0.03	0.77	0.01	182	1.30
	Sediment	Aerated	6.01	0.05	0.93	0.01	786	1.67
	Wastewater	Outlet	4.01	0.03	0.74	0.01	259	1.30
Winter (Jan)	Wastewater	Inlet	6.10	0.02	0.83	0.01	1670	2.23
	Sediment	Vertical	6.15	0.04	0.90	0.01	981	3.05
	Wastewater	Middle Point	5.84	0.04	0.87	0.01	861	0.54
	Sediment	Aerated	6.15	0.04	0.90	0.01	1071	3.33
	Wastewater	Outlet	5.89	0.03	0.87	0.01	881	1.37
Spring (May)	Wastewater	Inlet	5.34	0.03	0.79	0.01	883	3.61
	Sediment	Vertical	6.46	0.04	0.90	0.01	1296	8.31
	Wastewater	Middle Point	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Sediment	Aerated	6.20	0.04	0.91	0.01	937	6.02
	Wastewater	Outlet	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Summer (July)	Wastewater	Inlet	5.81	0.03	0.84	0.01	959	4.21
	Sediment	Vertical	6.41	0.03	0.90	0.01	1234	7.98
	Wastewater	Middle Point	3.33	0.02	0.60	0.01	260	2.11
	Sediment	Aerated	6.23	0.04	0.92	0.01	923	7.92
	Wastewater	Outlet	3.84	0.02	0.68	0.01	296	1.07

n.d.: no data.

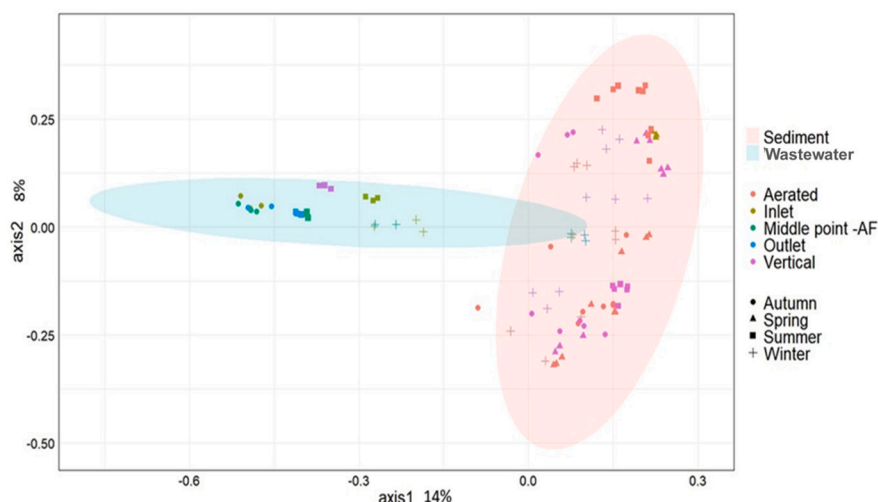


Fig. 5. Principal Coordinates Analysis (PCoA) of ASV distribution. Different colours represent sampling points (Vertical Flow bed, Aerated bed, Inlet, Middle and Outlet points), and different shapes represent sampling seasons: Autumn (November, circles), Winter (January, crosses), Spring (May, triangles) and Summer (July, squares). The ellipses group water and sediment samples.

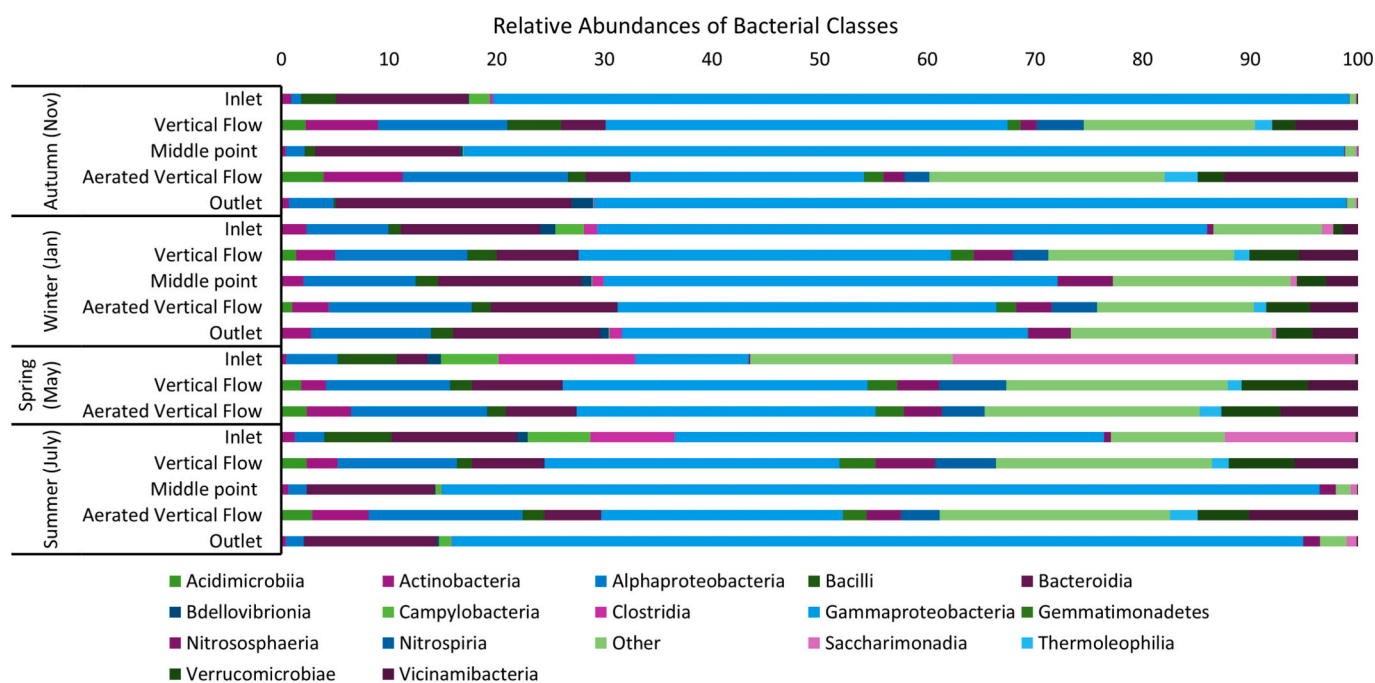


Fig. 6. Relative abundance (%) of bacterial classes in samples of water (Inlet, Middle point and Outlet) and sediment (Vertical Flow, Aerated Vertical Flow).

different between Inlet and Outlet. Although a small decrease in *Clostridia* and *Acidimicrobia* was observed from the Inlet (1.3 % and 0.9 %) to the Outlet (0.8 % and 0.5 %), other classes (e.g. *Nitrospiria* and *Vicinamibacteria*) did not show significant differences. In Spring (May) the predominant bacterial class was *Saccharimonadia* (37.3 %) followed by *Clostridia* (12.7 %). *Gammaproteobacteria* and *Bacteroidia* (10.5 % and 2.86 %) were significantly lower than those found at the Inlet of the two previous samplings; *Campylobacteria* (*Epsilonproteobacteria*) (5.37 %) were higher than in Autumn and Winter Inlet points. Data from the Middle and Outlet points were unavailable due to DNA extraction and amplification problems caused by low environmental DNA concentrations during this sampling.

In Summer (July) the abundance of *Gammaproteobacteria* increased from 39.9 % at the Inlet to 79.1 % at the Outlet. *Bacteroidia* were quite stable from the Inlet (11.67 %) to the Outlet (12.35 %); *Saccharimonadia*,

Clostridia, *Bacilli*, *Campylobacteria* decreased from the Inlet (12.10 %; 11.76 %; 6.23 %; 5.85 %) to the Outlet (0.90 %; 0.06 %; 0.03 %; 1.14 %).

Overall, *Saccharimonadia*, *Clostridia*, *Bacilli*, and *Campylobacteria* decreased from the Inlet to the Outlet in the Autumn (November), Winter (January), and Summer (July) samplings. These classes include several pathogens and antibiotic resistant bacteria (ARB).

Gammaproteobacteria were the predominant bacterial class in the sediment samples, followed by *Alphaproteobacteria* (Fig. 6). In Autumn (Nov), *Gammaproteobacteria* (which includes *Enterobacteriaceae*, *E. coli*, *Salmonella*, etc.) were more abundant in the VF than in AE and other classes (e.g. *Vicinamibacteria* and *Acidimicrobia*) were also detected. The second most abundant bacterial class, *Alphaproteobacteria*, was 11.9 % in the Vertical Flow bed and 15.3 % in the Aerated bed. *Actinobacteria* had higher relative abundance in the VF (7.4 %) than in the AE (6.8 %) bed. *Nitrososphaeria* and *Nitrospiria* were present in both the VF (1.5 % and

4.3 %, respectively) and the AE (1.9 % and 2.3 %, respectively) beds.

In Winter (Jan), *Alphaproteobacteria* showed a relative abundance of ca. 12.5 %, followed by *Bacteroida* (9.7 %); *Nitrososphaeria* and *Nitrospira* were detected in the VF at 3.7 % and 2.3 %, respectively, and in the AE at 3.3 % and 4.2 %, respectively. In Spring (May), *Alphaproteobacteria* had relative abundance of ca. 12.0 % followed by *Bacteroida* (7.5 %). *Verrucomicrobiae* and *Vicinamibacteria* were detected in all samples with similar relative abundances. *Nitrososphaeria* and *Nitrospira* were detected in both the VF (3.9 % and 6.3 %) and the AE (3.6 % and 3.9 %) beds.

In Summer (July), *Alphaproteobacteria* showed an average of 12.7 % followed by *Vicinamibacteria* (8.1 %). *Nitrososphaeria* and *Nitrospira* were detected both in the VF (5.6 % and 5.7 %) and the AE (3.2 % and 3.7 %) beds. Finally, *Verrucomicrobiae* and *Bacteroida* were detected in all the samples with similar relative abundances.

Significant seasonal differences in the relative percentages of microbial classes were observed in both wastewater and sediment, due to changes in both biotic factors (e.g. bacteria entering with wastewater in the system) and abiotic conditions (e.g. temperature), which directly or indirectly influenced the overall plant-microbiome system performance. The TW efficiently reduced bacterial abundances and ARGs, as well as micropollutants thanks to the synergistic actions in rhizosphere between the resident sediment microbial community and reed roots.

Fig. 7 shows the heatmap of the main genera identified in water samples, illustrating how seasonal changes partially affected their distribution. Results from Spring and Summer are displayed on the left, while those from Autumn and Winter are shown on the right. *Acinetobacter* and *Flavobacterium* showed higher abundances during Autumn and Winter than in Spring and Summer. *Pseudomonas* was consistently one of the most abundant genera, but showed lower percentages at the Inlet during Spring and Summer, where high abundances of *Saccharimonadales* were observed. *Aquabacterium* and *Rheinheimera* were detected at the Autumn and Winter samplings. In Summer, a high abundance of *Limnohabitans* at the Middle point and *Malikia* at the Outlet were also found. *Acinetobacter*, *Aeromonas*, *Arcobacter*, *Comamonas*, *Pseudoarcobacter*, *Rheinheimera*, *Saccharimonadales* and *Saccharofermantas* (genera which include pathogens and ARB; Howard et al., 2012; Sciortino et al., 2021; Ryan et al., 2022; Neil et al., 2024)

significantly decreased ($p < 0.05$) from the Inlet to the Outlet in Autumn, Winter, and Summer samplings, demonstrating the effectiveness of the TW in reducing AMR during these seasons.

Fig. 7 reveals several genera with key roles in nutrient cycling. Genera involved in the nitrogen cycle, such as *Denitromonas*, *Nitrosarchaeum*, *Nitrospira* and *Nitrospira* were present (Schramm et al., 1999; Nogueira and Melo, 2006; Kalu et al., 2021; Wang et al., 2022). Notably, *Nitrospira* was detected in all seasons, with the highest abundances in the VF bed during the Spring and Summer samplings. The genus *Vicinamibacteriaceae*, known for its ability to remove phosphorus (Kristensen et al., 2021; Wang et al., 2022; Zhang et al., 2024), was also detected in the AE bed.

Overall, bacterial genera in both wastewater and sediment (e.g. *Pseudomonas*, *Flavobacterium*, and *Acidovorax*) capable of degrading sulphonamides (Wang et al., 2022; Li et al., 2024) and other micro-contaminants, such as bisphenol A, organochloride pesticides, and herbicides (Bodour et al., 2004; Kalu et al., 2021; Wang et al., 2022; Sánchez-Castro et al., 2024) were identified. Interestingly, the fact that *Acinetobacter* and *Aeromonas*, comprising common ARB pathogens (Igbino et al., 2012; Bouki et al., 2013; Almasaudi, 2018), decreased in percentages from the Inlet to the Outlet (particularly in Autumn) suggests an active role of the sediment microbial community in cooperation with plant roots in removing these waterborne bacteria. Partial adsorption of some OMPs onto the sediment cannot be excluded; however, the extensive sampling and chemical analyses conducted over more than a year indicate that only a very small amount of chemicals is likely present in the effluent water.

Overall results show how the plant-microbiome system was effective in both outcompeting allochthonous bacteria from wastewater, including those carrying ARGs, and in OMP removal. However, this effect was less evident in winter because low temperatures (winter dormancy) affected not only plant physiology and exudate production (which favours microbial metabolism), but also slowed the activity of their associated microbiome. Moreover, the possibility that some ARB survived in winter and maintained plasmids (in this case containing ARGs for *sul2*) as a response to the stress for low-temperatures cannot be excluded. This phenomenon has been observed in some pathogenic

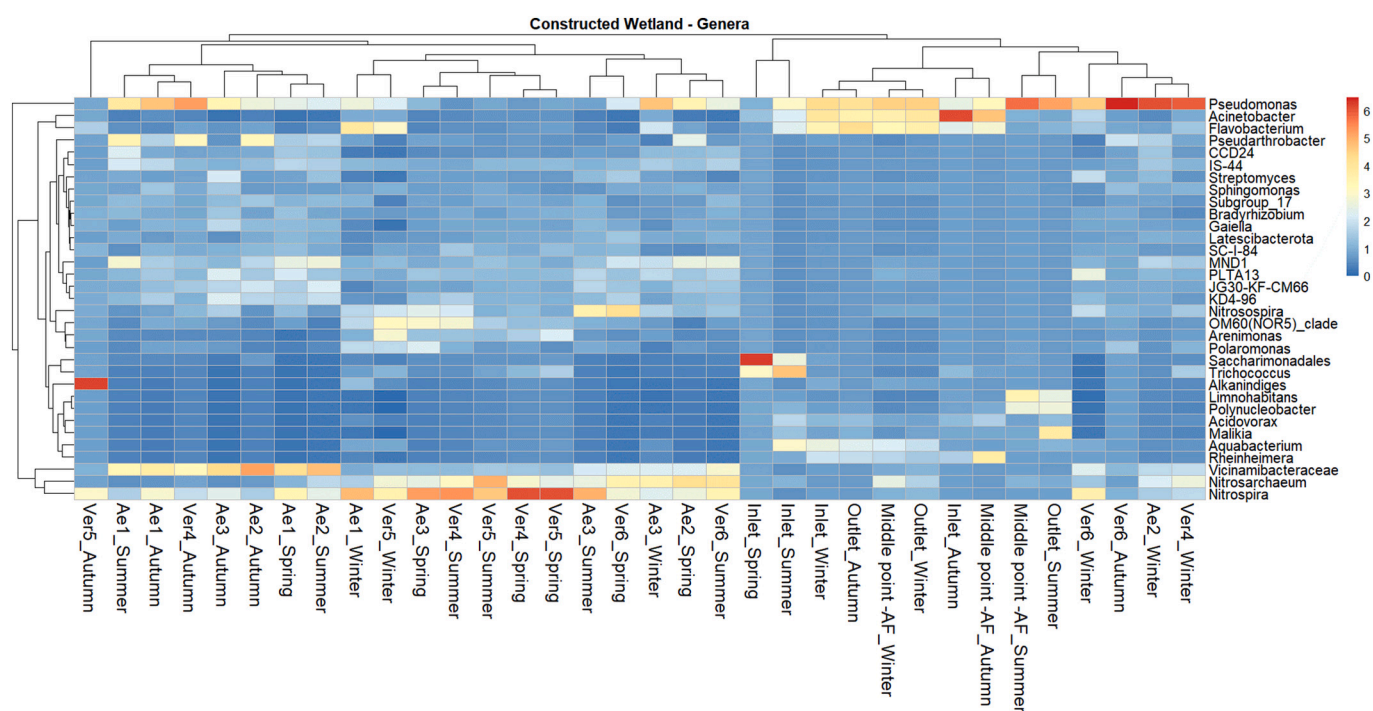


Fig. 7. Heatmaps of genus abundances in water and sediment samples during Autumn (November), Winter (January), Spring (May), and Summer (July) samplings. The colour scale indicates the abundance of each genus, ranging from the lowest (blue) to the highest (red).

species of *E. coli* (Wein et al., 2019; Vega et al., 2021) and *Flaviobacterium* (Satcioglu et al., 2019) and in some WWTPs (Miller et al., 2014; Harnisz et al., 2020).

ARG removal in conventional centralized WWTPs are quite variable with percentages from 40 % to 80 % for *sul1* and *sul2*, depending on operational conditions, influent composition, and microbial community dynamics (Michael et al., 2013; Pallares-Vega et al., 2019; Luo et al., 2025). Our values are comparable to or even exceeded the highest values reported for conventional centralized WWTPs. In fact, the removal efficiencies of N. gene copies/16S ranged from 47.2 to 99.9 % for *sul1*, from 64.1 to 99.9 % for *sul2* (excluding Winter) and from 86.5 to 99.6 % for *int1*. Moreover, considering the gene copies number and wastewater volume (mL) ratio (Fig. S2, Supplementary Material 1), the removal resulted higher (i.e. 86.4–99.9 % for *sul1*, 91–99.9 % for *sul2* and 97.2–99.9 % for *int1*). Our results are also in line with those reported for constructed wetlands from other Authors (Ávila et al., 2021; Abou-Kandil et al., 2021) supporting the useful application of these systems, with a suitable configuration and combination of vegetation and substrate type, for ARG removal.

TWs and conventional WWTPs differ substantially in design, capacity, and microbial mechanisms, this comparison underscores that mature, well-maintained TWs can achieve quite high ARG removal efficiencies, making them suitable for decentralized applications where advanced infrastructure is unfeasible.

3.5. Major findings, limitations and future perspectives

This study makes an important contribution by evaluating a full-scale TW operating over ten years, compared to previous studies often based on laboratory-scale systems (Vymazal, 2010; Parde et al., 2021). Bai et al. (2022) demonstrated that full-scale constructed wetlands can successfully remove antibiotics and manage ARGs, supporting our findings on TWs' long-term performance. Our results for secondary wastewater treatment are in line with those found previously by Sabri et al. (2021) for a full-scale system performing tertiary treatment. The studied system performed well overall, and the observed winter reduction in efficiency for conventional pollutants aligned with the know how in the field and best practices for design (Kadlec and Wallace, 2008). Despite the seasonal decline, our system maintained significant ARG removal, potentially due to the resilience of *Phragmites australis* and its rhizosphere microbiome, a key factor also highlighted by Liu et al. (2019), Vymazal (2010) and Ma et al. (2022), who emphasized the critical role of plants in pollutant removal and ARG suppression. While interesting to observe the seasonal decline affecting to antibiotics and other pharmaceuticals, the current experimental approach cannot exclude that other factors beyond temperature, or a combined effect of temperature and plant dormancy can influence its efficiency. It should be named also the key limitations of the present study: the lack of detailed loading rates and hydraulic loading rates that limit comparison with detailed laboratory studies, as well as sporadic occurrence of compounds that provides lower statistical robustness. Nevertheless, the winter sampling provided valuable insight into the system's seasonal behavior, confirming that ARGs can persist despite the absence of detectable antibiotics, as suggested by both Bai et al. (2022) and Wein et al. (2019), who highlighted that environmental stressors, rather than antibiotic presence alone, influence ARG dynamics. Our hybrid bed system with aerated vertical flow beds likely provided superior oxygenation and stability for microbial communities, making it possible a wide variety of bacterial development and metabolism. This hybrid system may explain why our study found no ARG proliferation, a concern noted by Song et al. (2018), who reported that wetlands could sometimes induce ARGs in specific conditions.

In terms of future research, exploring plant species selection and design elements to improve cold-weather performance seems relevant. For instance, using novel substrates with enhanced sorption capacity like biochars (Duan et al., 2022) and/or exploring different hybrid

configurations and potentially combining with microbial fuel cells (Colares et al., 2022) to improve performance under varying environmental conditions. Expanding the range of targeted compounds assessed in TWs, as the presented study does, offers a broader understanding of TWs' capabilities in wastewater treatment. Additionally, more research is needed for different types of TW operating under real world conditions, e.g. investigating horizontal and other hybrid designs could further enlighten the optimization of TW performance for the treatment of emerging contaminants.

4. Conclusions

The findings of this study offer new insights, with an ecological approach which combined chemical determinations with different molecular methodologies, on TW performance in removing OMPs and ARGs under field conditions. Our results align with previous studies that highlight the efficacy of TWs in wastewater treatment. Notably, this study provides long-term, full-scale data, which is limited in the existing literature. The treatment wetland was effective in decreasing emerging contaminants, including antibiotics and ARGs, due to the synergic interactions between *P. australis* (common reed) and its rhizosphere microbiome. The rhizosphere microbiome was able to decrease significantly total bacterial burden, counteracting resistant bacteria from the wastewater and remove OMPs, antibiotics and ARB. The removal efficiency decreased in winter, which negatively affected microbial activity and plant-microbiome interactions. These results provide some of the first data on a real-world application of a functioning treatment wetland, supporting the use of this eco-technology not only in rural areas but encouraging future adaption of such systems as decentralized ones for more densely populated areas.

CRediT authorship contribution statement

Vaidotas Kisieličius: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Anna Barra Caracciolo:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration. **Arianna Mingoli:** Visualization, Methodology, Investigation, Data curation. **Edoardo Parisi:** Visualization, Methodology, Investigation, Data curation. **Alba Martinez i Quer:** Writing – review & editing, Writing – original draft, Investigation. **Gian Luigi Garbini:** Supervision, Investigation, Data curation. **Paola Grenni:** Supervision, Investigation, Funding acquisition. **Pedro N. Carvalho:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

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.scitotenv.2025.180406>.

Data availability

All raw 16S rRNA amplicon sequencing data and associated meta-data have been deposited in the NCBI database under accession number PRJNA1260927. Additional datasets generated or analysed during this study are included in the Supplementary Materials of this manuscript.

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