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Nature-based solutions for antibiotics and antimicrobial resistance removal in tertiary wastewater treatment: Microbiological composition and risk assessment

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ABSTRACT

This field-scale study evaluates the seasonal effectiveness of employing nature-based solutions (NBSs), particularly surface flow and horizontal subsurface flow constructed wetland configurations, as tertiary treatment technologies for the removal of antibiotics (ABs) and antibiotic resistance genes (ARGs) compared to a conventional treatment involving UV and chlorination. Out of the 21 monitored ABs, 13 were detected in the influent of three tertiary wastewater treatments, with concentrations ranging from 2 to 1218 $ng \cdot L^{-1}$. The ARGs *sul1* and *dfrA1* exhibited concentrations ranging from 1×10^5 to 9×10^6 copies/100 mL. NBSs were better at reducing ABs (average 69 to 88 %) and ARGs (2–3 log units) compared to the conventional tertiary system (average 36 to 39 % and no removal to 2 log units) in both seasons. Taxonomic compositions in influent water samples shifted from wastewater-impacted communities (*Actinomycetota* and *Firmicutes*) to a combination of plant rhizosphere-associated and river communities in NBS effluents (*Alphaproteobacteria*). In contrast, the conventional technology showed no substantial differences in community composition. Moreover, NBSs substantially reduced the ecotoxicological risk assessment (cumulative RQs). Furthermore, NBSs reduced the ecotoxicological risk (cumulative RQs) by an average of over 70 % across seasons, whereas the benchmark technology only achieved a 6 % reduction. In conclusion, NBSs present a robust alternative for minimizing the discharge of ABs and ARGs into surface water bodies.

1. Introduction

Climate change together with the high demand for water resulting from human population growth and the continued expansion of industry and agriculture reduce considerably the quality and quantity of available freshwater resources (Mosquera-Romero et al., 2023). Recognition of the need for better stewardship of water resources has led to protective legal frameworks in multiple countries (e.g. Clean Water Act in the United States, Water Framework Directive in the European Union (EU), Water Law of China). Increasingly, these regulatory frameworks aim to protect watersheds from an expanded battery of contaminants, including those related to antimicrobial resistance (AMR) such as antibiotics (AB) and antimicrobial resistance genes (ARG), as their presence may pose a risk to aquatic ecosystems and ultimately human health (Kuster et al., 2008). For example, several broad-spectrum ABs such as clindamycin (CLI), miconazole (MIC), ofloxacin (OFL), sulfamethoxazole (SMX), and trimethoprim (TMP) are included in the EU Watchlist 2022 as compounds that may have ecotoxicological relevance (European Commission and Directorate-General for Environment, 2022). Moreover, the continuous exposition of aquatic bacteria to ABs can enhance ARG prevalence, thereby contributing to the substantial role of the aquatic environment as a reservoir and distribution pathway for AMR (EU/EEA, 2021). This is a critical issue as mortality rates due to AMR pathogens are increasing worldwide (World Health Organization, 2024).

ABs can reach aquatic systems through multiple sources such as urban and industrial wastewater (Kümmerer, 2009). Wastewater treatment plants (WWTPs) are significant contributors to the introduction of ABs and ARG into the environments (Rizzo et al., 2013; Subirats et al., 2017). Without counteracting measures, the concentrations of

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AMR-related contaminants in many surface waters are likely to increase due to the growing consumption of ABs in food animal production and human medicine (Klein et al., 2018), coupled with higher fractions of wastewater (treated and untreated) in rivers as a consequence of more frequent droughts. This double jeopardy is of particular concern in several southern European countries including Spain, which have comparatively high average AB effluent concentrations (Rodriguez-Mozaz et al., 2020), and treated wastewater fractions in rivers of up to 100 % in summer due to water scarcity that affect up to 29 % of the population (European Union, 2023).

To address these challenges, treatment technologies such as advanced oxidation processes (e.g. ozonation), photocatalysis, chlorination, and membrane processes (e.g. ultrafiltration) have been proposed as tertiary wastewater systems for reducing the presence of ABs and ARGs in the aquatic environment (Gorito et al., 2017; Pazda et al., 2019). However, all of these systems have high costs for construction, maintenance, and energy (Dhangar and Kumar, 2020). Nature-based solutions (NBS) such as constructed wetlands (CWs) are alternative low-cost energy technologies (Thorslund et al., 2017). These systems have already been shown to improve general water quality parameters and to reduce several emerging pollutants such as pharmaceuticals from wastewater at pilot- and field-scale (Ilyas et al., 2020; Matamoros et al., 2008). However, the presently available information on ABs and ARGs is still limited, as it mainly pertains to some pilot-scale studies with synthetic wastewater (Chen et al., 2019; Xu Huang et al., 2017) or to full-scale configuration but without considering seasonality (Ávila et al., 2021; Du et al., 2022). Seasonality in NBS is pivotal because changes in temperature and daylight hours directly influence biodegradation and photodegradation rates which, in turn, affect pollutant reduction (Escolà Casas and Matamoros, 2020).

In addition to the presence of pollutants such as ARGs and ABs, the microbiological composition of water effluent has a significant impact on natural ecosystems health, specially when this water is utilized for river recovery or agricultural irrigation (Esser et al., 2023). However, there is a scarcity of studies focusing on the microbiological composition of water effluents from CWs at field scale (Zhu et al., 2021), and none have explored them as tertiary wastewater treatment approaches.

In this study, we evaluate the seasonal effectiveness (in summer and winter) of two full-scale CW configurations—Surface Flow (SFCW) and Horizontal Flow (HFCWs)—in enhancing general water quality parameters and reducing concentrations of ABs and ARGs. Furthermore, we compare the performance of these NBS with that of a conventional tertiary WWTP for reference. Lastly, we conduct an analysis of microbiological composition and perform theoretical risk assessments, comparing inlet points and effluents for each system. The novelty of this study is twofold: firstly, the seasonal monitoring of field-scale tertiary wastewater NBS for the removal of ABs and ARGs and the assessment of microbiological shifts; and secondly, in the comparison of its findings with those of a prevailing reference tertiary wastewater technology.

2. Material and methods

2.1. Site description and sampling

To evaluate the effectiveness of NBS as a tertiary system, two configurations of CWs were monitored. In brief, the SFCW (Fig. 1**a**) is located in NE Spain (Can Cabanyes) and consists of one cell built with compacted clay, spanning an area of 10,000 m^{2}. There are two different water depths, 0.3–0.4 m and 1.5 m. The SFCW is planted by *Typha* sp. and *Phragmites australis*. The total surface area is 1 ha. This NBS received secondary-treated effluent of the Granollers WWTP (112,154 PE with a capacity of 1250 m^{3}·day^{-1}) at a water flow rate of 100 m^{3}·day^{-1}. The hydraulic retention time (HRT) of this NBS was approximately 15 days. Three sampling points were monitored (inlet point, intermediate point and effluent point), one day per week during one month for the summer (July 2022) ($n = 12$) and the winter campaigns (February 2023) ($n = 12$).

The other NBS monitored are 60 parallel HFCWs built along a 3.2 km stretch of the Besòs basin river (Fig. 1**b**). The HFCW were constructed with gravel with granulometric grain size between 6 and 25 mm. Each HFCW had a width of 50 m and a length ranging from 19 to 35 m. The water depth was 0.6 m at the inlet points and 0.7–0.8 m at the outlet point with a 0.5 % slope and the total surface area was 18,000 m^{2}. In all HFCWs, the main plant was *P. australis*, which covered the entire wetland surface. The HFCWs received roughly 30 % (21,800 m^{3}·d^{-1}) of the secondary-treated effluent from the Montcada and Reixac conventional WWTP (423,500 population equivalent (PE) with a capacity of 72,600 m^{3}·day^{-1}), with a HRT of approximately 4 days. The influent point and 13 HFCWs were monitored during the summer and autumn campaigns with a sampling period of 1 day per week for 2 consecutive weeks in the summer (July 2022) ($n = 14$) and autumn (October 2022) ($n = 14$). The HFCW systems were closed during winter. Additional information on the HFCW and SFCW can be found elsewhere (Huertas et al., 2006; Matamoros et al., 2008, 2017).

To compare these two NBSs, a conventional tertiary T-WWTP (Fig. 1**c**) in Lloret de Mar (185,000 PE and a capacity of 36,000 m^{3}·day^{-1}) was selected as a reference technology. This T-WWTP consisted of a chlorination stage (4 mg·L^{-1}) and UV radiation. Three sampling points were monitored: the influent point, which corresponds to the effluent of the secondary WWTP (P1), after chlorination (P2), and the effluent after UV radiation (P3). The reference site was monitored for two weeks ($n = 6$) in the summer campaign (July 2022) and one week ($n = 3$) during the winter campaign (February 2023).

2.2. Analytical methodologies

2.2.1. General water quality parameters

Twelve conventional wastewater quality parameters were monitored for all the sampling sites. Conductivity, redox, dissolved oxygen (DO), and temperature were determined in situ using HQ4300 Portable

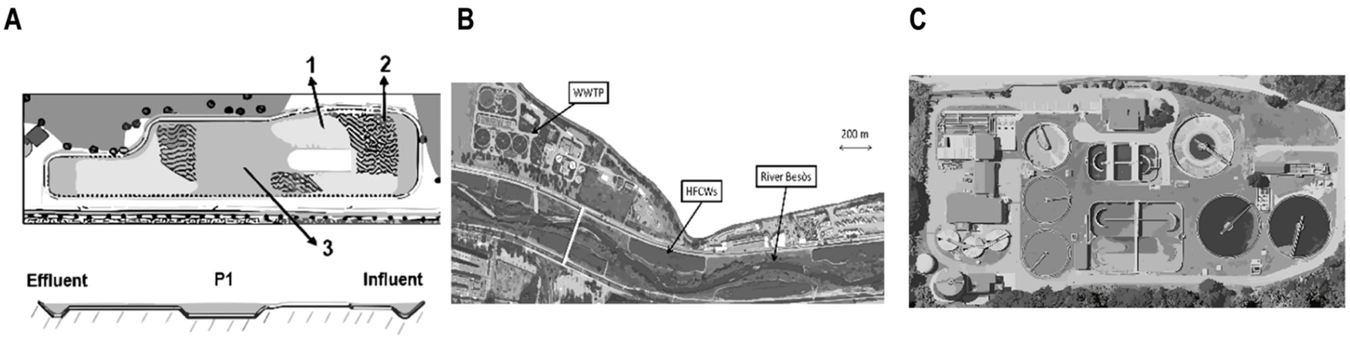

Fig. 1. Schematic map of the sampled tertiary systems. (A) SFCW system (1: *Thypa* sp.; 2: *Phragmites australis;* 3: Unplanted deep zones). (B) Aerial image of Montcada i Reixac WWTP, a selection of some HFCWs.(C) The conventional T-WWTP (reference technology).

multimeter with their corresponding probes (Intellical CDC401, Intellical MTC301 and LDO10105) from HACH (Loveland, USA). On the same day, pH was measured using a probe from Hach and turbidity with a portable turbidimeter M10 from Labbox (Premià de Dalt, Spain). Photometric tests with a Hach Lange DR 1900 portable Spectrophotometer were used to determine seven water quality parameters: ammonia (NH_4^+) (LCK 303 and 304), chemical oxygen demand (COD) (LCK 614), nitrates (NO_3^-) (LCK 340 and 339), nitrites (NO_2^-) (LCK 341), phosphates (PO_4^{-3}) (LCK 349), sulfate (SO_4^{-2}) (LCK 153), and total organic carbon (TOC) (LCK 380). Concentrations of total solid suspended (TSS) were gravimetrically determined with Whatman® (Maidstone, United Kingdom) glass microfiber filters, grade 394-AH®.

2.2.2. *Antibiotics analysis*

ABs were determined with a methodology previously described by Rodriguez-Mozaz et al., 2015. Briefly, 100 mL of filtered water samples were percolated through polymeric SPE cartridges, then eluted and injected into a UPLC-QToF device. Further details are described in the SM section.

2.3. *DNA extraction, 16S rRNA gene amplicon sequencing, and quantitative analyses of ARGs*

Water samples were filtered (Omnipore™ filters 0.2 μm PTFE (Merck, Massachusetts, USA) and kept in the freezer at –20 °C until analysis. DNA extraction, 16S rRNA gene amplicon sequencing, and data analysis were carried out as previously described (Haenelt et al., 2023). In brief, biomass was removed from the filters by agitation with zirconium beads in BE buffer, and DNA extraction was carried out using a NucleoSpin Microbial DNA Kit (Macherey Nagel, Germany) following the manufacturer's protocol. The 16S rRNA gene amplicon libraries were prepared with PCR primers targeting the variable region V3 of the 16S rRNA gene using forward primer 341bf. The libraries were generated with an Illumina NextSeq 500/550 High Output Kit v2.5 and sequenced on an Illumina Nextseq-System (Illumina, San Diego, CA, USA). Data analysis was carried out with the software package QIIME 2 (Bolyen et al., 2019). and DADA2 (Callahan et al., 2016). Taxonomic assignments were carried out in QIIME 2 using a pretrained classifier "Silva 138.1 99 % OTU full-length sequences", and manually curated in R using phyloseq (McMurdie and Holmes, 2013). Alpha diversity was assessed by computing the Observed Richness and Shannon diversity indices. For beta diversity, a Principal Coordinates Analysis (PCoA) plot using Emporer was generated from Bray Curtis dissimilarities in QIIME 2 (Bolyen et al., 2019). Aliquots of the same extracted DNA were used for qPCR-based determination of AMR indicator gene abundances. Further details are described in the SM section.

2.4. *Risk assessment*

Here, the risk quotient (RQ) was selected as a parameter by the European Environment Agency for risk assessment, aiming to identify priority substances with a highly protective approach against impacts on organisms across multiple trophic levels (Finckh et al., 2022). Predicted no-effect concentration (PNEC) values used were determined by experimental eco-toxicity studies with cyanobacteria and algae, which are considered to be more sensitive to antibiotics (Rodriguez-Mozaz et al., 2020). European Medicines Agency (EMEA) for Environmental Protection Agency (EPA) guidelines was used to determine a theoretical risk characterization for ABs detected in influents and effluent points for the NBSs and reference T-WWTP. Individual (eq. (1)) and mixture (eq. (2)) risk quotients (RQs) were estimated by dividing predicted environmental using average concentrations detected in influent and effluents points (PEC) with PNEC from freshwater as risk assessment studies have been reported (Finckh et al., 2022; Gros et al., 2010). PNEC values were extracted from the NORMAN Ecotoxicology Database for green algae and fish (NORMAN Ecotoxicology Database).

$$RQ_{\mathrm{individual}} = \frac{\mathrm{PEC}}{\mathrm{PNEC}} \tag{1}$$

$$RQ_{\mathrm{mixture}} = \sum_{i=1}^{n} RQ_{\mathrm{individual}} \tag{2}$$

The lowest PNECs based on experimental eco-toxicity data were used to achieve the most protective scenario for all organisms. In case experimental PNEC values were unavailable, the quantitative structure-activity relationship (QSAR) predictions were used to estimate provisional PNEC values. Three categories to evaluate the RQ values were previously defined (European Commission Joint Research Center and TGD PART II, 2003):

RQ ≤ 0.1	→ Insignificant environmental risk
0.1 < RQ < 1	→ Moderate environmental risk
RQ > 1	→ High environmental risk

To assess potential risk selection (PMRS) in each monitored water sample, we employed PNEC values (resistance selection) previously documented in (Bengtsson-Palme and Larsson, 2016). These values have been widely utilized in various studies to assess resistance development (Monahan et al., 2021). RQs were estimated as described above, using Eqs. (1) and (2).

2.5. *Data analysis*

The target compounds list included 22 ABs for 9 different categories (beta-lactams, dihydrofolate reductase inhibitors, fluoroquinolones, ionophore, lincosamides, macrolides, rifamycin, sulfonamides and tetracyclines) and one metabolite (acetyl-sulfamethoxazole) (**Table S1**). ABs were selected based on several studies developed by authors that have been devoted mainly to these AB categories selected in wastewater samples (Hazra et al., 2022; Michael et al., 2013). The data obtained were processed with TASQ® software version 6.0.0.1 (Bruker).

Average removal efficiencies for parameters and ABs (%) were calculated using the effluent concentration of the WWTP (C_{effluent}) and treated concentration (C_{treated}) for each sampling point along the seasons using Eq. (3).

$$Removal\ effiency\ (\%) = \left(1 - \frac{C_{effluent} - C_{treated}}{C_{effluent}}\right) x\ 100 \tag{3}$$

AB removal values were treated with R Studio Version 4.3.1 with the "rapportools" package, and the ANOVA test was used to detect statistical differences between removals observed by the treatments monitored per each season. Additionally, Tukey's Honestly Significant Difference (Tukey's HSD) was used as a statistical post hoc test to identify the specific group that differs from the others. Moreover, the removal of the same AB detected during the season in SFCW and HFCWs were treated with the "dplyr" and the non-parametric Mann-Whitney U test. For all the statistical tests were consider a p-value lower than 0.05 was assumed to indicate the significance of an analysis.

3. Results and discussion

3.1. *General water parameters for each treatment system*

Table S2 shows all general quality parameters of water at influent and effluent points during the sampling campaigns conducted in both seasons for each of the assessed tertiary wastewater treatment technologies. On average, SFCW had positive redox values at the water effluent throughout the seasons, showing aerobic conditions (61.9 to 145.9 mV) with positive DO values (2.8 to 9.7 $mg \cdot L^{-1}$). Redox potential and DO were lower at the effluent during summer, suggesting greater oxygen consumption, likely due to increased organic matter degradation associated with higher temperatures. On the other hand, HFCWs had negative redox values in the water effluent, indicating anaerobic conditions (−133.5.0 to −182.0 mV, on average) (Hussein and Scholz, 2017) with low DO values (< 0.3 mg/L). The concentration of NH_4^+ was reduced in the SFCW, indicating high to moderate nitrification capacity in both seasons, 88 % and 57 % on average in the warm and cold seasons, respectively. The reduction in nitrification during the cold period could be explained by the lower plant growth and microbial activity during this season (Matamoros and Rodríguez, 2017). On the other hand, the monitored HFCW systems had low nitrification capacity with < 15 % ammonia removal on average for both seasons and NO_3^- concentrations were equal to or slightly higher than the influent values. This is consistent with the low DO concentration and negative redox potential observed for HFCWs. An increase in COD effluent values was observed during the warm seasons in the SFCW. This type of wetland configuration enhances the proliferation of microalgae, phytoplankton and zooplankton, increasing the COD values in the effluent point. Nevertheless, during the cold season, when plant growth activity is low, COD values remain similar to those at the influent point (Almuktar et al., 2018). This behaviour is directly linked with TOC values, which showed low removal or no removal. HFCWs maintained similar values for COD and TOC in both sampling campaigns. Furthermore, the concentrations of PO_4^{-3} values were minimized at the effluent points of the SFCW during the warm and cold seasons with more than 60 % removal on average. However, HFCWs did not reduce phosphates and maintained similar values as the influent point. Various hypotheses can explain the diverse behaviors observed among NBS. For example, SFCW and HFCWs had the same major plant (*P. australis)* but the materials used for the construction were different. The compacted clay used in the SFCW has a high capacity to adsorb PO_4^{3-}, which helped to remove more phosphate than the HFCWs built with gravel (Bolton et al., 2019; Xu et al., 2022). On the other side, the proliferation of microalgae within the SFCW can act as an effective means of phosphate removal (Singh et al., 2018). The other water quality parameters conductivity, turbidity, pH, NO_2^-, SO_4^{-2} and TSS values were constant along the NBSs between seasons (Supplementary Material: Table S2). The reference T-WWTP did not demonstrate any improvement in the evaluated water quality parameters, as it was primarily designed for disinfection. The findings indicate that NBS, particularly in the SFCW configuration, offer a more effective solution for nutrient removal (N and P). Consequently, the implementation of NBS may contribute to a reduction in the eutrophication of the receiving surface water systems.

3.2. *Occurrence and attenuation of ABs*

The concentration of ABs in the monitored secondary-treated wastewater effluents ranged from 5 to 1218 ng·L^{-1} for the summer season and 5 to 803 ng·L^{-1} for the cold season (Table 1). Thirteen of the 22 ABs monitored were detected at least in two sampling campaigns. Azithromycin (AZI) and sulfamethoxazole (SMX) were the antibiotics detected at the highest concentration range, aligning with findings from other published studies on the assessment of AB occurrence in WWTP effluents (Solaun et al., 2021). Regarding fluoroquinolones, ofloxacin (OFL), levofloxacin (LVX) and ciprofloxacin (CIP) were detected in all sampling sites except for OFL during the winter campaign in SFCW and CIP in T-WWTP for the same season. The continuous detection of these fluoroquinolones could be explained by their constant use as broad-spectrum ABs for veterinary and human use (Janecko et al., 2016). NBS were able to achieve a high attenuation efficiency (85 to 100 %, on average). Despite their low log k_{ow}, fluoroquinolones have a high tendency to be adsorbed to the substrate according to their reported high K_D values, ranging from 2100 to 2300 L·Kg^{-1} (Fabregat-Palau et al., 2023). This observed behavior helps to understand their reduction in both NBSs compared to the reference technology, where removal efficiencies were not higher than 31 % on average. Concerning lincosamides, clindamycin (CLI) showed a higher concentration than lincomycin (LIN) in most of the sampling points. Different removal

Table 1
Average influent concentration [ng·L^{-1}], removal efficiencies (%) and respective standard deviations of ABs detected in the warm and cold season for the two studied NBSs (SFCW and HFCW) and the reference technology (T-WWTP).

Category	AB	SFCW[a]				HFCWs[a]				T-WWTP[b]			
		SUMMER		WINTER		SUMMER		AUTUMN**		SUMMER		WINTER	
		Concentration [ng·L^{-1}]	Removal (%) *	Concentration [ng·L^{-1}]	Removal (%) *	Concentration [ng·L^{-1}]	Removal (%)*	Concentration [ng·L^{-1}]	Removal (%)*	Concentration [ng·L^{-1}]	Removal (%)	Concentration [ng·L^{-1}]	Removal (%)
Dihydrofolate Reductase inhibitor	TMP	176 ± 16	100 ± 1[a]	269 ± 90	79 ± 2[a]	107 ± 38	100 ± 1[a]	5 ± 1	91 ± 6[b]	< LOD	–	209 ± 9	52
Fluoroquinolones	CIP	40 ± 7	100 ± 1[a]	235 ± 71	100 ± 1[a]	211 ± 86	100 ± 1[a]	38 ± 6	97 ± 4[a]	< LOD	–	360 ± 24	31
	LVX	168 ± 40	100 ± 1[a]	116 ± 15	85 ± 3[a]	149 ± 10	92 ± 15[a]	456 ± 17	90 ± 4[a]	377 ± 188	36 ± 25	294 ± 5	n.r
	OFL	206 ± 50	100 ± 1[a]	8 ± 4	100 ± 1[a]	155 ± 39	100 ± 1[a]	9 ± 2	94 ± 1[a]	68 ± 3	12 ± 5	31 ± 1	n.r
Lincosamides	CLI	336 ± 32	66 ± 6	115 ± 4	32 ± 11	103 ± 57	n.r	62 ± 29	n.r	213 ± 57	100 ± 1	96 ± 13	100
	LIN	121 ± 29	73 ± 5[a]	5 ± 2	100 ± 1[a]	24 ± 7	64 ± 24[a]	23 ± 6	57 ± 15[a]	< LOD	–	< LOD	–
Macrolides	AZI	587 ± 131	97 ± 4[a]	444 ± 206	74 ± 13[a]	966 ± 56	93 ± 8[a]	529 ± 45	96 ± 1[b]	1218 ± 715	n.r	803 ± 128	n.r
	CLA	5 ± 1	100 ± 1[a]	39 ± 7	76 ± 6[a]	68 ± 12	46 ± 26[b]	6 ± 1	21 ± 6[b]	130 ± 48	46 ± 24	90 ± 8	n.r
Sulfonamides	ASMX	< LOD	–	< LOD	–	62 ± 26	70 ± 16	61 ± 24	88 ± 6	< LOD	–	7 ± 1	n.r
	SMX	800 ± 183	70 ± 6[a]	289 ± 30	61 ± 7[a]	329 ± 101	77 ± 31[a]	204 ± 84	60 ± 3[a]	< LOD	–	281 ± 22	72
Rifamycin	RIF	< LOD	–	12 ± 2	100 ± 1	< LOD	–	< LOD	–	< LOD	–	13 ± 1	81
Tetracyclines	DOX	< LOD	–	482 ± 290	68 ± 27	< LOD	–	< LOD	–	< LOD	–	182 ± 10	17
	OXY	304 ± 28	78 ± 22[a]	17 ± 9	77 ± 14	114 ± 13	72 ± 12[a]	< LOD	–	< LOD	–	11 ± 1	77
TOTAL		2743	88	2031	79	2288	74	1393	69	2006	39	2377	36

[a,b] Letters show statistically significant differences; LOD: Limit of detection indicated in Table 1 Supplementary material for each AB; n.r: no removal observed;.

[*] Values were corrected by the evapotranspiration for both seasons;.

[**] Sampling campaigns were performed in autumn before the HFCWs system closed.

behaviors were observed for both ABs. SFCW was efficient to attenuate LIN (73 to 100 % on average) but had moderate to low capacity to reduce CLI (32 to 66 % on average). HFCWs were able to attenuate LIN but not CLI. However, the reference T-WWTP was able to remove CLI in both seasons (100 % on average). Tran et al., 2016 reported that attenuation of lincosamides was possible through biodegradation performed by nitrifying bacteria. This evidence could explain how SFCWs could remove more lincosamides than HFCWs with a low nitrification capacity. On the other hand, advanced processes such as advanced filtration could be helped to have high removal values for lincosamides with similar behavior observed in the reference site monitored that was able to remove CLI (Michael et al., 2013). Furthermore, 2 macrolides were detected (AZI and clarithromycin (CLA)) during the monitored study. AZI was the most abundant AB detected (> 350 ng·L^{-1} on average) in all the sampling sites along the seasons with similar concentrations in influents and effluents as previously reported (Fonseca et al., 2020; Ng et al., 2023). Macrolides are mainly used in the treatment of human infections and for animals for the dual function of treating infections and preventing diseases (Dinos et al., 2017; Li et al., 2022). AZI and CLA were easily removed during the warm campaign (46 to 100% on average). During the winter period, SFCW and HFCWs had reduced CLA removal capacities but not AZI. It was considered that biodegradation, photodegradation, and the presence of microalgae and plants enhance the effectivity of removing macrolides in both types of NBSs (Li et al., 2022). On the other hand, in the T-WWTP there was moderate removal of CLA (46 % on average) during the warm season whereas no removal was observed for the cold season for any macrolide. Regarding sulfonamides and trimethoprim (TMP), which is commonly administrated with SMX in the same dose (5 (SMX): 1 (TMP) ratio) and applied to humans and animals with poor/moderate metabolization (Dan A et al., 2013), the SMX can be metabolized in warm-blooded animals, producing an N-glucuronide conjugate and ASMX, which can be reversed to SMX in the environment. The presence of macrophytes and water biodegradation has proven to be significant for the reduction of these ABs (Garcia-Rodríguez et al., 2014). Indeed, it has been documented that both antibiotics can be eliminated under both aerobic and anaerobic conditions (Sabri et al., 2021; Vo et al., 2018). This accounts for the observed moderate to high effectiveness of SFCWs and HFCWs in attenuating TMP, SMX, and acetyl-sulfamethoxazole (ASMX) throughout various seasons (60 to 100 %, on average). On the contrary, the reference T-WWTP, employing a combination of UV irradiation and chlorination, proved ineffective in attenuating these antibiotic residues, consistent with prior studies (Hazra et al., 2022). Rifaximin (RIF), from the Rifacymin category, was detected only in the winter campaign in SFCWs and T-WWTP with low concentration (<15 ng/L) compared to other detected ABs. The main use of RIF is for gastrointestinal issues such as gastroenteritis. The detection of RIF in winter could be explained by the high prevalence of gastroenteritis during the wintertime with a peak in February and March (Calduch et al., 2021; Rohayem, 2009). RIF was removed during warm and cold periods, but further information on the degradation mechanism needs to be obtained. Tetracyclines (TCs) are other broad-spectrum ABs used for humans and animals. For winter samples, there was an increase in the concentration of doxycycline (DOX) and oxytetracycline (OXY) in the influents of SFCW and T-WWTP. However, the attenuation efficiency for TCs were nevertheless high in both seasons (68 to 78% on average). For NBSs, the biodegradation process in the rhizosphere and adsorption to sediments were suggested to be the main ways of removal (Garcia-Rodríguez et al., 2014; Hijosa-Valsero et al., 2011). The reference technology also achieved high attenuation for OXY (77 % on average), but not for DOX (17 % on average).

Considering the overall attenuation efficiencies observed, SFCWs achieved better removal in summer (88 % on average) and winter (77 % on average) than HFCWs (74 % and 67 % on average for summer and winter respectively), a significant difference was observed only during the summer for CLA and during the winter for TMP, AZI and CLA. However, considering the significant difference in HRTs among NBS (4 vs 15 days), we recommend the use of HFCW for effective AB removal. NBSs exhibited a statistical significantly superior attenuation capacity (67 to 88 %) compared to conventional T-WWTP (39 % and 42 % on average during warm periods and cold periods respectively).

3.3. Occurrence and behaviour of ARGs

The treatment performance of the NBS regarding AMR prevalence was assessed by SYBR Green-based qPCR analysis of the indicator genes *sul1*, which confers resistance to sulfonamides such as SMX and has been used as an indicator for almost two decades (Pruden et al., 2006), *dfrA1*, an indicator gene for resistance against TMP (Berendonk et al., 2015), *intI1*, coding for the integrase of the clinical class 1 integron which is a mobile carrier of resistance genes against folate inhibitors and other antimicrobials and an indicator for anthropogenic pollution (Gillings et al., 2015), and the 16S rRNA gene as proxy for bacterial abundance.

Overall, the recorded gene prevalence at the study sites was similar to previous investigations of the behaviour of these genes downstream of municipal WWTPs in Europe (Haenelt et al., 2023; Liang et al., 2023) (Table 2). Both NBS achieved average reductions of AMR indicator gene copy numbers of at least ~2 log, which is in a similar range to costlier treatment technologies such as activated sludge-based systems and membrane reactors (Munir et al., 2011). The reductions here were higher than reported for several models CW operated for a shorter period of time (Chen et al., 2016; Liu et al., 2019). A higher removal rate of mature CW systems compared to early-stage CWs has been described previously (Knecht et al., 2022). The mechanistic reason(s) for the better treatment performance of mature systems has not been scientifically addressed but could be a result of a greater extent of the rhizosphere. Noteworthy, the reduction in average copy numbers of *sul1, dfrA1* and *intI1* was approximately 0.5 to 1 log units better in SFCW than in HFCW. This finding resembles the result of our previous study that showed better removal of the same genes in an intensified CW compared to a standard HFCW at the pilot scale (Knecht et al., 2023). A likely explanation is a greater replacement of the incoming microbial community including bacterial hosts of the AMR genes in the SFCW and intensified CW with the resident community. Mechanistically, this enhanced replacement could be a consequence of the combination of higher redox potential and lower availability of organic carbon as an electron donor, which is a cause of stress to organisms not adapted to such conditions (Dukan and Nyström, 1999). Furthermore, there was no statistically significant difference in gene abundance across the seasons per NBS type. The elucidate further the mechanistic reason for the treatment performances of the NBS, we recorded microbial community profiles that are described in the following section.

3.4. Microbial community structure

Microbial community profiling was carried out by Illumina sequencing of 16S rRNA gene amplicons generated with aliquots of the same DNA extracts used for qPCR. A total of about 8.9 million high-quality paired-end reads were generated from 78 samples (51 HFCW, 18 SFCW, 9 T-WWTP) and used for further analysis. The lowest alpha diversity, as measured by the number of observed amplicon sequence variants (ASVs), were in the influent samples with an average of 864. This value increased to a statistically higher average of 1430 ASVs in the effluent samples ($p = 0.016$, Kruskal-Wallis test). Overall, most ASVs were from the HFCW samples (on average 1631), while ASV numbers from SFCW (759) and T-WWTP were similar (859; $p < 0.01$ for pairwise comparisons between HFCW versus SFCW and T-WWTP; $p = 0.44$ SFCW vs. T-WWTP). Similarly, the average Shannon index, which estimates the entropy of a community by combining species richness and evenness, was lowest in the influent samples with an average value of 8.08 compared to an average of 9.16 in the effluent samples ($p = 0.023$). On average, HFCW had the highest diversity based on the Shannon index

Table 2
Seasonal occurrence (copies/ 100 mL) and attenuation efficiency (log units) of selected ARGs in the 2 NBS and reference T-WWTP monitored.

Category	Gene	SFCW				HFCWs				T-WWTP			
		SUMMER		WINTER		SUMMER		AUTUMN[**]		SUMMER		WINTER	
		Copies[a] Influent Effluent	Log removal	Copies[a] Influent Effluent	Log removal	Copies[a] Influent Effluent	Log removal	Copies[a] Influent Effluent	Log removal	Copies[a] Influent Effluent	Log removal	Copies[a] Influent Effluent	Log removal
TMP	*dfrA1*	$4.3 \pm 13 \times 10^5$ $1.2 \pm 8 \times 10^3$	2.8[*]	$6.2 \pm 9 \times 10^5$ $3.5 \pm 7 \times 10^3$	2.3[*]	$3.2 \pm 10 \times 10^5$ $4.6 \pm 8 \times 10^3$	1.9[*]	$1.2 \pm 8 \times 10^5$ $2.7 \pm 4 \times 10^3$	1.6[*]	$9.7 \pm 10 \times 10^5$ $4.2 \pm 8 \times 10^5$	0.4	$8.2 \pm 3 \times 10^5$ $1.2 \pm 3 \times 10^5$	0.8
Sulfonamides	*sul1*	$8.5 \pm 9 \times 10^6$ $2.9 \pm 7 \times 10^4$	2.7[*]	$7.2 \pm 4 \times 10^6$ $1.7 \pm 8 \times 10^4$	2.7[*]	$7.7 \pm 18 \times 10^6$ $4.8 \pm 9 \times 10^4$	2.2[*]	$8.3 \pm 9 \times 10^6$ $5.1 \pm 8 \times 10^4$	2.2[*]	$4.3 \pm 14 \times 10^6$ $1.5 \pm 9 \times 10^6$	0.5	$6.3 \pm 23 \times 10^6$ $3.9 \pm 9 \times 10^6$	0.2
Class 1 integron	*intI1*	$7.5 \pm 12 \times 10^6$ $2.4 \pm 11 \times 10^4$	2.8[*]	$4.9 \pm 5 \times 10^6$ $1.6 \pm 11 \times 10^4$	2.6[*]	$7.5 \pm 12 \times 10^6$ $8.3 \pm 19 \times 10^4$	2.0[*]	$4.9 \pm 7 \times 10^6$ $6.6 \pm 9 \times 10^4$	1.9[*]	$4.5 \pm 3 \times 10^7$ $7.1 \pm 5 \times 10^6$	0.8	$4.9 \pm 7 \times 10^7$ $8.1 \pm 5 \times 10^6$	0.8
Bacterial abundance	16S rRNA	$5.2 \pm 4 \times 10^9$ $6.9 \pm 8 \times 10^7$	2.2[*]	$3.8 \pm 9 \times 10^9$ $8.0 \pm 4 \times 10^7$	1.8[*]	$4.0 \pm 9 \times 10^9$ $1.3 \pm 7 \times 10^7$	2.5[*]	$3.8 \pm 5 \times 10^9$ $8.0 \pm 4 \times 10^7$	1.7[*]	$5.2 \pm 4 \times 10^8$ $1.1 \pm 17 \times 10^8$	0.7	$1.2 \pm 4 \times 10^9$ $4.2 \pm 16 \times 10^8$	−2.5

[a] per 100 mL;.

[*] Values were corrected by the evapotranspiration for both seasons;.

[**] Sampling campaigns were performed in autumn before the HFCWs system closed.

(9.36 versus 8.24 for SFCW and 8.44 for T-WWTP; $p < 0.01$). These values are similar to previous microbial community profiling using 16S rRNA gene amplicon sequencing of CW samples (Knecht et al., 2022) Comparing the beta diversity at all sites and time points, displayed as a PCoA plot of Bray-Curtis dissimilarities revealed a shift from the influent to the effluent samples (Fig. 2). The influent samples to both NBS clustered with the effluent of the T-WWTP irrespective of the season. Furthermore, the microbial communities at HFCW formed another cluster irrespective of the season. In contrast, the communities at SFCW were more dissimilar over the seasons. A potential explanation for the greater beta diversity in the SFCW samples could be the greater differences in temperature and light intensity between summer and winter during the SFCW sampling campaign versus smaller differences in these parameters between summer and autumn during the HFCW campaign (**Table S2**).

The analysis of the taxonomic composition of the community profiles revealed coherent pictures fitting each particular habitat (**Table S3**). In the following, we highlight the abundance of selected phylotypes that are frequently found in a given habitat or that are indicative of a particular ecophysiology, being aware that 16S rRNA gene-based taxonomy is not necessarily proof for in situ physiology. The profiles in the samples from the reference site T-WWTP are described first, as there were also compositional similarities to the wastewater-impacted influent samples of the SFCW and HFCW reported further below.

At T-WWTP, there were no substantial differences in community composition at the phylum among the seasons. There were comparably

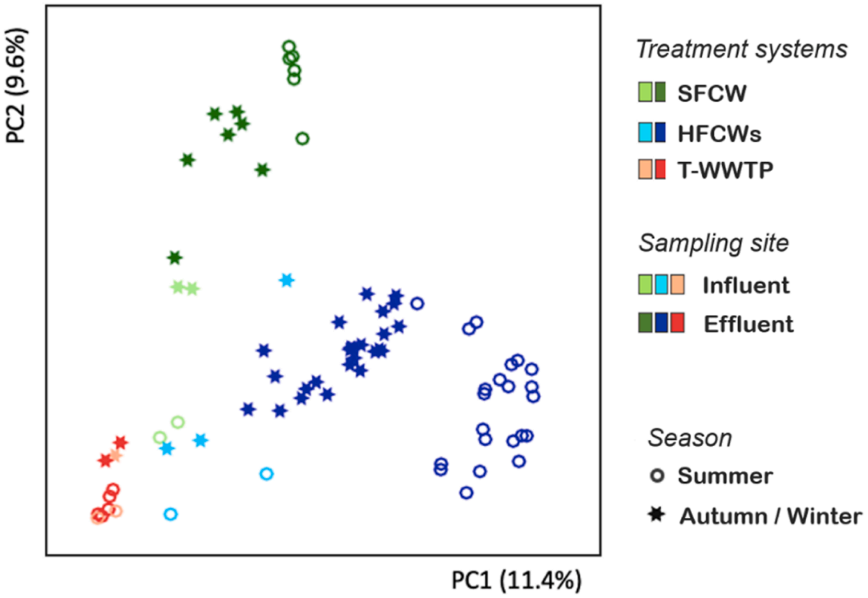

Fig. 2. Beta diversity of microbial communities at the HFCW (blue shades) and SFCW (green shades) systems and the reference T-WWTP (red shades) based on 16S rRNA amplicon sequencing data, visualized as PCoA of Bray-Curtis dissimilarities. Communities from influent samples are in light shade and communities from effluent samples are in dark shades. Circles represent samples taken during summer campaigns, stars represent samples taken during autumn or winter. The greatest differences were between samples from the three different treatment systems (ADONIS test, R2 = 0.148).

high relative abundances of sequences derived from members of the phyla *Actinomycetota* (14 %–16 % of total sequences) and *Firmicutes* (19 %). At the genus level, for example, sequences affiliated with the genus *Bifidobacterium*, a member of the *Actinomycetota* and abundant inhabitant of the mammalian colon (Duranti et al., 2021), had relative abundances of about 1 %, while the genus *Trichococcus*, a member of the *Firmicutes* frequently found in WWTP effluent (Kristensen et al., 2020), was detected at a relative abundance of 2 %. Predominant families of the *Proteobacteria* (15–21 %) were *Aeromonadaceae, Comamonadaceae, Enterobacteriaceae, Moraxellaceae* (mostly *Acinetobacter*), and *Rhodocyclaceae*, all of which are commonly found in WWTPs and their effluents (Wang et al., 2020; Yang et al., 2011). Sequences affiliated with *Escherichia-Shigella* had relative abundances of around 0.5 %. The community profiles obtained in this study were compared with previously described profiles of carriers of the ARGs and *intI1* present in sewage-impacted water and in CWs (Knecht et al., 2022, 2023). Here, the most prevalent phylotypes known to be frequent carriers of the indicator genes were *Acinetobacter, Aeromonadaceae, Arcobacteriaceae*, which had a high relative abundance of 5–6 % in the effluent of the T-WWTP, *Enterobacteriaceae* and *Rhodocyclaceae*.

At SFCW, the taxonomic compositions shifted from a mixture of WWTP-impacted and river communities in the influent samples to a mixture of communities associated with the rhizosphere of plants and river communities in the effluent samples. For example, the aforementioned *Bifidobacterium* was detected at 0.9 % in the influent but was not detected in the effluent samples. Similarly, members of the *Peptostreptococcacea*, which are typical of WWTP-impacted water (Haenelt et al., 2023), had a relative abundance of 2 % in the influent but had less than 0.6 % in the effluent samples. Conversely, phylotypes associated with the rhizosphere of wetland plants such as members of the *Rhizobiales* including *Rhizobiaceae* and *Xanthobacteraceae* (Nguyen et al., 2022; Zhu et al., 2021) were detected in the effluent at a relative abundance of 7.5 % but only at 1 % in the influent at SFCW. Members of the *Escherichia-Shigella* group were detected in half of the influent samples at a relative abundance of 0.7 %, whereas they were not found in the remaining influent samples. *Escherichia-Shigella* were detected only in one-fifth of the effluent samples (0.2 % relative abundance) without an apparent pattern in terms of sample time and location, thereby with to previously reported (Gonzalez-Flo et al., 2023). The sporadic detection of *Escherichia-Shigella* in the CW may have been due to prevalence variation in the influent together with the long HRT of 15 days. Its infrequent detection compromised the accurate calculation of the attenuation rate. Potential carriers of the ARGs and *intI1* present at relative abundances >0.5 % in most samples were *Acinetobacter, Aeromonas, Arcobacter*, members of the *Rhodocyclaceae* family, and, when present, *Escherichia-Shigella* and other *Enterobacteriaceae.*

At HFCW, the community profile also changed from a mixture of WWTP-impacted and river communities in the influent to a mixture of a community associated with the rhizosphere of plants and a river community in the effluent. Members of the *Peptostreptococcacea* had a relative abundance of 3 % in the influent but were not detected in the effluent samples, and *Bifidobacterium* was detected at 1 % in the influent but had less than 0.1 % relative abundance in the effluent. There were however some gradual community profile differences between the samples from the HFCWs and the SFCW, most notably for members of the *Alphaproteobacteria*. In the HFCWs influent and effluent samples, members of this phylum had an average relative abundance of 3 % and 5.8 %, respectively, which was quite similar to the 4.4 % relative abundance in the T-WWTP samples and to the 4.5 % in the influent to the SFCW. In contrast, sequences affiliated with the *Alphaproteobacteria* constituted on average 14.9 % of the total in the effluent of SFCW. Prominent alphaproteobacterial phylotypes were e.g. the *Rhizobiales*, which had on average a relative abundance of 7.5 % in the effluent of the SFCW versus 2.8 % in the HFCWs. Furthermore, *Sphingomonadaceae*, which are generally aerobes and are commonly isolated from various environmental habitats including the plant rhizosphere (Lee et al., 2005; Rosenberg et al., 2014), were found at relative abundances of 0.5 % in the HFCWs effluent, whereas they were present at SFCW at 2.3 %, which matches the higher redox potential in the SFCW water sample. The difference in redox potential recorded for the water samples from HFCWs and SFCW was also mirrored by a higher relative abundance of strict and obligate anaerobes such as *Anaerolinea* and *Desulfobacterota* at the HFCWs. Members of the *Escherichia-Shigella* were detected in the influent samples from the HFCWs at a relative abundance of 2 %, whereas they were not detected in any of the effluent samples of seven CW units, and at 0.1 % to 1 % in the effluent samples of the other six CW units. In the latter units, the relative abundances of *Escherichia-Shigella* were fractions of a per cent higher during the autumn campaign compared to the summer campaign. This may likely have been due to the reduced impact of abiotic and biotic processes that negatively affect the abundances of *Escherichia-Shigella*, such as lower UV radiation levels and lower predation rates in autumn than in summer. Potential carriers of the ARGs and *intI1* present at relative abundances >0.5 % in most samples were *Acinetobacter, Aeromonas, Arcobacter*, members of the *Rhodocyclaceae* family, and *Escherichia-Shigella* and other *Enterobacteriaceae* when present.

3.5. Risk assessment approach

Following the treatment processes of NBSs and T-WWTP, when the treated wastewater is discharged into an aquatic ecosystem, it becomes pertinent to assess the potential risk of ABs to environmental and human health. Individual RQ (RQ_i) values for each specific AB and removal efficiencies are detailed in **Table S4** whereas Fig. 3 shows the cumulative RQ values for each AB category. The cumulative risk exceeded the threshold of 1 for all samples, indicating the presence of risk even at outlet points. Influent points in the warm season achieved cumulative (ΣRQ_i) values ranging from 41 to 71 and 30 to 55 in the cold season for green algae, whereas those values were lower for fish (5.0 to 9.8). These high ΣRQ_i values are comparable with those observed in other studies (from 1 to up to 52) for ABs in wastewater for green algae (Figuière et al., 2022; Hayes et al., 2022). Nevertheless, our findings indicate that NBSs were able to minimize the risk assessment values in both seasons with an average removal of 70 % for both tested organisms. On the other hand, T-WWTP barely reduced the RQ value between influent and effluent points in the summer season (12 %, on average), but no removal was observed during the winter period. AZI, from the macrolide category, was the compound that contributed more to the accumulative final value of RQ with a range of 3 to 64 in all the sampling. Other studies reported high RQ values from AZI along to Ebro River with values of <1 to 141 (Gusmaroli et al., 2019). The lowest PNEC value ($0.019\ \mu g \cdot L^{-1}$) determined by cyanobacteria (*M. aeruginosa*) indicated that AZI is a priority compound to be monitored. Other main categories were lincosamides with the presence of CLI enhancing the RQs values with a range of 1 to 8, RIF during the winter season raised influent values but it was reduced in the effluents and fluoroquinolones with main presence in influents points had values up to the threshold when CIP was detected. For LVX and OFL, low RQ values were observed (< 0.2) along the seasons. Other AB categories (sulfonamides, tetracyclines and TMP) had moderate to low environmental risk (< 0.6), except for sulfonamides in influent points from SFCWs in the warm period (> 1).

Regarding PMRS (Fig. 2), inlet values generally exhibited lower levels than the ecotoxicological risk. Macrolides and fluoroquinolones were the antibiotic categories with the highest values at the inlet points, ranging from 1.6 to 6.9, consistent with previously reported findings (Mortimer et al., 2020) and other antibiotic categories, the risk values remained below 1. Among the NBS systems, SFCW demonstrated the ability to reduce ΣRQs to < 1 at the effluent point during both warm (0.7) and cold (0.9) periods, with removal efficiencies exceeding 87 %, indicating the absence of risk. For HFCWs, the removal efficiency ranged from 36 % to 85 %. Nevertheless, effluent points exhibited $\Sigma RQs > 1$. In contrast, conventional T-WWTP displayed a limited reduction capacity,

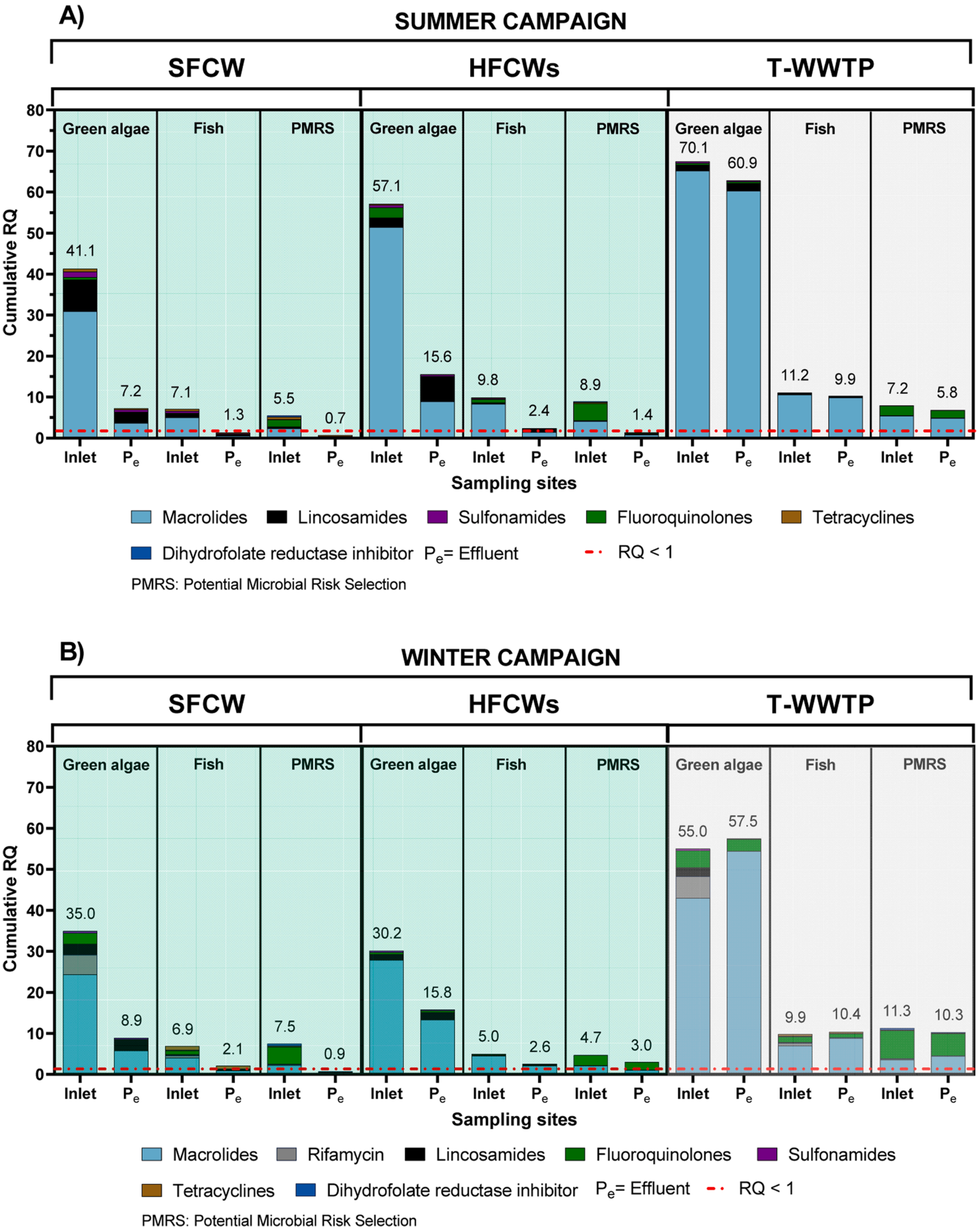

Fig. 3. Distribution of the cumulative RQ at the influents and effluents for each sampling point during the summer (A)) and the winter (B)) campaigns for all studied ABs classified by categories considering high to low RQ values from the bottom to the top.

with removal efficiency ranging from 9 % to 19 %. Both inlet and effluent points monitored showed values ranging from 7.2 to 11.3. In summary, the NBS demonstrated a greater capacity to mitigate the risk associated with resistance selection compared to conventional T-WWTP. However, it’s noteworthy that only the water effluents from the SFCW exhibited no discernible risk.

As with other water treatment processes, it is difficult to accurately quantify the risk to human health that is associated with the prevalence of resistance genes in NBS effluent (Larsson and Flach, 2022). The risk ultimately depends on the pathogenicity of the bacterial hosts carrying a particular resistance gene. Molecular and culture-dependent methods are available to identify the gene hosts (Knecht et al., 2023), but either they do not provide a comprehensive picture or are seldom applied in studies with dozens of samples due to their extensive labour requirement and consumable costs or do not provide a comprehensive picture. Furthermore, the resistance genes can be propagated again through vertical or horizontal transfer downstream of the treatment system when present in a suitable host. Since different hosts can have different survival and gene transfer potentials in the environment, an improved risk assessment will require the development of cost-effective high-throughput methods for the comprehensive identification of resistant gene hosts.

4. Conclusions

The current study, conducted at field scale, demonstrates that employing NBS as tertiary treatment technologies is a suitable strategy for mitigating ABs and ARGs. 13 of 21 ABs monitored were detected at the influent of the 3 studied tertiary wastewater treatments with concentration ranging from 2 up to 1218 $ng\cdot L^{-1}$, whereas ARGs such as Sul1 and dfrA ranged from 1×10^5 to 9×10^6 copies/100 mL. NBSs showed a greater capacity to attenuate ABs (67 to 88 % on average) and ARGs (2–3 log units) than the conventional tertiary system (36 to 39 % and no removal to 2 log units, on average) in both seasons. Taxonomic compositions in influent samples shifted from a mix of WWTP-impacted and river communities to a blend of plant rhizosphere-associated and river communities in NBS effluent. In contrast, the reference T-WWTP showed no substantial differences in community composition. Furthermore, NBSs reduced the ecotoxicological and resistance selection risks (cumulative RQs) by an average of over 70 % across seasons, whereas the benchmark technology only achieved a 6 % reduction. These results indicate that NBSs offer a significant alternative for minimizing the discharge of ABs and ARGs into surface water bodies compared to conventional treatments. Furthermore, these findings are crucial for enhancing the use of NBS in wastewater treatment sector and shaping future legislative policies. However, further studies are required to comprehend the true impact of these pollutants on aquatic ecosystems through the conduct of biological assays.

Data availability

The raw 16S rRNA gene amplicon sequences are available in the NCBI Short Read Archive under BioProject PRJNA1100746.

CRediT authorship contribution statement

Edward J. Pastor-Lopez: Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Mònica Escola Casas:** Writing – review & editing. **Dominik Hellman:** Formal analysis. **Jochen A. Müller:** Writing – review & editing, Writing – original draft, Visualization, Resources. **Víctor Matamoros:** Writing – review & editing, Supervision, Resources, 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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.122038.

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