Evaluation of a constructed wetland for wastewater treatment: Addressing emerging organic contaminants and antibiotic resistant bacteria

Stavros Christofilopoulos[1], Andreas Kaliakatsos[1], Konstantina Triantafyllou[1], Iosifina Gounaki[1], Danae Venieri[1], Nicolas Kalogerakis[1,21]

[1] School of Environmental Engineering, Technical University of Crete, 73100 Chania, Greece
[2] Department of Chemical Engineering, American University of Sharjah, Sharjah, United Arab Emirates

*Corresponding author. E-mail address: nicolas.kalogerakis@enveng.tuc.gr

Abstract

The occurrence of emerging organic contaminants (EOCs) in wastewaters and the inability of the conventional wastewater treatments plants to deal with them have been pointed out several times over the last few years. As a result, remnants of those compounds released into the aquatic environment present a potential risk for public health. Constructed wetlands (CWs) have been proposed as environmentally friendly, low-cost alternative systems with satisfactory results for different types of contaminants. This study aimed to evaluate the efficiency of a CW system, planted with the halophyte *Juncus acutus,* to eliminate bisphenol A (BPA) and two antibiotics, namely ciprofloxacin (CIP) and sulfamethoxazole (SMX) under different operating conditions. The behavior of *Escherichia coli* and enterococcal populations in terms of changes in their resistance profile for the selected antibiotics and the abundance of two resistance genes (*qnrA* and *sul1*) were also examined. BPA and CIP were significantly removed by the CW, with an overall removal of 76.2% and 93.9% respectively and with the plants playing a vital role. In contrast, SMX was not significantly eliminated. Moreover, fluctuations in the antibiotic resistance profile of bacteria were observed. Treatment processes affected the response of the two selected bacterial indicators, depending on the conditions employed in each case. Furthermore, increased levels of resistance genes were monitored in the system effluent. This study indicates that CWs, as tertiary wastewater treatment systems, may demonstrate high removal rates for some but not all EOCs. This implies that each EOC identified in the feed stream should be tested assiduously by analyzing the final effluents before their reuse or discharge into water bodies.

Keywords: wastewater treatment; wetland; BPA; antibiotics; antibiotic resistance genes

ABBREVIATIONS
Antibiotic resistant bacteria (ARB)
Antibiotic resistance genes (ARGs)
Bisphenol A (BPA)
Constructed wetland (CW)
Ciprofloxacin (CIP)
Emerging organic contaminants (EOCs)
Endocrine disrupting compounds (EDCs)
Evapotranspiration (ET)
Horizontal subsurface flow constructed wetland (HSF-CW)
Pharmaceuticals and personal care products (PPCPs)
Subsurface flow constructed wetland (SSF-CW)
Sulfamethoxazole (SMX)
Wastewater treatment plant (WWTP)

Introduction

Emerging organic contaminants (EOCs) constitute a group of natural and synthetic compounds contained in municipal wastewater well known for their impact on aquatic ecosystems worldwide [1]. The group includes diverse pollutants with notable negative effects on living organisms and the environment. Endocrine disrupting compounds (EDCs), pesticides, flame retardants, pharmaceuticals and personal care products (PPCPs), surfactants and industrial additives are part of the growing list of EOCs and their occurrence in the environment has raised concerns in terms of public health protection [2,3]. Among them, EDCs and antibiotics have gained considerable attention, as their detection and high concentration in aquatic matrices pose an increasing threat to aquatic organisms, as well as to human health [4,5].

It has been documented that exposure to EDCs such as plasticizers, detergents and dioxins may induce alterations of endocrine system functions [3,6,7]. In particular, bisphenol A (BPA) is considered a representative of EDCs and a synthetic compound commonly used in the manufacture of chemical products and electronic equipment. The main release of BPA into the environment is through the effluents of wastewater treatment plants (WWTPs), as a result of incomplete degradation [8]. Generally, EDCs and BPA are only partially removed in conventional WWTPs, remaining soluble in the treated effluents and finding their way to aquatic bodies [3,9]. The detection of BPA in environmentally relevant concentrations in water bodies and secondary treated wastewater effluents confirms this trend and necessitates the development of treatment processes for its effective and efficient biodegradation [10].

Similar attention should be paid to antibiotics, taking into account the enormous amounts discharged into the environment. A fraction of consumed antibiotics is excreted by the body almost unmetabolized and enters WWTPs, where partial degradation takes place upon physical and chemical treatments [11]. Ultimately, remnants of antibiotics are released through effluents, inducing multiple resistance in microbial communities and the generation of antibiotic resistant bacteria (ARB) [11]. The promotion of co-resistance among isolates to different antibiotics of the same class is also important, as many have structural similarities and share common modes of action. Thus, the occurrence of ARB may not necessarily be after their exposure to certain drugs [13]. Their proliferation is achieved by the rapid propagation and spread of the respective antibiotic resistance genes (ARGs), whose presence in the aquatic environment has already been highlighted in many studies [14–16]. Moreover, wastewater treatment adds to the overall dispersal of ARGs, as the applicable conditions in WWTPs may favor the growth of ARB. What is yet to be explored is the potential of the various tertiary treatment processes to eliminate those microorganisms and control the spread of ARGs [17].

Given the persistence of these contaminants, various biological, chemical and physical technologies have been proposed for their degradation with a view to reaching safe levels for environmental protection. Among them, advanced tertiary treatment technologies seem to provide the most favorable results. Nonetheless, implementation of these techniques remains an energy- and cost-intensive task [18]. Although these technologies tend to reduce bacterial load, data regarding ARB elimination and possible changes in their antibiotic resistance profile post treatment are limited [19].

In this aspect, constructed wetlands (CWs) are proposed as an environmentally friendly, low-cost alternative that enjoy high public acceptance. They are engineered systems that take advantage of the synergistic relationship of plants with their associated microorganisms, as well as other biotic and abiotic processes, for treatment of various types of wastewater, stormwater, landfill leachate and other pollution sources [20]. The role of wetlands on EOC elimination has been highlighted and their overall process efficiency is of particular concern, in terms of the removal mechanisms involved [21], but the influence of certain parameters has not yet been fully elucidated [22]. Although the overall process efficiency of a CW is highly dependent on the selected plant species the number of macrophyte species tested is considered rather limited. A comprehensive key study compiled the prominent role of microbes in the rhizosphere, the gas exchange or transport inside the plants and the processes on the root zone in CWs [23]. The role of plants has been also highlighted by others working on the role of CW planting on the treatment process, providing an insight into salt remediation[24]. As a general conclusion, it was agreed that further research is required on the plant suitability in relation to the remediation application.

In this perspective, the objectives of the present study were:

(i) Investigation of a horizontal subsurface flow CW (HSF-CW), planted with *Juncus acutus* L. for the elimination of selected EOCs from real municipal secondary treated wastewater. The choice of *J. acutus* as the plants in the CW was based on its highly desirable remediation capabilities [25]. Removal rates of BPA and the antibiotics ciprofloxacin (CIP) and sulfamethoxazole (SMX) were assessed.

(ii) Investigation of possible changes in resistance profile of ARB post treatment, and

(iii) Assessment of ARG elimination.

The process efficiency of CW was studied under various operating conditions.

Materials and methods

Chemicals and solvents

Separation and quantification of the organic compounds was carried out using a high performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan), equipped with LC-10AD VP solvent delivery modules, SPD-M10 AVP Diode Array Detector (DAD), RF-10AXL Fluorescence Detector and SIL-10AD VP autosampler. Bisphenol A (BPA) (97% purity) and acetonitrile (99.99% purity, HPLC gradient grade) were from Sigma–Aldrich (Germany). Ciprofloxacin (CIP) (≥98.0%), HPLC grade) and sulfamethoxazole (SMX) were from Fluka (Germany). Ethyl acetate (99.9%, HPLC grade), methanol (≥99.9%, GC) and acetone (HPLC, ≥99.9%), used for the extraction of BPA from soil, were from Sigma–Aldrich (USA). Deionized water was produced on a Barnstead/Thermolyne Easypure RF purification system (Dubuque, IO, USA).

BPA concentrations used in this study were chosen in order to be in the range of concentrations found in WWTPs [9]. Concentrations of CIP and SMX were higher than environmentally relevant levels, due to quantification limitations.

Sampling site and experimental set-up

An HSF-CW was developed in the WWTP of the city of Chania (60.000 inhabitants), 35° 32' 19.666" N, 24° 3' 7.330" E. The CW was fed with secondary treated wastewater and spiked with the endocrine disruptor BPA and CIP and SMX. Five *J. acutus* plants were collected from their natural environment (Souda Bay, Chania), transplanted to the wetland mesocosm and irrigated with secondary treated wastewater for 4 weeks. A schematic diagram of the CW set up is presented in **Figure 1**. A stainless steel tank (2 m x 0.5 m x 0.5 m) was filled with pea gravel (size 0.8 – 1.25 cm) of total volume of 0.4 m^3. Secondary treated wastewater was collected in 200L polyethylene tank (raw wastewater tank) with the assistance of an electric submersible pump. The mesocosm was fed with wastewater from the settling tank in a continuous mode using a peristaltic pump. A stock solution of a mixture of the target compounds was diluted in a 50L stainless steel tank and kept homogenized with the use of a submerged pump. A second peristaltic pump was used to inject the organic compounds to the CW system, before entering the wetland system, allowing flow rates from 0.25 – 0.5 L h^{-1}. The total volume of treated wastewater inside the wetland was kept constant at 157L, corresponding to a level of 10 cm below the gravel surface, due to a pipe from which the effluent overflowed from the bottom of the pilot to the specific height. Meteorological parameters (temperature and relative humidity) were monitored every 3h, by a DT-171 data logger. The duration of each treatment was set at 14 d and corresponded to different influent concentrations and hydraulic residence times (HRT), as described in **Table 1**. Another non-vegetated stainless steel tank of 0.25 m^3 (1 x 0.5 x 0.5 m) and working volume of 57L, was operated in parallel with the planted wetland and used as a control. Both wetlands were covered with HDPE film to prevent rainwater input

Sampling and analytical methods

Wastewater samples from the influent and the effluent of the CW were taken on a daily basis for analysis of the organic contaminants and every 2 or 3 d for physicochemical properties and nutrient analysis. Antibiotics and BPA concentrations were determined by HPLC as above.

Analyses were conducted according to the methods described elsewhere [25,26]. All wastewater samples were passed through glass fiber filters of 1 μm, acidified to pH 2.5 (± 0.2) and stored refrigerated at 4°C before injection into the HPLC.

Analyses of total suspended solids (TSS), chemical oxygen demand (COD), biological oxygen demand (BOD_5), total organic carbon (TOC) and total nitrogen (TN) were conducted according to Apha standard methods [27]. Total carbon (TC), inorganic carbon (IC) and TN were measured by multi N/C 2100S (Analytik Jena AG) from unfiltrated samples. TOC was determined as the numerical difference between TC and IC. Electrical conductivity (EC) and pH were measured by a Hach HQ40d multi parameter meter.

Microbiological analysis

The microbiological quality of wastewater (influent and effluent) during treatment was assessed by detection and quantification of fecal bacterial indicators *E. coli* and enterococci. Samples of 500 mL were taken using sterile bottles. Isolation of bacterial indicators was performed by filtration through nitrocellulose membranes (0.45 μm pore size, 47 mm diameter, Whatman® Germany) followed by plating on selective media and incubation at 37°C. The media used were Hi Crome Agar (HiMedia, Germany) and Slanetz & Bartley Medium (HiMedia) for *E. coli* and enterococci isolation, respectively. Viable counts were performed after 24 h for *E. coli* and 48 h for enterococci. The latter were also confirmed by transferring the membranes onto Bile Aesculin Agar (HiMedia) followed by incubation at 44°C. Enterococci hydrolyse aesculin on this medium in 2h.

Assessment of antibiotic resistance

Selected colonies of *E. coli* and enterococci were tested for antibiotic resistance using the broth microdilution method and estimating the Minimum Inhibitory Concentration (MIC_{60}) of CIP and SMX. (MIC_{60} is the minimum concentration sufficient for 60% reduction of a bacterial population). 96-well sterile microtiter plates were labeled with the appropriate concentrations of each antibiotic in the range of 20-0.08 mg L^{-1} for CIP and 128-0.5 mg L^{-1} for SMX. The concentration ranges were chosen according to EUCAST (European Committee on Antimicrobial Susceptibility Standards [28]). Each well was inoculated with the bacterial strain, with a final concentration of 10^5 CFUs mL^{-1}. Microtiter plates were incubated at 37°C for 18-24 h, followed by optical density measurement at 630 nm, using a microplate reader (Labtech LT-4000 Plate Reader) and Manta LML software. Susceptibility/resistance breakpoints were determined according to EUCAST criteria. *E. coli* isolates were considered resistant to SMX and CIP when the corresponding MIC_{60} values were ≥ 8 and ≥ 0.5 μg mL^{-1}, respectively. Enterococci were characterized similarly when MIC_{60} values were ≥ 1 and ≥ 4 μg mL^{-1}, for SMX and CIP, respectively.

Nucleic acid extraction and q-PCR-detection of target ARGs

DNA was extracted from viable cells of *E. coli* and enterococci, isolated from the CW before and after treatment, by chemical lysis and phenol/chloroform/isoamyl alcohol (25:24:1) extraction [29]. Chemical lysis was performed with proteinase K (20 mg mL^{-1}) (Sigma-Aldrich) and lysozyme (10 mg mL^{-1}) (AppliChem). The quantity and purity of extracted DNA were determined by absorbance at 260 and 280 nm (Eppendorf BioPhotometer® D30). All samples stored at −20°C before analysis.

Real time PCR assays were used to quantify the target resistance genes for SMX and CIP, namely, *sul1* and *qnrA*. Primers for *sul1* were sul1-F 5′-GCAAGGCGGAAACCCGCGCC-3′ and sul1-R 5′-CTTCGATGAGAGCCGGCGGC-3′ with a product size of 417 bp [29]. The respective primers for *qnrA* were qnrA-F 5′-GATAAAGTTTTTCAGCAAGAGG-3′ and qnrA-R 5′-ATCCAGATCGGCAAAGGTTA-3′, while the size of the product was 543 bp [30]. The concentration of the target ARGs was estimated by the SYBR green method using the StepOne Plus System (Applied Biosystems). All PCR reactions were run in triplicate in SYBR Green Master Mix (KAPA Biosystems) to a final volume of 20 μL. The reaction mixture consisted of 1.0 X Master Mix, 200 nM/400 nM of each primer for *sul1/ qnrA* and 2 μL of DNA template. Amplification was accomplished according to previous studies [29,30]. Melt curve analysis was performed by slowly heating the PCR mixtures from 64 for *qnrA* and *sul1* to 95°C (1°C per cycle of 10 s) with simultaneous measurements of the SYBR Green signal intensities. Standard curves were generated using bacterial reference strains, namely *E. coli* DSM 498 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures) and *K. pneumoniae* NCTC 5056 (Public Health England Culture Collections) for *qnrA* and *sul1*, respectively. Copies of each gene in samples were estimated based on the standard curves.

Calculation of removal efficiencies

For the optimal design of a CW and the assessment of system performance, certain calculations are required. The wetland evapotranspiration (ET), that may be more significant for wetland design projects, was evaluated in order to estimate the outlet flow rate (Q_{out}), the adjusted concentration of the contaminant (C_{out}^{adj}) at the outflow, the determination both of the actual efficiency in the mass concentration abatement (m_i^{rem}) and the overall rate of the mass removal of the organic contaminants (r_A). During the control experiment the ET value was considered to be zero. The adjusted concentration at the outflow in all cases was calculated using Equations (1) and (2), according to [31].

$$R = \frac{\tau Q_{in} - V_0}{\tau Q_{in}} \quad \text{(Equation 1)}$$

or $$R = \frac{Q_{in} - Q_{out}^{adj}}{Q_{in}} \quad \text{(Equation 2)}$$

where factor R expresses the losses due to the evapotranspiration, Q_{in} is the actual inlet flow rate (L h^{-1}), τ is the duration (h), V_{out} is the cumulative volume (L) of the outlet and Q_{out}^{adj} is the adjusted flow rate in the outflow of the wetland L h^{-1}.

According to Equation (3), the adjusted flow rate for the outflow is estimated by the difference of the inlet flow rate (Q_{in}) minus the average hourly ET.

$$Q_{out}^{adj} = Q_{in} - ET \quad \text{(Equation 3)}$$

where ET is in L h^{-1}.

The value for the measured concentration in the outlet was obtained using Equation (4):

$$C_{out}^{adj} = (1 - R)C_{out} \quad \text{(Equation 4)}$$

Where C_{out}^{adj} and C_{out} are the adjusted and the actual (measured) concentration in the outflow of the CW, respectively (expressed in μg L^{-1} for BPA and mg L^{-1} for both antibiotics).
The percent removal of any organic contaminant was obtained as:

$$Contaminant\ Removal\ (\%) = \frac{C_{in} - (1-R)C_{out}}{C_{in}} \quad \text{(Equation 5)}$$

where C_{in}, C_{out} are the concentrations in the influent and the effluent, respectively, in μg L^{-1} for BPA and in mg L^{-1} for the antibiotics.
The overall rate of mass removal of organic contaminant A (r_A, in μg h^{-1} or mg h^{-1}, depending on the compound) was calculated by the difference between BPA mass in the influent and the effluent (Equation 6).

$$r_A = \dot{m}_1 - \dot{m}_2 = Q_{in}C_{in} - Q_{out}^{adj}C_{out} \quad \text{(Equation 6)}$$

where $\dot{m}_1$ and $\dot{m}_2$ are the rates of contaminant mass transferred by the influent and effluent streams, Q_{in} and Q_{out}^{adj} are the volumetric flow rates of influent and effluent and C_{in}, and C_{out} are the concentrations at the influent and the effluent, respectively.
Another indicator that proved to be equally important for assessing system performance was obtained by combining Equations (7) and (8) in order to calculate the ratio of the organic contaminant mass removed divided by the input mass (m_p) [32].

$$m_i^{rem} = m_i^{in} - m_i^{out} \quad \text{(Equation 7)}$$

$$m_i^{out} = Q_{out,i}^{adj}C_{out,i}\Delta t_i \quad \text{(Equation 8)}$$

$$m_p(\%) = \frac{m_i^{rem}}{m_o^{in}}100(\%) \quad \text{(Equation 9)}$$

where m_i^{rem} is the mass of the organic compound that is removed from the system during the time estimated in mgs per hour (mg h^{-1}), m_i^{out} the mass of the compound at the same time in mgs per hour (mg h^{-1})[1], Q_{out}^{adj} the adjusted flow rate at the outlet(L h^{-1}), $C_{out,i}$ the measured concentration of the compound at the effluent of the system (mg L^{-1}) and Δt_i the time elapsed expressed in hours (h). Considering that in some cases the residence time was 2 d, the Δt_i was doubled for the implementation of m_i^{out}, in order for the inlet mass to be kept the same as the one calculated with residence time of 1 d.

Statistical analysis

The Shapiro-Wilks test was used to test the normality of samples. The data obtained were analyzed using the paired T-test, the Mann–Whitney U test and the Independent t-test, applied for BPA results, antibiotic removal and HRT and seasonality, respectively. P-values of <0.05 were considered significant. Statistical analysis was performed using IBM SPSS statistics software package version 20.0.

Results and Discussion

Impact of vegetation on organic contaminants removal

A comparison between the control wetland (absence of vegetation) and the vegetated one was first attempted in order to evaluate the contribution of plants on contaminant removal. **Table 2** illustrates the time period, meteorological data as monitored in 3h steps, daily total estimated ET, measured influent and estimated effluent flow rates.

The role of vegetation on EOCs removal from wastewater with CWs has been assessed in several studies. In a recent review [21] regarding the efficiency of biologically-based wastewater treatment systems for the removal of EOCs, the positive contribution of macrophytes in subsurface flow CWs (SSF-CWs) was outlined. The oxygen release from plant roots is highlighted as a vital process in the enhancement of biodegradation in the rhizosphere, due to the enhancement of aerobic pathways. The amount of oxygen released may reach 90% of the total oxygen in the substrate [33]. It is also known that plants are able to oxidize phytotoxic reduced compounds in the rhizosphere (Fe^{2+}, Mn^{2+}, S^{2-}), by transporting oxygen into their roots-[34]. Moreover, helophytes excrete root exudates into the rhizosphere (sugars, amino acids, vitamins and other organic compounds) that stimulate microbial growth, a process known as rhizodeposition. In addition, root exudates influence degradation of organic contaminants by increasing the bioavailability of the latter, as a result of the growth substrate provided and the increased population and activity of microorganisms. Nevertheless, plant uptake is considered a mechanism of minor importance for removal of organics by CWs in comparison with biodegradation, especially for the moderate hydrophobic BPA [23].

BPA removal

Planted and non-planted mesocosms were operated under the same nominal influent BPA concentration (100 $\mu g L^{-1}$) and HRT (1 d), as well as under similar environmental conditions (air temperature and humidity). Average temperature and humidity during treatments differed by 1.8 ^{o}C and 1.7%, respectively. Results are summarized in **Figure 2**, in which concentration of BPA for both wetlands during the winter period is plotted as a function of time. The positive effect of vegetation is apparent. BPA concentration of wastewater before spiking was measured weekly, but was found to be unstable and negligible in contrast with the spiked one (0.1 $\mu g\ L^{-1}$). As a result, it is assumed that the major role in the oscillation of the influent concentration was played by the flow rate of the pump.

Results showed that the BPA concentrations in the effluent were only significantly lower than in the influent for the vegetated unit ($p=0.028$, **Table 2**). They indicate the significant contribution of plants and their associated microorganisms in the removal of the endocrine disruptor. The rhizosphere, the reactive zone of CWs, is the area where the interactions of plants, microorganisms, xenobiotics and the substrate take place [23]. Previous studies on subsurface flow CWs have shown that oxygen plays an important role in EDCs' removal, and hence the advantage of vertical flow systems. However, both aerobic and anaerobic processes that take place near the roots in HSF CWs could lead to multiple degradation pathways [35]. Moreover, although sorption onto the biofilm produced in the gravel and root surface was not favored by the relatively low HRT applied to the CW, it appeared to be the prevailing abiotic mechanism for contaminant removal [20].

Antibiotics removal

As shown in Table 2 and **Figure 3**, the efficiency of the wetlands in terms of antibiotic removal differs between CIP and SMX. Removal of CIP in the vegetated unit was 94%, although there was also a significant removal in the absence of plants (60%). These results are consistent with our primary hydroponic experiment, in which natural attenuation of CIP in the control treatment varied from 59 to 54% [25]. Mass removal rate was 7.4 mg h^{-1} for the vegetated and 1.1 mg h^{-1} for the control wetland respectively. Biodegradation in the substrate, as well as sorption onto the biofilm of gravel medium are proposed as possible mechanisms of the removal, in accordance with previous studies [36]. However, given the fact that CIP dissipation by microorganisms is yet to be fully understood, microbiota have been identified that could utilize CIP as their sole carbon and nitrogen source and four biodegradation pathways proposed [35]. Overall, this review revealed that fluoroquinolone antibiotics have complex behavior during wastewater treatment and exhibit incomplete removal [37]. The sulfonamide antibiotic displayed a different behavior in the CWs. Figure 3 illustrates the observed variations in concentration and the insufficient removal of SMX, even in the vegetated wetland. This is reflected by the percent removal (m_p) below 20%. In the case of the control treatment no removal was noted, with some individual values of negative removals. This phenomenon has been also observed in previous studies and is discussed below. Statistical analysis indicated no significant difference between the two wetlands regarding SMX concentration removal ($p = 0.074$).

Operation under different conditions

Impact of HRT on removal of organic contaminants

Removal of BPA and possible mechanisms involved

Figure 4 shows a scatter diagram of BPA concentration measured in the influent and effluent of the CW with time. It is important to mention that HRT values were not adjusted in respect of ET, but refer to the influent flow rate of the wetland. Mean BPA concentration removal was 48% at HRT of 1 d and reached 92% when HRT was doubled (influent measured concentrations were 4.1 and 3.1 μg L^{-1}, respectively) (**Table 3**). Mass removal rate at the HRT of 2 d was found to be less than in the case of operation with half HRT. Specifically, BPA removal was estimated to be 12.8 μg h^{-1} in the low and 9.4 μg h^{-1} in the high HRT. Considerable variation in influent concentration has also been reported in studies on EDC treatment from municipal wastewater with CWs, at concentrations up to an order of magnitude greater than that spiked in the case of BPA [6]. The superior treatment performance of the wetland when HRT was 2 d is emphasized by m_p ratio estimated as 92%, compared to 48% at low HRT.

HRT is known to have a substantial role in removal efficiency of organic contaminants [23]. Others [6] also used three HSF-CW (9 x 3 x 0.6 m) for the investigation of the removal of BPA and nonylphenol (NP) from urban wastewater: wetlands were operated at an HRT of 1.8 d and resulted in removal efficiencies for BPA of 73, 70 and 62% of the influent 8.8 μg L^{-1} for the two CWs planted with *Heliconia psitacorum* and *Phragmites australis* and one non-planted CW, respectively. Pilot scale HSF-CWs for treatment of municipal wastewater, were also applied in [35], which indicated moderate removal efficiencies for BPAof 49.6%, 50.0% and 55.4% for mesocosms planted with *Phragmites australis*, *Typha latifolia* and the non-planted unit, respectively. Another pilot system, consisting of two 0.65 m^2 HSF-CWs working in parallel, followed by a 1.65 m^2 HSF-CW in series and planted with *Phragmites australis,* was used for wastewater treatment from a mix of EOCs, including BPA [38]. At an

HRT of 3.5 d, the system demonstrated 70-90% removal of the 1.5 μg L^{-1} BPA influent concentration after the two small units and 85-99% at the final effluent. It is worth noting that in this study as well, granular medium of the wetland consisted by small-sized gravel.

Removal of antibiotics

In contrast to BPA, analysis of the impact of HRT on CIP concentration removal was not statistically significant. Mean concentration removal was further increased by only 10%, after doubling HRT (Table 3). Mass removal rate was 1.5 and 0.7 mg h^{-1} at HR1 and HR2 treatments, respectively, a difference attributed to the reasons mentioned above for BPA. As far as SMX concentration removal is concerned, although statistical analysis showed a significant difference with the change of HRT ($p=0.008$), the overall efficiency of the removal of the sulfonamide antibiotic was not satisfactory, as illustrated in **Figure 5**, especially in the case of HRT of 1 d, where negative removal was observed. SMX is generally characterized as being resistant to biodegradation and there are several reports of negative removal efficiencies [12] . This is ascribed to deconjugation of conjugated metabolites during the treatment process [39,40]. The removal of nine antibiotics by WWTPs [39] also indicated a different approach to this issue: a possible adsorption of an amount of antibiotics onto the organic matter that is then removed during filtration of the sample, leading to an underestimation of the actual concentration [41]. Additionally, reversible transformation of this group of antibiotics that led to apparent higher concentrations at the effluent, has been described in another constructed wetland study [42].

Impact of seasonality on BPA removal

The effect of seasonality on BPA concentration removal was also tested by the comparative evaluation of two experiments conducted during June (HR2 treatment) and December of 2017 (OB treatment), under the same experimental conditions (HRT, influent concentration) as shown in **Figure 6**. The findings indicated high removal efficiency in both treatments (Table 3), with a slightly better performance, as anticipated, in the warm period. In this respect, no statistically significant difference was found for BPA removal between the two experimental runs. Higher removal efficiencies of EDCs have been also measured in the summer period [32], highlighting that apart from the type of organic substance and design criteria of the wetland, additional factors are of importance for the removal of EOCs in HSF systems, such as sewage composition and environmental parameters (e.g. temperature and sunshine).

Temperature has a great influence on the rate of biological and chemical processes in CWs, including nitrification, denitrification and BOD_5 decomposition. High temperatures, promote ET rate, which is directly associated with removal of organic contaminants with $\log K_{ow}$ between 0.5–1 and 3–3.5 [21], through plant uptake [32]. In the well-documented review [41], seasonality and temperature were discussed as they directly influence plant and microbial growth, stating that in temperatures of 15-25 ^{o}C, optimal activity of the latter is achieved. However, the optimum temperature range for efficient growth of microbes is related to both their species and the growth medium [43]. In the warm climate of Crete, average temperatures of experimental runs HR1 and HR2 well exceeded 25 ^{o}C, while that of cold season was not far from the lower limit of 15 ^{o}C, as noted in [41] (Table 1). Finally, [44] different WWTP technologies were tested for EOC treatment from wastewater [43]. Among them were included two gravel based HSF-CWs of 600 and 1000 m^2, planted with *Phragmites australis* and working intermittently at HRT of 4-6 d. Statistical analysis demonstrated the dependence of EOC removal on seasonality, with efficiencies of 24 and 49% in the cold and warm seasons, respectively, probably due to the higher activity of plant roots and the greater biofilm in the gravel bed in the warm season. In another study investigating

pesticide removal with CWs planted with macrophytes, lower efficiency was revealed in winter than in summer, pointing out the strong contribution of a pesticide removal with ET in the summer [31].

Possible removal mechanisms of EOCs in the HSF – CW system

Various mechanisms could be incorporated to the depuration of pharmaceuticals in a CW, including physical (retention, volatilization and adsorption onto the substrate's biofilm and roots), chemical (break down of the contaminants) and biological (plant assisted rhizoremediation, plant uptake, oxygen and exudates release into the rhizosphere) [45]. Hydrolysis is not expected to contribute to attenuation under environmental conditions, since the endocrine disruptor does not contain susceptible functional groups [46]. It is considered that optimum conditions for removal are not obtained in the HSF-CW, since although biodegradation is demonstrated to be the major removal mechanism, these conditions do not prevail in a bed of a horizontal system [38]. Furthermore, adsorption onto solid particles is noted as the key removal mechanism of BPA in HSF-CWs planted with macrophytes, due to its relatively high hydrophobicity ($\log K_{ow} > 3.5$) [21]. The vital role has been pointed out of retention onto particulate matter in the substrate of HSF-CWs (filtration, sedimentation and adsorption), with emphasis on the presence of oxygen and the coexistence of aerobic and anaerobic degradation pathways, due to the presence of both aerobic and anaerobic conditions in the system [35].

For removal of antibiotics, there is agreement that the predominant removal mechanism of fluoroquinolones (FQs) is sorption to sludge in WWTPs and not biodegradation. Hydroponic experiments confirmed the contribution of plants to the removal of CIP. Hydrolytic action and photodegradation were described as the two key mechanisms of CIP concentration removal [47,48]. In consequence, it is stated that free water systems (FWS) tackle pharmaceutical pollution more efficiently compared to the subsurface ones. Based on the literature, a plausible hypothesis of CIP attenuation in the non-planted wetland could be due to the substrate: gravel provides good hydraulic conductivity and is more resistant to clogging. The gravel bed of the non – planted HSF appears to facilitate biofilm production, microbial growth and redox conditions that contribute to the removal, while most manufactured organic compounds are considered to be enhanced under aerobic conditions [20]. Moreover, gravel media facilitate oxygen transfer to the lower layers of the wetland. For soil substrate, hydraulic conductivity would be lower, also resulting in lower treatment facilities [49].

With respect to SMX removal mechanisms, the high pKa values of sulfonamides result in ehanced electrostatic interaction and thus efficient adsorption onto soil particles. Microcosm experiments have revealed the important role of microorganisms for SMX degradation in CWs through the presence of plants (*P. australis*). Indeed it was suggested that biodegradation by microorganisms present in plant material, adsorption to soil particles and plants, are the main mediated removal mechanisms [39].

Antibiotic resistance profiles of E. coli and enterococci

MICs of the tested antibiotics were determined for the selected bacteria at the different sampling points, in order to examine any changes in their resistance profiles during treatment processes. Results regarding the response of *E. coli* and enterococci in the presence of SMX and CIP are presented in **Table 4**. The results showed that *E. coli* isolated from the influent of the WWTP exhibited resistance to SMX. This could be attributed to the acquired resistance, when released from individuals or other human activities. Generally, bacteria may develop resistance on their way to

the WWTP, as the latter offers ideal conditions for their proliferation and the exchange of ARGs. In the secondary treatment effluent (In Wet) the level of resistance was reduced for SMX, but still remained in the spectrum of resistance breakpoints while 75% of the isolates showed resistance. Focusing on the CW, the average MIC_{60} values for *E. coli* isolates from the effluent of the planted system (*J. acutus* out) were within the determined limits and 40% were characterized as resident, while this percentage rose to 66% for isolates derived from the effluent of the unplanted wetland (Control out). This demonstrates that plants may have a role in the mechanisms occurring for the elimination of WRB during treatment. Given that plants are able to take up antibiotics or provide biodegradation conditions [45], the selective pressure towards bacteria is reduced, with the latter tending to discard the acquired resistance genes through mutation procedures in order to save energy. Planted CWs could potentially contribute to the elimination of ARB and ARGs in water matrices, while other advanced and conventional disinfection methods have been suspected for the dispersal of resistance within bacterial populations. Chlorination may increase the resistance to tetracycline of tetracycline resistant *E. coli* strains [50], while ozonation produces adverse effects on antibiotic resistant *E. coli*, staphylococci and enterococci [49,50]. Noteworthy results were obtained from the CW spiked with low concentrations of antibiotics, in which *E. coli* isolates isolated from the effluent were shown to be susceptible to both antibiotics tested. In contrast, *E. coli* isolates were determined as susceptible to the fluoroquinolone antibiotic CIP in all stages examined. Nevertheless, average MIC_{60} values were higher in samples from the effluent of the CWs, planted and unplanted, and in the case of CW spiked with low concentrations of antibiotics.

For enterococci, the MIC_{60} was 64 mg L^{-1} for SMX in the influent of WWTP and remained steady during all processes, with all the examined isolates characterized as resistant. Instead, enterococci isolated from the influent of the WWTP were susceptible to CIP, but 50% of the isolates showed resistance. The resistance level was even lower in the effluent of the secondary treatment with 40% of the isolates resistant. Focusing on CWs, the MIC_{60} indicated susceptibility in enterococci of the effluent of the planted wetland and all isolates examined were susceptible, but the value increased compared to the previous stage (In WET). Nevertheless, this change was not observed in the case of the control CW. Interestingly, when the CW was spiked with a low concentration of antibiotics, enterococci generated resistance and the MIC_{60} value rose from 0.315 mg L^{-1} to 10 mg L^{-1}. This value was out of the breakpoint limits and characterized the enterococcal isolates from the effluent of the CW as resistant. This highlights the evidence that the exposure to low concentrations may accelerate the selective pressure in bacterial populations and lead to the generation of ARB [51].

ARGs occurrence and quantification

Quantitative real-time PCR was applied to quantify the *qnrA* and *sul1* resistance genes in DNA extracted from samples. In terms of presence/absence, the *sul1* gene was present in all samples of the examined bacteria, while the fluoroquinolone resistance gene *qnrA*, was more frequently detected in enterococcal isolates. Specifically, in bacterial colonies isolated from the CW effluent, *sul1* was detected in all samples while 25% of the bacteria were found to carry *qnrA*.

The mean concentration of the gene copies per μg of bacterial DNA for each gene is presented in **Figure 7**. In general, the results showed that sulfamethoxazole resistance gene *sul1* was more abundant than *qnrA* in *E. coli* isolates. In the CW treatment, *sul1* copies increased in the effluent of planted wetland (*J. acutus* out) while in effluent of the unplanted one remained at the same level as the influent. *qnrA* was detected and quantified

in CW effluent samples, whereas it was not detected either in the influent of WWTP nor in the effluent of secondary treatment (In WET). Likewise, *qnrA* was more abundant in the planted CW compared to unplanted. These findings suggest that the synthesis and prevailing conditions in a CW could favor the propagation of ARGs and make these systems a pool of ARGs [52].

For enterococci, *sul1* was detected in all samples examined, whereas *qnrA* was found in only 31% . In CW, *qnrA* was detected in 25% of the effluent samples (*J. acutus* out). The concentration of the genes varied between the sampling points as illustrated in Figure 7. For *sul1*, concentrations decreased in the effluent of the CW for both planted and unplanted, with this reduction being significantly higher for the planted CW. This could be explained by the fact that the population of bacterial indicators persisted in lower numbers in the CW effluents, limiting horizontal gene transfer mechanism [53]. On the other hand, the concentration of *qnrA* increased in the samples collected from the CW effluent both wetlands and the increase was higher for the unplanted one. For enterococci, the planted CW contributed to the reduction of *sul1*. These results are in agreement with another study [53], which highlights the role of plants in the elimination of ARGs, as they offer sorption and biological processes.

Taking the above results into consideration, CW presents complicated behavior in the elimination of the resistance genes. Screening *E. coli* strains, the concentration of both selected ARGs increased during the course of treatment in the CW, while in enterococci this occurred only for *qnrA*. In contrast to other studies, which indicated that plants have a role in the elimination of resistance genes [52], these results showed that macrophytes combined with the current operating conditions could have adverse effects on the abundance of ARGs in fecal bacterial populations.

Conclusions

- The performance of the HSF-CW for removal of BPA was efficient, with a significant contribution of the selected plants. HRT was an important factor as BPA showed sensitivity in longer HRT.
- *J. acutus* could be effectively used in CWs for bioremediation and the elimination of emerging organic contaminants.
- CIP exhibited high removal rates in the planted wetland while the operating conditions had adverse effects on SMX elimination. Variations in the antibiotic resistance profile of bacteria were observed during treatment, depending on the bacterial strain and the class of antibiotic tested.
- There was no substantial effect of the CW on abundance of ARGs. Their increase observed in the CW effluent indicated that the overall construction and the operating conditions may contribute o the propagation of resistance genes.
- ARGs should be examined not only in the treated effluents but also in the residual bacterial cells after treatment, which are considered the virulent carriers of waterborne diseases. Their high numbers in the effluents alongside the carried ARGs raise many concerns about the dispersal of the latter into the aquatic environment and their subsequent potential negative effect on public health.

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Table 1. Operating conditions of each experimental run at the HSF-CW

Experimental run	**HRT (d)**	**Month**	**Air temperature** (°C) / **Relative humidity** (%)	ET (L h^{-1})	Q_{in} (L h^{-1})	Q_{out}^{adj} (L h^{-1})	BPA (µg L^{-1})	CIP (mg L^{-1})	SMX (mg L^{-1})
Control	1	February	14.4 / 67.6	-	2.37	2.37	100	1	5
T	1	March	16.2 / 65.9	0.83	6.54	5.71	100	1	5
HR1	1	April	21.9 / 57.5	1.04	6.54	5.50	5	0.25	0.5
HR2	2	June	29.7 / 53.1	1.35	3.27	1.92	5	0.25	0.5
OB	2	December	18.3 / 66.1	1.04	3.27	2.23	5	-	-

Time period, meteorological conditions, evapotranspiration (ET) and the calculated input (Q_{in}) and output flow rates (Q_{out}^{adj}) corrected in respect to evapotranspiration (T: treatment with high concentration of BPA, OB: treatment only with BPA spike)

Table 2. Comparative presentation of the efficiency of the two CWs

Organic compound	Treatment	C_{in} BPA (μg L^{-1}) CIP, SMX (mg L^{-1})	C_{out}^{ET} BPA (μg L^{-1}) CIP, SMX (mg L^{-1})	Concentration removal (%)	Concentration removal (%) Evap. adjusted	Significant Difference (Cin-Cout) (p-value)	Rate of mass removal ET adjusted BPA (μg h^{-1}) CIP, SMX (mg L^{-1})	m_p (%)
BPA	Control	146.0	106.7	26.8	26.8	No (0.115)	93.0	26.9
	T	131.0	41.7	68.2	76.2	Yes (0.028)	618.5	73.2
CIP	Control	0.79	0.32	59.8	59.8	Yes (0.006)	1.12	59.98
	T	1.21	0.10	91.9	93.9	Yes (0.003)	7.38	93.13
SMX	Control	2.8	2.77	1.1	1.1	No (0.466)	0.07	1.2
	T	3.6	3.52	3.0	27.2	No (0.180)	3.64	18.5

Control: absence of plants; T: *J. acutus* planted wetland; HRT = 1 d; C_{in} and C_{out}^{ET} are the mean measured concentrations at the influent and the effluent of the wetlands, respectively (12<n<13); ET indicates that corresponded values have been corrected in respect to evapotranspiration; m_p is the removed mass of the contaminant over the mass entering the system. In the parenthesis is p-value, with significance level set at $p < 0.05$.

Table 3. The impact of different operating conditions on CW removal efficiency

Contaminant	Parameter	Paired treatments	P-value	Significant difference
BPA	Vegetation	C-T	0.046	YES
	HRT	HR1-HR2	0.049	YES
	Seasonality	HR1-OB	0.099	NO
CIP	Vegetation	C-T	0.004	YES
	HRT	HR1-HR2	0.360	NO
SMX	Vegetation	C-T	0.074	NO
	HRT	HR1-HR2	0.008	YES

C: control-absence of plants; T: *J. acutus* planted wetland; HR1: HRT = 1 d; HR2: HRT = 2 d; OB: treatment only with BPA spike. Results of statistical analysis of paired treatments: control (C) – T, HR1 – HR2 and HR1 – OB ($p < 0.05$).

Table 4. Average MIC_{60} values of *E. coli* and Enterococci after exposure to SMX and CIP antibiotics.

Bacteria	Antibiotic	In WWTP (mg mL^{-1})	In WET (mg mL^{-1})	Control out (mg mL^{-1})	J. acutus out (mg mL^{-1})	J. acutus out -Low conc. (mg mL^{-1})
E. coli	SMX	32^{R}	8^{R}	32^{R}	4	2^{S}
	CIP	0.08^{S}	0.04^{S}	0.16^{S}	0.16^{S}	0.16^{S}
Enterococci	SMX	64^{R}	64^{R}	64^{R}	64^{R}	64^{R}
	CIP	2.5^{S}	0.315^{S}	0.315^{S}	1.25^{S}	10^{R}

S = susceptible and R = resistance, HRT 2 days

In WWTP: Influent in WWTP, In WET: Secondary wastewater effluent, Control out: unplanted CW effluent, *J. acutus* out: Planted CW effluent, *J. acutus* low conc.: Planted CW spiked with low concentration of CIP and SMX

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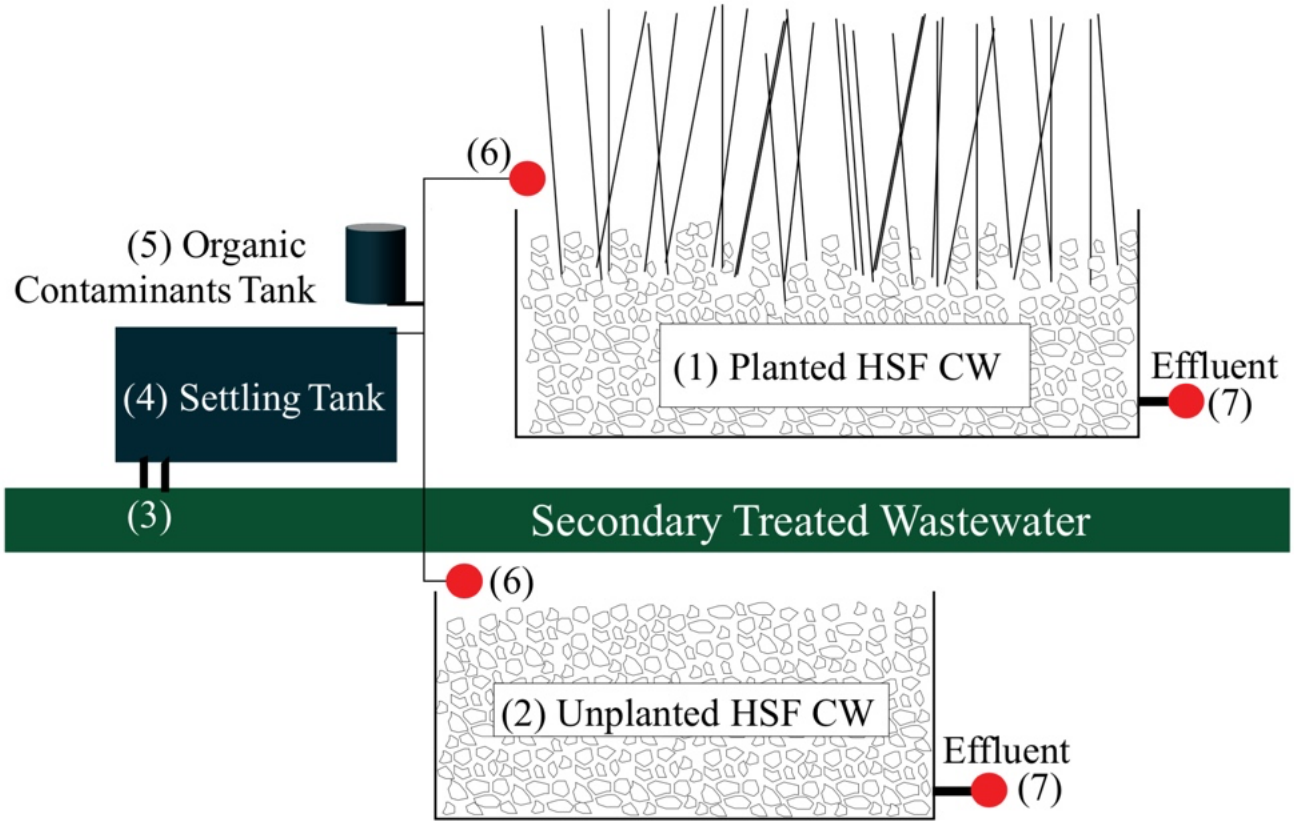

Figure 1. Schematic of the horizontal subsurface flow system (HSF). (1) Horizontal flow constructed wetland, (2) secondary treated wastewater, (3) wastewater inlet to the settling tank, (4) settling tank, (5) organic contaminants tank, (6) wetland input (sampling point), (7) wetland output / treated wastewater overflow (sampling point).

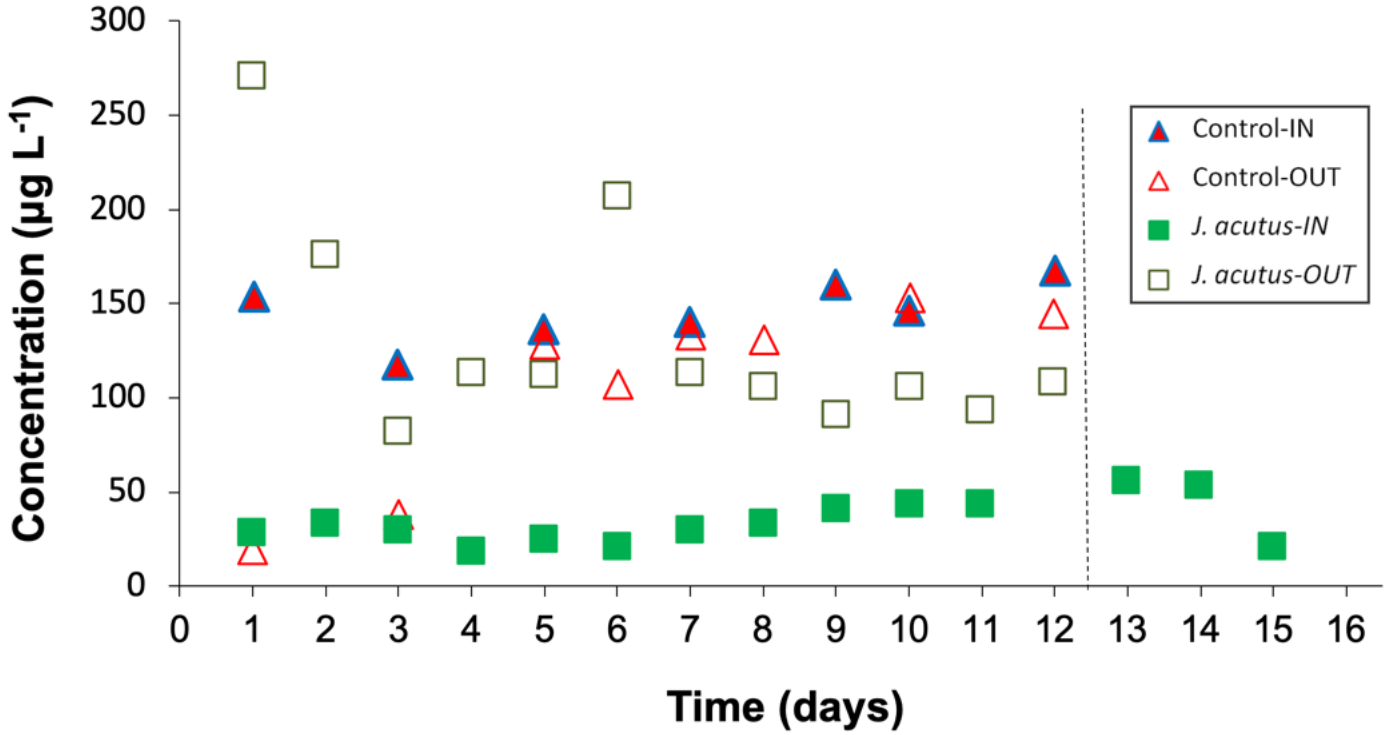

Figure 2. BPA concentrations in the influent (*J. acutus* – IN) and the effluent (*J. acutus* - OUT) of the planted and non-planted (Control - IN, Control - OUT) horizontal subsurface flow wetlands, during winter. Green squares represent BPA concentration, corrected in respect to evapotranspiration (ET). Dotted line indicates interruption of spiking.

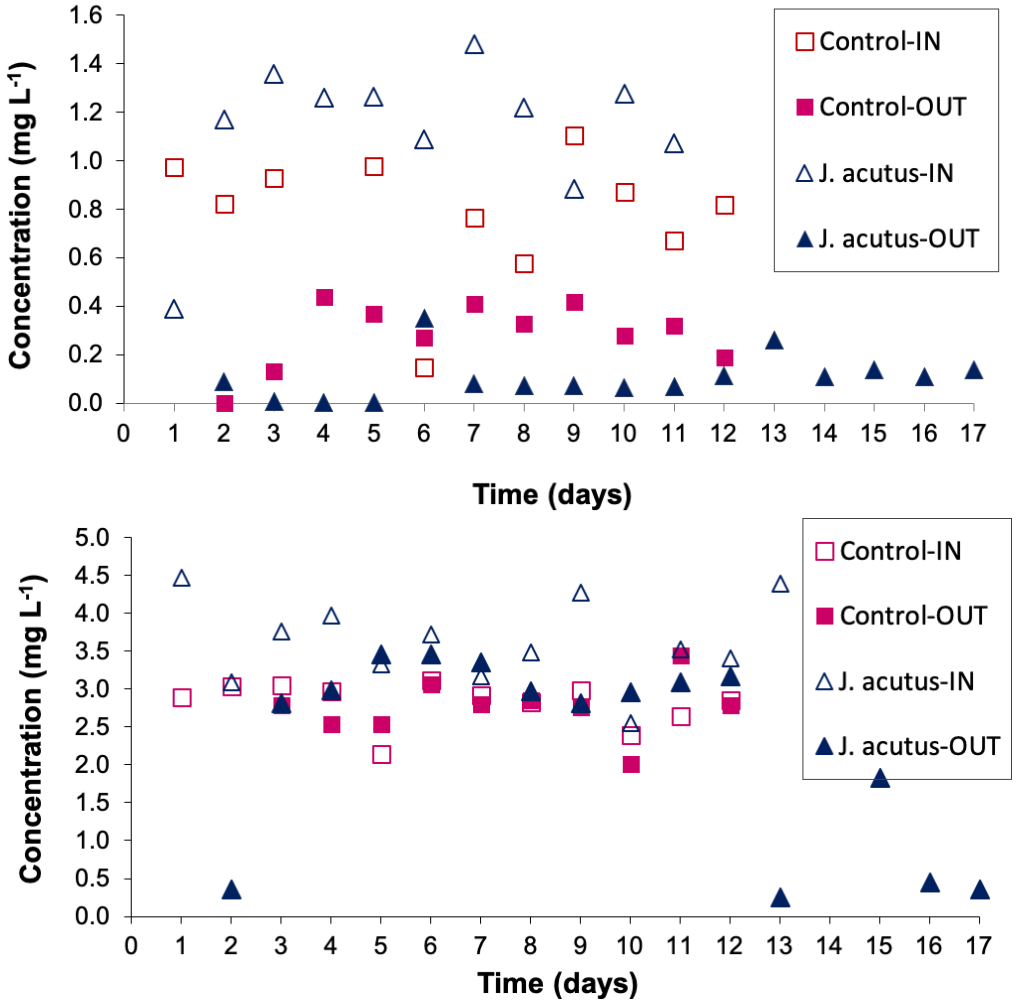

Figure 3. Comparative presentation of antibiotics a) CIP and b) SMX concentrations versus time, for the planted and non-planted mesocosms. Values have been corrected with respect to evapotranspiration. Dotted lines indicate the interruption of spiking (*J. acutus* – IN: Influent of planted CW; *J. acutus* – OUT: effluent of the planted CW; Control – IN: influent of non-planted CW; Control – OUT: effluent of non-planted CW).

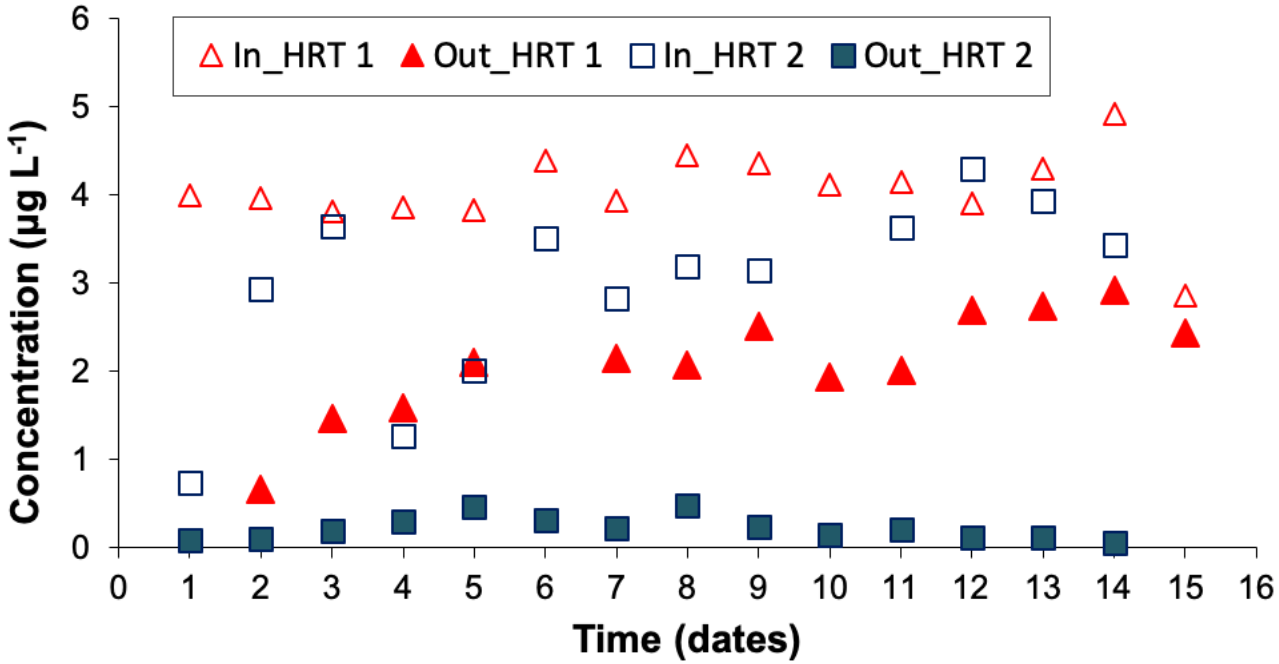

Figure 4. Comparison of the influent and effluent BPA concentration versus time, with respect to different HRTs (In_HRT 1: Influent of planted CW; Out HRT1: Effluent of planted CW after HRT of 1 d; In_HRT 2: Influent of planted CW; Out HRT2: Effluent of planted CW after HRT of 2 d).

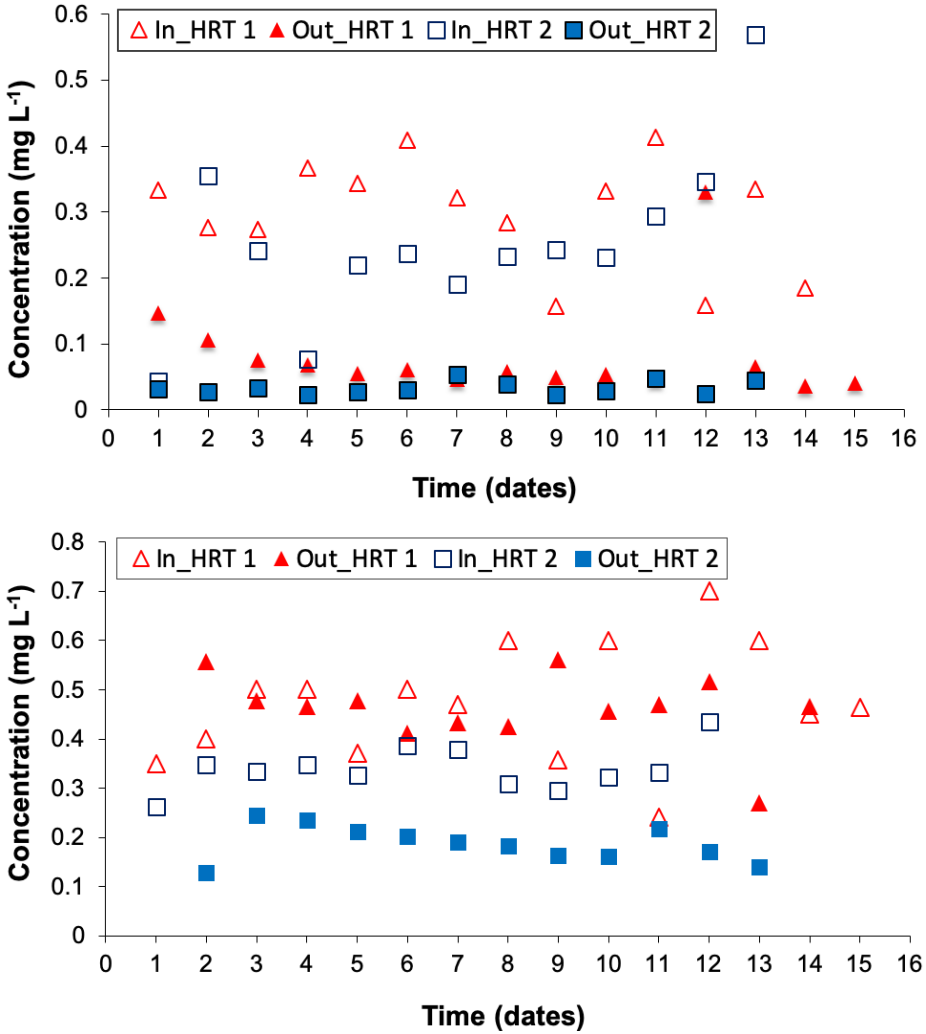

Figure 5. Comparison of the influent and effluent concentration of antibiotics a) CIP and b) SMX versus time, with respect to different HRTs. Effluent concentrations have been corrected with respect to evapotranspiration (In_HRT 1: Influent of planted CW; Out HRT1: Effluent of planted CW after 1 d; In_HRT 2: Influent of planted CW; Out HRT2: Effluent of planted CW after 2 d).

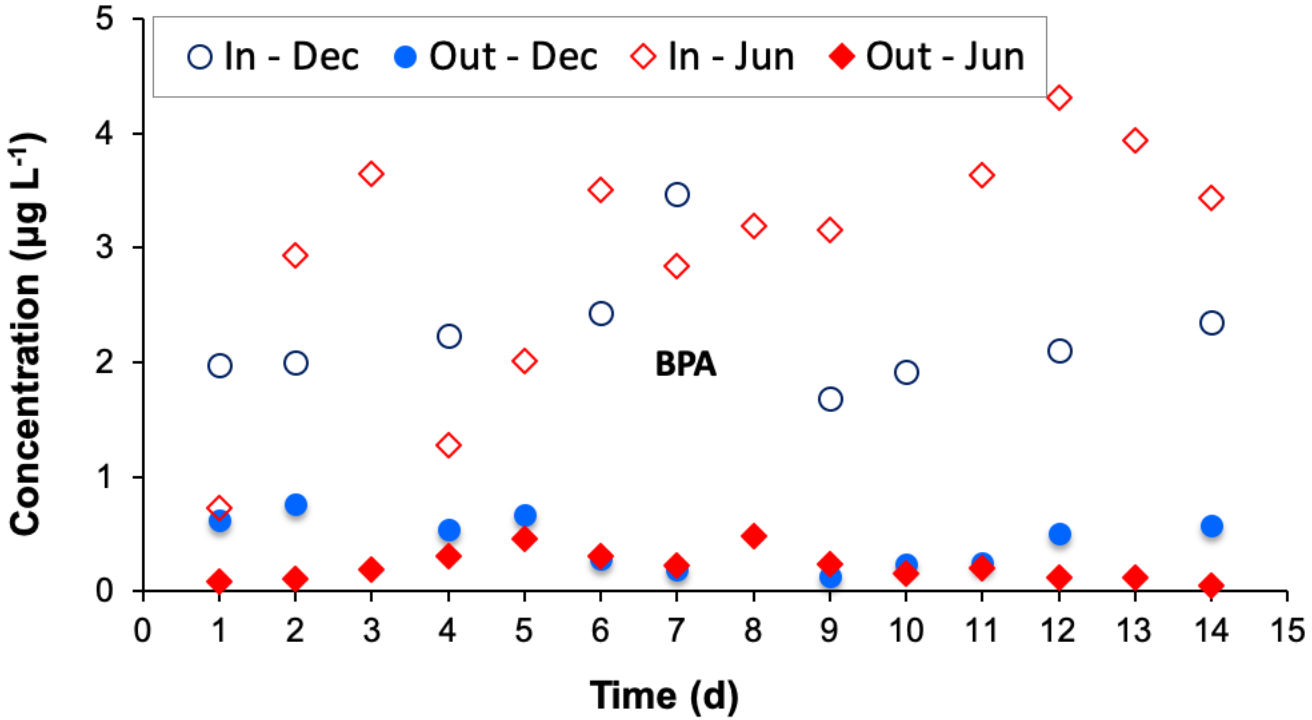

Figure 6. Comparison of the influent and effluent concentration of BPA versus time, during June and December of 2017. Effluent concentrations have been corrected with respect to evapotranspiration (In - Dec: Influent of planted CW in December; Out - Dec: Effluent of planted CW in December; In - June: Influent of planted CW in June; Out - Jun: Effluent of planted CW in June).

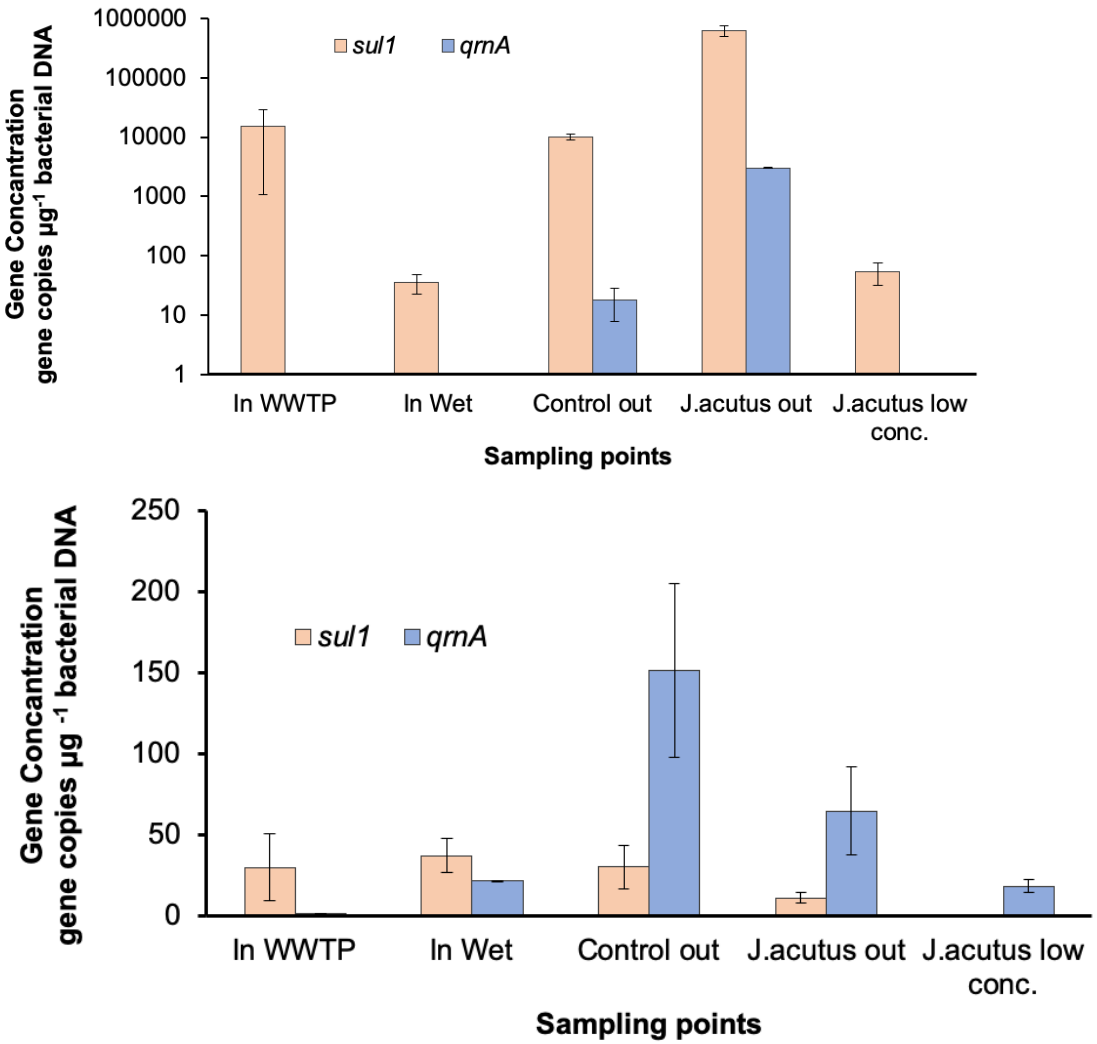

Figure 7. Absolute concentrations of ARGs (gene copies μg^{-1} bacterial DNA) in a) *E. coli* and b) enterococcal isolates with standard deviations (In WWTP: Influent in WWTP; In WET: Inflow in CW with secondary wastewater effluent; Control out: unplanted CW effluent; *J. acutus* out: Planted CW effluent; *J. acutus* low conc.: Planted CW spiked with low concentration of CIP and SMX).