New insights on the combined removal of antibiotics and ARG in urban wastewater through the use of two configurations of vertical subsurface flow constructed wetlands

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Abstract

The occurrence and removal of 49 antibiotics (including fluoroquinolones, quinolones, penicillins, cephalosporins, macrolides, tetracyclines, lincosamides, sulfonamides, dihydrofolate reductase inhibitor and nitroimidazole antibiotics) as well as 11 genes conferring resistance to sulfonamides (*sul1* and *sul2*), fluoroquinolones (*qnrS*), macrolides (*ermB*), glycopeptides (*vanA*), tetracyclines (*tetM* and *tetW*), β-lactams (bla_{TEM}, bla_{KPC}, bla_{NDM}, bla_{OXA-48}) and the Class I integron integrase gene (*intI1*) were investigated in 2 vertical subsurface flow (VF) constructed wetlands (1.5 m^2 each): an unsaturated (UVF) unit and a partially saturated (SVF) unit (0.35 m saturated out of 0.8 m) operating in parallel and treating urban wastewater in Barcelona (Spain). The determination of antibiotics in water was carried out by UPLC-MS/MS in 4 alternate sampling campaigns, whereas the occurrence of ARGs was evaluated by qPCR in water and biofilms from the top (5-15 cm) and bottom (70-80 cm) layers of both wetlands at the end of the operational period. 13 antibiotics were quantified in the primary-settled wastewater, 6 of which were present in all samples (i.e. ciprofloxacin, ofloxacin, pipemidic acid, azithromycin, clarithromycin and clindamycin). The SVF showed statistical significance on the removal of 4 compounds (namely ciprofloxacin, ofloxacin, pipemidic acid and azithromycin), suggesting that the wider range of pH and/or redox conditions of this configuration might promote the microbial degradation of some antibiotics. In contrast, the concentration of the latter (except pipemidic acid) and clindamycin was higher in the effluent than in the influent of the UVF. ARGs detected in influent wastewater in descending order were *sul1* and *sul2,* bla_{TEM}, *ermB* and *qnrS*. Except the latter, all these ARGs were detected in the biofilm of both wetlands. Average removal rates of ARGS showed no statistical differences between both units, and ranged between 46-97% for *sul1*, 33-97% for *sul2*, 9-99% for *ermB*, 18-97% for *qnrS* and 11-98% for bla_{TEM}. The distribution of ARGs was similar in both wetland units, and also in the top and bottom

layers of both wetlands, except for *sul2,* that showed significantly higher abundance in the SVF unit.

Keywords

Contaminants of emerging concern, pharmaceuticals, wastewater treatment, green treatment systems, nature-based solutions, treatment wetlands

1. Introduction

Antibiotic resistance (AR) has become a serious and growing threat at all ecological levels, affecting human, animal, and environmental health (Hernando-Amado et al., 2019). Although the origins of the problem are diverse and complex (Laxminarayan et al., 2013), pollution of surface waters by antibiotic residues from anthropogenic wastes (e.g. raw and treated wastewater discharges) has been underlined as one of the major causes of the rapid dissemination of AR in the environment (Martinez, 2009; Berglund, 2015).

Despite the numerous informative warning campaigns on the increasing prevalence of AR among bacterial pathogens and the need for a more rational use of antibiotics, Spain is among the top three countries with the highest antibiotic consumption in Europe, as demonstrated in the Surveillance report from the European Centre for Disease Prevention and Control (ECDC, 2018). However, overall consumption was reduced in a 4.78% between 2016 and 2017 (Horcajada et al., 2018). In the ECDC report, penicillins and β-lactams are the antibiotic families most consumed in Europe (including Spain); whereas the fluoroquinolone ciprofloxacin is the antibiotic most consumed in Spain (18 MT per year), followed by sulfamethoxazole and clarithromycin (10 MT per year) (Ortíz de García et al., 2013). Antibiotics enter the environment through different routes, namely: *i)* the excretion from medicated cattle in animal husbandry facilities (Tasho and Cho, 2016; Wei et al., 2011); *ii)* the discharge of hospital wastewater effluents (Hocquet et al., 2016; Rodríguez-Mozaz et al., 2015); *iii)* the direct application of antibiotics in fish farms (Sapkota et al., 2008); *iv)* the application of manure in land for fertilization purposes (and also the use of biosolids to the same end) (Chee-Sanford et al., 2009; Dolliver and Gupta, 2008; Sabourin et al., 2009); and *v)* the regular discharge of urban wastewater treatment plants (uWWTP) effluents into aquatic bodies (e.g. rivers, lakes) (Gracia-Lor et al., 2012; Michael et al., 2013; Rizzo et al., 2013; Zhang et al., 2015). Regarding the latter, it is well known that uWWTP are inefficient in the

77 removal of many pharmaceutical compounds (including antibiotics), antibiotic resistant
78 bacteria (ARB), and antibiotic resistance genes (ARGs), thus polluting the receiving
79 ecosystems with a combination of selective agents and selected biological entities (Cacace et
80 al., 2019; Corno et al., 2019; Manaia et al., 2018, 2016). Under this scenario, there is a
81 critical need to find alternative treatment technologies to efficiently remove antibiotics and
82 resistance determinants (both ARB and ARGs) from wastewater while maintaining low
83 operational and maintenance costs (O&M) (Sharma et al., 2016).

84 Constructed wetlands (CWs) are nature-based wastewater treatment systems that have proven
85 to be a sustainable alternative at decentralized scale for the treatment of different kinds of
86 wastewater worldwide (Álvarez et al., 2017; Paing et al., 2015). The necessary infrastructure
87 for their construction is simple and their O&M costs are well below those of conventional
88 uWWTPs. Besides, CWs do not require the addition of chemical reagents, there is not sludge
89 production, and the energy requirements are very low or close to zero. CWs have shown to be
90 highly efficient decreasing the concentration of conventional water quality parameters (such
91 as COD, BOD, NH_4^+), meeting the requirements of the Council Directive 91/271/EEC
92 regarding quality of wastewater effluents prior to their discharge in the environment. The
93 quality of these effluents is often suitable for reuse in agriculture or other environmental
94 purposes (Verlicchi and Zambello, 2014; Ávila and García, 2015). The capacity of these
95 systems to remove contaminants of emerging concern, including pharmaceuticals, has also
96 been demonstrated in different studies (Ávila et al., 2014; Matamoros et al., 2017; Paz et al.,
97 2019). Multiple design and operational characteristics have been developed over the last
98 decades. In particular, vertical subsurface flow (VF) wetlands are operated by providing
99 intermittent feeding from the top, which drains to the bottom of the filter bed, thus remaining
100 unsaturated between pulses. This configuration has proven to perform better than horizontal
101 subsurface flow (HF) wetlands on the removal of organic matter, and especially ammonia,

due to an enhanced nitrification capacity provided by their unsaturated conditions (Nivala et al., 2013; Kahl et al., 2017). However, the denitrification activity is generally limited in this type of wetlands due to the lack of anaerobic conditions necessary for the establishment of denitrifying bacteria. Recently, the use of VF with a partial saturation of the lower part of the gravel media yielded an enhanced removal of total nitrogen (TN) by promoting the generation of redox gradients throughout the filter bed (Dong and Sun, 2007; Silveira et al., 2015; Pelissari et al., 2017). The presence of more diverse and metabolically versatile bacterial communities in this type of wetland facilitates the occurrence of diverse N transformation pathways that enable the complete nitrification-denitrification process (Pelissari et al., 2018). This configuration has also led to higher elimination rates of various contaminants of emerging concern, including the antibiotics trimethoprim and sulfamethoxazole (Sgroi et al., 2018).

Although the occurrence and removal of antibiotics and ARGs in VF wetlands has recently received scientific attention (Chen et al., 2016a,b; Huang et al., 2015; Liu et al., 2014, 2013; Song et al, 2018), further research is needed to discern the different mechanisms involved in their fate, depending also on the effect of the wetland's operational characteristics. The present study evaluated the performance of VF wetlands, operating under different configurations, on the removal of both antibiotics and ARGs as a low-cost treatment to reduce the impact of these pollutants in the receiving water bodies. Two identical 1.5-m^2 VF wetlands (one unsaturated and another partially saturated) were set up in parallel and intermittently fed with urban wastewater during 6 months. The occurrence and removal of 49 antibiotic and 11 ARGs (conferring resistance to sulfonamides, fluoroquinolones, macrolides, glycopeptides, tetracyclines, and b-lactams) were evaluated in both water and bed media samples at the end of the operational period during two weeks.

2. Materials and Methods

2.1. Description of the experimental setup

The current study was carried out at the experimental CW system located outdoors at the facilities of the Universitat Politècnica de Catalunya (UPC) in Barcelona, (NE Spain). The system treated urban wastewater from the surrounding residential area, which was pumped directly into the system. The treatment line consisted of an Imhoff tank (0.2 m^3, HRT = 12 h) as primary treatment, followed by two vertical subsurface flow (VF) constructed wetlands (1.5 m^2 each) operating in parallel. The two CWs were identical except for the saturation status; whereas one of them was partially saturated with water (0.35 out of 0.8 m) (SVF), the other one was unsaturated (UVF) (Figure 1).

The VF beds were constructed inside polyethylene tanks. Each wetland unit had a surface area of 1.5 m^2 (1.0 m W × 1.5 m L), and a filter media depth of 0.8 m, consisting of a 0.1 m sand layer (Ø = 1-2 mm) on top, and a 0.7 m layer of fine gravel (Ø = 3-8 mm) underneath. A polyethylene pipe distributed the pumped water uniformly 0.1 m above the bed surface. The planted macrophyte in both wetlands was *Phragmites australis*, which was well developed at the time of the study. Both wetlands were intermittently and simultaneously fed, in pulses of about 25 L, providing about 8 pulses per day. The average organic loading rate (OLR) and hydraulic loading rate (HLR) applied to each wetland unit during the current study were of 30 g COD m^{-2} d^{-1} and 133 mm d^{-1}, respectively. Before this operational setup, the two VF wetlands had been in operation alternating cycles of feed and rest since their start-up in May 2010 (Ávila et al., 2017), thus the filter media and rhizosphere were well established and colonized by microorganisms. In January 2016, the VF wetlands started to operate in parallel, and sampling of antibiotics and ARGs took place after six months. Whereas the UVF wetland

150 operated typically unsaturated (i.e. 0.8 m free drainage), in the SVF wetland the bottom part
151 (0.35 m) remained saturated by leveling of the outlet pipe. An electromagnetic flow meter
152 (Sitrans F M Magflo®) was installed at the inlet and the outlet of the wetlands to monitor flow
153 values in the system and enable the assessment of the evapotranspiration in both units.

154 **2.2. Sampling**

155 Influent and effluent samples of both CW units were taken (as grab samples) on alternate
156 days during two consecutive weeks in June-July (n = 4). Urban wastewater samples (influent)
157 were taken from the outlet of the Imhoff tank, and effluent samples from the outlets of both
158 VF wetlands, as shown in Figure 1. All samples were taken every day at the same time (10
159 am). For chemical characterization of the water, samples were taken in PVC bottles and
160 analyzed in the laboratory on the same day of collection. Samples for the determination of
161 antibiotics were collected in amber glass bottles, filtered through PVDF 0.45 µm filters and
162 pre-concentrated by solid phase extraction (SPE) also on the same day; SPE cartridges were
163 frozen at -18 ºC until analysis. For the analysis of ARG, water samples (100 mL) were
164 collected in sterile bottles and filtered through 0.2 µm pore-size membranes (ISOPORE 0.2
165 µm GTTP 47 mm diameter, Millipore, Ref. GTTP04700) using an appropriate filtration
166 device. Filters were then carefully folded and introduced separately into 2 mL sterile
167 Eppendorf tubes and safely stored at –30 ºC until DNA extraction. Biofilm samples from the
168 CW filter media were collected by sampling the gravel filter of both VF wetlands in
169 triplicate, using a sterile plastic core. These biofilm samples (10–20 g of gravel) were taken
170 from the top (0–15 cm depth) and bottom (70–80 cm depth) layers at the end of the treatment
171 period (July), placed into 50 ml sterile Falcon tubes and stored at –30ºC until DNA extraction
172 (see below).

173 **2.3. Chemicals and reagents**

All antibiotic standards, including 6 cephalosporines, 1 dihydrofolate reductase inhibitor, 8 fluoroquinolones, 2 lincosamides, 6 macrolides, 2 nitroimidazole antibiotics, 3 penicillins, 4 quinolones, 15 sulfonamides (including 3 acetylated metabolites) and 4 tetracyclines, together with the corresponding isotopically labelled compounds (internal standards and surrogates during the SPE process) were purchased from Sigma-Aldrich with high purity grade (>90%) and Toronto Research Chemicals (Ontario, Canada). The whole list of target compounds is given in Table S1 of the Supplementary Information (SI).

Individual stock standard solutions and working standard solutions were prepared and stored as described by Gros et al. (2013). Similarly, separated mixtures of isotopically labelled internal standards, used for internal standard calibration, and surrogates were prepared in MeOH; ampicillin-15N was prepared in HPLC water/ACN (50:50, v/v). Further dilutions were also prepared in a MeOH/water (50:50, v/v) mixture.

SPE for pre-concentration and purification of the different samples was performed using Oasis HLB cartridges (60 mg, 3 mL) from Waters Corporation (Milford, MA, U.S.A.). Glass fiber filters (1 μm) and nylon membrane filters (0.45 μm) were purchased from Whatman (U.K.). HPLC water, acetonitrile and formic acid (98%) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) (37%) and ethylenediaminetetraacetic acid disodium salt solution (Na_2EDTA) at 0.1 mol L^{-1} were purchased from Panreac.

2.4. Analysis of conventional water quality parameters

On-site measurements at the time of the samples collection were performed for water temperature (Checktemp-1 Hanna thermometer, US), dissolved oxygen (DO) (EcoScan DO 6, ThermoFisher Scientific, US), pH (Crison 506, Spain), redox potential (E_H) (Thermo Orion 3 Star redox meter, ThermoFisher Scientific, US) and electrical conductivity (EC) (CLM-381, EH, Germany). Grab water samples were taken immediately to the adjacent laboratory to

198 measure total suspended solids (TSS), alkalinity and chemical oxygen demand (COD),
199 following the corresponding Standard Methods (APHA-AWWA-WEF, 2012);
200 NH_4^+-N according to Solórzano method (Solórzano, 1969); nitrate and nitrite nitrogen (NO_x-
201 N), orthophosphate (PO_4-P) and sulfate (SO_4^{2-}) were analyzed by ion chromatography (ICS-
202 1000, Dionex Corporation, USA), performed in isocratic mode with carbonate-based eluents
203 at 30°C and a flow of 1 mL min^{-1}.
204 Total carbon (TC) and total nitrogen (TN) were determined using a multi N/C (2100S)
205 analyzer (Analytik Jena, Germany). All the analyses were done in triplicate and results were
206 given as average values. For analysis of all these dissolved forms, samples were previously
207 filtered through 1–3 μm pore glass microfiber filters.

208 **2.5. Analysis of antibiotics in water**

209 Solid phase extraction (SPE) was performed in triplicate for each sample and on the same day
210 of collection. Extraction recovery efficiencies were obtained for each type of water sample
211 (influent and effluent), spiking the corresponding samples with a mixture of the target
212 compounds at a concentration of 500 and 250 ng L^{-1} for influent and effluents, respectively.
213 The surrogate mix solution was added in all the samples at a concentration of 100 ng L^{-1} prior
214 to SPE, in order to evaluate the performance of the method (considering potential losses
215 during extraction and clean up).

216 Analysis of the target antibiotics in the water samples was performed following the
217 methodology by Gros et al. (2013). Briefly, chromatographic separation was carried out with
218 a Waters Acquity Ultra-Performance™ liquid chromatograph (UPLC) system (Milford, MA,
219 USA), using an Acquity HSS T3 column (50 mm x 2.1 mm i.d., 1.8 μm particle size). The
220 UPLC instrument was coupled to a 5500 QTRAP hybrid triple quadrupole linear ion trap
221 mass spectrometer (QqLIT-MS/MS) (Applied Biosystems, Foster City, CA, USA) with a
222 turbo Ion Spray source. For increased sensitivity, two selected reaction monitoring (SRM)

transitions were monitored per compound, one for quantitation and the other for positive confirmation. Time-specific SRM windows were adjusted to the retention times of each target compound (windows of 20 s), and target scan time was set to 0.25 s. Further information is given elsewhere. Quantification was performed based on the internal standard calibration approach. Concentrations were estimated for the most abundant SRM transition selected. All data were acquired and processed using Analyst 1.6.3 software. Linearity, LOD and LOQ for all antibiotic compounds are shown in the Table S2 of SI.

2.6. Quantification of antibiotic resistance genes

DNA from filters (water samples) and gravel filter (biofilms) was extracted using the FastDNA SPIN kit for soils (MP Biomedicals, CA, US) and following the manufacturer's instructions. The concentration of DNA in the resulting extracts was analyzed using QUBIT® (Life Technologies; Carlsbad, CA, USA) and purity was determined by measuring A_{260}/A_{230} and A_{260}/A_{280} absorbance ratios using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific; Wilmington, DE, USA).

The concentration of 11 ARGs as well as the integrase gene of class 1 integron (*intI1*) in water and biofilm samples was determined using quantitative PCR (qPCR) as previously described by Subirats et al. (2017). Target ARGs were selected according to their clinical relevance and prevalence in wastewater, and for being representatives of resistance against main antibiotic families, namely: sulfonamides (*sul1* and *sul2*), fluoroquinolones (*qnrS*), macrolides (*ermB*), tetracyclines (*tetM* and *tetW*), glycopeptides (*vanA*) and β-lactams (bla_{TEM}, bla_{KPC}, bla_{NDM} and $bla_{\mathrm{OXA\text{-}48}}$). Furthermore, copy numbers of bacterial 16S rRNA genes were determined from the same extracts to allow normalization across samples and to compare bacterial abundance between different sites/compartments. Primers and PCR conditions used for the quantification of target ARGs are compiled in SI, Table S3.

2.7. Statistical analysis

All statistical tests were performed in R version 3.3.1 (R Core Team, 2014; http://www.Rproject.org). In brief, all data were tested for normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test) using package *stats*. Significant differences in the absolute or relative abundance of target genes between systems were assessed using One-way ANOVA or its non-parametric equivalent (i.e. Kruskal-Wallis test, package *npar*) when data deviated from normality. Significance level was set at $\alpha = 0.05$.

3. Results and Discussion

3.1. Effect of partial saturation on conventional water quality parameters

Table 1 shows average values of conventional water quality parameters in the influent and effluent of the two CW units. DO and redox values were significantly higher in the effluent of the UVF (2.2 mg O_2 L^{-1} and +108 mV) than in the SVF (0.2 mg O_2 L^{-1} and –5 mV), due to the higher oxygenation of the UVF bed. Consequently, sulfate reduction occurred only in the SVF wetland (60%), which was also observed in a study conducted simultaneously at this treatment plant (Pelissari et al., 2018), who reported the enrichment of sulfate-reducing bacteria (SRB) such as *Desulfobacterales* and *Desulforomonadales* in this CW. Electric conductivity (EC) showed a slight increase in the SVF, which could be associated to the higher evapotranspiration in this unit (16% *vs.* 2% in SVF and UVF units), concomitant with a better adaptation of the vegetation (greater plant height and growth). Despite the pH remained close to neutrality at all sampling points (6.9–7.5), values were somehow lower in the SVF wetland during all the sampling campaigns, which could be related to the production of organic acids by fermentation under anoxic conditions within the saturated zone (Lettinga, 1995). The higher removal of organic matter in the UVF compared to the SVF unit (60% *vs.* 36% COD, respectively) could be attributed to the higher oxygen transfer capacity of the

UVF unit due to the complete drainage of water in the filter bed between feeding events (Nivala et al., 2013). The removal of solids was also superior in the UVF than in the SVF (79% *vs.* 68% TSS removal, respectively), which is in agreement to previous results by Saeed and Sun (2017).

Table 1. Average concentration (± SD) of conventional water quality parameters at the influent (Imhoff tank) and effluent of the unsaturated (UVF) and saturated (SVF) vertical subsurface flow constructed wetlands during the study period (June and July 2016; n=12), and removal efficiency (in parenthesis).

Variable/parameter	Influent	UVF	SVF
***T (ºC)**	23.2 ± 2.2	22.1 ± 2.1	24.3 ± 1.7
***DO (mg L^{-1})**	0.2 ± 0.2	2.2 ± 0.7	0.2 ± 0.3
***EC (mS cm^{-1})**	2.3 ± 0.6	2.3 ± 0.3	2.8 ± 0.5
***E_H (mV)**	-65 ± 49	+108 ± 34	-5 ± 41
***pH**	7.2 ± 0.1	7.5 ± 0.2	6.9 ± 0.2
TSS (mg L^{-1})	97 ± 52	20 ± 13 (79%)	31 ± 13 (68%)
***COD (mg L^{-1})**	232 ± 64	93 ± 33 (60%)	148 ± 36 (36%)
***TOC (mg L-1)**	71 ± 29	32 ± 13 (55%)	51 ± 23 (28%)
TN (mg L^{-1})	56 ± 11	37 ± 7 (34%)	32 ± 5 (43%)
NH_4-N (mg L^{-1})	19 ± 4	6 ± 4 (68%)	4 ± 1 (79%)
***NO_x-N (mg L^{-1})**	<LOD	26.9 ± 6.6	1.1 ± 1.0
***SO_4^{2-}**	85 ± 18	111 ± 14 (-30%)	34 ± 22 (60%)
PO_4-P	5.5 ± 1.3	6.0 ± 1.4 (-9%)	7.5 ± 3.1 (-36%)

<LOD: below limit of detection
* Statistical significance observed between UVF and SVF wetlands.

The saturation of the bottom compartment in the SVF (43% of total depth) did not limit the nitrification capacity of the filter bed, and the concentrations of ammonia in the SVF and UVF effluents were similar. Moreover, a high denitrification activity probably occurred in the SVF unit as suggested by the negligible concentration of NO_x-N in the effluent, compared to the UVF (1.1 NO_x-N L^{-1} in SVF vs 27 mg NO_x-N L^{-1} in UVF). Consequently, the removal of TN was higher in the SVF (43%) than in the UVF (34%), which is in agreement with

previous studies (Silveira et al., 2015; Saeed and Sun, 2017). Pelissari et al. (2018) observed a higher difference after a larger operation period that included the winter season (January-July) (56% *vs.* 34%). These data confirmed, altogether with microbiological assessments previously carried out within this experimental plant, that the partial saturation of the filter bed of the SVF wetland enabled the occurrence of a greater denitrifying activity.

3.2. Behavior of antibiotics in constructed wetlands

3.2.1. Occurrence of antibiotics in influent wastewater

13 antibiotics were quantified in influent wastewater samples at least 1 of the 4 days of sampling (Table 2). 6 of which were present in all influent samples, namely all the fluoroquinolones (ciprofloxacin, ofloxacin) and macrolides (azithromycin and clarithromycin) detected, pipemidic acid and clindamycin. Concentrations in the influent ranged from 1.8 ng L^{-1} for clindamycin to 1190.8 ng L^{-1} for pipemidic acid. The latter has been previously found in other uWWTPs of Spain up to a concentration of 2500 ng L^{-1} (Dorival-García et al., 2013a; Gracia-Lor et al., 2012). For macrolides, azithromycin was the second antibiotic detected at the highest concentration, at concentration in the range 379–627 ng L^{-1}, and clarithromycin was found at lower concentrations (29–266 ng L^{-1}). Azithromycin concentrations were slightly higher than those reported by Rodríguez-Mozaz et al. (2015) in an uWWTPs of NE Spain, whereas clarithromycin levels were in agreement to those measured by Dorival-García et al. (2013a) in two uWWTPs. Ciprofloxacin (105–278 ng L^{-1}) and ofloxacin (130–214 ng L^{-1}) have frequently been found at similar values in urban wastewater in Spain (Dorival-García et al., 2013; Rodríguez-Mozaz et al., 2015), across Europe (Castiglioni et al., 2005; Lindberg et al., 2006), and worldwide (Ghosh et al., 2009; Shi et al., 2009). Moreover, of the two lincosamides studied, clindamycin was the only one detected (lincomycin was not present in any influent sample) and at relatively low concentrations (1.8-40 ng L^{-1}), results which are in agreement with those reported by Fatta-

313 Kassinos et al. (2011). Three sulfonamides (i.e. sulfadiazine, sulfathiazole and
314 sulfapyridine) were detected two of the four days of sampling at concentrations ranging
315 from 71.5 ng L^{-1} (sulfadiazine) to 186.1 ng L^{-1} (sulfathiazole); sulfamethoxazole was found
316 just at a single sampling campaign (33 ng L^{-1}) and sulfisoxazole was detected but non-
317 quantified, which agrees with the low frequency of detection and levels observed a recent
318 study in the same area (García-Galán et al., 2020).

319 **Table 2**. Average concentration of antibiotics in influent wastewater and removal efficiencies
320 (%) of the two vertical flow wetland configurations (UVF: unsaturated unit; SVF: saturated unit)

Group / Compound	Frequency of detection (%)	Influent concentration (ng L^{-1})	RE (%) UVF	RE (%) SVF
Sulfonamides				
Sulfamethoxazole	25	33	>98	>94
Sulfadiazine	50	76±6	NA	61±52
Sulfapyridine	50	90±8	NA	64±50
Sulfathiazole	50	176±14	NA	75±33
Fluoroquinolones				
*Ciprofloxacin	100	213±80	-28±73	77±14
*Ofloxacin	100	165±35	<-200	98±0.4
*Pipemidic acid	100	780±400	80±12	>99
ß-lactams				
Ampicillin	75	160±17	55±5	49±8
Macrolides				
*Azithromycin	100	455±117	-17±30	53±10
Clarithromycin	100	105±109	58±31	65±34
Lincosamides				
Clindamycin	100	12±18	<-200	<-200
Nitroimidazoles				
Metronidazole	25	101	82	>99
Dihydrofolate reductase inhibitors				
Trimethoprim	50	32±4	>93	>89

321 *Statistical significance between the UVF and SVF wetlands.
322 NA: not available because of recovery problems in the analysis of this matrix.
323 Note: Only those compounds with influent concentrations > LOQ were used for calculation of removal efficiencies.
324 RE% calculated for non-detected effluent values were determined by using the LOD/2, and indicated as >X%.
325

326 However, sulfamethoxazole has frequently been considered a water quality indicator due to
327 its ubiquity and recalcitrance (García-Galán et al., 2011; Gros et al., 2010; Osorio et al.,

328 2016). Furthermore, among sulfonamides it has been identified as the main responsible for
329 the increase of sulfonamide resistance in WWTP (Guo et al., 2017). Previous studies have
330 measured sulfamethoxazole in urban wastewater in Spain in concentrations of about one
331 order of magnitude higher, ranging 200–600 ng L^{-1} (García-Galán et al., 2012; Gros et al.,
332 2013; Rodríguez-Mozaz et al., 2015). Regarding the 4 penicillins evaluated, only ampicillin
333 was detected at concentrations ranging from 146 ng L^{-1} to 179 ng L^{-1}. These levels are in
334 accordance to those measured in a previous study conducted in a uWWTP in NW Spain
335 (Hijosa-Valsero et al., 2011). Metronidazole was present only in one sample (101 ng L^{-1}),
336 and its metabolite metronidazole-OH was not detected. Both compounds are frequently
337 detected at higher concentrations in urban wastewaters (García-Galán et al., 2020; Gros et al.,
338 2013; Rodríguez-Mozaz et al., 2015). Finally, trimethoprim was detected two of the four
339 sampling days at a range of 29-35 ng L^{-1}. These levels are slightly lower than those published
340 in previous studies in Spain (Gracia-Lor et al., 2012; Dorival-García et al., 2013; Rodríguez-
341 Mozaz et al., 2015; Gros et al., 2013). Tetracyclines and cephalosporins were not detected in
342 the present study.

343 *3.2.2. Removal of antibiotics in vertical subsurface flow wetlands*

344 The removal efficiencies (RE%) calculated in the two VF wetland systems for the 4 sampling
345 campaigns are shown in Table 2. The partially saturated wetland (SVF) showed a robust
346 performance throughout the study period with efficiencies >50% for all antibiotics, except for
347 clindamycin whose removal was negative. Excellent elimination rates (>90%) were achieved
348 for sulfamethoxazole, ofloxacin, pipemidic acid, metronidazole and trimethoprim, whereas
349 moderate removals (60-75%) were achieved for sulfadiazine, sulfapyridine, sulfathiazole,
350 ciprofloxacin and clarithromycin. Ampicillin and azithromycin were removed on average by
351 49 and 53%, respectively.

352 Conversely to the SVF system, the UVF showed a comparatively lower performance,
353 exhibiting negative RE% (higher concentrations in the effluent than in the influent) for 4
354 antibiotics (*i.e.* ciprofloxacin, ofloxacin, azithromycin and clindamycin). This was especially
355 severe for ofloxacin and clindamycin and observed each day of sampling, with average
356 removals <-200% in both cases. Negative removals for azithromycin and clindamycin were
357 previously reported by Breitholtz et al. (2012) in free water surface wetlands operating as
358 tertiary treatment. As in the present study, influent concentrations were already low and close
359 to the LOQ. Negative removals are oftentimes attributed to the desorption or release of
360 compounds sorbed onto the particulate matter that gets dissolved during the biological
361 treatment under certain pH and redox conditions (Ávila et al., 2017). Moreover, most
362 antibiotics are mainly excreted with bile and feces partially attached to the particulate matter
363 and may be released back to the aqueous phase when solids are trickled within the filter bed
364 (Lindberg et al., 2005; Plósz et al., 2010; Verlicchi et al., 2012). Another possible cause
365 might be a result of the transformation of the conjugated forms into the original parent
366 compound (Göbel et al., 2007; García-Galán et al., 2012; Dong et al., 2016; Mamo et al.,
367 2018).

368 It has been widely reported that sorption seems to be the major removal mechanism of
369 fluoroquinolones during biological wastewater treatment (Golet et al., 2003; Verlicchi et al.,
370 2012). Both ciprofloxacin and ofloxacin have been found in the literature in the particulate
371 phase of influent and effluent wastewater at relative abundances higher than 20% of the total
372 concentration (Petrie et al., 2014; Marx et al., 2015). In CW systems, these compounds
373 generally present a low solubility and a high tendency to adsorb to the gravel in the filter
374 media; furthermore, they are also very prone to photodegradation (Jia et al., 2012). Binding
375 mechanisms involved in the sorption of contaminants onto a solid matrix depend on much
376 more than only its K_{ow} value. Other interactions specific to the functional groups of sorbates

and sorbents, which in turn depend on factors such as pH or the ionic strength (Hörsing et al., 2011; Svahn and Björklund, 2015; Wu et al., 2015) contribute to the strength of the sorption of a molecule to particulate matter. In fact, the sorption of fluoroquinolones is supposed to be highly pH-dependent (Kümmerer, 2009). The fact that high negative RE% values were observed for ciprofloxacin and ofloxacin in the UVF wetland at every sampling event, whereas good removals occurred in the SVF (77±14% and 98±1%, respectively), suggest that specific pH and/or redox conditions may have affected sorption processes within the filter biofilm of the UVF unit. On the other hand, similar results than those observed in the SVF have been obtained for ofloxacin in HF wetlands at mesocosms scale (Chen et al., 2016a), for ciprofloxacin and ofloxacin in HF wetlands at full-scale, functioning as tertiary treatment units (Verlicchi et al., 2013), and for ciprofloxacin in VF wetlands receiving highly-loaded swine wastewater (Liu et al., 2013).

The concentration of clindamycin in the effluent of both wetlands increased almost every sampling day. Negative removal of clindamycin is frequently reported in studies conducted in uWWTPs (Kovalova et al., 2012; Gros et al., 2013; Nielsen et al., 2013) and also in CWs (Breitholtz et al., 2012). This lincosamide has been considered as a rather 'inert' substance, exhibiting a low capacity to be mineralized but forming persistent transformation products, as sulfoxide and N-desmethyl clindamycin. Indeed, several authors have proposed to draw special attention on the determination of the human metabolite sufisoxide clindamycin in influent samples to correct measured input loads of clindamycin (Oertel et al., 2014; Marx et al., 2015; Ooi et al., 2017). Clindamycin has also been found in sewage sludge, although at relatively low concentrations (2-11 ng g^{-1}) (Marx et al., 2015; Subedi et al., 2017), thus deconjugation of its human metabolites seems the most plausible explanation for its negative removal (Marx et al., 2015).

401 Regarding macrolides, high K_{ow} values and low water solubilities usually lead to high
402 adsorption by plants and the material of the filter media. Average removal efficiencies of
403 82% have been reported for these antibiotics in VF wetlands (Liu et al., 2019). In the present
404 study, however, the negative removal obtained for azithromycin in the UVF unit is in
405 agreement with the literature in CWs, which reports a high variability on its removal,
406 observing from negative up to 86% elimination (Verlicchi and Zambello, 2014). Negative to
407 negligible RE% were also observed in three uWWTPs evaluated by Gros et al. (2013), as
408 well as after fungal treatment with *Trametes versicolor* (Lucas et al., 2016). Negative
409 removal (12%) and low sorption capacity was also found by Marx et al. (2015) in uWWTP.
410 However, 20-66% removal of this compound was observed by Yan et al. (2014) in the largest
411 uWWTP of southwest China, where sorption onto sludge represented about 9-27% of the
412 initial loading. Thus, the increase of its concentration in the UVF unit might be due to its
413 desorption from the rhizosphere of the wetland under specific physical and chemical
414 conditions. Regarding clarithromycin, its elimination was similar in the SVF (65±34%) and
415 the UVF (58±31%), being in accordance with the moderate removal shown in the literature in
416 CWs under different configurations (Hijosa-Valsero et al., 2011; Rühmland et al., 2015; Chen
417 et al., 2016a). Moreover, Marx et al. (2015) observed low removal (11%) of this antibiotic
418 and no sorption capacity to the sludge of uWWTPs.

419 The saturation of the filter bed was also beneficial for the removal of pipemidic acid, which
420 was reduced by >99% in the SVF unit at all sampling campaigns and by 80±12% in the UVF
421 unit. This elimination was higher than that observed in a conventional uWWTP (45%) in NE
422 Spain (influent concentration of 80±47 ng L^{-1}) (Rodríguez-Mozaz et al., 2015). Batch
423 experiments showed that despite the low K_{ow} of this substance, its sorption capacity to sludge
424 was much higher than expected (>500 L Kg^{-1}) (Dorival-García et al., 2013b), indicating that
425 sorption plays a major role on the removal of this and other quinolones from the aqueous

426 phase in uWWTPs (Jia et al., 2012). To the best of our knowledge, there is no previous
427 literature about the behavior of this quinolone in CWs.

428 The removal of ampicillin was similar in the UVF (55±5%) and SVF (49±8%) wetlands.
429 These results agree with a study conducted in experimental CWs with different
430 configurations, where a floating system planted with *Typha angustifolia* was the only CW out
431 of several configurations that removed this penicillin though at low RE% (29%) (Hijosa-
432 Valsero et al., 2011). Regarding sulfonamides, these could only be determined for the SVF
433 effluent, as only sulfamethoxazole could be analyzed in the UVF effluent sample. As
434 explained in the previous section, sulfamethoxazole was detected only on one day of
435 sampling and the removals achieved were similar in the UVF (>98%) and SVF (>94%) units.
436 These values were significantly higher than those observed in the same UVF wetland the
437 preceding fall season (Ávila et al., 2017), indicating that higher temperatures promote higher
438 microbial degradation rates of these organic compounds, as observed by Rühmland et al.
439 (2015). The high RE% values are in accordance with previous studies in mesoscale UVF
440 wetlands, which reported values ranging from 68% to 98% (Liu et al., 2014; Chen et al.,
441 2016b). Sulfadiazine, sulfapyridine and sulfathiazole were removed by 60-75% in the SVF
442 unit. Although sorption onto particulate matter seems to be another relevant pathway for
443 sulfonamides (Marx et al., 2015), this could be rather reversible (Yang et al., 2011).

444 Metronidazole was also detected only in one sampling campaign, achieving similar RE% in
445 both wetlands (82 and >99% for UVF and SVF). These values are higher than removals
446 obtained in CAS systems, which are usually <30% (Dolar et al., 2012; Jelic et al., 2011).
447 Almost complete removal of metronidazole was detected in a HF wetland planted with *P.*
448 *australis* and operating at tertiary treatment level (Li et al., 2014). This nitroimidazole has
449 been found to be weakly adsorbed to soil (Rabølle and Spliid, 2000) and barely biodegraded
450 in uWWTPs. On the contrary, and due to its high photosensitivity, photodegradations appears

as its principal removal pathway. This was recently observed by García-Galán et al. (2020) in a microalgae-based wastewater treatment system (high rate algae ponds) fed with urban wastewater. In this study, metronidazole was not detected in any biomass sample, and its overall removal was >90%. The high RE% of trimethoprim agrees studies in the literature in HF and VF wetlands (Verlicchi and Zambello, 2014; Chen et al., 2016b; Sgroi et al., 2018).

All in all, statistical results indicated that the partial saturation of the filter bed of the SVF wetland configuration seemed to enhance the elimination of 4 out of the 10 antimicrobials detected in both CWs (i.e. ciprofloxacin, ofloxacin, pipemidic acid and azithromycin). This could be attributed both to the higher microbial community diversity developed in its filter biofilm (Pelissari et al., 2018; Sgroi et al., 2018), together with the pH and redox conditions occurring within the filter bed of this wetland (Saeed and Sun, 2012). The larger variation in redox conditions occurring within this wetland configuration should allow the existence of both aerobic and anaerobic degradation pathways, thus promoting the removal of relatively complex organic molecules.

3.3. Occurrence and removal of antibiotic resistance genes

Comparison of 16S rRNA gene copy numbers (used here as a proxy for bacterial abundance) between influent wastewater and the effluents from both wetlands showed similar values ($\approx 1x10^8$ 16S rRNA copies/mL) indicating that both systems did not reduce the overall bacterial load in the flowing water despite their different operational conditions (Figure 2). These results contrast with those by Chen et al. (2016b), which observed a significant reduction in the absolute concentration of 16S rRNA when treating domestic sewage using CWs operating with different configurations. These authors obtained aqueous removal rates of total ARGs ranging from 64% to 84%. Despite their similarity in quantitative terms in the current study, the composition of effluent bacterial communities from both CWs qualitatively differed from that of the influent wastewater (see Pelissari et al., 2018).

476 Out of the 12 genes targeted in our qPCR analysis, only *intI1*, *sul1*, *sul2*, *ermB*, bla_{TEM} and
477 *qnrS* were identified in both water and biofilm samples. Remarkably, genes conferring
478 resistance to glycopeptides (*vanA*), tetracyclines (*tetM* and *tetW*) and carbapenems (bla_{KPC},
479 bla_{NDM}, $bla_{OXA-048}$) were always below the limit of detection of the qPCR assays in both
480 sample types (data now shown). This result agrees with the lack of detection of the antibiotics
481 to which these genes confer resistance to in the wastewater. The fact that genes *tetM* and *tetW*
482 were below the detection limit in all samples is not surprising considering the reduced use of
483 tetracyclines in Spain (Ortíz de García et al., 2013).

484 Among the measured ARGs, genes conferring resistance to sulfonamides (i.e. *sul1* and *sul2*)
485 showed the highest abundance in influent wastewater (4.25×10^6 and 4.43×10^6 copies/mL,
486 respectively), followed by bla_{TEM} (2.82×10^6 copies/mL), *ermB* (9.06×10^5 copies/mL) and
487 *qnrS* (1.30×10^5 copies/mL) (Figure 2). These concentrations are one (*ermB*, *qnrS*) or two
488 (*sul1*, *sul2*) orders of magnitude lower than those measured by Chen et al. (2016a, b) in
489 sewage feeding similar CWs. In both CW units, the absolute concentrations of measured
490 ARGs in effluents were lower than those measured in influent wastewater although the
491 observed differences were not statistically significant (Figure 2). The only exception was
492 *sul2*, which showed a significant decrease in its absolute concentration in the effluent of the
493 UVF wetland when compared to the influent wastewater (One-way ANOVA, Tukey HSD
494 post-hoc test $p<0.02$). Overall, these results suggest that under the operational conditions
495 tested, any of the wetlands was efficient enough to significantly reduce the abundance of
496 detected ARGs per unit volume of water (being *sul2* the exception in UVF). Similar results
497 were obtained when comparing the relative abundance of most of the studied ARGs (gene
498 copies normalized by 16S rRNA gene copies) (Suppl. Figure S2). No differences were
499 observed between influent wastewater and effluents from both wetlands in the relative
500 abundance of genes *intI1*, *sul2*, bla_{TEM} and *ermB* and only genes *sul1* and *qnrS* showed a

slight but significant decrease in their prevalence within the bacterial community in effluents from the SVF system.

The abundance pattern of the studied genes in the gravel media of both CWs was similar to that found in the flowing wastewater, being *intI1*, *sul1,* and *sul2* the ARGs showing the highest abundance, followed by *ermB* and bla_{TEM} (Figure 3). Remarkably, however, the gene *qnrS* was not detected in gravel media (data not shown) suggesting that bacteria carrying this gene were unable to develop in gravel biofilms under the conditions tested. Moreover, the fact that we did not observe differences in the absolute abundance of the detected ARGs between the top (5–15 cm depth) and the bottom (70–80 cm depth) layers of both wetlands, suggests a homogeneous distribution of ARGs across the filter bed. Nevertheless, the comparison of absolute abundances of ARGs between wetland systems yielded significative differences for *intI1*, *sul1* and *ermB*, which showed lower concentrations in biofilms developed under unsaturated conditions (Figure 3). Furthermore, when concentrations were normalized by the bacterial abundance in both gravel media, differences between both CWs were even more pronounced for all genes except bla_{TEM} (Suppl. Figure 3), suggesting that conditions in the unsaturated wetland did not favor the colonization of gravel particles by bacteria carrying ARGs. Aydin et al. (2015) also reported that anaerobic conditions (prevalent in the saturated CW) favor the accumulation of ARGs.

For the detected ARGs, the estimated removal rates ranged between 21% to 93% in both wetland systems (Table 3). These values fairly agree with those obtained by Chen et al. (2016a, b) in CWs using different substrates, flow configurations, and plant species. Remarkably, yet no differences were observed in the removal of any of the detected ARGs between both wetland configurations, although the elimination of 16S rRNA and *intI1* genes showed contrasting results in UVF and SVF. Indeed, whereas the SVF effluent showed a positive enrichment in *16S rRNA* (average removal rate of –2.4%) and *intI1* (–7.7%) gene

copies, the effluent of the UVF system showed an average removal rate of 44.9% and 21.1% for these genes, respectively (Table 3). Overall, these results indicated that the studied wetlands were not able to significantly reduce either the bacterial load of the influent wastewater or the prevalence of most ARGs within the bacterial community.

Table 3. Influent average concentration (± SEM) and their average (± SEM) removal efficiencies (%) for the detected ARGs at the two vertical subsurface flow constructed wetlands (UVF: unsaturated unit; SVF: saturated unit).

Gene	Influent concentration (gene copies mL^{-1})	RE (%) UVF	RE (%) SVF
16S rRNA	$1.69\times10^{8} \pm 7.92\times10^{6}$	44.9 ± 15.5	–2.40 ± 20.9
intI1	$3.12\times10^{6} \pm 4.32\times10^{5}$	21.1 ± 23.4	–7.67 ± 25.8
sul1	$4.25\times10^{6} \pm 1.23\times10^{6}$	59.6 ± 14.1	67.2 ± 8.85
sul2	$4.43\times10^{6} \pm 1.04\times10^{6}$	81.8 ± 5.36	45.0 ± 9.77
bla_{TEM}	$2.82\times10^{6} \pm 2.43\times10^{6}$	69.9 ± 10.4	92.9 ± 0.72
ermB	$9.06\times10^{5} \pm 4.34\times10^{5}$	75.5 ± 15.1	69.7 ± 6.35
qnrS	$1.30\times10^{5} \pm 4.10\times10^{4}$	78.8 ± 9.52	80.6 ± 4.59

4. Conclusions

The occurrence and removal of 49 antibiotics and genes resistant to sulfonamides, fluoroquinolones, macrolides, glycopeptides, tetracyclines, β-lactams, and Class I integron were evaluated in an unsaturated (UVF) and a partially saturated (SVF) vertical subsurface flow constructed wetland operating in parallel. The main conclusions were:

– The occurrence of a saturated zone in the SVF wetland bed promoted the occurrence of a different redox conditions that enabled an enhanced capacity for complete nitrogen removal within a single wetland unit. However, the reduction of organic matter and solids

544 was lower in this wetland.

545 – 13 antibiotics were quantified in the influent wastewater at least in one of the 4 sampling
546 days, including 4 sulfonamides (sulfamethoxazole, sulfadiazine, sulfapyridine and
547 sulfathiazole), 2 fluoroquinolones and 1 quinolone (ciprofloxacin, ofloxacin and pipemidic
548 acid), 1 ß-lactam (ampicillin), 2 macrolides (azithromycin and clarithromycin), 1
549 lincosamide (clindamycin), 1 nitroimidazole (metronidazole) and 1 dihydrofolate
550 reductase inhibitor (trimethoprim). The removal of antibiotics was significantly higher in
551 the SVF than in the UVF for all the fluoroquinolones, azithromycin, metronidazole and
552 trimethoprim. On the contrary, all the sulfonamides, ampicillin and clarithromycin had
553 RE% values similar in both units or slightly higher in the UVF. These results could be due
554 to the higher microbial diversity given specific redox and/or pH conditions in this wetland
555 type. Clindamycin was present at higher concentration in the effluent than in the influent
556 of both wetland units. Negative removals were also observed for four antibiotics at the
557 UVF wetland, suggesting that desorption/ deconjugation processes could be taking place
558 under certain pH and/or redox conditions.

559 – The removal of ARGs from wastewater in both absolute and relative terms showed a high
560 variability between sampling days, but no statistical differences were observed between
561 both CW units. The distribution of ARGs was similar in the top and bottom layers of both
562 wetland units, as well as between the two CW units, except for *sul2*, that showed
563 significantly higher abundance in the SVF unit.

564

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576

References

APHA-AWWA-WEF, 2012. Standard methods for the examination of water and wastewater, 22nd ed. Published jointly by the American Water Works Association, the American Public Health Association, and the Water Environment Federation., Washington, D.C.

Álvarez, J.A., Ávila, C., Otter, P., Kilian, R., Istenič D., Rolletschek, M., Molle, P., Khalil, N., Ameršek, I, Mishra, V.K., Jorgensen, C., Garfi, A., Carvalho, P., Brix H., Arias, C.A., 2017. Constructed wetlands and solar-driven disinfection technologies for sustainable wastewater treatment and reclamation in rural India: SWINGS project. Water Sci. Technol., 76, 1474-1489.

Ávila, C., Matamoros, V., Reyes-Contreras, C., Piña, B., Casado, M., Mita, L., Rivetti, C., Barata, C., García, J., Bayona, J.M., 2014. Attenuation of emerging organic contaminants in a hybrid constructed wetland system under different hydraulic loading rates and their associated toxicological effects in wastewater. Sci. Total Environ. 470–471, 1272–1280.

Ávila, C., García, J., 2015. Pharmaceuticals and personal care products (PPCPs) in the environment and their removal from wastewater through constructed wetlands. In: Zeng, E. (Ed.), Comprehensive Analytical Chemistry; Persistent Organic Pollutants: Analytical Techniques, Environmental Fate and Biological Effects vol. 67. Elsevier, pp. 199-244 (chapter 6). (ISBN: 978-0-444-63299-9).

Ávila, C., Pelissari, C., Sezerino, P.H., Sgroi, M., Roccaro, P., García, J., 2017. Enhancement of total nitrogen removal through effluent recirculation and fate of PPCPs in a hybrid constructed wetland system treating urban wastewater. Sci. Total Environ. 584-585, 414-425.

Aydin, S., Ince, B., Ince, O., 2015. Development of antibiotic resistance genes in microbial communities during long-term operation of anaerobic reactors in the treatment of pharmaceutical wastewater. Water Res. 83, 337-344.

Breitholtz, M., Näslund, M., Stråe, D., Borg, H., Grabic, R., Fick, J., 2012. An evaluation of free water surface wetlands as tertiary sewage water treatment of micro-pollutants. Ecotoxicol. Environ. Saf. 78, 63–71.

Berglund, B., 2015. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. Infect. Ecol. Epidemiol. 5: 28564.

Cacace, D., Fatta-Kassinos, D., Manaia, C.M., Cytryn, E., Kreuzinger, N., Rizzo, L., Karaolia, P., Schwartz, T., Alexander, J., Merlin, C., Garelick, H., Schmitt, H., de Vries, D., Schwermer, C.U., Meric, S., Ozkal, C.B., Pons, M.N., Kneis, D., Berendonk, T.U., 2019. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings. Water Res. 162, 320–330.

Carvalho, I.T., Santos, L., 2016. Antibiotics in the aquatic environment: A review of the European scenario. Environ. Int. 94, 736-757.

Casas, M.E., Chhetri, R.K., Ooi, G., Hansen, K.M.S., Litty, K., Christensson, M., Kragelund, C., Andersen, H.R., Bester, K., 2015. Biodegradation of pharmaceuticals in hospital wastewater by staged Moving Bed Biofilm Reactors (MBBR). Water Res. 83, 293-302.

Castiglioni, S., Bagnati, R., Calamari, D., Fanelli, R., Zuccato, E., 2005. A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. J. Chromatogr. A, 1092, 206-215.

Chee-Sanford, J.C., Mackie, R.I., Koike, S., Krapac, I.G., Lin, Y., Yannarell, A.C., Maxwell, S., Aminov, R.I., 2009. Fate and Transport of Antibiotic Residues and Antibiotic Resistance Genes following Land Application of Manure Waste. J. Environ. Qual. 38, 1086-1108.

Chen, J., Liu, Y.S., Su, H.C., Ying, G.G., Liu, F., Liu, S.S., He, L.Y., Chen, Z.F., Yang, Y.Q., Chen,

F.R., 2015. Removal of antibiotics and antibiotic resistance genes in rural wastewater by an integrated constructed wetland. Environ. Sci. Pollut. Res. 22, 1794–1803.

Chen, J., Wei, X., Liu, Y., Ying, G., Liu, S., He, L., Su, H., Hu, L., Chen, F., Yang, Y., 2016a. Removal of antibiotics and antibiotic resistance genes from domestic sewage by constructed wetlands: Optimization of wetland substrates and hydraulic loading. Sci. Total Environ. 565, 240–248.

Chen, J., Ying, G., Wei, X., Liu, Y., Liu, S., Hu, L., He, L., Chen, Z., Chena, F., Yang, Y., 2016b. Removal of antibiotics and antibiotic resistance genes from domestic sewage by constructed wetlands: Effect of flow configuration and plant species. Sci. Total Environ. 571, 974-982.

Corno, G., Yang, Y., Eckert, E.M., Fontaneto, D., Fiorentino, A., Galafassi, S., Zhang, T., Di Cesare, A., 2019. Effluents of wastewater treatment plants promote the rapid stabilization of the antibiotic resistome in receiving freshwater bodies. Water Res. 158, 72–81.

Dolar, D., Gros, M., Rodriguez-Mozaz, S., Moreno, J., Comas, J., Rodriguez-Roda, I., Barceló, D., 2012. Removal of emerging contaminants from municipal wastewater with an integrated membrane system, MBR-RO. J. Hazard. Mater. 239–240, 64–69.

Dolliver, H., Gupta, S., 2008. Antibiotic losses in leaching and surface runoff from manure-amended agricultural land. J. Environ. Qual. 37, 1227–1237.

Dong, H., Yuan, X., Wang, W., Qiang, Z., 2016. Occurrence and removal of antibiotics in ecological and conventional wastewater treatment processes: A field study. J. Environ. Manag. 178, 11-19.

Dong, Z., Sun, T., 2007. A potential new process for improving nitrogen removal in constructed wetlands – promoting coexistence of partial-nitrification and ANAMMOX. Ecol. Eng. 31, 69-78.

Dorival-García, N., Zafra-Gómez, A., Cantarero, S., Navalón, A., Vílchez, J.L., 2013a. Simultaneous determination of 13 quinolone antibiotic derivatives in wastewater samples using solid-phase extraction and ultra performance liquid chromatography–tandem mass spectrometry. Microchemical Journal 106, 323–333.

Dorival-García, N., Zafra-Gómez, A., Navalón, A., González, J., Vílchez, J.L., 2013b. Removal of quinolone antibiotics from wastewaters by sorption and biological degradation in laboratory-scale membrane bioreactors. Sci. Total Environ. 442, 317-328.

European Centre for Disease Prevention and Control, 2018. Antimicrobial consumption in the EU/EEA. Annual epidemiological report for 2018.

Fatta-Kassinos, D., Meric, S., Nikolaou, A., 2011. Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. Anal. Bioanal. Chem. 399, 251–275.

García-Galán, M.J., Arashiro, L., Santos, L.H.M.L.M., Insa, S., Rodríguez-Mozaz, S., Barceló, D., Ferrer, I., Garfí, M., 2020. Fate of priority pharmaceuticals and their main metabolites and transformation products in microalgae-based wastewater treatment systems. J. Hazard. Mater. 121771

García-Galán, M. J., Díaz-Cruz, M. S., Barceló, D., 2011. Occurrence of sulfonamide residues along the Ebro river basin. Removal in wastewater treatment plants and environmental impact assessment. Environ Int. 37, 462-473.

García-Galán, M. J., González Blanco, S., López Roldán, R., Díaz-Cruz, S., Barceló, D., 2012. Ecotoxicity evaluation and removal of sulfonamides and their acetylated metabolites during conventional wastewater treatment. Sci. Total Environ. 437, 403-412.

Ghosh, G. C., Okuda, T., Yamashita, N., Tanaka, H., 2009. Occurrence and elimination of antibiotics at four sewage treatment plants in Japan and their effects on bacterial ammonia oxidation. Water Sci Technol. 59, 779-786.

Göbel, A., McArdell, C.S., Joss, A., Siegrist, H., Giger, W., 2007. Fate of sulfonamides, macrolides,

670 and trimethoprim in different wastewater treatment technologies. Sci. Total Environ. 372, 361-371.

671 Golet, E., Xifra, I., Siegrist, H., Alder, A.C., Giger, W., 2003. Environmental Exposure Assessment of
672 Fluoroquinolone Antibacterial Agents from Sewage to Soil. Environ. Sci. Technol. 37, 3243-3249.

673 Gracia-Lor, E., Sancho, J.V., Serrano, R., Hernández, F., 2012. Occurrence and removal of
674 pharmaceuticals in wastewater treatment plants at the Spanish Mediterranean area of Valencia.
675 Chemosphere 87, 453-462.

676 Gros, M., Petrovic, M., Ginebreda, A., Barceló, D., 2010. Removal of pharmaceuticals during
677 wastewater treatment and environmental risk assessment using hazard indexes. Environ. Int. 36,
678 15–26.

679 Gros, M., Rodríguez-Mozaz, S., Barceló, D., 2013. Rapid analysis of multiclass antibiotic residues
680 and some of their metabolites in hospital, urban wastewater and river water by ultra-high-
681 performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass
682 spectrometry. J. Chromatogr. A, 1292, 173– 188.

683 Guo, X. Pang, W., Dou, C., Yin, D., 2017. Sulfamethoxazole and COD increase abundance of
684 sulfonamide resistance genes and change bacterial community structures within sequencing batch
685 reactors. Chemosphere 175, 21-27.

686 He, Y., Nurul, S., Schmitt, H., Sutton, N.B., Murk, T.A.J., Blokland, M.H., Rijnaarts, H.H.M.,
687 Langenhoff, A.A.M., 2018. Evaluation of attenuation of pharmaceuticals, toxic potency, and
688 antibiotic resistance genes in constructed wetlands treating wastewater effluents. Sci. Total Enviro.
689 631–632, 1572–1581.

690 Hernando-Amado, S., Coque, T.M., Baquero, F., Martinez, J.L., 2019. Defining and combating
691 antibiotic resistance from One Health and Global Health perspectives. Nat. Microbiol. 4, 1432–
692 1442.

693 Hijosa-Valsero, M., Fink, G., Schlüsener, M.P., Sidrach-Cardona, R., Martín-Villacorta, J., Ternes,
694 T., Bécares, E., 2011. Removal of antibiotics from urban wastewater by constructed wetland
695 optimization. Chemosphere 83, 713–719.

696 Hocquet, D., Muller, A., Bertrand, X., 2016. What happens in hospitals does not stay in hospitals:
697 antibiotic-resistant bacteria in hospital wastewater systems. J. Hosp. Infect. 93, 395-402.

698 Horcajada, J.P., Grau, S., Paño-Pardo, J.R., López, A., Oliver, A., Cisneros, J.M., Rodriguez-Baño, J.,
699 2018. Antimicrobial stewardship in Spain: Programs for optimizing the use of antibiotics
700 (PROA) in Spanish hospitals. Germs 8, 109–112.

701 Hörsing, M., Ledin, A., Grabic, R., Fick, J., Tysklind, M., Jansen, J., Andersen, H.R., 2011.
702 Determination of sorption of seventy-five pharmaceuticals in sewage sludge. Water Res. 45, 4470–
703 4482.

704 Huang, X., Liu, C., Li, K., Su, J., Zhu, G., Liu, L., 2015. Performance of vertical up-flow constructed
705 wetlands on swine wastewater containing tetracyclines and tet genes. Water Res. 70, 109-117.

706 Jia, A., Wan, Y., Xiao, Y., Hu, J., 2012. Occurrence and fate of quinolone and fluoroquinolone
707 antibiotics in a municipal sewage treatment plant. Water Res. 46, 387-394.

708 Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M., Barcelo, D.,
709 2011. Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during
710 wastewater treatment. Water Res.

711 Kahl, S., Nivala, J., van Afferden, M., Müller, R.A., Reemtsma, T., 2017. Effect of design and
712 operational conditions on the performance of subsurface flow treatment wetlands: Emerging
713 organic contaminants as indicators. Water Res. 125, 490-500.

714 Kovalova, L., Siegrist, H., Singer, H., Wittmer, A., McArdell, C.S., 2012. Hospital wastewater
715 treatment by membrane bioreactor: performance and efficiency for organic micropollutant
716 elimination. Environ. Sci. Technol. 46, 1536-1545.

717 Kümmerer, K., 2009. Antibiotics in the aquatic environment – A review – Part I. Chemosphere 75,
718 417-434.

719 Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A.K.M., Wertheim, H.F.L., Sumpradit, N., Vlieghe,
720 E., Hara, G.L., Gould, I.M., Goossens, H., Greko, C., So, A.D., Bigdeli, M., Tomson, G.,
721 Woodhouse, W., Ombaka, E., Peralta, A.Q., Qamar, F.N., Mir, F., Kariuki, S., Bhutta, Z.A.,
722 Coates, A., Bergstrom, R., Wright, G.D., Brown, E.D., Cars, O., 2013. Antibiotic resistance-the
723 need for global solutions. Lancet Infect. Dis. 13, 1057–1098.

724 Lettinga, G., 1995. Anaerobic digestion and wastewater treatment systems. Antonie van
725 Leeuwenhoek 67, 3-28.

726 Li, Y., Zhu, G., Ng, W.J., Tan, S.K., 2014. A review on removing pharmaceutical contaminants from
727 wastewater by constructed wetlands: design performance and mechanism, Sci. Total Environ. 468–
728 469, 908–932.

729 Lindberg, R.H., Wennberg, P., Johansson, M.I., Tysklind, M., Andersson, B.A.V., 2005. Screening of
730 human antibiotic substances and determination of weekly mass flows in five sewage treatment
731 plants in Sweden. Environ. Sci. Technol. 39, 3421-3429.

732 Lindberg, R. H., Olofsson, U., Rendahl, P., Johansson, M. I., Tysklind, M., Andersson, B. A. V.,
733 2006. Behavior of fluoroquinolones and trimethoprim during mechanical, chemical, and active
734 sludge treatment of sewage water and digestion of sludge. Environ. Sci. Technol. 40, 1042-1048.

735 Liu, L., Liu, C., Zheng, J.Y., Huang, X., Wang, Z., Liu, Y.H., Zhu, G.F., 2013. Elimination of
736 veterinary antibiotics and antibiotic resistance genes from swine wastewater in the vertical flow
737 constructed wetlands. Chemosphere 91, 1088–1093.

738 Liu, L., Liu, Y., Wang, Z., Liu, C., Huang, X., Zhu, G., 2014. Behavior of tetracycline and
739 sulfamethazine with corresponding resistance genes from swine wastewater in pilot-scale
740 constructed wetlands. J. Hazard. Mater. 278, 304-310.

741 Liu, X., Guo, X., Liu, Y., Lu, S., Xi, B., Zhang, J., Wang, Z., Bi, B., 2019. A review on removing
742 antibiotics and antibiotic resistance genes from wastewater by constructed wetlands:
743 Performance and microbial response. Environ. Pollut. 254, 112996.

744 Liu, Y.S., Ying, G.G., Shareef, A., Kookana, R.S., 2011. Biodegradation of three selected
745 benzotriazoles under aerobic and anaerobic conditions. Water Res. 45, 5005-5014.

746 Lucas, D., Badía-Fabregat, M., Vicent, T., Caminal, G., Rodríguez-Mozaz, S., Balcázar, J.L., Barceló,
747 D., 2016. Fungal treatment for the removal of antibiotics and antibiotic resistance genes in
748 veterinary hospital wastewater. Chemosphere 152, 301-308.

749 Luprano, M.L., De Sanctis, M., Del Moro, G., Di Iaconi, C., Lopez, A., Levantesi, C., 2016.
750 Antibiotic resistance genes fate and removal by a technological treatment solution for water reuse
751 in agriculture. Sci. Total Environ. 571, 809-818.

752 Mamo, J., García-Galán, M.J., Stefani, M., Rodríguez-Mozaz, S., Barceló, D., Monclús, H.,
753 Rodríguez-Roda, I., Comas, J., 2018. Fate of pharmaceuticals and their transformation products in
754 integrated membrane systems for wastewater reclamation. Chem. Eng. J. 331, 450-461.

755 Manaia, C.M., Macedo, G., Fatta-Kassinos, D., Nunes, O.C., 2016. Antibiotic resistance in urban
756 aquatic environments: can it be controlled? Appl. Microbiol. Biotechnol. 100, 1543–1557.

757 Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato,
758 G., Iakovides, I.C., Zammit, I., Kampouris, I., Vaz-Moreira, I., Nunes, O.C., 2018. Antibiotic
759 resistance in wastewater treatment plants: Tackling the black box. Environ. Int. 115, 312–324.

760 Marti, E., Jofre, J., Balcazar, J.L., 2013. Prevalence of antibiotic resistance genes and bacterial
761 community composition in a river influenced by a wastewater treatment plant. PloS One 8 (10).

762 Marti, E., Variatza, E., Balcazar, J.L., 2014. The role of aquatic ecosystems as reservoirs of antibiotic
763 resistance. Trends Microbiol. 22, 36-41.

764 Martinez, J.L., 2009 Environmental pollution by antibiotics and by antibiotic resistance determinants.
765 Environ. Pollut. 157, 2893–2902.

766 Martínez Bueno, M.J., Gomez, M.J., Herrera, S., Hernando, M.D., Agüera, A., Fernández-Alba, A.R.,
767 2012. Occurrence and persistence of organic emerging contaminants and priority pollutants in five
768 sewage treatment plants of Spain: Two years pilot survey monitoring. Environmental Pollution
769 164, 267-273.

770 Marx, C., Günther, N., Schubert, S., Oertel, R., Kuehn, V., 2015. Mass flow of antibiotics in a
771 wastewater treatment plant focusing on removal variations due to operational parameters. Sci.
772 Total Environ. 538, 779-788.

773 Matamoros, V., Rodríguez, Y., Bayona, J.M., 2017. Mitigation of emerging contaminants by full-
774 scale horizontal flow constructed wetlands fed with secondary treated wastewater. Ecol. Eng. 99,
775 222–227.

776 Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-
777 Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in
778 the environment: a review. Water Res. 47, 957-995.

779 Nielsen, U., Hastrup, C., Klausen, M.M., Pedersen, B.M., Kristensen, G.H., Jansen, J.L., Bak, S.N.,
780 Tuerk, J., 2013. Removal of APIs and bacteria from hospital wastewater by MBR plus O(3),
781 O(3)+H(2)O(2), PAC or ClO(2). Water Sci. Technol. 67, 854-862.

782 Nivala, J., Wallace, S., Headley, T., Kassa, K., Brix, H., van Afferden, M., Müller, R., 2013. Oxygen
783 transfer and consumption in subsurface flow treatment wetlands. Ecol. Eng. 61, 544-554.

784 Oertel, R., Schubert, S., Mühlbauer, V., Büttner, B., Marx, C., Kirch, W., 2014. Determination of
785 clindamycin and its metabolite clindamycin sulfoxide in diverse sewage samples. Environ. Sci.
786 Pollut. R. 21, 11764-11769.

787 Ooi, G.T.H., Casas, M.E., Andersen, H.R., Bester, K., 2017. Transformation products of clindamycin
788 in moving bed biofilm reactor (MBBR). Water Res. 112, 139-148.

789 Ortiz de García, S., Pinto Pinto, G., García Encina, P., Irusta Mata, R., 2013. Consumption and
790 occurrence of pharmaceutical and personal care products in the aquatic environment in Spain. Sci.
791 Total Environ. 444, 451, 465.

792 Osorio, M., Reis, S., Lima, J., Segundo, M., 2016. Analytical Features of Diclofenac Evaluation in
793 Water as a Potential Marker of Anthropogenic Pollution. Curr. Pharm. Anal. 13, 39–47.

794 Paing, J., Guilbert, A., Gagnon, V., Chazarenc, F., 2015. Effect of climate, wastewater composition,
795 loading rates, system age and design on performances of French vertical flow constructed
796 wetlands: A survey based on 169 full scale systems. Ecol. Eng. 80, 46-52.

797 Paz, A. de la, Salinas, N., Matamoros, V., 2019. Unravelling the role of vegetation in the attenuation
798 of contaminants of emerging concern from wetland systems: Preliminary results from column
799 studies. Water Res. 166, 115031.

800 Pelissari, C., Ávila, C., Trein, C.M., García, J., de Armas, R.D., Sezerino, P.H., 2017. Nitrogen
801 transforming bacteria within a full-scale partially saturated vertical subsurface flow constructed
802 wetland. Sci. Total Environ. 574, 390-399.

803 Pelissari, C., Guivernau, M., Viñas, M., García, J., Velasco-Galilea, M., Silva, S.S., Sezerino, P.H.,
804 Ávila, C., 2018. Effects of partially saturated conditions on the metabolically active microbiome
805 and on nitrogen removal in vertical subsurface flow constructed wetlands. Water Res. 141, 185-
806 195.

807 Petrie, B., McAdam, E.J., Lester, J.N., Cartmell, E., 2014. Obtaining process mass balances of
808 pharmaceuticals and triclosan to determine their fate during wastewater treatment. Sci. Total
809 Environ. 497-498, 553-560.

810 Plósz, B.G., Leknes, H., Thomas, K.V., 2010. Impacts of Competitive Inhibition, Parent Compound

811 Formation and Partitioning Behavior on the Removal of Antibiotics in Municipal Wastewater
812 Treatment. Environ. Sci. Technol. 44, 734-742.

813 Proia, L., von Schiller, D., Sànchez-Melsió, A., Sabater, S., Borrego, C.M., Rodríguez-Mozaz, S.,
814 Balcázar, J.L., 2016. Occurrence and persistence of antibiotic resistance genes in river biofilms
815 after wastewater inputs in small rivers. Environ. Pollut. 210, 121-128.

816 Rabølle, M., Spliid, N.H., 2000. Sorption and mobility of metronidazole, olaquindox, oxytetracycline
817 and tylosin in soil. Chemosphere 40, 715-722.

818 Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos,
819 D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes
820 spread into the environment: a review. Sci. Total Environ. 447, 345-360.

821 Rodríguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sánchez-Melsió, A., Borrego,
822 C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in
823 hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234-242.

824 Rühmland, S., Wick, A., Ternes, T.A., Barjenbruch, M., 2015. Face of pharmaceuticals in a
825 subsurface flow constructed wetland and two ponds. Ecol. Eng. 80, 125-139.

826 Sabourin, L., Beck, A., Duenk, P.W., Kleywegt, S., Lapen, D.R., Li, H., Metcalfe, C.D., Payne, M.,
827 Topp, E., 2009. Runoff of pharmaceuticals and personal care products following application of
828 dewatered municipal biosolids to an agricultural field. Sci. Total Environ. 407, 4596–4604.

829 Saeed, T., Sun, G., 2012. A review on nitrogen and organics removal mechanisms in subsurface flow
830 constructed wetlands: Dependency on environmental parameters, operating conditions and
831 supporting media. J. Environ. Manag. 112, 429-448.

832 Saeed, T., Sun, G., 2017. Pollutant removals employing unsaturated and partially saturated vertical
833 flow wetlands: a comparative study. Chem. Eng. J. 325, 332-341.

834 Sapkota, A., Sapkota, A.R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., Lawrence, R., 2008.
835 Aquaculture practices and potential human health risks: current knowledge and future priorities.
836 Environ. Int. 34, 1215-1226.

837 Sgroi, M., Pelissari, C., Roccaro, P., Sezerino, P.H., García, J., Vagliasindi, F.G.A., Ávila, C., 2018.
838 Removal of organic carbon, nitrogen, emerging contaminants and fluorescing organic matter in
839 different constructed wetland configurations. Chem. Eng. J. 332, 619-627.

840 Sharma, V.K., Johnson, N., Cizmas, L, McDonald, T.J., 2016. A review of the influence of treatment
841 strategies on antibiotic resistant bacteria and antibiotic resistance genes. Chemosphere 150, 702-
842 714.

843 Shi, L., Zhou, X.F., Zhang, Y.L., Gu, G.W. 2009. Simultaneous determination of 8 fluoroquinolone
844 antibiotics in sewage treatment plants by solid-phase extraction and liquid chromatography with
845 fluorescence detection. Water Sci. Technol. 59, 805-813.

846 Silveira, D.D., Belli Filho, P., Phlippi, L.S., Kim, B., Molle, P., 2015. Influence of partial saturation
847 on total nitrogen removal in a single-stage French constructed wetland treating raw domestic
848 wastewater. Ecol. Eng. 77, 257-264.

849 Song, H., Zhang, S., Guo, J., Yang, Y., Zhang, L., Li, H., Yang, X., Liu, X., 2018. Vertical up-flow
850 constructed wetlands exhibited efficient antibiotic removal but induced antibiotic resistance genes
851 in effluent. Chemosphere 203, 434-441.

852 Soucy, S.M., Huang, J., Gogarten, J.P., 2015. Horizontal gene transfer: building the web of life. Nat.
853 Rev. Genet. 16, 472-482.

854 Subedi, B., Balakrishna, K., Joshua, D.I., Kannan, K., 2017. Mass loading and removal of
855 pharmaceuticals and personal care products including psychoactives, antihypertensives, and
856 antibiotics in two sewage treatment plants in southern India. Chemosphere 167, 429-437.

857 Subirats, J., Triadó-Margarit, X., Mandaric, L., Acuña, V., Balcázar, J.L., Sabater, S., Borrego, C.M.,

2017 Wastewater pollution differently affects the antibiotic resistance gene pool and biofilm bacterial communities across streambed compartments. Mol. Ecol. 26, 5567–5581.

Svahn, O., Björklund, E., 2015. Describing sorption of pharmaceuticals to lake and river sediments, and sewage sludge from UNESCO Biosphere Reserve Kristianstads Vattenrike by chromatographic asymmetry factors and recovery measurements. J. Chromatogr. A 1415, 73–82.

Tasho, R. P., Cho, J.Y., 2016. Veterinary antibiotics in animal waste, its distribution in soil and uptake by plants: a review. Sci. Total Environ. 563-564, 366-376.

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment-A review. Sci. Total Environ. 429,123-155.

Verlicchi, P., Galletti, A., Petrovic, M., Barceló, D., Al Aukidy, M., Zambello, E., 2013. Removal of selected pharmaceuticals from domestic wastewater in an activated sludge system followed by a horizontal subsurface flow bed - Analysis of their respective contributions. Sci. Total Environ. 454–455, 411–425.

Verlicchi, P., Zambello, E., 2014. How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? A review. Sci. Total Environ. 470-471, 1281-1306.

Yan, Q., Gao, X., Huang, L., Gan, X.M., Zhang, Y.X., Chen, Y.P., Peng, X.Y., Guo, J.S., 2014. Occurrence and fate of pharmaceutically active compounds in the largest municipal wastewater treatment plant in Southwest China: Mass balance analysis and consumption back-calculated model. Chemosphere 99, 160–170.

Yang, S., Lin, C., Lin, A., Hong, P., 2011. Sorption and biodegradation of sulfonamide antibiotics by activated sludge: Experimental assessment using batch data obtained under aerobic conditions. Water Res. 45, 3389-3397.

Wei, R., Ge, F., Huang, S., Chen, M., Wang, R., 2011. Occurrence of veterinary antibiotics in animal wastewater and surface water around farms in Jiangsu Province, China. Chemosphere 82, 1408–1414.

Wu, W., Hu, Y., Guo, Q., Yan, J., Chen, Y., Cheng, J., 2015. Sorption/desorption behavior of triclosan in sediment-water-rhamnolipid systems: effects of pH, ionic strength, and DOM. J. Hazard. Mater. 297, 59–65.

Zhang, H., Du, M., Jiang, H., Zhang, D., Lin, L., Ye, H., Zhang, X., 2015. Occurrence, seasonal variation and removal efficiency of antibiotics and their metabolites in wastewater treatment plants, Jiulongjiang River Basin, South China. Environm. Sci.: Processes Impacts 17, 225-234.

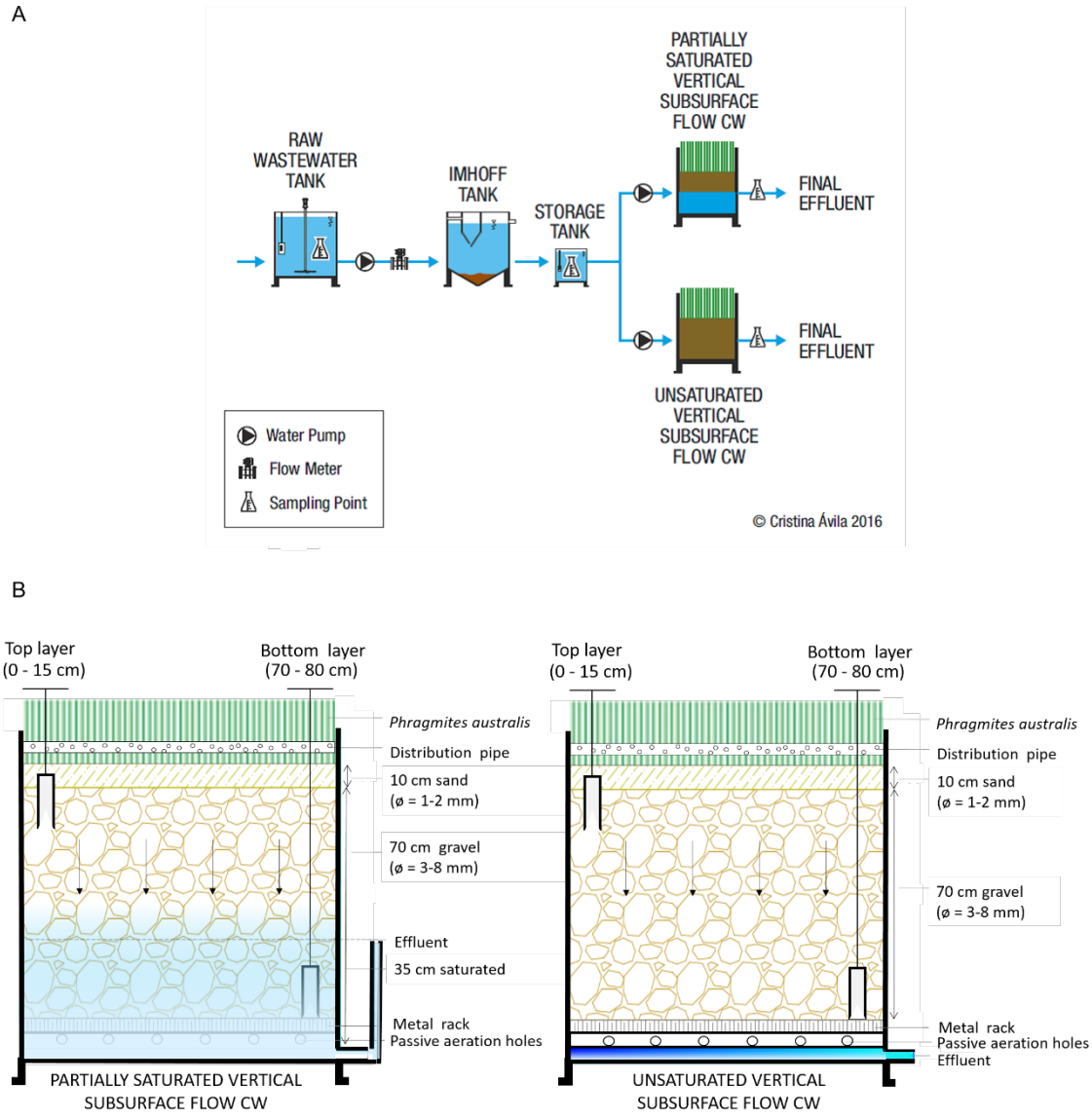

Figure 1. Wastewater treatment plant. (A) Diagram of the experimental treatment plant indicating water influent and effluent sampling points; (B) Cross-section of the two constructed wetlands indicating the gravel (biofilm) sampling depths for the determination of ARGs.

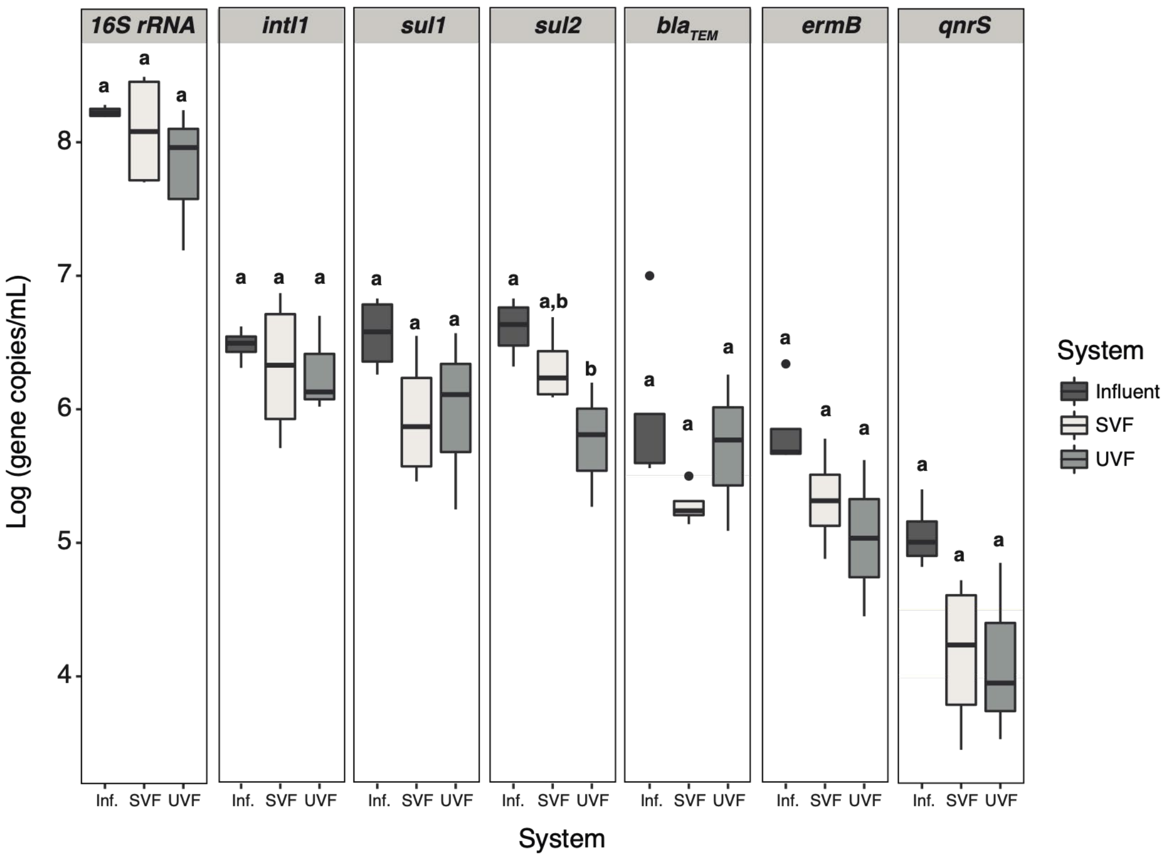

Figure 2. Boxplots showing the absolute concentrations of targeted genes in the influent and effluent wastewater from saturated (SVF) and unsaturated (UVF) constructed wetlands. Different letters above boxplots indicate significant differences (Post-hoc Tukey test after correction of *p*-values for multiple comparisons). The lower and upper edge of each boxplot are the first and third quartiles, the mid line shows the median and the whiskers extend from the minimal and maximal values.

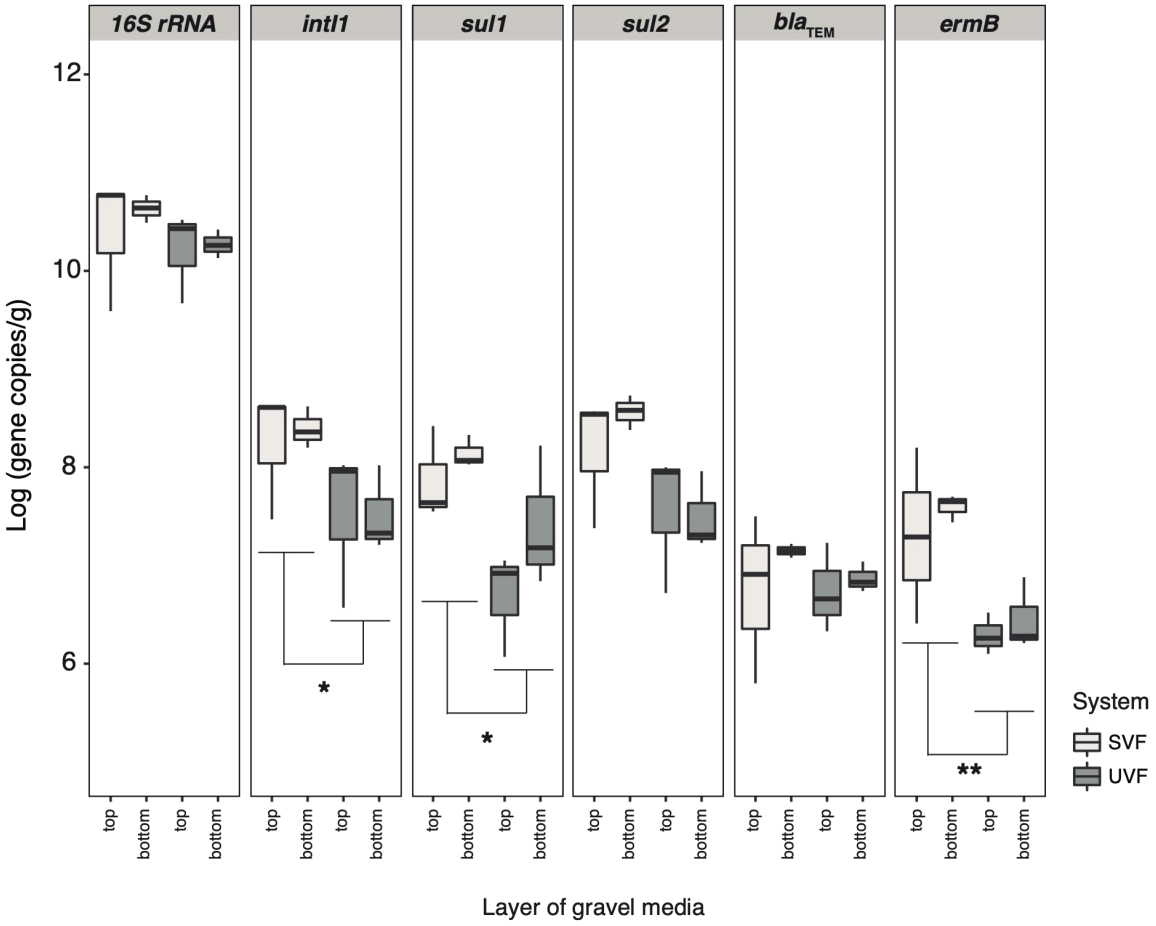

Figure 3. Boxplots showing the absolute concentrations of targeted genes at the different depth layers of gravels in saturated (SVF) and unsaturated (UVF) constructed wetlands. The lower and upper edge of each boxplot are the first and third quartiles, the mid line shows the median and the whiskers extend from the minimal and maximal values. Asterisks denote statistical significance between saturated and unsaturated CW as follows: * $p < 0.05$; ** $p < 0.01$.