URBAN WASTEWATER EFFLUENT INCREASES ANTIBIOTIC RESISTANCE GENE CONCENTRATIONS IN A RECEIVING NORTHERN EUROPEAN RIVER

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Urban wastewater effluent increases antibiotic resistance gene concentrations in a receiving Northern European river

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Abstract

Antibiotic resistant bacteria are an emerging global problem which threatens to undermine important advances in modern medicine. The environment is likely to play an important role in dissemination of antibiotic resistance genes (ARGs) among both environmental and pathogenic bacteria. Wastewater treatment plants (WWTPs) accumulate both chemical and biological waste from the surrounding urban milieu and have therefore been viewed as potential hotspots for dissemination and development of antibiotic resistance. To assess the effect of wastewater effluent on a river which flows through a Swedish city, sediment and water samples were collected from Stångån River, both upstream and downstream of an adjacent WWTP over three months. Seven ARGs and the integrase gene on class 1 integrons were quantified in the collected sediment using real-time PCR. Liquid chromatography-mass spectrometry was used to assess the abundance of ten different antibiotics in the water phase of the samples. The results showed an increase in ARGs and integrons downstream of the WWTP. The measured concentrations of antibiotics were low in the water samples from Stångån River, suggesting that selection for ARGs did not occur in the surface water. Instead, the downstream increase in ARGs is likely to be due to accumulation of genes present in the treated effluent discharged from the WWTP.

Keywords: Antibiotic resistance genes, Antibiotics, Integrons, Quantitative real-time PCR, Wastewater

INTRODUCTION

The increasing prevalence of antibiotic resistance among human pathogenic bacteria is a major global threat. Bacterial infections, which are currently cured readily by treatment with antibiotics, may become difficult, if not impossible, to treat. Furthermore, the lack of access to efficient antibiotics may make routine medical procedures such as surgery and chemotherapy in cancer treatment extremely risky [1]. Human use and misuse of antibiotics are likely to have significantly contributed to the emergence of antibiotic resistance. Recently, much attention has been directed to the role of environmental bacteria. Many antibiotic resistance genes (ARGs) carried by pathogenic bacteria are thought to have originated in environmental bacteria [2], and ARGs have been found to be ubiquitous in a large range of environments [3], including those considered pristine [4]. In particular, environments exposed to high concentrations of antibiotics have been demonstrated to also contain high concentrations of ARGs [5,6]. It seems plausible that perturbations of environmental ecosystems caused by human antibiotic contamination may play an important role in the dissemination of clinical antibiotic resistance [7,8].

Wastewater treatment plants (WWTPs) and their subsequent effluent are environments in which human bacteria and antibiotics from the urban milieu mix together with environmental bacteria, making them potential hot spots for both development and dissemination of ARGs [9,10]. WWTPs are not always efficient at removing antibiotics; these and other pharmaceuticals are often found in concentrations ranging from ng/L to low μg/L in wastewaters [11]. ARGs too, have been reported to be ubiquitous in wastewater [3,12]. Insufficiently treated industrial waste has also been observed to elevate levels of antibiotics in the environment [6,13].

Class 1 integrons are genetic assembly platforms capable of incorporating and utilising gene cassettes from the environment. These gene cassettes can encode a wide range of functions including antibiotic resistance. Class 1 integrons are widely associated with mobile genetic elements which make

them ideal for disseminating ARGs in a bacterial community [14]. Several studies have shown that class 1 integrons are more abundant in anthropogenically affected environments which indicate that these genetic elements are important in mediating ARGs in the environment [15,16].

In this study, we aimed to assess the impact of WWTP effluent on relative abundances of ARGs and integrons in the receiving river. Antibiotic and ARG concentrations were investigated in a river which flows through a Swedish city. Samples were taken in the winter 2011, upstream and downstream of the WWTP which receives wastewater from the city.

MATERIAL AND METHODS

Sampling site and collection of samples

Stångån is a river in the southern part of Sweden. It is 202 km in length and passes through the city of Linköping (population: 150,000) just before its outlet in the lake, Roxen. From its source to Linköping, Stångån passes through an area which is only lightly affected by human activities. As Stångån passes through Linköping, it receives effluent from the WWTP Nykvarnsverket. In 2011, the average flow of incoming and outgoing water of the WWTP was 46,000 m^3/d and the hydraulic retention time was 12-13 h. Water and sediment samples were gathered from five sampling locations (R1-R5) in the river. R1 was approximately 1 km upstream of the WWTP, and R2 was located just prior to the river passing the WWTP. R3 was located in the river just as it passed the WWTP, R4 was approximately 1 km downstream of the WWTP, and R5 approximately 2.5 km downstream of the WWTP. Grab-samples were collected in 2011, once in October, November and December each. The average flow of the river during these months was 6.6 m^3/s. Effluent from the WWTP was also collected at each time point. The sediment phase of the samples was pre-treated within 4 h after sampling whereas the water phase of the samples was frozen in -20 ˚C before chemical analysis.

Pre-treatment of samples and DNA extraction

Sediments were pelleted from each water sample by centrifugation of 2,000 mL of sample for 30 min in 5,000 *g*. Pellets were stored overnight in -20 ˚C before subsequent DNA extraction. DNA was extracted from the pellets accumulated from the water samples with the FastDNA SPIN Kit for Soil and the FastPrep Instrument (MP Biomedicals). Extracted DNA was stored in -20 ˚C before subsequent analyses.

Quantification of 16S rRNA genes, ARGs and intI1

Quantitative real-time PCR was used for gene quantification on the DNA extracted from the samples. The genes which were quantified were *sulI* (sulphonamide resistance gene), *dfr1* (trimethoprim resistance gene), *ermB* (macrolide/lincosamide/streptogramin B resistance gene), *tetA* and *tetB* (tetracycline resistance genes), *vanB* (vancomycin resistance gene), *qnrS* (quinolone resistance gene) and *intI1*, the integrase gene on class 1 integrons. 16S rRNA gene content was quantified and used to normalise the quantified number of genes in each sample. All PCRs were carried out on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories). Quantification method, primers, primer concentrations and thermal cycling protocols for each gene were used as described in Berglund et al. [17].

Antibiotic quantification

Antibiotic concentrations in the water samples were determined by chemical analysis using an in-line SPE column coupled to liquid chromatography-tandem mass spectrometry, as described in Khan et al. [6]. In short, a triple stage quadrupole MS/MS TSQ Quantum ULTRA EMR (Thermo Fisher Scientific) coupled with an Accela and a Surveyor LC Pump (Thermo Fisher Scientific) and a PAL HTC autosampler (CTC Analytics AG) were used as analytical system.

Statistical analysis

A Friedman test followed by a Dunn's Multiple Comparisons test was used to assess differences in ARG gene concentrations between the different sampling locations. t-tests using Welch's

correction were used to assess differences in concentration of specific genes between sites upstream and downstream of the WWTP. All statistical analyses were carried out using Prism 5 for Windows v.5.00.

RESULTS

Quantification of antibiotic resistance genes

ARGs were detected and quantified in water samples taken from all sampling points at all sampling times (Figure 1). Overall, ARG concentrations were lower at the upstream sites R1 and R2, than at the site R3, downstream of the WWTP ($p < 0.01$ and $p < 0.001$ respectively). The ARGs which were found in the highest concentrations were *sulI*, *tetA* and *ermB*. Concentrations of *ermB* were significantly higher downstream than upstream of the WWTP ($p < 0.01$), whereas concentrations of *sulI* and *tetA* were more than ten times higher downstream compared to upstream of the WWTP ($p < 0.01$). ARGs *tetB*, *dfr1* and *vanB* were found in comparatively lower concentrations, particularly at the upstream sites at which *tetB* and *dfr1* were detected below the quantification limit. *vanB* was only detected at one time point among the upstream sampling locations. ARG *qnrS* was not detected at any sampling location. All ARGs (except *qnrS*) were detected and quantified in the WWTP effluent at concentrations at similar levels as in the downstream sites.

The integrase gene *intI1* was detected and quantified in all samples (Figure 1). Concentrations were significantly higher downstream of the WWTP than upstream ($p < 0.001$). In general, *intI1* concentrations at the downstream sites were higher by approximately one order of magnitude (around 10^4 genes / 10^6 16S rDNA copies for the upstream sites and 10^5 genes / 10^6 16S rDNA copies for the downstream sites). *intI1* concentrations in the WWTP effluent were of similar magnitude to the concentrations found at the downstream sites.

Quantification of antibiotics and other pharmaceuticals

Antibiotics were quantified in the downstream locations and in the wastewater effluent (Figure 2). CIP, CLA and CLI were quantified at concentrations close to the detection limit in the treated wastewater effluent (10, 3 and 3 ng/L, respectively) while the average concentration of TRI was 24 ng/L, about an order of magnitude higher than the detection limit (3 ng/L). At the downstream sites, CLA, CLI and TRI were found sporadically, at concentrations similar to those in the wastewater effluent. No antibiotics were detected in any of the upstream sampling locations. NOR, OFX, OXY, ROX, SUL and TET were not detected at any sampling location.

Additionally, 83 non-antibiotic pharmaceuticals were analysed. Of these, only 19 were detected, mostly in effluent and downstream sampling locations. Three were detected in upstream sampling locations, very close to the detection limit (Supplemental Data, Table S1).

DISCUSSION

ARGs and integrons were quantified in sediments from Stångån River, Sweden, both upstream and downstream of a WWTP receiving wastewater from the adjacent city Linköping. Both for ARG abundance in general and when comparing abundances of specific genes, the locations downstream of the WWTP displayed significantly higher abundance than upstream locations. The difference was most pronounced for genes *sulI* and *tetA*. Several other studies have reported similar trends in ARG abundance upstream and downstream of anthropogenic perturbations. In [6], ARGs were quantified in river sediments in a river upstream and downstream of a large Pakistani city. ARG concentrations were consistently higher downstream than upstream. Abundances of *sulI* was approximately 10^3 genes / 10^6 16S rDNA copies upstream and 10^5 genes / 10^6 16S rDNA copies downstream which is higher compared to this study. The upstream abundances of *tetA* and *dfr1* were not high enough to be detected, which can be compared to the upstream abundances in this study in which *dfr1* was detectable but not quantifiable and *tetA* was found in the order of magnitude of 10^0 genes / 10^6 16S rDNA. The downstream abundances of these genes in [6], were notably higher than compared to this study; with *dfr1* being found at

189 approximately four orders of magnitude higher concentrations and *tetA* at almost two orders of magnitude
190 higher concentrations. The abundance of *sulI* in river sediments has been observed to increase in a river in
191 the United States, at a pristine site and downstream of a range of human activities [18]. Concentrations
192 increased from approximately 10^0 to 10^2 genes / 10^6 16S rDNA copies from the pristine site to the
193 perturbed sites, overall somewhat lower abundances than in this study. In [19], ARGs were measured in
194 sediments of a river upstream and downstream of a WWTP in Spain. *sulI* was found at similar
195 concentrations upstream and downstream, approximately 5×10^3 genes / 10^6 16S rDNA copies. *ermB* was
196 measured at higher concentrations downstream than upstream, although at both locations at lower
197 concentrations than in this study (by approximately one order of magnitude). It should be noted that these
198 studies were done in different areas of the world. Factors such as temperature and nutrient availability may
199 be important in resistance development, and these factors were likely different between the compared
200 locations.

201 Class 1 integron gene *intI1* was found in all samples with a significant increase in
202 abundance from upstream to downstream sites. Although integrons are ubiquitous in nature, several
203 studies have reported that human contamination increases the abundance of integrons [15,16, 20]. In [6],
204 *intI* abundances were reported to increase in river sediments as the river passed a large Pakistani city,
205 although concentrations were higher than in this study with downstream concentrations reaching as high
206 as 8×10^5 genes / 10^6 16S rDNA copies.

207 Antibiotics were not detected in the surface water at locations upstream of the WWTP.
208 However, antibiotics were detected in both wastewater effluent and in sample locations downstream of the
209 WWTP, although only four (CIP, CLA, CLI and TRI) of the ten analysed antibiotics, and at very low
210 concentrations. TRI, which was found at the highest concentrations, had a mean concentration (n=3) as
211 low as 38 ng/L (highest concentration quantified was 47 ng/L) in the effluent and 10 ng/L (n=3) in the
212 surface water. None of the other antibiotics quantifiable were found at concentrations above 20 ng/L. The
213 non-antibiotic pharmaceuticals analysed showed a similar trend to the antibiotics, the few pharmaceuticals

detected were quantified at low concentrations and only three were detected at the upstream locations. In [21], minimum selective concentrations for test strains of bacteria were found to be 10^6 ng/L, 1.5×10^4 ng/L and 10^2 ng/L for streptomycin, TET and CIP respectively. This can be compared to this study, where TET could not be detected with a detection limit at 20 ng/L, and CIP which was quantified at about half the minimum selective concentration. In [17], selection for ARGs could not be observed in a wetland bacterial community when exposed to a mixture of antibiotics including concentrations of CLA, CLI and TRI measured up to 250 ng/L, 66 ng/L and 420 ng/L, respectively. It may be reasonable to assume that the low antibiotic concentrations measured in the effluent and downstream sites do not select for ARGs. Consequently, the observed increase in ARG abundance from upstream to downstream sites likely stem from the WWTP. The ARG abundances in the wastewater may originate either from selection in the wastewater treatment process (e.g. due to exposure to antibiotics) or by accumulation of ARGs via the received waste from the urban environment.

It should be noted that, since the antibiotics are measured in the water phase, the concentrations represent only the concentrations in the water at the moment the samples were taken. As such, sedentary bacteria on the examined sediments may be exposed to a range of antibiotic concentrations well outside of the measured concentrations. The concentration of antibiotics in the untreated wastewater is also likely higher than the concentration in the effluent. This could mean that the bacteria in the WWTP are exposed to antibiotic concentrations higher than those measured in the effluent. On the other hand, the measured genes include both extracellular DNA and genes within living bacteria. Extracellular DNA can avoid environmental degradation by adhesion to sand and clay particles [22]. The ARGs from extracellular DNA have been reported to be greater than ARGs from intracellular DNA in a Chinese river basin [23]. In the case that a significant portion of the measured ARGs in the sediment are extracellular, the concentration of antibiotics in the surrounding water may have little to no effect on the selection and proliferation of ARGs.

It is becoming clear that the environment outside of clinical settings play an important role in the dissemination and spread of antibiotic resistance. Therefore it is important to elucidate the ecology and dynamics of ARG dissemination. Anthropogenic contamination and environmental perturbations have been linked to increases in ARGs and for this reason WWTPs have been regarded as potential hotspots for the dissemination of these genetic elements. The results of this study showed an increase in ARG abundances in a river downstream of a WWTP. The low antibiotic concentrations in the river and WWTP effluent indicate that selection for ARGs does not occur in the surface water. Instead, the WWTP is the likely point source of ARGs. Further studies are needed to assess the origins of these ARGs, to determine if selection for ARGs occurs in the wastewater treatment process or whether the accumulated ARGs originate in the recipient waste coming from other sources (e.g. hospitals).

SUPPLEMENTAL DATA

The concentrations of 93 different pharmaceuticals (including ten different antibiotics) were analysed in the surface water and WWTP effluent samples and are presented in Supplemental Data, Table S1.

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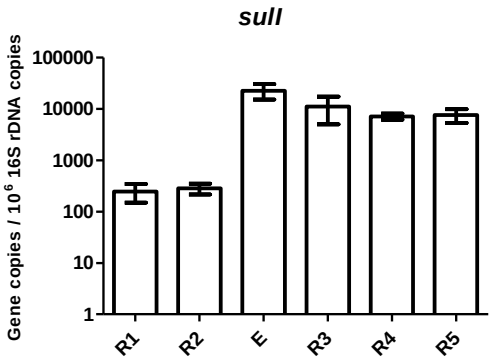
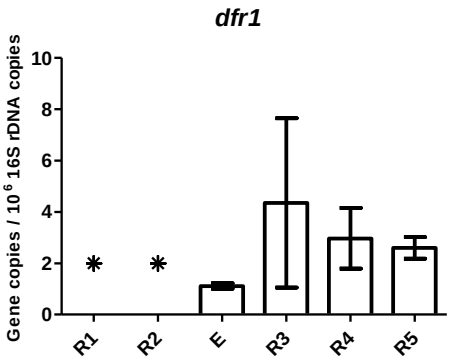
315 Figure 1. Antibiotic resistance genes (ARGs) were measured from collected sediments from Stångån River. Sites R1
316 and R2 are upstream, and sites R3, R4 and R5 are downstream of the wastewater treatment plant (WWTP). 'E'
317 sampling location denotes the wastewater effluent. Presented values are means over three months. Error bars
318 denote the standard error of the mean. Note that linearity and magnitude of the scales differ between the graphs.
319 '*' denotes; detected, below quantification limit.

320

321 Figure 2. Antibiotics were quantified from collected water samples from Stångån River. Sites R1 and R2 are
322 upstream, and sites R3, R4 and R5 are downstream of the wastewater treatment plant (WWTP). 'E' sampling
323 location denotes the wastewater effluent. CIP: ciprofloxacin, CLA: clarithromycin, CLI: clindamycin, TRI:
324 trimethoprim.

325


sull
Gene copies / 10^6 16S rDNA copies
100000
10000
1000
100
10
1
R1
R2
E
R3
R4
R5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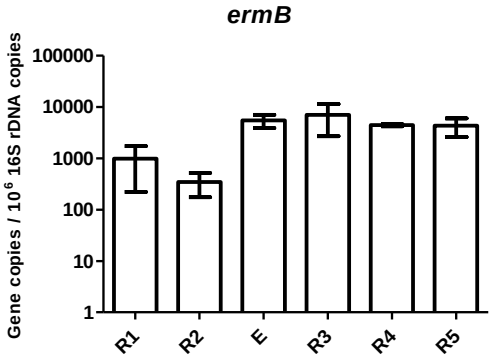
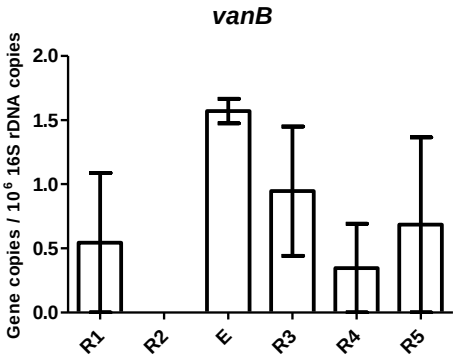
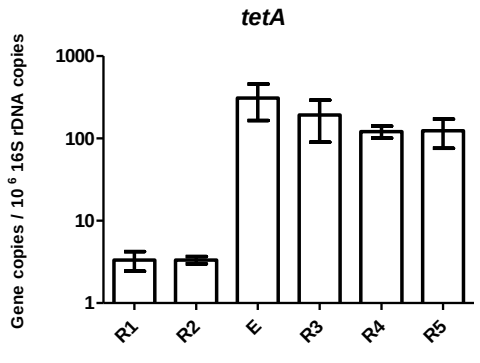
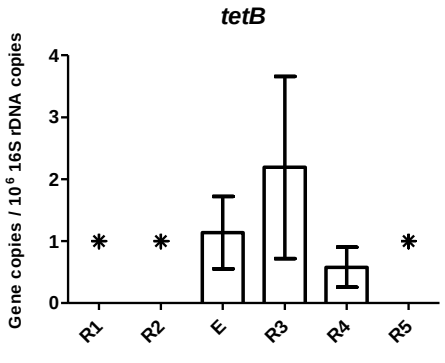
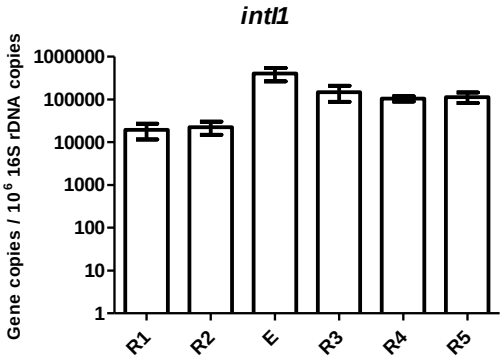
Gene copies / 10^6 16S rDNA copies
10
8
6
4
2
0
R1
R2
E
R3
R4
R5


ermB
Gene copies / 10^6 16S rDNA copies
100000
10000
1000
100
10
1
R1
R2
E
R3
R4
R5


vanB
Gene copies / 10^6 16S rDNA copies
2.0
1.5
1.0
0.5
0.0
R1
R2
E
R3
R4
R5


tetA
Gene copies / 10^6 16S rDNA copies
1000
100
10
1
R1
R2
E
R3
R4
R5


tetB
Gene copies / 10^6 16S rDNA copies
4
3
2
1
0
R1
R2
E
R3
R4
R5


intl1
Gene copies / 10^6 16S rDNA copies
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100000
10000
1000
100
10
1
R1
R2
E
R3
R4
R5

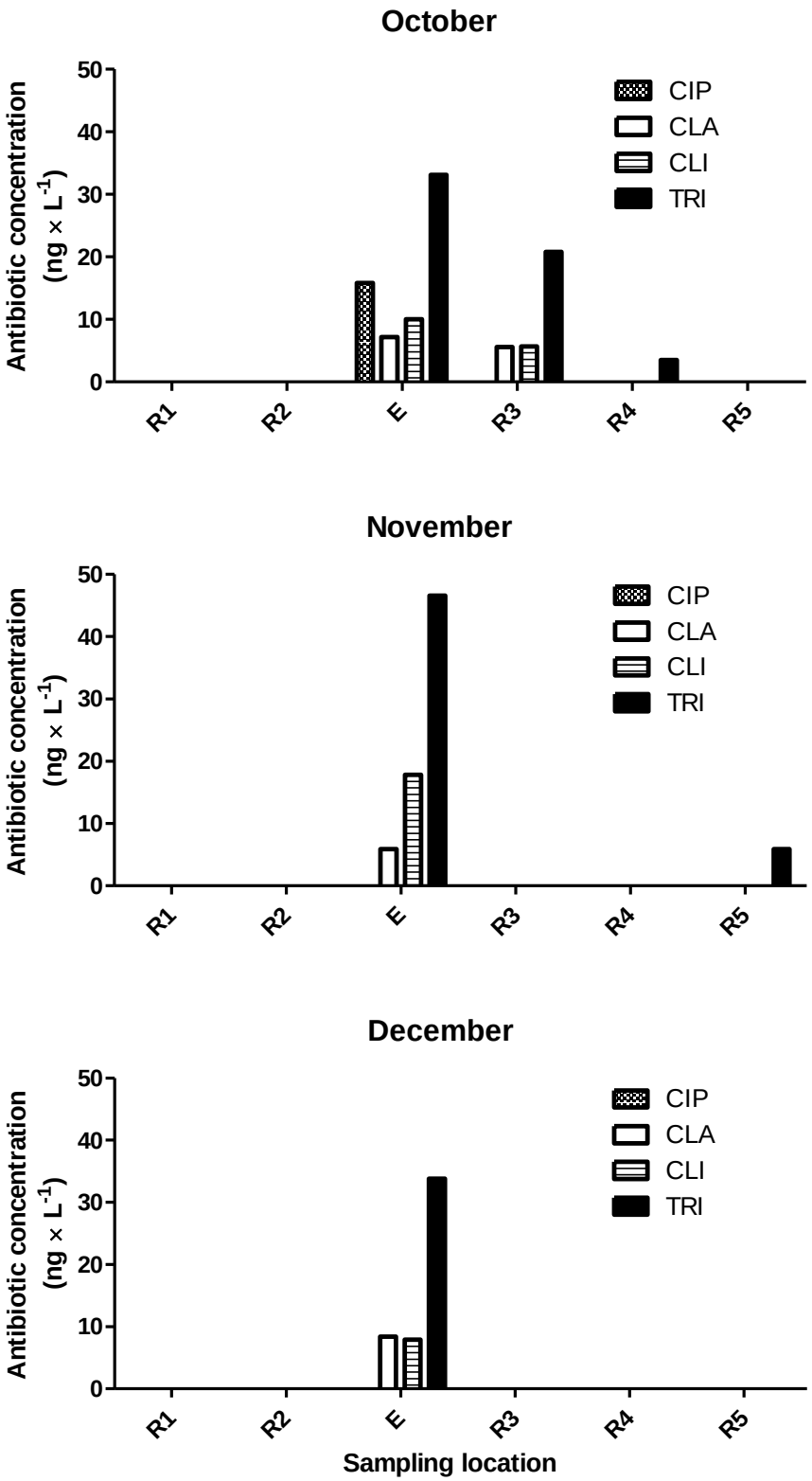
October
November
December
Antibiotic concentration (ng × L-1)
50
40
30
20
10
0
CIP
CLA
CLI
TRI
R1
R2
E
R3
R4
R5
Sampling location

Table S1. The abundance of 93 different pharmaceuticals were analysed as described in Grabic et al. [24], in the water phase of the samples from the surface water (R1-R5) and WWTP effluent (E). Concentrations are given in ng/L. '-' denotes that the concentration of the given pharmaceutical was below the limit of quantification. Pharmaceuticals which were below the limit of quantification in all sampling points are omitted from the table. These are: alfuzosin, alprazolam, amiodarone, amytriptyline, atorvastatin, atracurium, azelastine, biperiden, bromocriptine, buprenorphine, bupropion, chlorpromazine, chlorprothixene, cilazapril, citalopram, clemastine, clomipramine, clonazepam, clotrimazol, cyproheptadine, desloratidin, dicycloverine, dihydroergotamine, diphenhydramine, donepezil, duloxetine, eprosartan, fenofibrate, fentanyl, finasteride, flunitrazepam, fluoxetine, flupentixol, fluphenazine, flutamide, glibenclamide, glimepiride, haloperidol, hydroxyzine, ketoconazole, levomepromazine, loperamide, maprotiline, meclozine, memantine, mianserin, miconazole, nefazodone, norfloxacin, ofloxacin, orphenadrine, oxytetracycline, paracetamol, paroxetine, perphenazine, pizotifen, promethazine, ranitidine, repaglinide, rosuvastatin, roxithromycine, sertraline, sulfamethoxazole, tamoxifen, telmisartan, terbutaline, tetracycline, trihexyphenidyl, verapamil, zolpidem.

		Oct R1	Oct R2	Oct R3	Oct R4	Oct R5	Oct E	Nov R1	Nov R2	Nov R3	Nov R4	Nov R5	Nov E	Dec R1	Dec R2	Dec R3	Dec R4	Dec R5	Dec E
(ng/L)	LOQ[a]																		
Atenolol	15	-	-	195.6	22.3	19.9	255.3	-	-	-	-	27.1	314.9	-	-	-	-	-	268.2
Bisoprolol	3	-	-	4.4	-	-	6.5	-	-	-	-	-	9.5	-	-	-	-	-	4.9
Budesonide	20	-	-	-	-	-	-	22.3	-	-	-	-	-	-	-	-	-	-	-
Carbamazepin	8	-	-	77.8	9.1	-	93.3	-	-	-	-	12.0	124.2	-	-	-	-	-	83.8
Ciprofloxacin	10	-	-	-	-	-	15.8	-	-	-	-	-	-	-	-	-	-	-	-
Clarithromycine	3	-	-	5.6	-	-	7.2	-	-	-	-	-	5.9	-	-	-	-	-	8.4
Clindamycine	3	-	-	5.7	-	-	10.0	-	-	-	-	-	17.8	-	-	-	-	-	7.9
Codeine	15	-	-	41.3	-	-	53.3	-	-	-	-	-	68.4	-	-	-	-	-	45.6
Diclofenac	10	-	-	26.0	-	-	40.4	-	-	-	-	-	47.7	-	-	-	-	-	30.1
Diltiazem	2	-	-	-	-	-	-	-	-	-	-	-	2.2	-	-	-	-	-	-
Fexofenadine	10	-	-	15.7	-	-	11.1	-	-	-	-	-	16.6	-	-	-	-	-	13.7
Flecainide	2	-	-	15.0	2.4	1.9	23.2	-	-	-	-	2.3	36.1	-	-	-	-	-	26.9
Fluconazole	8	-	-	93.6	-	-	139.6	-	-	-	-	-	118.6	-	-	-	-	-	100.8
Irbesartan	3	-	-	7.4	-	-	8.8	-	-	-	-	-	12.1	-	-	-	-	-	9.7
Metoprolol	15	-	-	116.8	16.7	-	154.5	-	-	-	-	22.7	204.0	-	-	-	-	-	134.4
Mirtazapine	15	-	-	-	-	-	-	-	-	-	-	-	25.0	-	-	-	-	-	-
Naloxone	2	-	-	10.8	-	-	-	-	-	-	-	-	-	4.7	6.9	-	-	-	-
Oxazepam	10	-	-	11.6	-	-	14.2	-	-	-	-	-	17.9	-	-	-	-	-	13.3
Risperidone	4	6.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sotalol	15	-	-	32.7	-	-	44.7	-	-	-	-	-	53.4	-	-	-	-	-	39.4
Tramadol	15	-	-	84.4	-	-	112.1	-	-	-	-	-	140.6	-	-	-	-	-	96.8
Trimethoprim	3	-	-	20.8	3.5	-	33.1	-	-	-	-	5.9	46.6	-	-	-	-	-	33.8
Venlafaxine	20	-	-	21.7	-	-	34.7	-	-	-	-	-	50.2	-	-	-	-	-	24.6

[a] 'LOQ' denotes limit of quantification