



The impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes

Gabriela K. Paulus^{a,b,*}, Luc M. Hornstra^a, Nikiforos Alygizakis^{c,d}, Jaroslav Slobodnik^c, Nikolaos Thomaidis^d, Gertjan Medema^{a,b}

^a KWR Watercycle Research Institute, Groningenhaven 7, 3433PE, Nieuwegein, the Netherlands

^b Delft University of Technology, Faculty of Civil Engineering & Geosciences, Department of Water Management, Stevinweg 1, 2628CN, Delft, the Netherlands

^c Environmental Institute, Okružná 784/42, 97241, Koš, Slovak Republic

^d Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis Zografou, 15771, Athens, Greece

ARTICLE INFO

Keywords:

Advanced wastewater treatment
Contaminants of emerging concern
Pharmaceutical
Antibiotic resistance

ABSTRACT

This study quantified antibiotic and antibiotic resistance gene (ARG) concentrations in hospital and communal wastewaters as well as the influents and effluents of the receiving urban wastewater treatment plants (UWWTP) in two Dutch cities. In only one city, hospital wastewater was treated on-site using advanced technologies, including membrane bioreactor treatment (MBR), ozonation, granulated activated carbon (GAC) and UV-treatment.

On-site hospital wastewater (HWW) treatment reduced gene presence of hospital-related antibiotic resistance genes and antibiotic concentrations in the receiving urban wastewater treatment plant. These findings support the need for on-site treatment of high-risk point sources of antibiotic resistance genes.

13 antibiotic resistance genes, Integrase Class 1 and 16S rRNA concentrations were quantified using multiplex quantitative real-time PCR (qPCR) assays and the presence and/or concentration of 711 antibiotics were analyzed.

Hospital wastewater contained approximately 25% more antibiotics and gene concentrations between 0.4 log to 1.8-fold higher than communal wastewater (CWW). *bla*_{KPC} and *vanA* could be identified as hospital-related genes and were reduced to under the limit of detection (LOD) during on-site treatment. Advanced on-site treatment removed between 0.5 and 3.6-fold more genes than conventional biological urban wastewater treatment (activated sludge). Advanced on-site treatment was able to eliminate 12 out of 19 detected antibiotics, while urban waste water treatment eliminated up to 1 (out of 21 detected). Different advanced treatment technologies were able to target different pollutants to varying extents, making sequential alignment more effective. MBR treatment was most efficient in antibiotic resistance gene reduction and ozonation in antibiotic reduction.

*bla*_{KPC} could only be detected in the influent of the urban wastewater treatment plant receiving untreated hospital wastewater. Similarly, *vanA* was only consistently detected in this treatment plant. These results indicate a positive effect of on-site treatment of hospital wastewater on the communal sewage system.

1. Introduction

Antibiotic Resistance (AR) is a growing global threat which will require worldwide joint efforts to be conquered (ECDC/EMEA Joint Technical Report: The bacterial challenge, 2009; ECDC strategic multi-annual programme 2014–2020, 2014). Hospitals have been in the focus of AR research as one of the high-risk point sources of antibiotics

(Brown et al., 2006; Kümmerer, 2001; Lien et al., 2016) and ARGs (Berendonk et al., 2015; Harris et al., 2010; Harris et al., 2013a,b; Harris et al., 2013a,b; Lien et al., 2016; Rowe et al., 2017). Although, the release of untreated HWW might be posing a hazard to the environment and human health, there are still few studies investigating the release and direct impact of HWW into the environment or communal sewage system (Czekalski et al., 2014; Wang et al., 2018a). Due

* Corresponding author. KWR Watercycle Research Institute, Groningenhaven 7, 3433PE, Nieuwegein, the Netherlands.

E-mail address: gabriela.paulus@kwrwater.nl (G.K. Paulus).

<https://doi.org/10.1016/j.ijheh.2019.01.004>

Received 30 October 2018; Received in revised form 21 December 2018; Accepted 15 January 2019

1438-4639/ © 2019 Elsevier GmbH. All rights reserved.

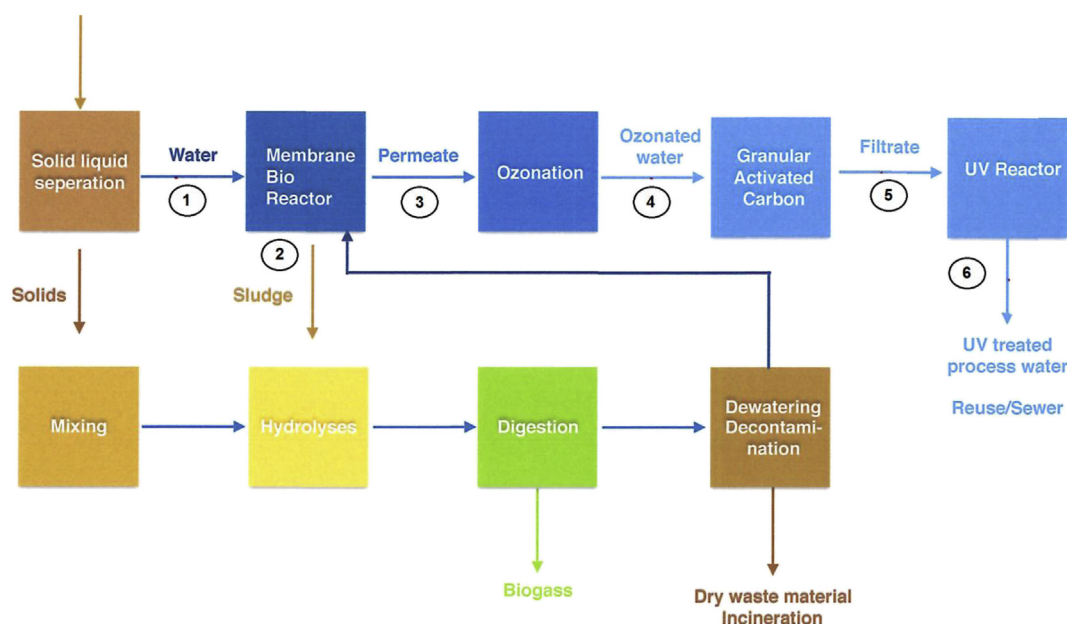


Fig. 1. Pharmafilter Installation and Process Steps; samples taken (1)–(6): (1) Untreated HWW, (2) Sludge, (3) MBR, (4) Ozonation, (5) GAC, (6) UV Treatment/Effluent.

to this gap in information, regulations for the treatment of HWW are absent in most countries (Aukidy et al., 2017; Liu et al., 2010; Rodriguez-Mozaz et al., 2015).

The release of untreated HWW could increase ARG prevalence in environmental water bodies. Antibiotic resistant bacteria were shown to survive in the HWW, in the UWWTPs and, subsequently, the UWWTP effluent (Thompson et al., 2013). The risk potential of HWW is further increased by the fact that hospitals use last-resort antibiotics (e.g. piperacillin and vancomycin) more frequently and thus their ARG profiles might be different when compared to other wastewaters (Kümmerer and Henninger, 2003). Overall, conventional wastewater treatment renders limited results in terms of antibiotic and ARG removal and might even increase the concentration of certain ARGs (Berendonk et al., 2015; Luo et al., 2014; Narciso-Da-Rocha et al., 2014; Szczepanowski et al., 2009; Szekeres et al., 2017).

The present study investigates the impact and efficiency of antibiotic, ARG and bacterial removal of advanced on-site treatment compared to urban wastewater treatment. The effect of different advanced treatment steps and their impact on the downstream urban wastewater system are studied. ARG occurrence and concentrations in HWWs and CWWs in the Netherlands are compared to identify potential differences. To this end genes conferring resistance to aminoglycosides (*aph(III)a*), β -Lactam Antibiotics (*bla_{KPC}*, *bla_{SHV}*, *bla_{OXA}*, *mecA*), macrolides (*ermB*, *ermF*), quinolones (*qnrS*), sulfonamides (*sul1*), tetracyclines (*tetB*, *tetM*) and vancomycines (*vanA*, *vanB*) as well as a class 1 Integrase (*int1*) were screened for and quantified. A total of 711 antibiotics were investigated, out of which 41 were quantified and 670 were screened for presence in the samples. Further, correlations between antibiotic and ARG concentrations were studied.

2. Materials and methods

2.1. Sampling

Samples were taken from two cities in the Netherlands, namely from Delft (location 1) and Nieuwegein (location 2). At location 1 HWW was treated on-site. The following samples were taken from each location: hospital wastewater, communal sewage (at a location not impacted by HWW), on-site hospital wastewater treatment plant (Pharmafilter, location 1 only) and samples from the receiving UWWTPs. Two sampling

rounds were conducted at all sampling locations with at least 6 months in between sampling rounds. The first sampling round took place in spring and the second in winter.

2.1.1. Hospitals

Samples were taken from combined HWW at location 1 (H1) and location 2 (H2). All samples were composite sample (12 h-composites – 1st sampling round; 8 h-composites – 2nd sampling round).

Both hospitals contained wards that typically have high antibiotic use.

2.1.2. Communal WW

CWW samples were taken from the urban sewage system, which was accessed by street manholes. Samples were taken at a location at which the sewage system was not impacted by HWW. Samples were combined grab samples consisting of at least 3 subsamples taken approx. 3 h apart, which were pooled together before analysis.

2.1.3. Urban WWTs

Samples were taken from two UWWTPs: 1) W1 (location 1) and 2) W2 (location 2).

W1 (built in 2006): The treatment plant processes a quantity of water which compares to a population equivalent of 1.260.000 (PE) and has an average in- and outflow of 180.000 m³/d. W1 receives CWW including wastewater from H1. Wastewater treatment consists of primary and secondary treatment, including: influent screening (6 mm bars), primary sedimentation, biological (activated sludge) treatment, final clarification and biological phosphorus removal.

W2 (built in 1975 and renovated in 2010): The treatment plant has a volume capacity which compares to 144.000 PE and an average in- and outflow of 25.700 m³/d. W2 receives CWW including wastewater from H2. Wastewater treatment consists of primary and secondary, including: influent screening, primary sedimentation, biological (activated sludge) treatment and biological Nitrogen and phosphorus removal.

24 h-composite samples (taken by automatized composite samplers) were obtained from each UWWTP (influent and effluent wastewater).

2.1.4. Pharmafilter

HWW at location 1 was treated on-site by an installation called the

Pharmafilter. Pharmafilter treatment consists of 4 sequentially aligned treatment steps (see Fig. 1):

- Membrane Bioreactor (Microfiltration) (MBR)
- Ozonation (Ozon.)
- Granulated Activated Carbon (GAC)
- UV Treatment (UV)

24 h-composite samples were taken after each treatment step, as well as from the MBR-Sludge (see Fig. 1).

2.2. Sample preparation

Samples were processed immediately after arrival to the laboratory.

2.2.1. Biological analysis

Samples were filtered using 0.22- μ m-pore-size polycarbonate track-etch filter membranes (Sartorius). DNA was extracted from the filters using DNeasy PowerSoil Kit (QIAGEN Benelux B.V). Extraction was performed according to manufacturer instructions, with one exception:

An internal control (IC) plasmid was added to the samples (concentration: 2.5×10^4 gene copies/ μ L) to quantify the DNA loss caused by the extraction process (Wullings et al., 2007).

Extraction blanks yielded negative results. DNA loss was corrected for, based on IC concentrations measured by qPCR.

2.2.2. Chemical analysis

The sample preparation protocol involved clean-up and 4000x pre-concentration on an Atlantic HLB-M Disk, using a HORIZON SPE-DEX 4790 (USA) with 47 mm disk holder. Conditioning and extraction programs used for the preparation of the wastewater samples can be found in the SI (Table SC.2). The extract was evaporated using a gentle stream of nitrogen and was reconstituted with 250 μ L of 50:50 methanol:water mixture for instrumental analysis. Before analysis, extracts were filtered through RC syringe filters of 4 mm diameter and 0.2 μ m pore size (Phenomenex, USA). See SI (SC.1) for information on chemicals and reagents.

2.3. ARG detection and quantification – biological analysis

2.3.1. Multiplex qPCR assays

DNA extracts were stored at -20°C prior to qPCR analysis. All qPCR assays were performed at least twice using technical duplicates each time. 16S rRNA was quantified using a SYBR Green qPCR assay. The following genes were quantified by qPCR: *aph(III)a*, *bla_{KPC}*, *bla_{OXA}*, *bla_{SHV}*, *ermB*, *ermF*, *int1*, *mecA*, *qnrS*, *sul1*, *tetB*, *tetM*, *vanA* and *vanB*. Multiplex qPCR assays were performed under the conditions described in the SI (SB 1–3). Standards, a positive and a negative control were included in every assay to confirm multiplex qPCR quality. Standards were made up of 5 subsequent dilutions with concentrations ranging from 2.5×10^4 to 2.5×10^0 gene copies/ μ L. Multiplex qPCR assays were performed using the iQ™ Multiplex Powermix (Bio Rad, München, DE) and qPCR reactions were performed using a CFX96™ Real-Time PCR Detection System (Bio Rad, München, DE). CFX96™ Real-Time PCR Detection System data was interpreted by CFX Manager v.3.1.1517.0823.

2.3.2. Data analysis

Python 3.6.0 (Hunter, 2007; McKinney, 2010) executed in Jupyter Notebooks was used to clean and analyze raw data, to calculate descriptive statistics and correlations and to create data visualizations. R version 3.5.0 was used to perform inferential statistical analysis. Significant differences between experiments and/or measurements were detected by employing paired or unpaired Student's t-Tests, or Welch's t-Tests for the case that the sample variances were not comparable and data transformation not possible. Two samples/measurements were

defined to be significantly different from each other for $p < 0.05$. Correlations between antibiotic and ARG concentrations were calculated using Pearson's rank correlation coefficient. An ARG and antibiotic were considered correlated for $R^2 > 0.5$, $p < 0.05$ and if there were ≥ 4 common data points available. Relatedness with values of $0.5 < R^2 < 0.7$ was considered a 'moderate correlation', while $R^2 > 0.7$ was considered a 'strong correlation'.

2.4. Antibiotic detection and quantification – chemical analysis

2.4.1. Instrumental analysis

Instrumental analysis was performed with a Thermo UHPLC Accela system connected to a TSQ Quantum Access triple quadrupole mass spectrometer from Thermo Electron Corporation (San Jose, CA, USA) equipped with an electrospray ionization source (Thermo IonMAX) in positive mode. Chromatographic separation was achieved on an Atlantis T3 C18 (100 mm $\times$ 2.1 mm, 3 μ m) column from Waters Corporation (Milford, MS, USA) at a constant flow rate of 100 μ L/min. The mobile phase, the gradient elution programs and the ESI parameters are presented (SI Table SC.3). Identification and quantification were performed under selected reaction monitoring (SRM) mode, recording the transitions between the precursor ion and the two most abundant product ions for each target analyte, thus achieving 4 identification points per compound (2002/657/EC). SRM transitions for each compound were optimized by infusion of standard solutions at mean concentration 1 mg/L. The optimized ionization mode, fragmentation voltages, collision energies for each antibiotic (41 in total) are summarized in (SI SC.4). To assure that as many antibiotics as possible were captured, extracts were also injected in a UHPLC-QTOF-MS system, equipped with a UHPLC apparatus (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Dreieich, Germany), coupled to the QTOF-MS mass analyzer (Maxis Impact, Bruker Daltonics, Bremen, Germany). Chromatographic separation was performed on an Acclaim RSLC C18 column (2.1 $\times$ 100 mm, 2.2 μ m) from Thermo Fisher Scientific (Dreieich, Germany) preceded by a guard column of the same packaging material, kept at 30°C . Gradient program, ESI parameters and mobile phases are summarized in (SI SC.5). See SI SC.8 for detailed data analysis.

3. Results and discussion

3.1. Antibiotics and ARGs in the urban WW cycle

3.1.1. Hospital wastewater had higher prevalence and concentrations of antibiotics and ARGs than communal wastewater

HWW samples showed 0.4–1.8-fold higher relative ARG concentrations than CWW samples (Fig. 2). No ARGs were observed in significantly higher concentration in CWW samples. Similarly, absolute ARG concentrations (meaning: ARG concentrations per mL sample) which significantly differed from each other between HWW and CWW samples showed between 0.8 and 2.3-fold increase in HWW samples (SI Fig.SB9). The higher ARG pollution of HWWs suggests higher incidences of AR and can potentially suggest multi-drug-resistant bacteria, as has been found previously in several studies (Amador et al., 2015; Magalhães et al., 2016; Vaz-Moreira et al., 2015) or a larger proportion of resistant organisms compared to CWW.

bla_{KPC} and *vanA* were not found in any of the analyzed CWW samples, suggesting that these genes are hospital-related ARGs and that occurrences at other location of the urban wastewater cycle originate from health care facilities. *VanA* has previously been suggested as an indicator gene to monitor AR of anthropogenic origins in the environment (Narciso-Da-Rocha et al., 2014). *VanA* and *bla_{KPC}* have repeatedly been detected in HWW (Chagas et al., 2011; Cuzon et al., 2011; Gootz et al., 2009; Hu et al., 2012; Iversen et al., 2002; Mascini and Bonten, 2005; Novais et al., 2005; Sahlström et al., 2009; Zhang et al., 2012). Occurrences of *bla_{KPC}* in the environment were only recently and rarely

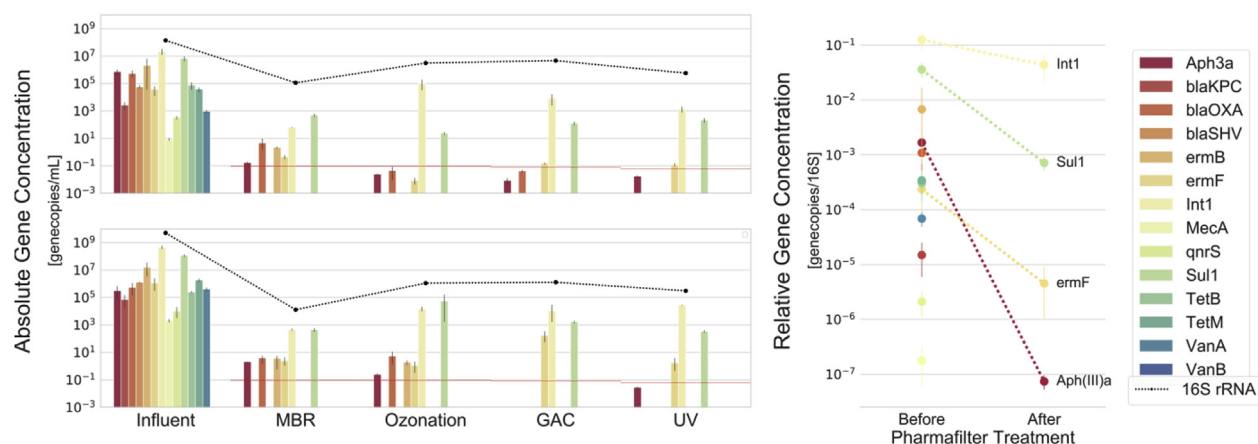
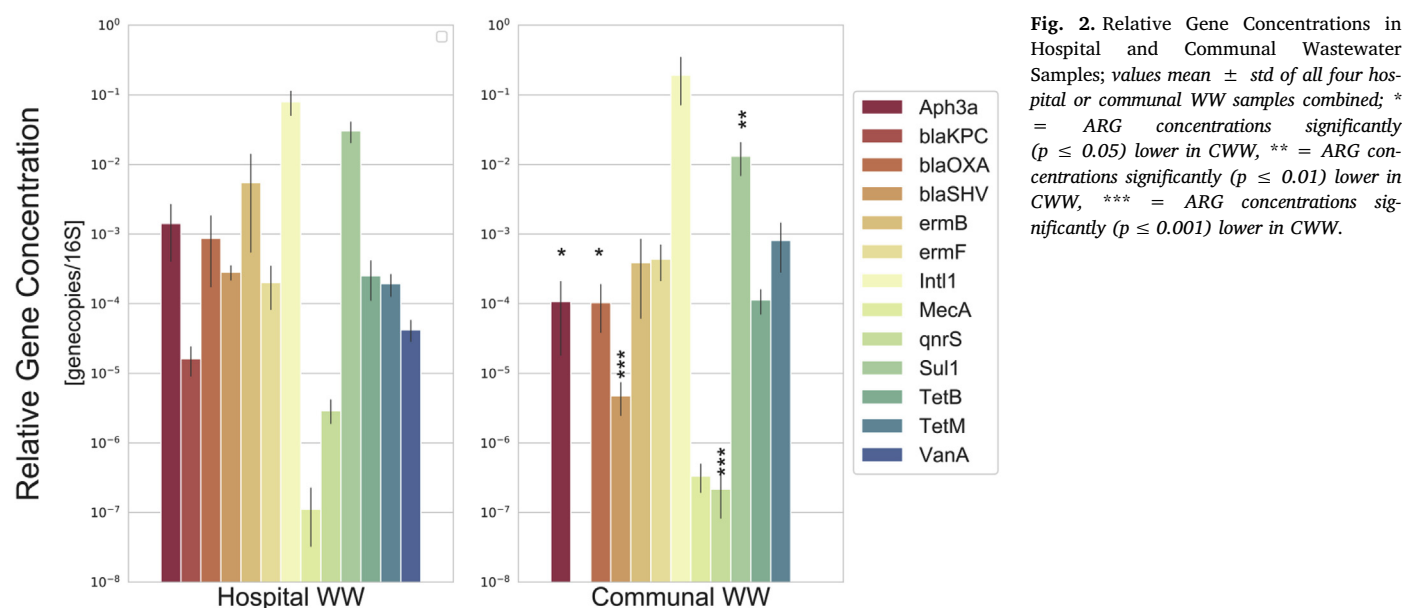


Fig. 3. Gene Reduction During Pharmafilter Treatment; left: impact of different treatment steps (1st (top) and 2nd (bottom) sampling rounds), dotted black line – 16S rRNA concentration; right: overall impact of Pharmafilter on HWW; red line – LOQ. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

shown (Sellera et al., 2017). Some of these occurrences could be traced back to hospital-associated bacterial strains (Cerqueira et al., 2017). The assumption of association of HWW with *bla*_{KPC} and *vanA* is strengthened by previous findings that these genes are more prevalent in hospitals which use more carbapenems (Nasri et al., 2017) or vancomycin (Iversen et al., 2002). *VanA*, is found downstream of hospital sewage release with higher prevalence (Novais et al., 2005). The potential risks of these specific genes would be exacerbated by the possibility to be transferred horizontally between strains. At least in case of *bla*_{KPC}, transconjugants were detected after horizontal gene transfer (HGT) (Hu et al., 2012), suggesting a heightened transfer risk potential of this gene.

Ciprofloxacin (2706 ng/L – H1, 3752 ng/L - H2) and sulfamethoxazole (367 ng/L - H1, 269 ng/L - H2) were detected at concentration levels of up to several orders of magnitude higher than in CWW samples. Metronidazole with a frequency of detection of 92% across all samples, reached concentrations as high as 4 ng/L (H1) and 7500 ng/L (H2) (see Table SC.6). While antibiotic concentrations in HWWs can vary widely (Lien et al., 2016), concentrations within the same dimensions have been previously recorded, with ciprofloxacin, sulfamethoxazole and metronidazole frequently being detected (Baquero et al., 2008; Brenner et al., 2011; Lien et al., 2016; Martins et al., 2008,

2010; Vasconcelos et al., 2009).

Some antibiotics could only be detected in HWW (Fluconazole, Sulfaclozine, Trimethoprim) or were detected in HWW with disproportionately higher concentration than in CWW (Sulfamethoxazole, Ciprofloxacin; both detected at concentrations over 2-fold higher in HWWs). Ampicillin and Amoxicillin, on the other hand, were only detected in CWW. These findings are consistent with previous reports, that β -lactam antibiotics are largely used inside and outside hospitals with Amoxicillin being one of the most frequently used antibiotics for outpatient prescription (Durkin et al., 2018; Hicks et al., 2015; European Centre For Disease Prevention And Control, 2018). Quinolones and sulfonamides are more frequently used in hospitals than for outbound patients in the Netherlands (European Centre For Disease Prevention And Control, 2018).

3.2. Reduction of antibiotics and ARGs during communal and on-site treatment

3.2.1. On-site treatment eliminated antibiotics and ARGs efficiently

*Bla*_{KPC}, *bla*_{SHV}, *mecA*, *qnrS*, *tetB*, *tetM* and *vanA* were reduced < LOD from HWW during MBR treatment (Fig. 3). The following genes could not be detected in the MBR permeate but were

Table 1

Mean and Standard Deviation for log₁₀-fold Gene Reduction and Increase in W1, W2, and Pharmafilter (combined data from two sampling rounds); ¹ – only detected in first sampling round, ² – only detected in second sampling round, ³ – reduced to < LOD, ⁴ – only detected during 2nd sampling round of W2 effluent, ⁵ – non-quantifiable reduction from < LOQ to < LOD, * – Pharmafilter reduction significantly higher than UWWTP reduction, **bold**: gene concentrations significantly increased.

ARG	W1	W2	Pharmafilter
<i>aph(III)a</i>	0.4 ± 0.9	0.5 ± 0.1	> 3.8 ^{*, 3}
<i>bla_{KPC}</i>	n.s	1.0 ± 2.6	> 1.7 ^{*, 3}
<i>bla_{OXA}</i>	0.7 ± 0.1	n.s	> 3.6 ^{*, 3}
<i>bla_{SHV}</i>	1.0 ± 0.1	0.6 ± 0.1	> 3.1 ^{*, 3}
<i>ermB</i>	0.8 ± 0.1	n.s	> 4.4 ^{*, 3}
<i>ermF</i>	1.1 ± 0.3	n.s	1.7 ± 0.8
<i>intI1</i>	n.s	n.s	0.5 ± 0.9 [*]
<i>mecA</i>	> 1.7 ³	> 2.0 ^{2, 3}	⁵
<i>qnrS</i>	1.0 ¹	> 1.3 ³	> 0.9 ³
<i>sul1</i>	n.s	0.5 ± 0.1	1.7 ± 0.4 [*]
<i>tetB</i>	1.3 ± 0.1	0.9 ± 0.1	> 3.1 ^{*, 3}
<i>tetM</i>	1.2 ± 2.2	0.5 ± 0.2	> 3.1 ³
<i>vanA</i>	n.s ¹	> 2.2 ³	> 2.4 ³
<i>vanB</i>	–	⁴	–

detected in the MBR sludge: *blaSHV*, *tetB*, *tetM* and *vanA* (see Supplementary Material SB.5). No genes were consistently eliminated during the ozonation treatment step. GAC treatment showed some variation between the two sampling rounds, with some genes being significantly reduced or increased. *IntI1* and *sul1* were consistently detected in the highest and second-highest concentration, respectively.

Overall changes in gene concentrations showed high consistency between the two sampling rounds. All detected genes were significantly reduced in absolute concentration and most also in relative concentration during the Pharmafilter treatment (Table 1). 9 out of 13 initially detected ARGs in HWW were reduced < LOD during Pharmafilter treatment, including *bla_{KPC}*, *bla_{OXA}*, *bla_{SHV}*, *ermB*, *mecA*, *qnrS*, *tetB*, *tetM* and *vanA*. *Aph(III)a*, *ermF*, *intI1* and *sul1* stayed detectable but were significantly reduced.

Notably, the bacterial load increased during ozonation treatment. This can be explained by hydraulic retention times up to 2 h before ozonation, during which the microbial community has time to adjust to the new conditions and propagate.

Pharmafilter treatment reduced ciprofloxacin from 2706 ng/L to 62 ng/L. Sulfamethoxazole was reduced from 367 ng/L to 0.9 ng/L (Fig. 5). Ozonation was the crucial treatment step for the elimination. MBR treatment seems to release cleavage forms of certain types of antibiotics thus increasing concentrations of certain antibiotics such as metronidazole, which is increased from 4 ng/L to 1203 ng/L during this step. The same trend was observed for other compounds: sulfamethoxazole (concentration difference after MBR treatment: +96%), ofloxacin (+110%), fluconazole (+289%) and erythromycin (17-fold difference). In some cases, concentrations of pharmaceutical residues appear to increase through MBR treatment, a documented phenomenon (Snyder et al., 2007), and might be explained by the cleavage of conjugated residues. For example, sulfamethoxazole can be generated during treatment by cleavage of its human metabolite N4-acetylsulfamethoxazole in WWTPs (Radjenović et al., 2009). Moreover, it is known that antibiotics are absorbed onto negatively charged surface of sewage sludge through ionic interactions. In case of malfunction of membranes or poor maintenance it is possible that desorption phenomena may happen (Radjenović et al., 2009).

3.2.2. Urban wastewater treatment plants show low efficiency in antibiotic reduction and varying efficiency in ARG reduction

ARG concentrations did not uniformly show significant decrease during urban wastewater treatment (Fig. 4). Significant gene reductions varied between 0.5 ± 0.1 (*aph(III)a*) to > 2.2-fold (*vanA*) in UWWTPs

(see Table 1). ARG reduction efficiency varied between the two UWWTPs. Significant changes in relative gene concentration were uniformly reductions in W1, while three ARGs significantly increased in concentration in W2. Genes which did not significantly decrease in concentration were *intI1* and *bla_{SHV}* (during both sampling rounds), *bla_{KPC}*, *bla_{OXA}* and *sul1* (during the 2nd sampling round). On the other hand only one ARG was reduced < LOD in W1; *mecA* and *tetM* during the 1st and 2nd sampling round, respectively; while 3 ARGs were reduced < LOD in W2; *qnrS* and *vanA* (in both sampling rounds) and *mecA* (1st round) or *bla_{KPC}* (2nd round).

In W1 50–67% of present ARGs could be significantly reduced, while only 23–36% of present ARGs were significantly reduced in W2. A large proportion of genes did not show a significant change in relative concentration after treatment at W2. *IntI1* is the only gene that does not show any significant changes in relative concentration in any of the different WWTPs and sampling rounds (see Fig. 4). Previous studies showed that secondary wastewater treatment decreased half of 78 detected ARGs by < 94% in concentration (Yang et al., 2014), while tertiary treatment has been found to retain 2%–50% of ARG raw influent concentrations (Mao et al., 2015). Generally UWWTPs were shown to have varying effects on ARG concentrations depending on wastewater treatment conditions and the type of ARG, even for wastewater treatment plants with tertiary treatment steps (Du et al., 2014; Rodriguez-Mozaz et al., 2015).

Conventional urban wastewater treatment was not capable of removing ciprofloxacin effectively (removal efficiency: 41% for W1, 39% for W2). Similarly, sulfamethoxazole could not be eliminated effectively (removal efficiency: 25% for W1, 19% for W2). Both investigated UWWTPs fail to remove most of the detected antibiotics. Other antibiotics with poor removal efficiency were ofloxacin, trimethoprim, clarithromycin, sulfachloropyridazine, fluconazole, azithromycin, erythromycin and lincomycin (Fig. 5). Low biodegradability of many antibiotics might explain the inefficient antibiotic removal (Kümmerer et al., 2000).

Conventional urban wastewater treatment might therefore not be the most efficient method to reduce antibiotic and ARG concentrations from CWW, contaminated with HWW, prior to release into the environment. Due to substantial fluctuations in antibiotic and ARG concentrations and CWW quality, the resulting effluent will be of variable quality with unknown environmental impact.

3.3. Advanced on-site treatment is more efficient and constant than regular urban wastewater treatment

While relative ARG concentrations did not uniformly decrease in UWWTPs and increased for approximately 10–30% of all ARGs detected, all relative ARG concentrations were consistently significantly reduced during the Pharmafilter process (Fig. 3). Only *intI1* was not consistently significantly removed during Pharmafilter treatment. Pharmafilter treatment reduced approx. 70% of all detected ARGs to < LOD, while regular urban wastewater treatment reduced between 10% (W1) and 22% (W2) of detected ARGs to < LOD. Furthermore, the reduction of ARG concentrations, of genes which were still quantifiable after the respective treatments, was 0.5–4.4-fold during Pharmafilter treatment and 0.5–2.2-fold during UWWT. Pharmafilter reduces individual genes with efficiencies between 0.5-fold (*intI1*) to more than 3.6-fold (*ermB*) higher than that of UWWTPs. This discrepancy in efficiency is further increased considering that UWWTPs could increase certain ARG concentrations more than 1-fold.

The increased ability of the Pharmafilter treatment compared to urban wastewater treatment is, with high probability, due to several interconnected factors: Conventional wastewater treatment has a limited capacity to remove resistance genes (Bouki et al., 2013; Narciso-Da-Rocha et al., 2014; Szekeres et al., 2017) while advanced wastewater treatment (including MBR, Ozone and UV treatment) has been shown to have a better efficiency (Zhang et al., 2015; Zhuang et al.,

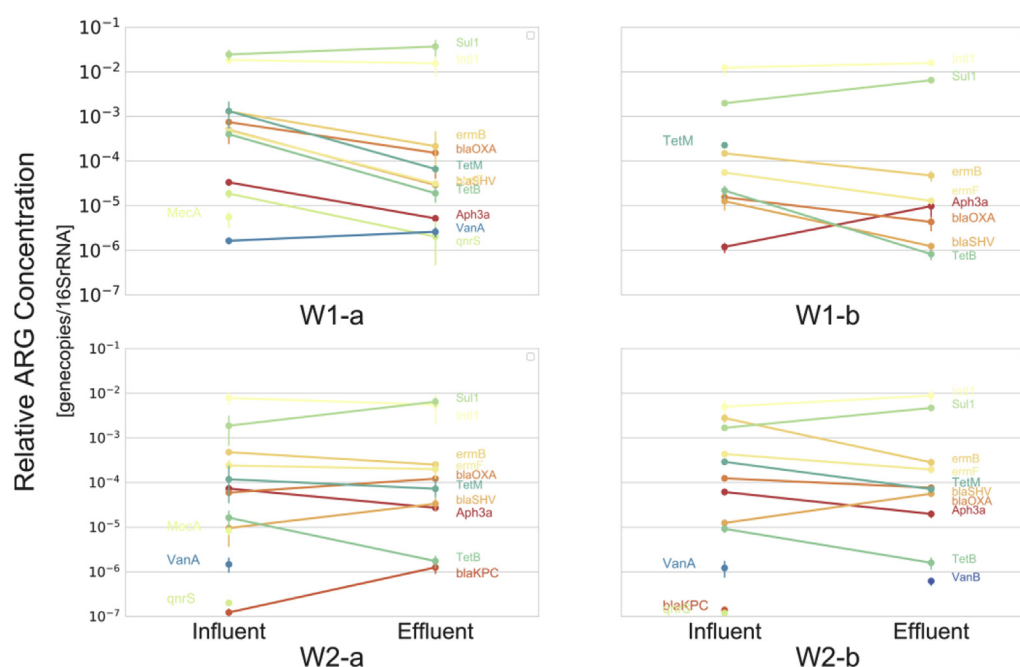


Figure 4. Change in Relative Gene Concentration During Communal Wastewater Treatment in W1 and W2 at Different Times, (a – 1st sampling round in spring, b – 2nd sampling round in winter).

2015). The sequential set-up of the Pharmafilter treatment steps seems to be of importance, as single treatment steps, seem to have the potential to increase the relative ARG concentrations when applied alone (Mao et al., 2015; Sousa et al., 2017). This study showed similar findings. While MBR seems to be the single most effective treatment step to eliminate ARGs, only 7 out of 13 detected ARGs were reduced < LOD during this step. Two ARGs (*bla_{OXA}*, *ermB*) were reduced < LOD, *aph* (III)a was significantly reduced in concentration in the subsequent treatment steps. The subsequent treatment steps therefore accounted for approximately 1/4 of the overall removal efficiency of the Pharmafilter. UV treatment had the least positive impact. Each of these advanced treatment types have their benefits and disadvantages (Aukidy et al., 2017) with ARG removal efficiency strongly depending on the type of ARGs present, the quality of wastewater influent and the applied treatment processes (Barancheshme and Munir, 2018; Sun et al., 2016). The high efficiency of MBR treatment is likely to be due largely to size exclusion, thus filtering out ARG-carrying microorganisms (Visvanathan et al., 2000; Judd, 2010). MBRs have been shown to develop characteristic communities, which differ from the influent community (Judd, 2010). Subsequent partial detachment of microorganisms from this characteristic community might explain why some ARGs are eliminated to a higher extent than others during this treatment step.

Further, antibiotics and other pharmaceutical compounds which might exert selective pressure and increase HGT of ARGs (Bengtsson-Palme and Larsson, 2015; Wintersdorff et al., 2016; Xiong et al., 2015) are thoroughly eliminated by the Pharmafilter process. Correlations between antibiotic and ARG concentrations have been shown (Li et al., 2015b; Mao et al., 2015). Elevated concentrations of β -lactam antibiotics, glycopeptides and trimethoprim were detected in untreated HWW (Szekeres et al., 2017). In contrast to the Pharmafilter, UWWTPs were shown to eliminate a much lower percentage of chemicals, including antibiotics. Elimination of antibiotics can be as low as 20% for sulfamethoxazole, 69% for trimethoprim and 70% for ofloxacin (Brown et al., 2006).

Correlations between antibiotic and ARG concentrations were detected during the present study. Of the 41 quantified antibiotics, concentrations of two antibiotics correlated strongly with ARG

concentration ((rifaximin, metronidazole), two correlated moderately (azithromycin and norfloxacin) and ciprofloxacin correlated moderately to strongly (depending on the ARG). Antibiotics correlated with different numbers of ARG (azithromycin(3), rifaximin(7), metronidazole(6), ciprofloxacin(3), norfloxacin(5) (SI Table SB.8)). While most correlations were observed between unrelated antibiotic-ARG pairs, azithromycin (a macrolide) and *ermF* ($R^2 = 0.66$), ciprofloxacin (a fluorquinolone) and *qnrS* ($R^2 = 0.56$) and norfloxacin (a quinolone) and *qnrS* ($R^2 = 0.64$) correlated moderately. Interestingly, rifaximin and metronidazole concentrations correlated with ARG concentrations of a large number of unrelated ARGs. This could indicate that selective pressure of antibiotics on unrelated ARGs might be a larger problem than selective pressure on related ARGs. Antibiotics like metronidazole which do not largely cause resistance (Otte et al., 2017; Regnath et al., 2017) might then have a larger impact on AR. Another explanation for these correlations could be co-selection. Co-selection of related and unrelated genes can be caused by co-occurrence on plasmids or other mobile genetic elements (Baker-Austin et al., 2006; Di Cesare et al., 2016; Stepanauskas et al., 2006; Gaze et al., 2011; Seiler and Berendonk, 2012; Li et al., 2015a). Finally, (non-antibiotic) pharmaceuticals which have not been investigated but are largely present in wastewaters, could be further driving HGT thus increasing AR (Hegstad et al., 2010; Wang et al., 2018b). It is to be noted that correlation does not necessarily imply causation and that further research will be needed to conclude if one of the described mechanisms are responsible for the observed correlations. Nevertheless, these observations are of interest, in case future research can find similar relationships between the respective antibiotics and ARGs.

3.4. The impact of pharmafilter treatment on ARG concentration in hospital wastewater effluents and the urban wastewater system

On-site wastewater treatment with the Pharmafilter reduces the number of quantified ARG present in hospital wastewater discharge from 13 to 4 and the number of quantified antibiotics from 17 to 7. ARG concentrations of the four genes still detectable after treatment are reduced between 0.5 ± 0.9 to > 3.8 -fold (Fig. 3). Similarly, relative gene concentrations are reduced for genes detectable after treatment.

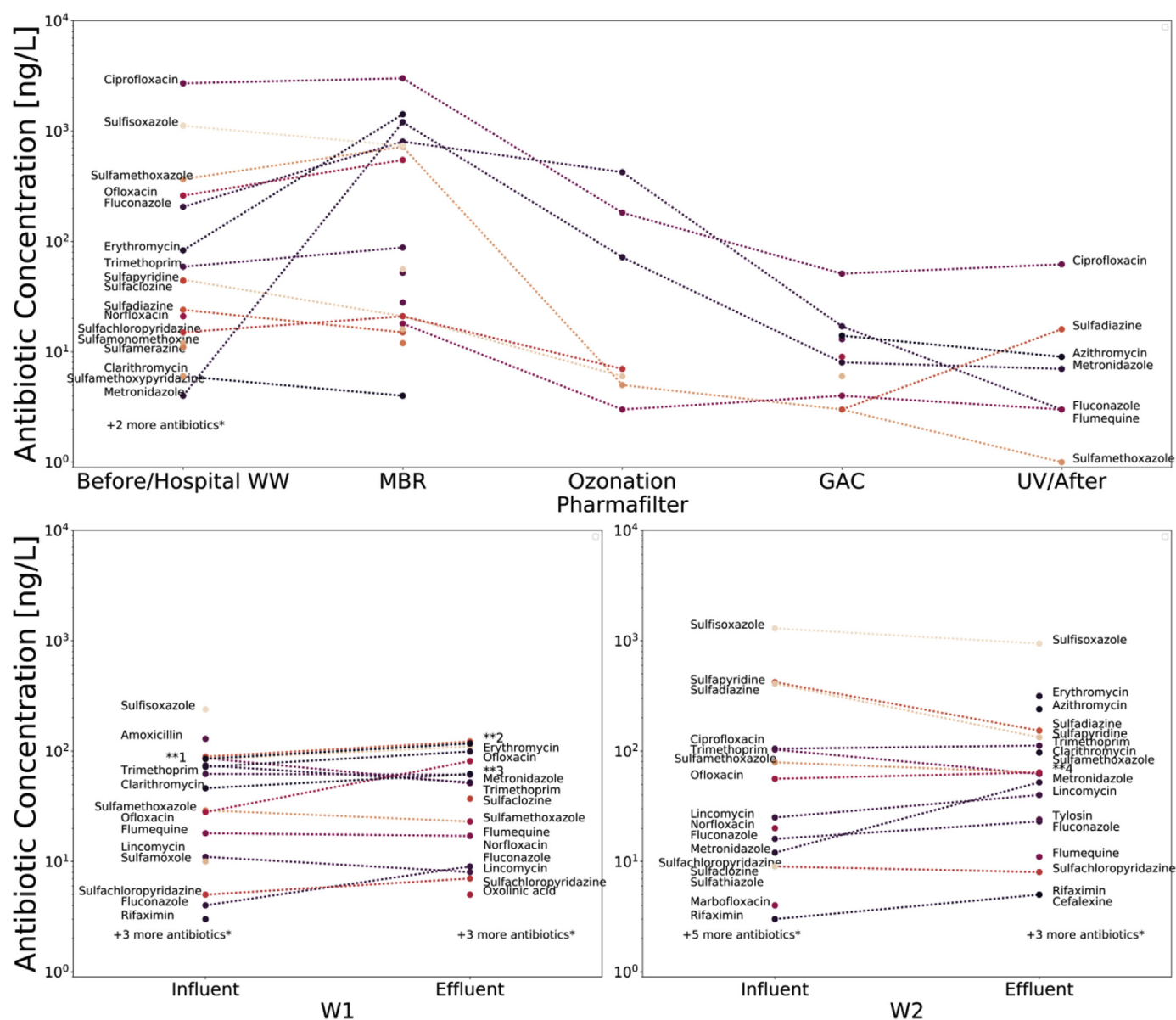


Fig. 5. Antibiotic Concentrations Compared During Pharmafilter Process and During Conventional Urban Wastewater Treatment; * - number of antibiotics detected but not quantified; **1 – Azithromycin, Erythromycin, Metronidazole, Ciprofloxacin, Sulfadiazine, Sulfapyridine (72–89 ng/L); **2 – Azithromycin, Sulfapyridine, Sulfadiazine (110–120 ng/L); **3 – Ciprofloxacin, Clarithromycin (51–62 ng/L); **4 – Ciprofloxacin, Ofloxacin (62–62 ng/L).

Int1 has been identified as a measurement of HGT potential and gene acquisition (Narciso-da-Rocha et al., 2014) and it has been proposed as an indicator for anthropogenic pollution (Gillings et al., 2015). *Int1* was found to show the highest relative concentrations of all genes in all analyzed HWW samples. A study had previously found that high antibiotic concentrations increase *int1* rearrangement, thus increasing the likelihood of HGT (Barraud and Ploy, 2015). The overall discharge of ARGs concentrations, including *int1*, from HWW to the communal sewage system is therefore greatly reduced by Pharmafilter treatment, decreasing the potential for HGT events induced by this otherwise high pollution point source for ARGs.

There are indications of a positive impact of the Pharmafilter treatment on the downstream urban wastewater system and, as a consequence, a benefit in terms of downstream environmental pollution. A lower number of genes was detected in influent samples of W1 (receiving treated HWW) than of W2 (receiving untreated HWW). Interestingly, hospital-related genes (not found in CWW) eliminated during Pharmafilter treatment could rarely be detected in W1. *bla*_{KPC}

could not be detected in W1 samples and *vanA* could only be detected during one of the two sampling rounds (Fig. 6). Both genes were consistently detected in W2 samples (Fig. 6). Similar results could be found for hospital-related antibiotics, which were consistently detected at elevated concentrations in W2, with concentrations up to > 5-fold higher (Fig. 6). Antibiotics only detected in W1 influent (amoxicillin, azithromycin, clarithromycin, erythromycin, flumequine and sulfamoxole) were not detected in treated HWW (location 1) and must therefore originate from other sources. Antibiotics detected only in W2 influent (marbofloxacin, norfloxacin and sulfathiazole) were similarly only detected in H2, with the exception of norfloxacin, which was also detected in CWW, albeit at low concentrations.

4. Conclusion

On-site treatment was substantially more efficient in reducing antibiotic and ARG concentrations than UWWTPs. On-site treatment of HWW did also reduce UWWTP influent loads with hospital-related

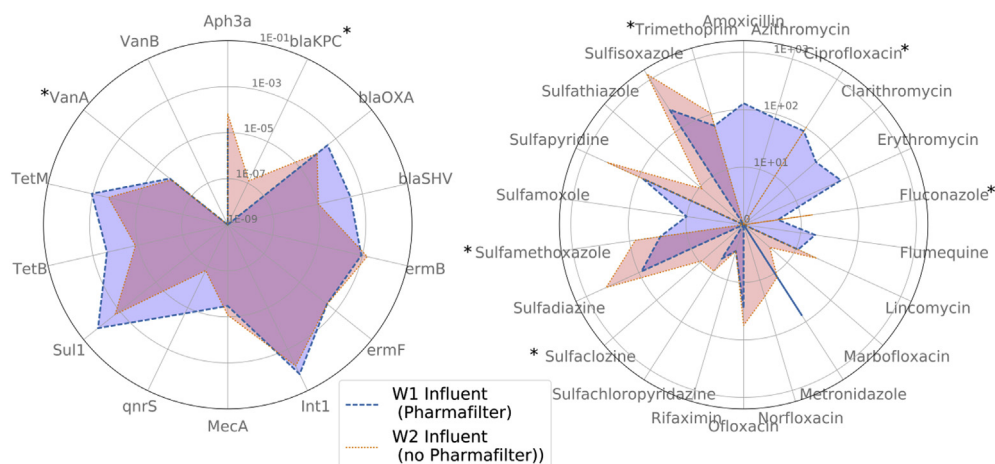


Fig. 6. Antibiotic and ARG Presence and Concentration in UWWTP Influent; left - ARG concentrations in Influent; right – antibiotic concentrations in Influent; * - hospital-related antibiotics or ARGs found in HWW of both location (H1 and H2) at comparable concentrations.

pollutants. *Int1* concentrations were reduced to a considerably larger extent, which could subsequently reduce HGT potential in wastewaters. Combining these findings with elevated levels of antibiotics and ARGs in HWWs (compared to CWW), on-site treatment of HWWs with sequentially aligned advanced treatment technologies is an important step to decrease the risk potential of HWWs and to decrease the impact of wastewater effluents on the environment and subsequently on human health. Alternatively, upgrading existing UWWTPs to include more advanced treatment technologies could mimic the benefits of on-site wastewater treatment of high-risk point sources.

Pharmafilter treatment results in the reduction of pharmaceuticals, including antibiotics, in the treated wastewaters. Correlations between antibiotic and ARG concentrations, suggest potential interactions between these two factors. This reduction could further decrease HGT events as potential sources of selective pressure are diminished, especially for last-resort antibiotics frequently used in hospitals.

Summarizing it can be said that on-site treatment of high-risk wastewater sources was proven to be highly advantageous in regard to antibiotic and ARG reduction. Legislative guidelines and requirements would be conducive to create incentives and increase practical implementation of on-site wastewater treatment.

Declarations of interest

None.

Acknowledgements

We are grateful to Dr. Stefan Kools (KWR Watercycle Research Institute) for facilitating contact to Pharmafilter and to Mr. Erwin Koetse (Pharmafilter) for granting access to all Pharmafilter facilities for sampling.

We would like to warmly thank Danielle van der Linde (KWR Watercycle Research Institute) and Goffe Elsinga (KWR Watercycle Research Institute) for their very valuable assistance during lab analysis.

We further thank Dr. Peter Oswald (Environmental Institute, SK) and Martina Oswaldova (Environmental Institute, SK) for their contributions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijheh.2019.01.004>.

Funding

"The work presented in this publication is part of a project that has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 675530".

References

- Amador, P.P., Fernandes, R.M., Prudêncio, M.C., Barreto, M.P., Duarte, I.M., 2015. Antibiotic resistance in wastewater: Occurrence and fate of Enterobacteriaceae producers of Class A and Class C β -lactamases. *J. Environ. Sci. Health, Part A* 50 (1), 26–39. <https://doi.org/10.1080/10934529.2015.964602>.
- Aukidy, M.A., Chalabi, S.A., Verlicchi, P., 2017. Hospital Wastewater Treatments Adopted in Asia, Africa, and Australia. In: *Hospital Wastewaters*. Springer, Cham. https://doi.org/10.1007/978-94-007-698-2_5.
- Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J.V., 2006. Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 14 (4), 176–182. <https://doi.org/10.1016/j.tim.2006.02.006>.
- Baquero, F., Martínez, J.-L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19 (3), 260–265. <https://doi.org/10.1016/j.copbio.2008.05.006>.
- Barancheshme, F., Munir, M., 2018. Strategies to Combat Antibiotic Resistance in the Wastewater Treatment Plants. *Front. Microbiol.* 8. <https://doi.org/10.3389/fmicb.2017.02603>.
- Barraud, O., Ploy, M.-C., 2015. Diversity of Class 1 Integron Gene Cassette Rearrangements Selected Under Antibiotic Pressure. *J. Bacteriol.* JB 02455-14. <https://doi.org/10.1128/JB.02455-14>.
- Bengtsson-Palme, J., Larsson, D.G.J., 2015. Antibiotic resistance genes in the environment: prioritizing risks. *Nat. Rev. Microbiol.* 13 (6), 396. <https://doi.org/10.1038/nrmicro3399-c1>.
- Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.-N., co-authors, 2015. Tracking antibiotic resistance: the environmental framework. *Nat. Rev. Microbiol.* 13 (5), 310–317. <https://doi.org/10.1038/nrmicro3439>.
- Bouki, C., Venieri, D., Diamadopoulos, E., 2013. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review. *Ecotoxicol. Environ. Saf.* 91, 1–9. <https://doi.org/10.1016/j.ecoenv.2013.01.016>.
- Brenner, C.G.B., Mallmann, C.A., Arsand, D.R., Mayer, F.M., Martins, A.F., 2011. Determination of Sulfamethoxazole and Trimethoprim and Their Metabolites in Hospital Effluent. *Clean. - Soil, Air, Water* 39 (1), 28–34. <https://doi.org/10.1002/clen.201000162>.
- Brown, K.D., Kulis, J., Thomson, B., Chapman, T.H., Mawhinney, D.B., 2006. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. *Sci. Total Environ.* 366 (2), 772–783. <https://doi.org/10.1016/j.scitotenv.2005.10.007>.
- Cerdeira, L., Fernandes, M.R., Ienne, S., Souza, T.A., DE, O., Garcia, D., Lincopan, N., 2017. Draft genome sequence of an environmental multidrug-resistant *Klebsiella pneumoniae* ST340/CC258 harbouring blaCTX-M-15 and blaKPC-2 genes. *J. Global Antimicrob. Resist.* 8, 108–109. <https://doi.org/10.1016/j.jgar.2016.12.001>.
- Chagas, T.P.G., Seki, L.M., Silva, D.M. da, Asensi, M.D., 2011. Occurrence of KPC-2-producing *Klebsiella pneumoniae* strains in hospital wastewater. *J. Hosp. Infect.* 77 (3), 281. <https://doi.org/10.1016/j.jhin.2010.10.008>.
- Cuzon, G., Naas, T., Villegas, M.-V., Bermudez, A.C., Quinn, J.P., Nordmann, P., 2011. Wide dissemination of *Pseudomonas aeruginosa* producing β -lactamase blaKPC-2 gene in Colombia. *Antimicrob. Agents Chemother.* AAC 00297-11. <https://doi.org/10.1128/AAC.00297-11>.

- Czekalski, N., Gascón Díez, E., Bürgmann, H., 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. *ISME J.* 8 (7), 1381–1390. <https://doi.org/10.1038/ismej.2014.8>.
- Di Cesare, A., Eckert, E.M., D'urso, S., Bertoni, R., Gillan, D.C., Wattiez, R., CORNO, G., 2016. Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. *Water Res.* 94, 208–214. <https://doi.org/10.1016/j.watres.2016.02.049>.
- Du, J., Ren, H., Geng, J., Zhang, Y., Xu, K., Ding, L., 2014. Occurrence and abundance of tetracycline, sulfonamide resistance genes, and class 1 integron in five wastewater treatment plants. *Environ. Sci. Pollut. Control Ser.* 21 (12), 7276–7284. <https://doi.org/10.1007/s11356-014-2613-5>.
- Durkin, M.J., Jafarzadeh, S.R., Hsueh, K., Sallah, Y.H., Munshi, K.D., Henderson, R.R., Fraser, V.J., 2018. Outpatient Antibiotic Prescription Trends in the United States: A National Cohort Study. *Infect. Contr. Hosp. Epidemiol.* 39 (5), 584–589. <https://doi.org/10.1017/ice.2018.26>.
- Ecdc Strategic Multi-Annual Programme 2014–2020, 2014. ECDC strategic multi-annual programme 2014–2020. <http://ecdc.europa.eu/en/publications-data/ecdc-strategic-multi-annual-programme-2014-2020>.
- Ecdc/Emea Joint Technical Report: The Bacterial Challenge, 2009. ECDC/EMEA Joint Technical Report: The bacterial challenge: time to react. <http://ecdc.europa.eu/en/publications-data/ecdcemea-joint-technical-report-bacterial-challenge-time-react>.
- European Centre For Disease Prevention And Control, 2018. Antimicrobial consumption - Annual Epidemiological Report for 2016. EU. <http://ecdc.europa.eu/en/publications-data/antimicrobial-consumption-annual-epidemiological-report-2016>.
- Gaze, W.H., Zhang, L., Abdoulsam, N.A., Hawkey, P.M., Calvo-Bado, L., Royle, J., Brown, H., Davis, S., Kay, P., Boxall, A.B.A., co-authors, 2011. Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J.* 5 (8), 1253–1261. <https://doi.org/10.1038/ismej.2011.15>.
- Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.-G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J.* 9 (6), 1269–1279. <https://doi.org/10.1038/ismej.2014.226>.
- Gootz, T.D., Lescoe, M.K., Dib-Hajj, F., Dougherty, B.A., HE, W., Della-Latta, P., HUARD, R.C., 2009. Genetic Organization of Transposase Regions Surrounding blaKPC Carbapenemase Genes on Plasmids from Klebsiella Strains Isolated in a New York City Hospital. *Antimicrob. Agents Chemother.* 53 (5), 1998–2004. <https://doi.org/10.1128/AAC.01355-08>.
- Harris, A.D., Pineles, L., Belton, B., Johnson, J.K., Shardell, M., Loeb, M., Newhouse, R., Demby, L., Braun, B., Perencevich, E.N., co-authors, 2013a. Universal Glove and Gown Use and Acquisition of Antibiotic-Resistant Bacteria in the ICU: A Randomized Trial. *J. Am. Med. Assoc.* 310 (15), 1571–1580. <https://doi.org/10.1001/jama.2013.277815>.
- Harris, S.R., Cartwright, E.J., Török, M.E., Holden, M.T., Brown, N.M., Ogilvy-Stuart, A.L., Ellington, M.J., QUAIL, M.A., Bentley, S.D., Parkhill, J., co-authors, 2013b. Whole-genome sequencing for analysis of an outbreak of methicillin-resistant *Staphylococcus aureus*: a descriptive study. *Lancet Infect. Dis.* 13 (2), 130–136. [https://doi.org/10.1016/S1473-3099\(12\)70268-2](https://doi.org/10.1016/S1473-3099(12)70268-2).
- Harris, S.R., Feil, E.J., Holden, M.T.G., Quail, M.A., Nickerson, E.K., Chantratita, N., Gardete, S., Tavares, A., Day, N., Lindsay, J.A., co-authors, 2010. Evolution of MRSA During Hospital Transmission and Intercontinental Spread. *Science* 327 (5964), 469–474. <https://doi.org/10.1126/science.1182395>.
- Hegstad, K., Langsrud, S., Lunestad, B.T., Scheie, A.A., Sunde, M., Yazdankhah, S.P., 2010. Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health? *Microb. Drug Resist.* (Larchmont, N.Y.) 16 (2), 91–104. <https://doi.org/10.1089/mdr.2009.0120>.
- Hicks, L.A., Bartoces, M.G., Roberts, R.M., Suda, K.J., Hunkler, R.J., Taylor, T.H., Schrag, S.J., 2015. US Outpatient Antibiotic Prescribing Variation According to Geography, Patient Population, and Provider Specialty in 2011. *Clin. Infect. Dis.* 60 (9), 1308–1316. <https://doi.org/10.1093/cid/civ076>.
- Hu, Y., Cai, J., Zhang, R., Zhou, H., Sun, G., Chen, G., 2012. Emergence of *Proteus mirabilis* Harboring blaKPC-2 and qnrD in a Chinese Hospital. *Antimicrob. Agents Chemother.* AAC 05519-11. <https://doi.org/10.1128/AAC.05519-11>.
- Hunter, J.D., 2007. Matplotlib: A 2D Graphics Environment. *Comput. Sci. Eng.* 9 (3), 90–95. <https://doi.org/10.1109/MCSE.2007.55>.
- Iversen, A., Kühn, I., Franklin, A., Möllby, R., 2002. High Prevalence of Vancomycin-Resistant Enterococci in Swedish Sewage. *Appl. Environ. Microbiol.* 68 (6), 2838–2842. <https://doi.org/10.1128/AEM.68.6.2838-2842.2002>.
- Judd, S., 2010. *The MBR Book: Principles and Applications of Membrane Bioreactors for Water and Wastewater Treatment*. Elsevier, pp. 537.
- Kümmerer, K., Al-Ahmad, A., Mersch-Sundermann, V., 2000. Biodegradability of some antibiotics, elimination of the genotoxicity and affection of wastewater bacteria in a simple test. *Chemosphere* 40 (7), 701–710. [https://doi.org/10.1016/S0045-6535\(99\)00439-7](https://doi.org/10.1016/S0045-6535(99)00439-7).
- Kümmerer, Klaus, 2001. Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources – a review. *Chemosphere* 45 (6), 957–969. [https://doi.org/10.1016/S0045-6535\(01\)00144-8](https://doi.org/10.1016/S0045-6535(01)00144-8).
- Kümmerer, K., Henninger, A., 2003. Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clin. Microbiol. Infect.* 9 (12), 1203–1214. <https://doi.org/10.1111/j.1469-0691.2003.00739.x>.
- Li, B., Yang, Y., Ma, L., Ju, F., Guo, F., Tiedje, J.M., Zhang, T., 2015a. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME J.* 9 (11), 2490–2502. <https://doi.org/10.1038/ismej.2015.59>.
- Li, J., Cheng, W., Xu, L., Strong, P.J., Chen, H., 2015b. Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system. *Environ. Sci. Pollut. Control Ser.* 22 (6), 4587–4596. <https://doi.org/10.1007/s11356-014-3665-2>.
- Lien, L.T.Q., Hoa, N.Q., Chuc, Ntk, Thoa, N.T.M., Phuc, H.D., Diwan, V., Dat, N.T., Tamhankar, A.J., Lundborg, C.S., 2016. Antibiotics in Wastewater of a Rural and an Urban Hospital before and after Wastewater Treatment, and the Relationship with Antibiotic Use—A One Year Study from Vietnam. *Int. J. Environ. Res. Publ. Health* 13 (6). <https://doi.org/10.3390/ijerph13060588>.
- Liu, Q., Zhou, Y., Chen, L., Zheng, X., 2010. Application of MBR for hospital wastewater treatment in China. *Desalination* 250 (2), 605–608. <https://doi.org/10.1016/j.desal.2009.09.033>.
- Luo, Y., Yang, F., Mathieu, J., Mao, D., Wang, Q., Alvarez, P.J.J., 2014. Proliferation of Multidrug-Resistant New Delhi Metallo- β -lactamase Genes in Municipal Wastewater Treatment Plants in Northern China. *Environ. Sci. Technol. Lett.* 1 (1), 26–30. <https://doi.org/10.1021/ez400152e>.
- Magalhães, M.J.T.L., Pontes, G., Serra, P.T., Balieiro, A., Castro, D., Pieri, F.A., Crainey, J.L., Nogueira, P.A., Orlandi, P.P., 2016. Multidrug resistant *Pseudomonas aeruginosa* survey in a stream receiving effluents from ineffective wastewater hospital plants. *BMC Microbiol.* 16 (193). <https://doi.org/10.1186/s12866-016-0798-0>.
- Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., MU, Q., Alvarez, P.J.J., 2015. Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water Res.* 85, 458–466. <https://doi.org/10.1016/j.watres.2015.09.010>.
- Martins, A.F., Mallmann, C.A., Arsand, D.R., Mayer, F.M., Brenner, C.G.B., 2010. Occurrence of the Antimicrobials Sulfamethoxazole and Trimethoprim in Hospital Effluent and Study of Their Degradation Products after Electrocoagulation. *Clean. - Soil, Air, Water* 39 (1), 21–27. <https://doi.org/10.1002/clen.201000126>.
- Martins, A.F., Vasconcelos, T.G., Henriques, D.M., Frank, C. da S., König, A., Kümmerer, K., 2008. Concentration of Ciprofloxacin in Brazilian Hospital Effluent and Preliminary Risk Assessment: A Case Study. *Clean. - Soil, Air, Water* 36 (3), 264–269. <https://doi.org/10.1002/clen.200700171>.
- Mascini, E.M., Bonten, M.J.M., 2005. Vancomycin-resistant enterococci: consequences for therapy and infection control. *Clin. Microbiol. Infect.: Offic. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* 11 (Suppl. 4), 43–56. <https://doi.org/10.1111/j.1469-0691.2005.01164.x>.
- Mckinney, W., 2010. *Data Structures for Statistical Computing in Python* 6.
- Narciso-Da-Rocha, C., Varela, A.R., Schwartz, T., Nunes, O.C., Manaia, C.M., 2014. blaTEM and blaVas as indicator genes of antibiotic resistance contamination in a hospital–urban wastewater treatment plant system. *J. Global Antimicrob. Resist.* 2 (4), 309–315. <https://doi.org/10.1016/j.jgar.2014.10.001>.
- Nasri, E., Subirats, J., Sánchez-Melsió, A., Mansour, H.B., Borrego, C.M., Balcázar, J.L., 2017. Abundance of carbapenemase genes (blaKPC, blaNDM and blaOXA-48) in wastewater effluents from Tunisian hospitals. *Environ. Pollut.* 229, 371–374. <https://doi.org/10.1016/j.envpol.2017.05.095>.
- Novais, C., Coque, T.M., Ferreira, H., Sousa, J.C., Peixe, L., 2005. Environmental Contamination with Vancomycin-Resistant Enterococci from Hospital Sewage in Portugal. *Appl. Environ. Microbiol.* 71 (6), 3364–3368. <https://doi.org/10.1128/AEM.71.6.3364-3368.2005>.
- Otte, E., Nielsen, H.L., Hasman, H., Fuglsang-Damgaard, D., 2017. First report of metronidazole resistant, nimD-positive, *Bacteroides stercoris* isolated from an abdominal abscess in a 70-year-old woman. *Anaerobe* 43, 91–93. <https://doi.org/10.1016/j.anaerobe.2016.12.010>.
- Radjenović, J., Petrović, M., Barceló, D., 2009. Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. *Water Res.* 43 (3), 831–841. <https://doi.org/10.1016/j.watres.2008.11.043>.
- Regnath, T., Raacke, O., Enninger, A., Ignatius, R., 2017. Increasing metronidazole and rifampicin resistance of *Helicobacter pylori* isolates obtained from children and adolescents between 2002 and 2015 in southwest Germany. *Helicobacter* 22 (1), e12327. <https://doi.org/10.1111/hel.12327>.
- Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sánchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Res.* 69, 234–242. <https://doi.org/10.1016/j.watres.2014.11.021>.
- Rowe, W.P.M., Baker-Austin, C., Verner-Jeffreys, D.W., Ryan, J.J., Micallef, C., Maskell, D.J., PEARCE, G.P., 2017. Overexpression of antibiotic resistance genes in hospital effluents over time. *J. Antimicrob. Chemother.* 72 (6), 1617–1623. <https://doi.org/10.1093/jac/dkx017>.
- Sahlström, L., Rehinder, V., Albiñ, A., Aspan, A., Bengtsson, B., 2009. Vancomycin resistant enterococci (VRE) in Swedish sewage sludge. *Acta Vet. Scand.* 51, 24. <https://doi.org/10.1186/1751-0147-51-24>.
- Seiler, C., Berendonk, T.U., 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Front. Microbiol.* 3. <https://doi.org/10.3389/fmicb.2012.00399>.
- Sellera, F.P., Fernandes, M.R., Moura, Q., Souza, T.A., Cerdeira, L., Lincopan, N., 2017. Draft genome sequence of *Enterobacter cloacae* ST520 harbouring blaKPC-2, blaCTX-M-15 and blaOXA-17 isolated from coastal waters of the South Atlantic Ocean. *J. Global Antimicrob. Resist.* 10, 279–280. <https://doi.org/10.1016/j.jgar.2017.07.017>.
- Snyder, S.A., Adham, S., Redding, A.M., Cannon, F.S., DeCarolis, J., Oppenheimer, J., et al., 2007. Role of membranes and activated carbon in the removal of endocrine disruptors and pharmaceuticals. *Desalination* 202 (1), 156–181. <https://doi.org/10.1016/j.desal.2005.12.052>.
- Sousa, J.M., Macedo, G., Pedrosa, M., Becerra-Castro, C., Castro-Silva, S., Pereira, M.F.R., Silva, A.M.T., Nunes, O.C., Manaia, C.M., 2017. Ozonation and UV254nm radiation for the removal of microorganisms and antibiotic resistance genes from urban wastewater. *J. Hazard Mater.* 323, 434–441. Special Issue on Emerging Contaminants in engineered and natural environment. <https://doi.org/10.1016/j.jhazmat.2016.03.096>.

- Stepanaukas, R., Glenn, T.C., Jagoe, C.H., Tuckfield, R.C., Lindell, A.H., King, C.J., McArthur, J.V., 2006. Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environ. Microbiol.* 8 (9), 1510–1514. <https://doi.org/10.1111/j.1462-2920.2006.01091.x>.
- Sun, Y., Shen, Y., Liang, P., Zhou, J., Yang, Y., Huang, X., 2016. Multiple antibiotic resistance genes distribution in ten large-scale membrane bioreactors for municipal wastewater treatment. *Bioresour. Technol.* 222, 100–106. <https://doi.org/10.1016/j.biortech.2016.09.117>.
- Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K.-H., Gützkow, T., Eichler, W., Pühler, A., Schlüter, A., 2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* 155 (7), 2306–2319. <https://doi.org/10.1099/mic.0.028233-0>.
- Szekeres, E., Baricz, A., Chiriac, C.M., Farkas, A., Opris, O., Soran, M.-L., Andrei, A.-S., Rudi, K., Balcázar, J.L., Dragos, N., co-authors, 2017. Abundance of antibiotics, antibiotic resistance genes and bacterial community composition in wastewater effluents from different Romanian hospitals. *Environ. Pollut.* 225, 304–315. <https://doi.org/10.1016/j.envpol.2017.01.054>.
- Thompson, J., Gundogdu, A., Stratton, H., Katouli, M., 2013. Antibiotic resistant *Staphylococcus aureus* in hospital wastewaters and sewage treatment plants with special reference to methicillin-resistant *Stap*. *PubMed - NCBI* 114 (1), 44–54. <https://doi.org/10.1111/jam.12037>.
- Vasconcelos, T.G., Kümmerer, K., Henriques, D.M., Martins, A.F., 2009. Ciprofloxacin in hospital effluent: Degradation by ozone and photoprocesses. *J. Hazard Mater.* 169 (1), 1154–1158. <https://doi.org/10.1016/j.jhazmat.2009.03.143>.
- Vaz-Moreira, I., Varela, A.R., Pereira, T.V., Fochat, R.C., Manaia, C.M., 2015. Multidrug Resistance in Quinolone-Resistant Gram-Negative Bacteria Isolated from Hospital Effluent and the Municipal Wastewater Treatment Plant. *Microb. Drug Resist.* 22 (2), 155–163. <https://doi.org/10.1089/mdr.2015.0118>.
- Visvanathan, C., Aim, R.B., Parameshwaran, K., 2000. Membrane Separation Bioreactors for Wastewater Treatment. *Crit. Rev. Environ. Sci. Technol.* 30 (1), 1–48. <https://doi.org/10.1080/10643380091184165>.
- Wang, Q., Wang, P., Yang, Q., 2018a. Occurrence and diversity of antibiotic resistance in untreated hospital wastewater. *Sci. Total Environ.* 621, 990–999. <https://doi.org/10.1016/j.scitotenv.2017.10.128>.
- Wang, Y., Lu, J., Mao, L., Li, J., Yuan, Z., Bond, P.L., Guo, J., 2018b. Antiepileptic drug carbamazepine promotes horizontal transfer of plasmid-borne multi-antibiotic resistance genes within and across bacterial genera. *ISME J.* 1. <https://doi.org/10.1038/s41396-018-0275-x>.
- Wintersdorff, V., H. C.J., Penders, J., Niekerk, V., M. J., Mills, N.D., Majumder, S., Alphen, V., B. L., Savelkoul, P.H.M., co-authors, 2016. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Front. Microbiol.* 7. <https://doi.org/10.3389/fmicb.2016.00173>.
- Wullings, B., Wubbels, G., Veenendaal, H., Van Der Kooij, D., 2007. Snelle, kwantitatieve detectie van *Legionella pneumophila* met Q-PCR. *H twee O: tijdschrift voor water-voorziening en afvalwaterbehandeling* 40 (5), 39–41.
- Xiong, W., Sun, Y., Ding, X., Wang, M., Zeng, Z., 2015. Selective pressure of antibiotics on ARGs and bacterial communities in manure-polluted freshwater-sediment microcosms. *Front. Microbiol.* 6. <https://doi.org/10.3389/fmicb.2015.00194>.
- Yang, Y., Li, B., Zou, S., Fang, H.H.P., Zhang, T., 2014. Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Res.* 62, 97–106. <https://doi.org/10.1016/j.watres.2014.05.019>.
- Zhang, X., Lü, X., Zong, Z., 2012. Enterobacteriaceae producing the KPC-2 carbapenemase from hospital sewage. *Diagn. Microbiol. Infect. Dis.* 73 (2), 204–206. <https://doi.org/10.1016/j.diagmicrobio.2012.02.007>.
- Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Zhang, Y., Ding, L., Xu, K., 2015. Inactivation of antibiotic resistance genes in municipal wastewater effluent by chlorination and sequential UV/chlorination disinfection. *Sci. Total Environ.* 512–513, 125–132. <https://doi.org/10.1016/j.scitotenv.2015.01.028>.
- Zhuang, Y., Ren, H., Geng, J., Zhang, Y., Zhang, Y., Ding, L., Xu, K., 2015. Inactivation of antibiotic resistance genes in municipal wastewater by chlorination, ultraviolet, and ozonation disinfection. *Environ. Sci. Pollut. Control Ser.* 22 (9), 7037–7044. <https://doi.org/10.1007/s11356-014-3919-z>.