Environment International 150 (2021) 106436

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Evidence for selection of multi-resistant *E. coli* by hospital effluent

Nadine Kraupner [a,b], Marion Hutinel [a,b], Kilian Schumacher [a,b,c], Declan A. Gray [a,b], Maja Genheden [a,b], Jerker Fick [d], Carl-Fredrik Flach [a,b], D.G. Joakim Larsson [a,b,*]

[a] Centre for Antibiotic Resistance Research (CARe) at the University of Gothenburg, Gothenburg, Sweden
[b] Department of Infectious Diseases, Institute of Biomedicine, The Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden
[c] Department of Biology I, Microbiology, Ludwig-Maximilians-Universität München, Martinsried, Germany[1]
[d] Department of Chemistry, Umea University, Sweden

ARTICLE INFO

Handling Editor: Yong-Guan Zhu

Keywords:
Antibiotic resistance
Resistance selection
Hospital effluent

ABSTRACT

There is a risk that residues of antibiotics and other antimicrobials in hospital and municipal wastewaters could select for resistant bacteria. Still, direct experimental evidence for selection is lacking. Here, we investigated if effluent from a large Swedish hospital, as well as influent and effluent from the connected municipal wastewater treatment plant (WWTP) select for antibiotic resistant *Escherichia coli* in three controlled experimental setups. Exposure of sterile-filtered hospital effluent to a planktonic mix of 149 different *E. coli* wastewater isolates showed a strong selection of multi-resistant strains. Accordingly, exposure to a complex wastewater community selected for strains resistant to several antibiotic classes. Exposing individual strains with variable resistance patterns revealed a rapid bactericidal effect of hospital effluent on susceptible, but not multi-resistant *E. coli*. No selection was observed after exposure to WWTP effluent, while exposure to WWTP influent indicated a small selective effect for ceftazidime and cefadroxil resistant strains, and only in the *E. coli* mix assay. An analysis of commonly used antibiotics and non-antibiotic pharmaceuticals in combination with growth and resistance pattern of individual *E. coli* isolates suggested a possible contribution of ciprofloxacin and β-lactams to the selection by hospital effluent. However, more research is needed to clarify the contribution from different selective agents. While this study does not indicate selection by the studied WWTP effluent, there is some indications of selective effects by municipal influent on β-lactam-resistant strains. Such effects may be more pronounced in countries with higher antibiotic use than Sweden. Despite the limited antibiotic use in Sweden, the hospital effluent strongly and consistently selected for multi-resistance, indicating widespread risks. Hence, there is an urgent need for further evaluation of risks for resistance selection in hospital sewers, as well as for strategies to remove selective agents and resistant bacteria.

1. Introduction

Reducing the development and spread of antibiotic resistant bacteria poses a major global challenge. To respond effectively, it has become increasingly recognized that a one-health perspective is required, considering both humans, animals and the environment. While selection for resistance certainly occurs within humans and domestic animals given antibiotics, environmental bacterial communities are also frequently exposed to antibiotic residues, albeit generally at much lower concentrations (Martínez, 2008; Tran et al., 2018; Wang et al., 2020; Wellington et al., 2013). To inform actions, knowledge about specific environments that are likely to select for resistant bacteria is urgently needed (Andersson et al., 2020; Larsson et al., 2018).

The presence of antibiotic combinations in many different environments has been observed for decades (Chow et al., 2020), representing a concentration gradient where the highest levels are reported in

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; EUCAST, The European Committee on Antimicrobial Susceptibility Testing; LOEC, Lowest observed effect concentration; MIC, Minimal inhibitory concentration; MSC, Minimal selective concentration; NOEC, No observed effect concentration; WWTP, Wastewater treatment plant; PNEC, Predicted No Effect Concentration.

* Corresponding author at: Department of Infectious Diseases, Institute of Biomedicine, The Sahlgrenska Academy, University of Gothenburg, Guldhedsgatan 10, SE-413 46 Gothenburg, Sweden.
E-mail address: joakim.larsson@fysiologi.gu.se (D.G.J. Larsson).
[1] Present address.

https://doi.org/10.1016/j.envint.2021.106436
Received 22 December 2020; Received in revised form 28 January 2021; Accepted 29 January 2021
Available online 13 February 2021

environments impacted from industrial emissions (Bielen et al., 2017; Larsson, 2014). Untreated hospital effluents (Lindberg et al., 2004; Rodriguez-Mozaz et al., 2015; Verlicchi et al., 2012), untreated (Bengtsson-Palme et al., 2016; Sahlin et al., 2018; Östman et al., 2017) and treated (Rodriguez-Mozaz et al., 2020; Wang et al., 2020) municipal wastewaters typically contain stepwise lower concentrations, eventually leading to widespread, low-level contamination of surface waters and sediments (Chow et al., 2020; Kümmerer, 2009).

Selection of antibiotic resistance in wastewater treatment plants (WWTPs) has been studied primarily by comparing abundance of resistance genes or resistant bacteria between influents and effluents. Whereas some studies report selection of resistance (Ferreira da Silva et al., 2007; Lefkowitz and Duran, 2009; Mao et al., 2015), others could not observe any clear increase in resistance due to wastewater treatment (Bengtsson-Palme et al., 2016; Flach et al., 2018). In hospital wastewater, even higher proportions of resistance genes or resistant bacteria compared to municipal wastewater are often found (Hutinel et al., 2019; Paulshus et al., 2019; Rodriguez-Mozaz et al., 2015). A higher proportion of resistant bacteria in hospital wastewater could be a consequence of resistant fecal bacteria being more widespread at hospitals than in the general community. Additionally, higher prevalences of resistant bacteria or resistance genes could be a reflection of on-site selection due to elevated antibiotic concentrations there.

While field studies represent reality, causality is often difficult to establish without controlled laboratory experiments. Some of these have elegantly shown that very low concentrations of antibiotics are able to select for a resistant strain over a sensitive one in the lab (Gullberg et al., 2014, 2011; Liu et al., 2011). Also, models based on available effect data on growth (minimal inhibitory concentrations; MICs) can be used to predict no effect concentrations (PNECs) for resistance selection, as done for over 100 antibiotics by Bengtsson-Palme and Larsson (2016). More recently, minimal selective concentrations (MSCs) have been predicted by taking into account the full dose-response curves for growth (Greenfield et al., 2018).

While laboratory studies examining growth or pairwise competitions between two strains reveal important information, those that take into account competition between multiple strains and species are likely to better reflect the real world scenario (Bottery et al., 2020; Klümper et al., 2019; Kraupner et al., 2018, 2020; Lundström et al., 2016; Murray et al., 2018; Stanton et al., 2020). An additional step towards increased realism is to investigate the selective effect of mixtures. The accuracy of predicted mixture effects from an array of concentrations of single selective agents depends on that the concentrations of all important components of the real mixture is known, their bioavailability, their individual potencies as well as knowledge about how the components interact with each other. While empirical investigations of synthetic mixtures do consider interactions, they still rely on presumptions with respect to the other aspects (González-Pleiter et al., 2013; Yang et al., 2008). As we rarely know the concentrations of more than some of the selective agents in complex mixtures such as different wastewaters, a way to overcome this limitation is to investigate the effect of exposure to the real mixture itself.

The aim of this study was to test if the wastewater from a major Swedish hospital as well as untreated and treated municipal wastewater select for antibiotic resistant bacteria in controlled laboratory experiments. Therefore, sterile-filtered wastewaters were used to expose i) an artificial community of 149 *E. coli* strains, ii) a set of individual *E. coli* strains with different resistance patterns one by one, or iii) natural wastewater microbial communities. Effects on the relative prevalence of resistant *E. coli* strains, or just growth in the case of individual strains, were used as indicators for selection. If evidence of selection was found, a second aim was to find indications of which types of agent(s) could be the main drivers via analyses of resistance patterns in favored strains in combination with concentration measurements of different antibiotics.

2. Material and methods

2.1. Collection and preparation of wastewater samples to test for their selective ability

Wastewater effluent samples from Sweden's largest hospital (Sahlgrenska University hospital serving 1950 beds in Gothenburg, Sweden) and influent and effluent samples from the connected WWTP in Gothenburg (Ryaverket, Sweden) were obtained the same day on April 4th and 28th and June 11th 2019 (called sample 1, 2 and 3 in the following sections). Collection days were chosen to avoid recent heavy rain that could cause both considerable contribution of storm water mixed into the wastewater, and also lead to less optimal treatment. Composite samples from continuous subsampling during 24 h were used to assure a representative test water from different locations (subsamples during 24 h: hospital n = 160, municipal wastewater n = approximately 225 for both influent and effluent). Samples were sterile-filtered using 0.45 μm pore size S-Pak filters (Millipore Corporation, Bedford, USA), aliquoted in 50 mL falcon tubes and stored at −20 °C. No bacterial growth was observed when sterile-filtered samples where supplemented with 10% LB medium and incubated at 37 °C for 24 h.

2.2. Chemical analysis

LC-MS grade methanol and acetonitrile (Lichrosolv – hypergrade) were purchased from Merck (Darmstadt, Germany). Purified water was prepared using a Milli-Q Advantage system, including an ultraviolet radiation source (Millipore, Billerica, USA). Formic acid (Sigma-Aldrich, Steinheim, Germany) was used (at 0.1%) to prepare the mobile chromatographic phases. All standards and labeled standards were of analytical grade (≥98%). Samples were thawed and spiked with 5 ng of each internal labeled, and surrogate, standard before solid phase extraction and measurement by liquid chromatography coupled with triple quadropole mass spectrometry, described by Flach et al. (2018). In short, the liquid chromatography mass spectrometry analysis was made on a UHPLC system connected to a TSQ Quantitative triple quadrupole mass spectrometer (Thermo Scientific, San Jose, USA) equipped with a heated-electrospray ionization ion source operating in positive mode. Mass spectrometry (MS) settings for the included chemicals are shown in supplementary data 2 (table S5).

2.3. Establishing selective effect of wastewater with different origin

Three different test systems were used to assess the selective effects of sterile-filtered wastewaters. In the first, we used an artificial community of 149 *E. coli* isolates from untreated hospital wastewater (Sahlgrenska University hospital, Gothenburg, Sweden), as described earlier (Kraupner et al., 2020). All isolates originated from three different samples taken in 2016 (January, June, November) and were confirmed to belong to the species *E. coli* by MALDI-TOF. The composition of the *E. coli* community was chosen based on differing resistance pattern and individual phenotypes as tested by chemical fingerprinting (Hutinel et al., 2019; Kraupner et al., 2020). Resistance to 11 antibiotics and antibiotic combinations at EUCAST clinical breakpoint concentrations (EUCAST 2016) was determined as described in Section 2.5.1. In total, the community consisted of 61 antibiotic resistant *E. coli* isolates with varying resistance patterns as tested via broth screening, and 88 isolates susceptible to all 11 tested antibiotics (Table 1). Within this mix, 20 isolates were resistant against 1 antibiotic, 9 isolates against 2 antibiotics, 12 isolates against 3 antibiotics, 4 isolates against 4 antibiotics, 2 isolates against 5 antibiotics, 5 isolates against 6 antibiotics, 5 isolates against 7 antibiotics, 3 isolates against 8 antibiotics and 1 isolate was resistant against 10 antibiotics. Aliquots of the *E. coli* mix with a final concentration of 2.2×10^5 CFU/μL and 20% glycerol were stored at −80 °C.

In the second setup, we exposed complex microbial communities to

Table 1
Composition of the artificial *E. coli* community as determined by broth resistance screening as described in Hutinel et al. (2019).

Antibiotic	MEC	AMC	TZP	CFR	CTX	CAZ	CIP	TOB	NIT	TMP	SXT
Breakpoint concentration[1]	8 mg/L	AMX 8 mg/L CLA 2 mg/L	PIP 16 mg/L TZB 4 mg/L	16 mg/L	2 mg/L	4 mg/L	1 mg/L	4 mg/L	64 mg/L	TMP 4 mg/L	TMP 4 mg/L SMX 76 mg/L
# resistant isolates	6	39	3	19	11	13	25	13	2	36	32
% resistant isolates	4	26.2	2.0	12.8	7.4	8.7	16.8	8.7	1.3	24.2	21.5

[1] According to the European Committee on antimicrobial susceptibility testing (EUCAST) in 2016; Abbreviations: AMC: amoxicillin-clavulanic acid, AMX: amoxicillin, CAZ: ceftazidime, CFR: cefadroxil, CIP: ciprofloxacin, CLA: clavulanic acid, CTX: cefotaxime, MEC: mecillinam, NIT: nitrofurantoin, PIP: piperacillin, SMX: sulfamethoxazole, SXT: trimethoprim.

the different filtered effluents. The complex microbial test community was obtained from influent from Scandinavia's largest WWTP (Ryaverket in Gothenburg, Sweden). A one liter 24 h composite sample was filtered through 0.45 μm pore size S-Pak filters (Millipore Corporation, Bedford, USA). Individual filters were cut into pieces and transferred to 50 mL falcon tubes containing sterile glass beads and 25 mL of 100 mM PBS (pH 7). The tubes were vortexed for 10 min to resuspend the community in PBS. The liquid phase was pooled and transferred to an empty 50 mL falcon tube. The mixture was centrifuged at 3000*g* for 15 min and washed twice with PBS to remove potential soluble contaminants. The pellets were resuspended in PBS to an OD of 3.5 and glycerol was added to a final concentration of 20%. The complex community was stored in aliquots at −80 °C until further use. In the third setup, we exposed a selection of *E. coli* strains individually as described below under Section 2.3.2.

2.3.1. Selection of resistant E. coli in fully complex community or in an E. coli mix by different types of wastewaters

At the start of each selection experiment, 15 mL falcon tubes were filled with 4.5 mL filtered wastewater (or physiological saline as control) and 0.5 mL LB medium. After inoculating the tubes with 5 μL of the *E. coli* mix or 100 μL of the wastewater community (aimed final concentration of total bacteria in the tube: 5×10^5 CFU/mL), a small sample was transferred immediately after vortexing onto agar plates with or without antibiotics. The tubes were incubated at 37 °C and 170 rpm. After 24 h, 5 μL of the grown culture was transferred to a fresh tube containing the same ratio of wastewater and LB as described above. The outgrown culture was transferred on agar plates with and without antibiotics after three passages in liquid media. Antibiotic concentrations in agar plates were similar to the clinical breakpoints listed in Table 1, except for tobramycin (8 μg/mL). The agar plates were LB or CHROMagar™ ECC plates incubated at 37 °C for 20–24 h, for experiments with the artificial *E. coli* community or the complex wastewater community respectively. The percentage of resistance was calculated by determining the number *E. coli* colonies on antibiotic containing plates (CFU/mL) relative to the control plate without antibiotics using the median of three plating replicates. Analyses of variance (ANOVA) with Bonferroni's multiple comparisons test were used to compare the log-transformed resistance ratios at day 4 between hospital effluent and saline, WWTP influent and saline, and WWTP effluent against saline, respectively.

2.3.2. Growth of individual E. coli isolates with different resistance pattern

Individual growth curves of *E. coli* isolates were monitored in 96 well plates using the OmniLog™ system. For each plate 18 mL of sterile filtered wastewater or saline was mixed with 2 mL LB medium and 200 μL Biolog redox dye A, which turns purple in correlation to the ability of cells to convert extracellular metabolites into mitochondrial reducing equivalents. After transferring 200 μL of the described medium mix to every well of the 96 well plate, 2.5 μL of an overnight grown culture was inoculated to every second well, leaving the other wells as contamination controls. Incubation at 37 °C and recording of the phenotypic data was performed by the OmniLog™ system, which was set to take pictures every ten minutes for 20 h. The data was converted and exported to Microsoft Excel with the OmniLog™ Phenotype MicroArray™ software 1.30. The median of three technical replicates was used for illustrating graphs and statistical analysis. For the latter, we used the area under the growth curve to represent growth.

2.4. Time-kill test of individual E. coli isolates in hospital effluent

To resolve how fast bacteria were killed by the filtered hospital effluent, we performed a short-term time-kill test on susceptible *E. coli* isolates and *E. coli* isolates resistant against at least 6 antibiotics, largely following the standard guideline as described in the M26-A document of Clinical and Laboratory Standards Institute (CLSI) (Balouiri et al., 2016) with some modifications indicated below. A fresh batch of hospital effluent was collected on October 15th 2019, sterile filtered and treated similarly compared to the effluents collected earlier in the same year (see 2.1). At the start of each experiment, 15 mL falcon tubes were filled with either 5 mL hospital effluent or physiological saline as control. After inoculating with 5×10^5 CFU/mL, the tubes were incubated with 170 rpm shaking at 20 °C. Every 30 min 100 μL were sampled during the first five hours of incubation, diluted and transferred to LB agar plates with two plating replicates. The last sample point was taken after 24 h. LB agar plates were incubated at 37 °C for 24 h.

2.5. Susceptibility testing

2.5.1. Broth resistance screening at clinical antibiotic breakpoint concentrations

To compare the resistance pattern of the 149 isolates and isolates that were selected for after exposure to hospital effluent, resistance to 11 antibiotics and antibiotic combinations at EUCAST clinical breakpoint concentrations (EUCAST 2016) was determined as described by Hutinel et al. (2019) for: mecillinam, amoxicillin-clavulanic acid, cefadroxil, cefotaxime, ceftazidime, ciprofloxacin, nitrofurantoin, piperacillin-tazobactam, tobramycin, trimethoprim, trimethoprim-sulfamethoxazole. For each tested antibiotic a 96 well plate was prepared with 200 μL of cation-adjusted Mueller-Hinton (MH) containing antibiotic at the appropriate breakpoint concentration. One plate without any antibiotics served as positive control plate. Cells were inoculated to every second well to a final concentration of 5×10^5 CFU/mL from a freshly overnight incubated blood agar plate. Each plate contained one well inoculated with the antibiotic susceptible *E. coli* ATCC 25922 as negative growth control and two wells inoculated with *E. coli* sewage isolates (that in combination were resistant to all tested antibiotics) as positive growth control. Resistance/susceptibility was determined by visual assessment of growth after culturing the bacteria overnight at 37 °C with 170 rpm shaking.

2.5.2. MIC determination by broth microdilution

The Minimum Inhibitory Concentration (MIC) was tested with the broth microdilution method according to CLSI standards (CLSI, 2012), if not stated otherwise. Briefly, a 96 well plate was prepared with twofold dilutions of the antimicrobial agent. Overnight grown cultures were used as inoculum with a final well concentration 5×10^5 CFU/mL using MH broth as growth medium. Each plate contained one well inoculated

with the antibiotic susceptible *E. coli* ATCC 25922 as negative growth control and one well that was used to test for sterility. 96 well plates were incubated at 37 °C with 170 rpm shaking for 20 h. Resistance/susceptibility was determined by visual assessment of growth.

2.5.3. Etest antimicrobial susceptibility testing

To characterize *E. coli* isolates #105, #127 and #130, resistance to 30 antibiotics and antibiotic combinations were tested using Etest (bioMérieux SA, Marcy l'Etoile, France) according to the manufacturers guideline.

3. Results

3.1. Chemical analysis of hospital effluent, WWTP influent and effluent

Concentrations of 24 antibiotics, two antibacterial biocides and seven commonly used non-antibiotic pharmaceuticals were analyzed in samples of hospital effluent, WWTP influent and effluent that were obtained on the same day during three different occasions between April and June 2019 (Table 2). In general, concentrations for all tested substances were highest in hospital effluent with cefadroxil levels reaching on average 92, ciprofloxacin 67, norfloxacin 76, ofloxacin 67, linezolid 53, and amoxicillin 51 times higher concentrations when compared to WWTP influent. Treatment of incoming wastewater led to a further reduction of antibiotic concentrations, of which eight antibiotics were below the detection limit. Highest removal through wastewater treatment was observed for benzylpenicillin, ciprofloxacin, tetracycline and trimethoprim.

3.2. Selection of antibiotic resistant E. coli by different wastewaters

An artificial *E. coli* mix consisting of 149 isolates, each with a unique phenotypic profile, was exposed to different types of wastewaters and selection of resistance was monitored by comparing the proportion of resistant bacteria in the mixture at the start and end of each experiment. At the start of each experiment, resistance levels where similar to what was expected based on the resistant pattern of the individual strains (Fig. 1). A small portion of cells was transferred daily to a fresh wastewater and 10% LB mixture (1:1000 dilution). Even though extreme fast growers were not included in the artificial *E. coli* mix, growth in the control condition (physiological saline supplemented with 10% LB) resulted in a significant increase of ciprofloxacin and trimethoprim-sulfamethoxazole resistant isolates (Fig. 1). This increase in resistance in physiological saline should not be interpreted as a sign of selection caused by antibiotics or directly related to antibiotic resistance, but rather as natural consequence of the co-existence of multiple different genotypes that are characterized by different growth rates. No significant change in the percentage of resistant isolates was observed in the

Table 2
Results of the chemical analysis of hospital effluent, WWTP influent and effluent samples on the same day at three different occasions during April to June 2019 in ng/L.

		Hospital effluent			WWTP influent			WWTP effluent			Lowest MIC for *E. coli* [ng/L]	PNEC[ng/L][4]
		1	2	3	1	2	3	1	2	3		
Antibiotics	LOQ											
Amoxicillin	5.0	140	130	110	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1,000,000[1]	8000
Azithromycin	5.0	270	64	34	22	19	28	82	11	17	1,000,000[1]	250
Benzylpenicillin	1.0	280	<LOQ	440	58	57	61	<LOQ	<LOQ	<LOQ		250
Cefotaxim	5.0	99	130	<LOQ	39	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	16,000[1]	125
Cefadroxil	5.0	2300	790	3400	15	72	30	9.3	36	<LOQ	4,000,000[1]	2000
Cephalexin	5.0	24	20	<LOQ	14	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	2,000,000[1]	4000
Chlaritromycin	1.0	170	13	120	23	11	24	9.7	<LOQ	17		2,50
Ciprofloxacin	10	2100	2200	4400	16	120	86	48	<LOQ	<LOQ	40,001	64
Clindamycin	1.0	330	160	1900	25	17	23	82	19	94		1000
Erythromycin	50	43	130	200	<LOQ	<LOQ	70	<LOQ	<LOQ	<LOQ		1000
Linezolid	10	560	71	900	<LOQ	23	20	13	<LOQ	<LOQ		8000
Meropenem	100	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	80,001	64
Metronidazole	50	4000	1600	1900	73	76	76	<LOQ	<LOQ	<LOQ		125
Nitrofurantoin	50	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	4,000,000[1]	64,000
Norfloxacin	50	2500	1300	1900	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	16,000[1]	500
Ofloxacin/ Levofloxacin	10	610	260	140	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	16,000[1]	500
Oxytetracycline	5.0	130	<LOQ	160	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ		500
Phenoxyenicillin	10	<LOQ	180	97	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ		64
Piperacillin	0.1	130	580	950	86	27	70	73	76	76	500,000[1]	500
Roxithromycin	50	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ		1,000
Sulfadiazine	10	23	<LOQ	33	18	24	14	<LOQ	<LOQ	13		
Sulfamethoxazole	10	540	260	860	52	54	59	17	18	28	2,000,000[1]	16,000
Tetracycline	10	750	290	730	550	<LOQ	360	30	54	78	500,000[1]	1000
Trimethoprim	10	980	300	1400	140	150	150	22	29	37	60,000[1]	500
Other substances												
Carbamazepine	1.0	520	1400	1100	210	270	250	190	260	320		
Chlorhexidine	1.0	1200	2400	1400	110	15	42	78	11	12	1,000,000[2]	
Diclofenac	50	330	230	620	400	400	560	420	550	710		
Gemfibrozil	50	62	<LOQ	87	93	69	150	1100	2600	1200		
Ibuprofen	100	1800	2000	2000	1500	840	1500	140	230	470		
Naproxen	5.0	140	83	360	78	73	29	350	26	33		
Paracetamol	50	830,000	670,000	520,000	81,000	110,000	93,000	2900	130	180		
Propranolol	5.0	1700	1900	2200	1700	2100	1600	270	440	32		
Triclosan	50	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	780[3]	

LOQ-Limit of quantification.

[1] The lowest 1% MIC value observed for *E. coli* in the EUCAST database in [ng/L].

[2] Lutgring et al. (2020) measured the MIC for chlorhexidine of 158 antibiotic-resistant *E. coli* isolates.

[3] Cameron et al. (2019) measured the MIC for triclosan of 99 *E. coli* wastewater isolates.

[4] Predicted No Effect Concentration (PNEC) derived from Bengtsson-Palme and Larsson (2016) in [ng/L].

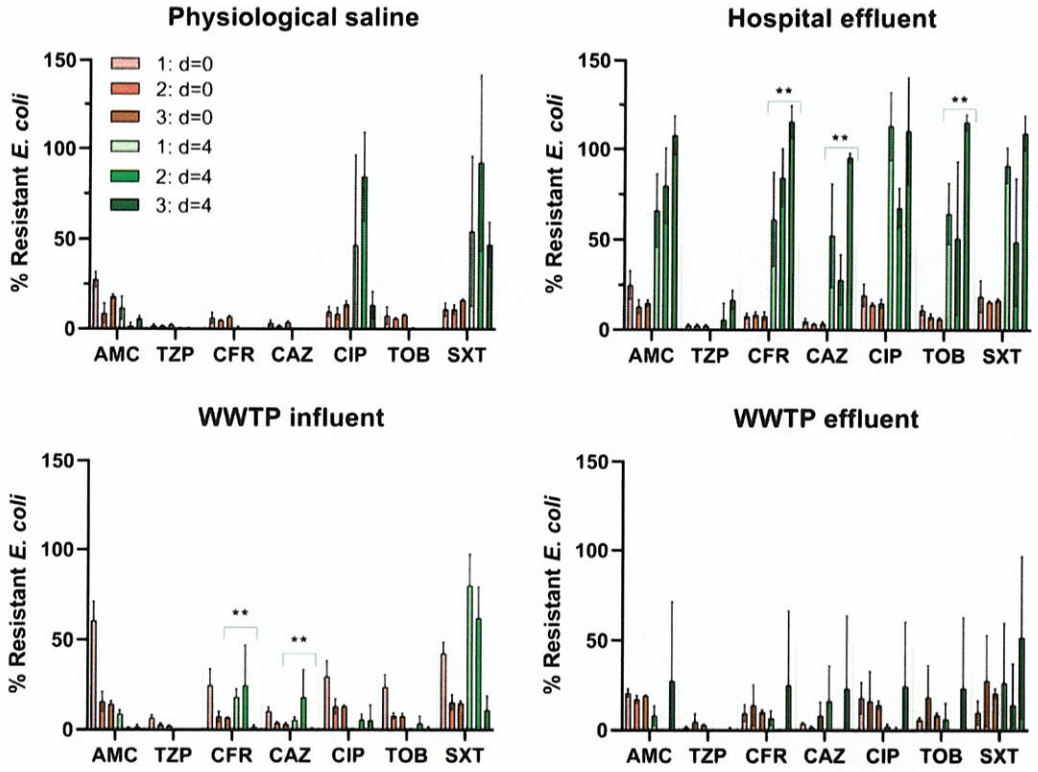

Fig. 1. Selection of resistant *E. coli* by WWTP influent and effluent or hospital effluent. An artificial mix of 149 *E. coli* isolates was exposed to different types of sterile filtered wastewater samples from three different sampling occasions and physiological saline as control for 4 days (3 passages). The percentage of resistant *E. coli* was determined by plating a fraction of the culture on LB plates with or without antibiotics. ANOVA followed by Bonferroni's multiple comparisons test was used to compare log-transformed resistance ratios at day 4 between hospital effluent and saline, WWTP influent and saline and WWTP effluent against physiological saline, respectively (**$p < 0.01$). Abbreviations: AMC: amoxicillin-clavulanic acid; CAZ: ceftazidime; CFR: cefadroxil; CIP: ciprofloxacin; SXT: trimethoprim-sulfamethoxazole; TOB: tobramycin; TZP: piperacillin-tazobactam.

mix grown in effluent from the studied WWTP. Exposure to WWTP influent resulted in a significantly increased proportion of *E. coli* isolates resistant against cefadroxil and ceftazidime compared to physiological saline after 4 days. Finally, exposure to hospital effluent resulted in a significant increase of isolates resistant to cefadroxil, ceftazidime and tobramycin. There was also an average increase in the proportion of strains resistant to amoxicillin-clavulanic acid (from 66 to 100%) and for ciprofloxacin (from 68 to 100%), but not significantly ($p = 0.107$ and >0.99, respectively). The lack of significance for ciprofloxacin should be seen in light of the fast-growing nature of some ciprofloxacin resistant strains also in the controls, as described above. Hence, the power of the statistical analyses was more limited for this antibiotic. The most drastic outcome was seen for sample three, which caused an increase of resistance rates for 6 out of 7 tested antibiotics to almost 100%. To test whether only one successful multi-resistant clone was selected for in all replicates, 45 single colonies isolated at the end of each experiment on LB agar (without antibiotics) were profiled regarding their susceptibility against all tested antibiotics (Fig. 2). However, the relative proportion of these isolates differed in all replicates indicating selection of several different multi-resistant *E. coli* isolates at all three sampling occasions.

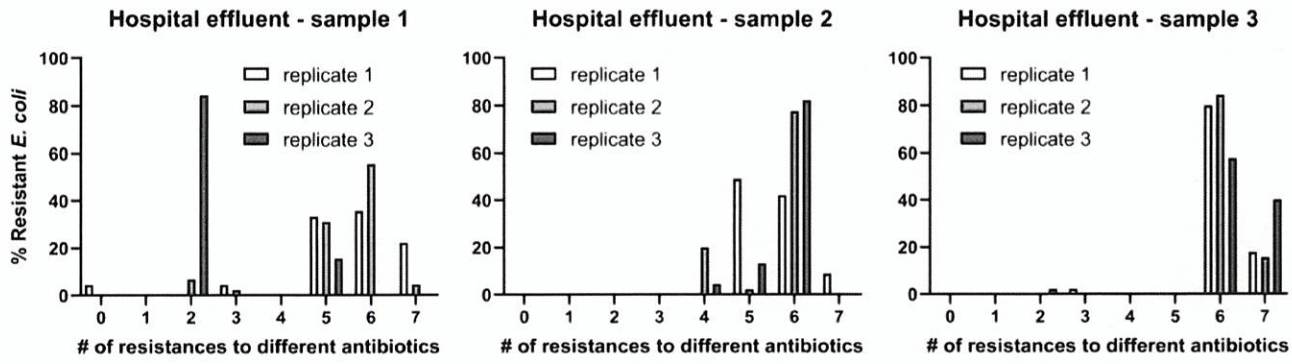

Fig. 2. Comparison of the number of resistances in *E. coli* isolated from cultures after 4 days exposed to hospital effluent as shown in Fig. 1. Forty-five isolates were picked from each of the technical replicates for resistance profiling from LB plates without any antibiotics. Resistance was profiled by broth resistance screening in the same manner as the initial isolates constituting the community (Hutinel et al., 2019) for amoxicillin-clavulanic acid, cefadroxil, ceftazidime, ciprofloxacin, piperacillin-tazobactam, tobramycin, trimethoprim-sulfamethoxazole.

3.3. Growth of individual E. coli isolates in different wastewater streams and ciprofloxacin

To monitor the growth of individual isolates in different types of wastewaters, 32 *E. coli* isolates were selected from the artificial mix and grouped in isolates with five or less resistances and isolates with at least six resistances to antibiotics included in the screen (supplementary data 1 table S1, Fig. 3). Only exposure to the hospital effluent resulted in a significantly different growth pattern as seen in reduced growth for isolates that are more sensitive to antibiotics (ANOVA with Tukeys posthoc test, $p < 0.05$). The growth of multi-resistant isolates on the other hand was not influenced by exposure to hospital effluent.

To test if only one particular resistance is needed to grow similarly in hospital effluent as compared to control conditions, *E. coli* isolates with only one resistance were further selected and grown in all types of wastewater or physiological saline as control supplemented with 10% LB medium (Fig. 4). Eleven isolates originated from the artificial *E. coli* community were selected, out of which two isolates were susceptible (#1, #5), two isolates were only resistant against the combination of amoxicillin-clavulanic acid (#13, #26), three isolates only against trimethoprim-sulfamethoxazole (#24, #28, #60), one isolate only against ciprofloxacin (#53) and three isolates were resistant against at least 6 antibiotics (#105, #127, #133) (Supplementary data 1 table S1). To further test the impact of ciprofloxacin resistance on improved survival in hospital effluent, one *E. coli* MG1655 lab strain was made resistant against fluoroquinolones by exposing the cells to low concentrations of ciprofloxacin by slowly increasing the concentration to 1 mg/L within 26 days (called GyrA). The evolved ciprofloxacin resistant strain harbored two well-characterized resistance-conferring mutations in the Gyrase A protein at nucleotide S83L and D87G identified by sequencing as described in Kraupner et al. (2018). Furthermore, growth of a tobramycin resistant *E. coli* wastewater isolate was tested (called TOB, collected on 28 September 2016 in Sahlgrenska hospital wastewater). No other resistance could be detected in this strain through broth resistance screening. Differences in growth of isolates exposed to hospital effluent sampled during three different occasions could be observed, indicating varying concentrations of selective agents over time. In general, hospital effluent had a negative effect on growth of the vast majority of susceptible and single resistant isolates. In the most extreme case (hospital effluent sample 3, Fig. 4) the outgrowth of susceptible isolates was completely inhibited, as well as isolates solely resistant to trimethoprim-sulfamethoxazole, ciprofloxacin and tobramycin. However, one out of two amoxicillin-clavulanic acid and two out of three multi-resistant isolates were able to grow in the presence of hospital effluent. To test whether outgrowth in this particular sample was associated with the resistance to amoxicillin-clavulanic acid, the MIC was determined through broth dilution for isolate #13, #26, #105, #127, #133. Isolate #26 and #105, which could not grow in hospital effluent (sample 3), had the lowest MIC for amoxicillin-clavulanic acid with 32–64 μg/mL. The remaining isolates had higher MICs, between 64 and 128 μg/mL, and were able grow when exposed to hospital effluent. The observed relation between a higher MIC for amoxicillin-clavulanic acid and the growth in hospital effluent could suggest pollution with β-lactams. A more comprehensive Etest screen of strains #105, #127 and #133 to 30 antibiotics or antibiotic combination supported that strain #105 was only more sensitive compared to #127 and #133 to amoxicillin/clavulanic acid, doripenem and imipenem (supplementary data 2, table S2).

Given our finding of ciprofloxacin at concentrations around the MIC for some susceptible *E. coli* strains, full dose response curves were

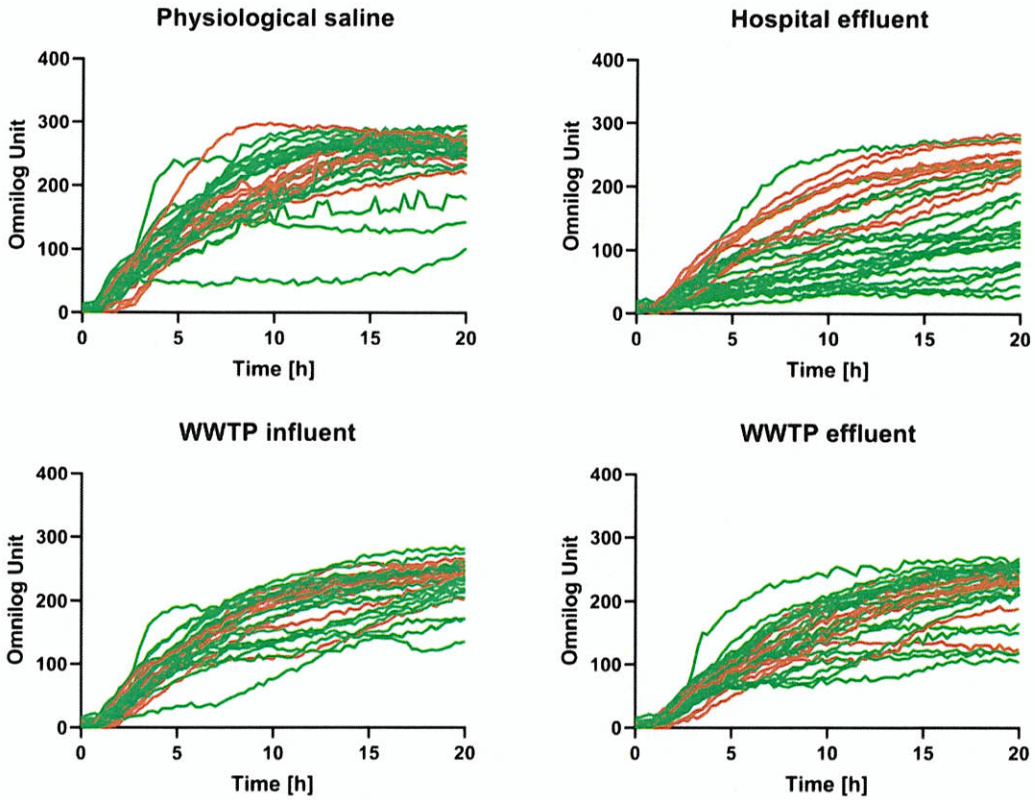

Fig. 3. Growth of 32 *E. coli* isolates grouped in ≤ 5 resistances out of 11 antibiotics tested (green) or isolates with ≥ 6 resistances out of 11 antibiotics tested (red). Isolates were exposed to WWTP influent and effluent or hospital effluent that has been collected on the same day, sterile filtered and supplemented with 10% LB to facilitate growth. Physiological saline containing 10% LB medium was used as control growth condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

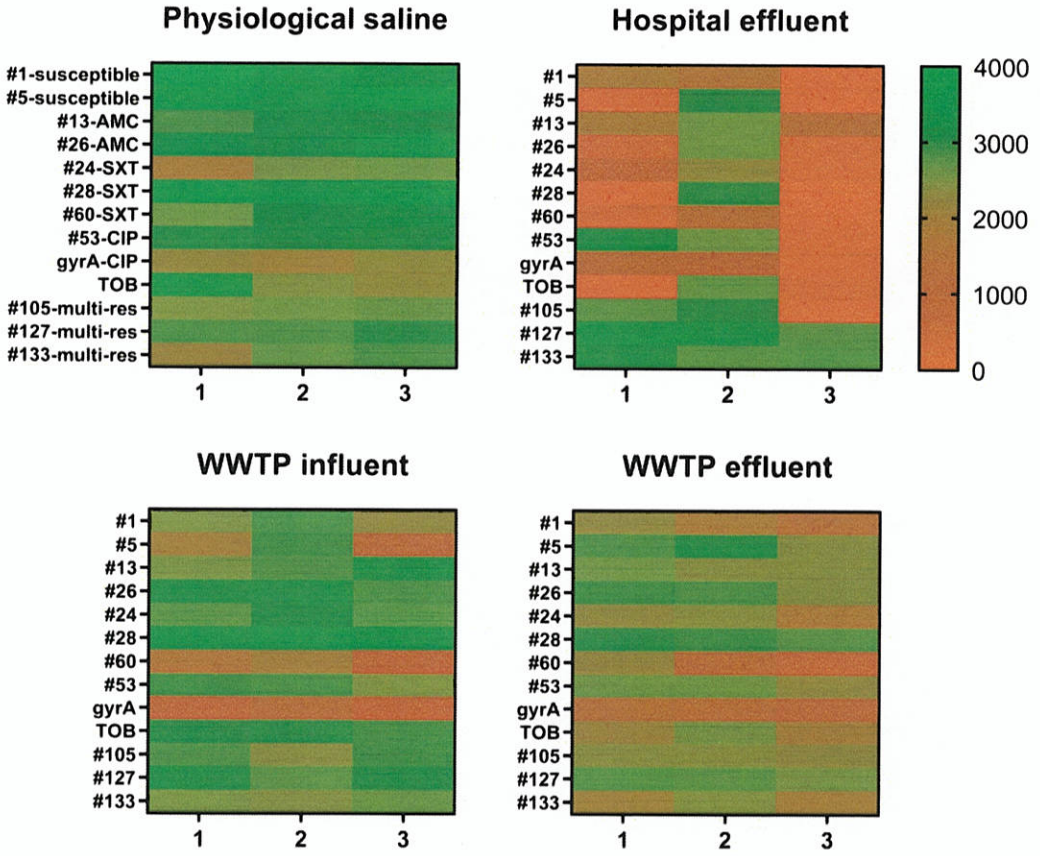

Fig. 4. Area under the growth curve of selected *E. coli* strains. Isolates were exposed to WWTP influent and effluent or hospital effluent that were collected on the same day at three different occasions (1–3), sterile filtered and supplemented with 10% LB to facilitate growth. Physiological saline containing 10% LB medium was used as control growth condition. AMC: amoxicillin-clavulanic acid resistant; CIP: ciprofloxacin resistant; multi-res: multi-resistant; SXT: trimethoprim-sulfamethoxazole resistant; TOB: tobramycin resistant.

generated for three strains (Fig. 5). These were *E. coli* MG1655, *E. coli* wastewater isolate #1 and wastewater isolate #26 with ciprofloxacin MICs of 0.012, 0.016 and 0.008 mg/L, respectively. The isolates were grown in physiological saline supplemented with 10% LB medium and ciprofloxacin using an experimental setup identical to when individual isolates were exposed to different wastewaters. The lowest concentration of ciprofloxacin that had a significant effect on the growth of susceptible isolates was 6.4 μg/L using a two-way ANOVA and Dunnett's post hoc test comparing the area under the curve (AUC) of exposed conditions to the control (0 μg/L ciprofloxacin) to determine statistically significant differences ($p < 0.05$).

3.4. Selection of *E. coli* within in a complex multispecies community

To test whether selection of resistant *E. coli* occurs also in a natural complex community upon exposure to hospital effluent, a fresh batch of WWTP influent community was collected on 18 October 2019, washed and concentrated as described under Section 2.3. This complex wastewater community was then exposed to sterile-filtered wastewater samples 2 (same samples as used in Section 3.1) supplemented with 10% LB medium (Fig. 5). After passaging the communities three times, a significant enrichment of isolates with resistances to amoxicillin-clavulanic acid, piperacillin-tazobactam, cefadroxil, ceftazidim, tobramycin and trimethoprim/sulfamethoxazole was observed in communities exposed to hospital effluent compared to those exposed to saline parallel in time

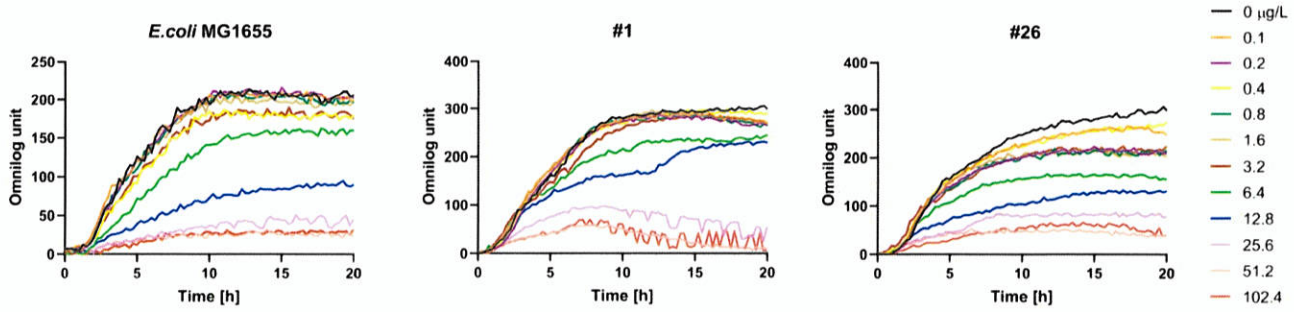

Fig. 5. Growth of ciprofloxacin susceptible *E. coli* isolates in physiological saline supplemented with 10% LB medium with increasing concentrations of ciprofloxacin. Each growth curve represents the median of three technical replicates.

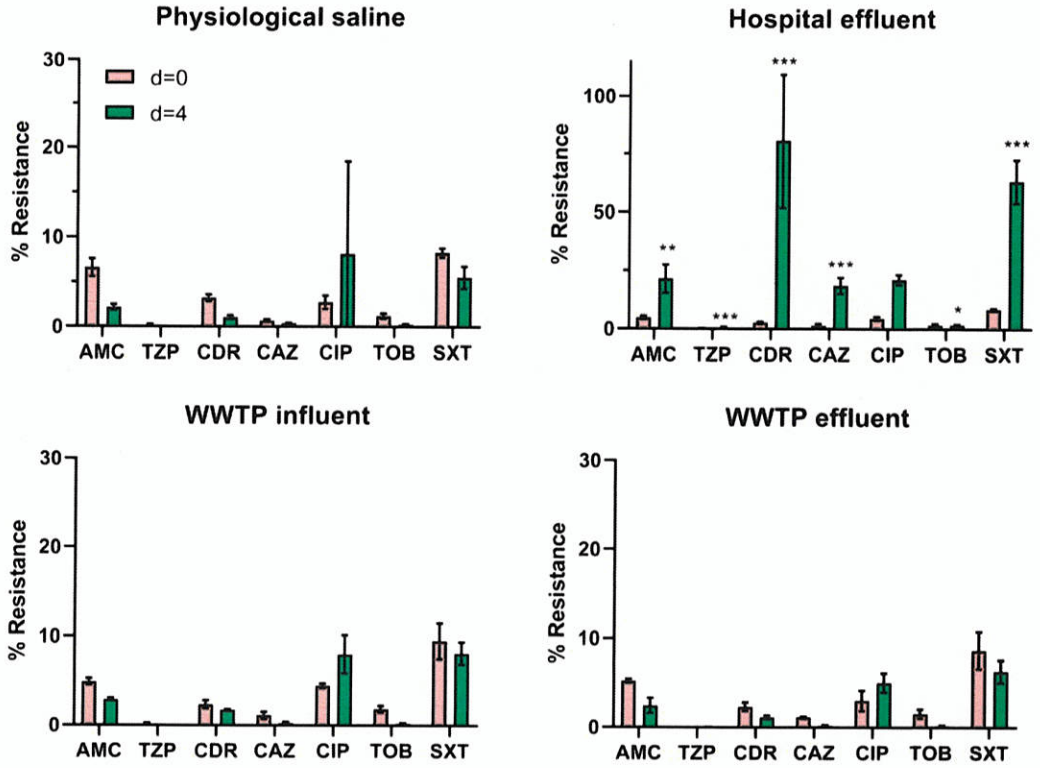

Fig. 6. Selection of resistant *E. coli* in WWTP influent and effluent or hospital effluent. A complex wastewater influent community was exposed to sterile filtered WWTP influent, effluent and hospital effluent supplemented with 10% LB medium for 4 days (3 passages). Growth in physiological saline with 10% LB medium served as control. The percentage of resistant *E. coli* was determined by plating a fraction of the culture on CHROMagar™ ECC agar media with or without antibiotics. ANOVA followed by Bonferroni's multiple comparisons test was used to compare log-transformed resistance ratios at day 4 between hospital effluent and saline, WWTP influent and saline and WWTP effluent against saline, respectively (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$). Abbreviations: AMC: amoxicillin-clavulanic acid; CAZ: ceftazidime; CFR: cefadroxil; CIP: ciprofloxacin; SXT: trimethoprim-sulfamethoxazole; TOB: tobramycin; TZP: piperacillin-tazobactam.

(Fig. 6). Hence, the selective effect of hospital wastewater on a single species *E. coli* community could be reproduced by using a complex community that consists of many different taxa, including *E. coli*. Exposure to wastewater influent and effluent did not significantly change the abundance of resistant *E. coli* after 4 days compared to physiological saline.

3.5. Time-kill test of sensitive and resistant E. coli isolates in hospital effluent

Exposure to hospital effluent has demonstrated both a clear selection for resistance, and a strong growth inhibition of sensitive isolates as described above. Next, we asked if hospital effluent is bactericidal to sensitive *E. coli* isolates. To test this, five susceptible strains and five strains resistant to at least six antibiotics were selected from the artificial community and exposed to a freshly sampled batch of hospital effluent sampled on 15 October 2019 that has not been used in earlier experiments (Fig. 7). During the time-kill test the cultures were incubated at 20 °C without LB medium in order to resemble conditions closer to the environment found for example in pipes running from the hospital to WWTPs. A clear bactericidal effect of hospital effluent exposure was observed for all tested susceptible *E. coli* isolates with a reduction of CFU counts between 65 and 98% within the first five hours. After 24 h of exposure to hospital effluent, susceptible isolates showed a log reduction

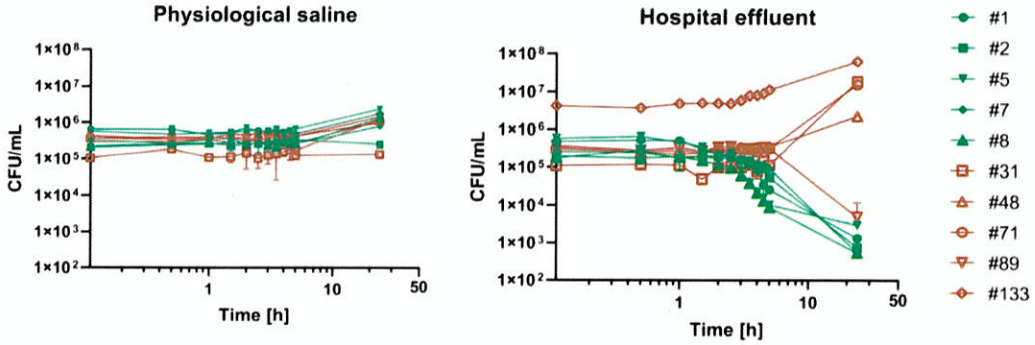

Fig. 7. Time-kill assay of susceptible *E. coli* strains (green) and strains resistant against ≥ 6 antibiotics (red) *E. coli* isolates exposed to either saline or hospital effluent at 20 °C. Strains were enumerated on LB agar plates that were incubated at 37 °C. The numbers of the isolates correspond to the *E. coli* isolates listed in supplementary data 1 table S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between 2-fold and 3-fold. Only multi-resistant cells were able to stably maintain CFU numbers, except for one isolate (#89 in Fig. 7). However, when the susceptible *E. coli* isolates were cultured in control conditions (physiological saline instead of effluent), stable CFU counts were observed over 24 h. Growth rates were calculated using CFU numbers between 2 and 5 h (Supplementary data table S3 and S4) and showed clear distinctions between sensitive cells exposed to hospital effluent compared to all other conditions (2-way ANOVA with Tukey's posthoc test $p < 0.001$).

4. Discussion

To our best knowledge, this is the first study that directly demonstrates selection of antibiotic resistance in hospital effluent via controlled experiments. Coherent results were obtained using three different phenotypic assays including comparisons of growth rates of single isolates, via studies of selection in an artificial *E. coli* mixture to complex, multispecies wastewater microbial communities. We show that multi-resistant *E. coli* isolates overtake the communities and simply grow better in hospital effluent compared to isolates that are more susceptible. The rapid kill-off of susceptible cells demonstrated that resistant strains not only have a slight growth advantage, but that selection can take place even under no growth conditions. While the underlying selective agents are still elusive, fluoroquinolones and β-lactams may have contributed, but if so, likely in combination with other compounds. This will require further research. Taken together, the results highlight that untreated hospital effluent and residual antibiotics therein are plausible arenas and drivers for the evolution of resistance. Some evidence was also found for selection of β-lactam resistant strains by the studied WWTP influent, but not the effluent. This suggests a risk for selection along the entire sewer system, possibly also in the WWTP plant, but it provides no support for selection in the recipient river.

Growth is needed for sub-inhibitory concentrations of antibiotics to elicit selective effects on bacterial populations. With the exception for stationary biofilms (see below), it is unknown to what extent bacterial growth occurs within the pipes of sewer systems, and hence what role sub-inhibitory selective agents might play here. Importantly, we show that the investigated hospital effluent does not only provide a growth advantage for resistant strains but actually kills all studied antibiotic-susceptible bacteria, leaving multi-resistant bacteria largely unaffected. Hence, no growth at all is needed to select for resistance in the hospital sewers, while any level of growth would exacerbate the selective effect further. The first killing effects on *E. coli* were observed after 2 h. We have not been able to establish retention times of bacteria in the pipes, thus it is unclear to what extent a kill-off of susceptible strains will occur in the free-flowing phase. Closer to the municipal WWTP, the hospital wastewater becomes more diluted with household wastewater and thus becomes less selective. This is supported by lower concentrations of antibiotics in the WWTP influent and the weaker selection observed in the assay with the *E. coli* mix. The bacterial biofilms that grow in hospital sewers will, on the other hand, be exposed over very long time periods. Hence, they are very likely to be shaped by the strong antibiotic selection pressure from the passing hospital wastewater. Ory et al. (2016) examined biofilms grown in hospital effluent and showed that more than 60% of the tested isolates were resistant to up to six antibiotics. Although that observation alone does not demonstrate selection, it is in line with the direct observations of selection of multi-resistant strains by hospital effluent as shown here. Flocks from growing biofilms will continuously detach from the pipes, and be transported to the local WWTP, and thus potentially spread further.

After identifying the direct selection of antibiotic resistance by untreated hospital effluent, we aimed to explore what could be causing the observed selection. A large variability in both the selective ability and the concentrations of antibiotics was observed in hospital effluent sampled at different days, despite the composite sampling strategy. We believe the variability is most likely caused by hospital effluent representing both fewer people and being less mixed compared to the corresponding WWTP influent. The strongest growth inhibiting effect for susceptible *E. coli* isolates or isolates with only one resistance was observed for sample 3 (Fig. 4), which also corresponds to the sample with the overall highest antibiotic concentrations e.g. for benzylpenicillin, cefadroxil, ciprofloxacin, clindamycin, piperacillin and trimethoprim (Table 2).

One of the analyzed antibiotics, ciprofloxacin, exceeded MICs for some antibiotic-sensitive *E. coli* in the hospital effluent (Table 2). Measured concentrations of 2.1–4.4 μg/L should be compared to a 1% lowest MIC for *E. coli* of 4 μg/L according to EUCAST, with 16 μg/L completely inhibiting growth of most clinical antibiotic-sensitive *E. coli* strains. Growth assays of susceptible isolates did not show any clear effect at concentrations up to 3.2 μg/L, while a significantly reduced growth was observed from 6.4 μg/L and higher. Norfloxacin was detected at 1.3–2.5 μg/L while the corresponding 1% lowest MIC for *E. coli* is 16 μg/L and the majority of *E. coli* strains are completely inhibited by 60 μg/L. None of the other analyzed antibiotics was close to the MICs for sensitive *E. coli*. In the community assay with 149 *E. coli* strains, there was a non-significant trend that ciprofloxacin resistant strains were favored. Given the fast-growing nature of some of the ciprofloxacin-resistant strains also in saline, the ability to detect relative increases in ciprofloxacin resistant strains as a result of wastewater exposure became somewhat blunted in this setup. In the multispecies community assay, we did not see an increase in the proportion of ciprofloxacin resistant *E. coli*. The strain made resistant to ciprofloxacin in the laboratory (called GyrA) did not grow at all in the hospital effluent sample 3, indicating that if fluoroquinolones provide selection pressure here, it is in combination with some other class(es) of selective agents. Indeed, mixtures of antibiotics, including ciprofloxacin, could act additively and thus further increase individual antibiotic effects (Yeh et al., 2006). Taken together, there is some support that fluoroquinolones, particularly ciprofloxacin, contributes to the selection of multi-resistant *E. coli* strains by the investigated hospital effluent, but more research is need. It should also be noted that other species could be more sensitive to fluoroquinolones than is *E. coli* and it is thus possible that studies of other species could provide stronger evidence for selection by this class of antibiotics.

While we found ciprofloxacin in concentrations between 2.1 and 4.4 μg/L in hospital effluent, some other studies report ranges between 0.08 and 26 μg/L (Brown et al., 2006; Lindberg et al., 2014; Varela et al., 2014; Verlicchi et al., 2012; Zorita et al., 2009). Considerably higher ciprofloxacin concentrations have been reported in hospital wastewater from Germany (up to 124.5 μg/L, 2 h composite sample in Hartmann et al. (1999)), Sweden (101 μg/L, grab sample in Lindberg et al. (2004)), Brazil (up to 155 μg/L, grab sample in Martins et al. (2008)) and India (up to 236 μg/L, grab sample in Diwan et al. (2010)). Gullberg et al. (2011) estimated the MSC for ciprofloxacin to select for a specific resistant strain in a sensitive pairwise competition assay to be 0.1 μg/L. Bengtsson-Palme and Larsson (2016) proposed a PNEC for resistance selection of 0.064 μg/L based on available MIC data from 70 species. A more elaborate biofilm assay with complex communities revealed a No Observed Effect Concentration (NOEC) of 0.1 μg/L based on within-species selection, taxonomic composition and the relative abundance of mobile quinolone resistance genes, the latter being the most sensitive endpoint (Kraupner et al., 2018). Murray et al. (2020) reported a NOEC of 0.98 μg/L as derived from overall reduced growth of suspended complex wastewater communities. The range of different effect concentrations reported, together with the ranges found in hospital effluent, thus indicate a rather widespread potential for selection by ciprofloxacin.

In both community assays, we found a clear and significant increase in strains resistant to different β-lactam antibiotics after exposure to hospital effluents. Similarly, the growth assays of individual strains showed that some of the β-lactam resistant strains grew relatively well in hospital effluents. Strains resistant to the combination of amoxicillin and

the β-lactamase inhibitor clavulanic acid were still inhibited both by sample 1 and 3. Hence, if β-lactams indeed are contributing to the selection pressure, we can conclude that either they are not the only type of selective agent present in potent concentrations, and/or the selection is caused by types of β-lactams that are not counteracted by this type of resistance. The strong growth of the multi-resistant strains #127 and #133 but not #105 in hospital effluent sample 3 is somewhat coherent with a β-lactam selection pressure, as the MIC to amoxicillin/clavulanic acid, doripenem and imipenem is slightly lower in strain #105, but the differences in MICs between the other two strains are very small (supplementary data 2). Cefadroxil was measured in hospital effluent at concentrations (0.79–3.4 μg/L) exceeding the PNEC of 2.0 μg/L reported by Bengtsson-Palme and Larsson (2016). However it should be noted that this PNEC is derived from very few (7) species, hence a relatively large safety factor is included in the PNEC. The 1% lowest observed MIC for all studied species was 125 μg/L (*Streptococcus pyogenes*) while *E. coli* is rather insensitive to cefadroxil, with a lowest reported MIC of 4 mg/L, i.e. 1000-fold above measured concentrations. Other β-lactams, such as benzyl-penicillin and piperacillin were detected in the hospital effluent with concentrations < 1 μg/L, but still exceeding estimated PNECs (Bengtsson-Palme and Larsson, 2016). Again, *E. coli* is not sensitive to benzyl-penicillin and it is not the species driving the PNEC for piperacillin. β-lactams are the most commonly used antibiotic class for human use, but in general, β-lactams are considered to be degraded fast, where the initial hydrolysis of the β-lactam ring leads to a complete loss of efficacy. Hence, despite their high usage, they are rarely pointed out as likely selective agents in the environment. However, it might be that the short time from excretion makes sewer systems an arena for selection also of relatively short-lived antibiotics. Our results suggest a possible contribution of β-lactam antibiotics as selective agents in the studied hospital effluents, but more research is needed to clarify if this indeed is the case.

Trimethoprim was measured at concentrations between 0.3 and 1.4 μg/L in the hospital effluents. The highest levels are higher than the theoretical derived PNEC of 0.5 μg/L by Bengtsson-Palme and Larsson (2016), although the lowest reported MIC for *E. coli* is 16 μg/L and also the experimentally validated NOEC of 1 μg/L reported by Kraupner et al. (2020). The latter NOEC was based on the NOEC/Lowest Observed Effect Concentration (LOEC) for providing a benefit of carrying different *dfr* genes, but disregarding costs, as costs would likely be strongly dependent on what genetically engineered strains and test conditions that were used. The NOEC for increasing the relative abundance of trimethoprim resistant *E. coli* in serially passaged communities or in continuously exposed biofilms was higher (10 μg/L). There was no indication of an increase of trimethoprim/sulfamethoxazole resistance in any of the assays. Together, we interpret this as rather weak evidence for trimethoprim being a major contributor to the selection of multi-resistant strains by the studied hospital effluents, which also contained trimethoprim levels similar to what has been measured in hospital wastewater elsewhere (Italy 0.068–1.8 μg/L; Verlicchi et al. (2012)), Australia (0.3 μg/L; Watkinson et al. (2009)) and the US (up to 5 μg/L; Brown et al. (2006)). In the investigated WWTP influent and effluent trimethoprim concentrations were consistently lower with 0.14–0.15 μg/L, in good agreement with several other studies (Lindberg et al., 2014; Rodriguez-Mozaz et al., 2020; Watkinson et al., 2009; Verlicchi et al., 2012; Östman et al., 2017).

Exposure of the bacterial community to WWTP effluent did not result in a significantly increased abundance of antibiotic resistant isolates, but the resistance profile resembled that after saline exposure in all setups. Similarly, no effects was seen by exposure to the WWTP influent on the growth of individual strain or on the fully complex community assay. However, in the assay with the *E. coli* mix the WWTP influent, significantly selected for both ceftazidime and cefadroxil resistant strains, similar to the hospital effluent but to a smaller degree. Because a selection by WWTP influent was not consistently found in all three setups, the evidence is somewhat weaker than for the hospital effluent, but this could also be a consequence of differences in sensitivity of the assays. Considering that growth assays of individual strains do not integrate growth differences between strains over such a large numbers of generations, it is expected to be less sensitive. A small selective effect for β-lactam resistant strains by WWTP influent is coherent with β-lactams as possible contributors to the much stronger selective effects provided by the hospital effluent, as discussed above. Together this suggest that there might be a selection pressure along the entire sewer system, following a gradient from the hospital all the way to the WWTP. It is considerably less clear if the relative small selective pressure provided by the influent is sufficient to select for resistance within the WWTP. A recent study by Flach et al. (2018) compared the resistance profiles of over 4000 *E. coli* isolates between the influent and effluent of the same WWTP as studied here over 18 months and did not find any indications for resistance selection. The lack of apparent selection is likely a result of consistently lower levels of antibiotics found there. For example, considerably lower levels of ciprofloxacin were measured in WWTP influent (0.016–0.12 μg/L) and effluent (up to 0.048 μg/L) in this study, which is comparable to some earlier reports (Rodriguez-Mozaz et al., 2020; Sahlin et al., 2018). The WWTP influent studied is a mix of primarily urban but also hospital wastewater that have been combined in the pipes before reaching the municipal treatment plant. In general, antibiotic levels observed in WWTP influent and effluent are low in Sweden and range between concentrations below the detection limit up to circa 1 μg/L (Bengtsson-Palme et al., 2016; Flach et al., 2018; Lindberg et al., 2014; Östman et al., 2017). While we found no support for selection in the studied WWTP effluent, sensitivity limitations of the assays could have prevented detection of a minor selection pressure. Hence, we cannot exclude the potential for resistance selection in Swedish WWTP influents. The considerably higher levels of antibiotics reported in other studies show a clear potential for selection in many other countries with higher antibiotic use. Ciprofloxacin, for example, was detected at considerably higher levels in influent wastewater in Australia (up to 4.6 μg/L; (Watkinson et al., 2007)) or even final effluent in Portugal with ciprofloxacin concentrations of 1.4 μg/L (Rodriguez-Mozaz et al., 2020).

We show that Swedish hospital effluent samples rapidly and strongly select for multi-resistant *E. coli* strains. This indicates apparent risks for resistance selection in hospital sewers, not only in Sweden but also elsewhere. It is plausible (but not proven) that fluoroquinolones, particularly ciprofloxacin, contributes to this selection, which further supports already initiated action in Sweden to reduce use and consequently also environmental exposure to this class of antibiotic (Sahlin et al., 2018). When selection of resistance was observed in our experiments, multi-resistant isolates were strongly selected for (Figs. 1 and 2), indicating co-selection and therefore a risk that reach far beyond just limiting the clinical usefulness of fluoroquinolones. To fully understand the underlying dynamics of selection, more details are needed about selective agents. Rather than using a targeted analytical approach, measuring individual candidate antibiotics as done in this study, a more explorative search could reveal which (additional) substance(s) that inhibit(s) growth. For example, bioassay-directed fractionations of hospital effluent, followed by ion-depth chemical characterization of active fractions, might be one approach to shed more light on selective agents.

Regulations concerning the treatment of hospital wastewater vary between different countries. Some consider it as "industrial waste" or as waste from sanitary activities (as e.g. Spain and France; Carraro et al. (2016)). This implies that hospital discharges must meet specific characteristics to be discharged into municipal WWTP, usually requiring pretreatment. Some countries, including Germany and Sweden, have not implemented such regulations and hence no particular authorization is needed for the emission of hospital wastewater into the municipal WWTP (Carraro et al., 2016; Naturvårdsverket, 2008). Studies investigating the mass flow and hence contribution of pharmaceuticals to the overall load of pharmaceuticals in WWTPs report varying contributions

from hospital discharges (Aydin et al., 2019; Azuma et al., 2019; Sörengård et al., 2019). Santos et al. (2013) estimated that fluoroquinolones discharged from a university hospital contributes to up to 40% of fluoroquinolones detected in WWTP influent in one particular study side in Portugal. For the WWTP investigated in this study, however, only a very small proportion of the incoming wastewater comes from hospitals. Resistance selection within the hospital sewer system itself, as it is supported by the presented data, would imply that separate treatment steps with chemical removal close to the hospitals might not be fully protective. Ideally, the treatment should include also the removal of resistant bacteria. The current study points to the need to further evaluate the management of risks associated with hospital wastewater.

CRediT authorship contribution statement

Nadine Kraupner: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Marion Hutinel:** Investigation, Writing - review & editing. **Kilian Schumacher:** Methodology, Investigation, Writing - review & editing. **Declan Alan Gray:** Writing - review & editing. **Maja Genheden:** Methodology, Writing - review & editing. **Jerker Fick:** Methodology, Investigation, Resources, Writing - review & editing. **Carl-Fredrik Flach:** Conceptualization, Methodology, Writing - review & editing, Supervision. **D.G. Joakim Larsson:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Gryaab for providing WWTP influent and effluent, personnel at the Sahlgrenska hospital for assisting with the sampling of hospital effluent, and Dr Maria-Elisabeth Böhm for providing the ciprofloxacin resistant strain GyrA. This work was funded by the Swedish Research Councils VR (2018-02835 and 2018-05771) and FORMAS (2108-00787), and the Region Västra Götaland under the ALF agreement (grant number ALFGBG-717901) to DGJL.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106436.

References

Andersson, D.I., Balaban, N.Q., Baquero, F., Courvalin, P., Glaser, P., Gophna, U., Kishony, R., Molin, S., Tønjum, T., 2020. Antibiotic resistance: turning evolutionary principles into clinical reality. FEMS Microbiol. Rev. 44, 171–188.

Aydin, S., Aydin, M.E., Ulvi, A., Kilic, H., 2019. Antibiotics in hospital effluents: occurrence, contribution to urban wastewater, removal in a wastewater treatment plant, and environmental risk assessment. Environ. Sci. Pollut. Res. 26, 544–558.

Azuma, T., Otomo, K., Kunitou, M., Shimizu, M., Hosomaru, K., Mikata, S., Ishida, M., Hisamatsu, K., Yunoki, A., Mino, Y., 2019. Environmental fate of pharmaceutical compounds and antimicrobial-resistant bacteria in hospital effluents, and contributions to pollutant loads in the surface waters in Japan. Sci. Total Environ. 657, 476–484.

Balouiri, M., Sadiki, M., Ibnsouda, S.K., 2016. Methods for in vitro evaluating antimicrobial activity: a review. J. Pharm. Anal. 6, 71–79.

Bengtsson-Palme, J., Hammaren, R., Pal, C., Ostman, M., Bjorlenius, B., Flach, C.F., Fick, J., Kristiansson, E., Tysklind, M., Larsson, D.G.J., 2016. Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. Sci. Total Environ. 572, 697–712.

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ Int 86, 140–149.

Bielen, A., Šimatović, A., Kosić-Vukšić, J., Senta, I., Ahel, M., Babić, S., Jurina, T., Plaza, J.J.G., Milaković, M., Udiković-Kolić, N., 2017. Negative environmental impacts of antibiotic-contaminated effluents from pharmaceutical industries. Water Res. 126, 79–87.

Bottery, M.J., Pitchford, J.W., Friman, V.-P., 2020. Ecology and evolution of antimicrobial resistance in bacterial communities. The ISME J. 1–10.

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, 772–783.

Cameron, A., Barbieri, R., Read, R., Church, D., Adator, E.H., Zaheer, R., McAllister, T.A., 2019. Functional screening for triclosan resistance in a wastewater metagenome and isolates of Escherichia coli and Enterococcus spp. from a large Canadian healthcare region. PLoS ONE 14 (e0211144).

Carraro, E., Bonetta, S., Bertino, C., Lorenzi, E., Bonetta, S., Gilli, G., 2016. Hospital effluents management: chemical, physical, microbiological risks and legislation in different countries. J. Environ. Manage. 168, 185–199.

Chow, L.K., Ghaly, T.M., Gillings, M.R., 2020. A survey of sub-inhibitory concentrations of antibiotics in the environment. J. Environ. Sci. 99, 21–27.

CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 9 Edition 2012; CLSI guideline M07-A9.

Diwan, V., Tamhankar, A.J., Khandal, R.K., Sen, S., Aggarwal, M., Marothi, Y., Iyer, R.V., Sundblad-Tonderski, K., Stålsby-Lundborg, C., 2010. Antibiotics and antibiotic-resistant bacteria in waters associated with a hospital in Ujjain, India. BMC Public Health 10, 414.

Ferreira da Silva, M., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O.C., Manaia, C.M., 2007. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. FEMS Microbiol. Ecol. 60, 166–176.

Flach, C.F., Genheden, M., Fick, J., Larsson, D.G.J., 2018. A comprehensive screening of Escherichia coli Isolates from Scandinavia's largest sewage treatment plant indicates no selection for antibiotic resistance. Environ. Sci. Technol. 52, 11419–11428.

González-Pleiter, M., Gonzalo, S., Rodea-Palomares, I., Leganés, F., Rosal, R., Boltes, K., Marco, E., Fernández-Piñas, F., 2013. Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: implications for environmental risk assessment. Water Res. 47, 2050–2064.

Greenfield, B.K., Shaked, S., Marrs, C.F., Nelson, P., Raxter, I., Xi, C., McKone, T.E., Jolliet, O., 2018. Modeling the emergence of antibiotic resistance in the environment: an analytical solution for the minimum selection concentration. Antimicrob. Agents Chemother. 62.

Gullberg, E., Albrecht, L.M., Karlsson, C., Sandegren, L., Andersson, D.I., 2014. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. MBio 5, e01918–01914.

Gullberg, E., Cao, S., Berg, O.G., Ilback, C., Sandegren, L., Hughes, D., Andersson, D.I., 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog 7, e1002158.

Hartmann, A., Golet, E., Gartiser, S., Alder, A., Koller, T., Widmer, R., 1999. Primary DNA damage but not mutagenicity correlates with ciprofloxacin concentrations in German hospital wastewaters. Arch. Environ. Contamin. Toxicol. 36, 115–119.

Hutinel, M., Huijbers, P.M.C., Fick, J., Ahren, C., Larsson, D.G.J., Flach, C.F., 2019. Population-level surveillance of antibiotic resistance in Escherichia coli through sewage analysis. Euro Surveill 24.

Klümper, U., Recker, M., Zhang, L., Yin, X., Zhang, T., Buckling, A., Gaze, W.H., 2019. Selection for antimicrobial resistance is reduced when embedded in a natural microbial community. ISME J. 13, 2927–2937.

Kraupner, N., Ebmeyer, S., Bengtsson-Palme, J., Fick, J., Kristiansson, E., Flach, C.F., Larsson, D.G.J., 2018. Selective concentration for ciprofloxacin resistance in Escherichia coli grown in complex aquatic bacterial biofilms. Environ. Int. 116, 255–268.

Kraupner, N., Ebmeyer, S., Hutinel, M., Fick, J., Flach, C.F., Larsson, D.G.J., 2020. Selective concentrations for trimethoprim resistance in aquatic environments. Environ. Int. 144, 106083.

Kümmerer, K., 2009. Antibiotics in the aquatic environment–a review–part I. Chemosphere 75, 417–434.

Larsson, D.G.J., 2014. Pollution from drug manufacturing: review and perspectives. Philos. Trans. R. Soc. B: Biol. Sci. 369, 20130571.

Larsson, D.G.J., Andremont, A., Bengtsson-Palme, J., Brandt, K.K., de Roda Husman, A. M., Fagerstedt, P., Fick, J., Flach, C.F., Gaze, W.H., Kuroda, M., Kvint, K., Laxminarayan, R., Manaia, C.M., Nielsen, K.M., Plant, L., Ploy, M.C., Segovia, C., Simonet, P., Smalla, K., Snape, J., Topp, E., van Hengel, A.J., Verner-Jeffreys, D.W., Virta, M.P.J., Wellington, E.M., Wernersson, A.S., 2018. Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. Environ. Int. 117, 132–138.

Lefkowitz, J.R., Duran, M., 2009. Changes in antibiotic resistance patterns of Escherichia coli during domestic wastewater treatment. Water Environ. Res. 81, 878–885.

Lindberg, R., Jarnheimer, P.-Å., Olsen, B., Johansson, M., Tysklind, M., 2004. Determination of antibiotic substances in hospital sewage water using solid phase extraction and liquid chromatography/mass spectrometry and group analogue internal standards. Chemosphere 57, 1479–1488.

Lindberg, R.H., Ostman, M., Olofsson, U., Grabic, R., Fick, J., 2014. Occurrence and behaviour of 105 active pharmaceutical ingredients in sewage waters of a municipal sewer collection system. Water Res. 58, 221–229.

Liu, A., Fong, A., Becket, E., Yuan, J., Tamae, C., Medrano, L., Maiz, M., Wahba, C., Lee, C., Lee, K., Tran, K.P., Yang, H., Hoffman, R.M., Salih, A., Miller, J.H., 2011. Selective advantage of resistant strains at trace levels of antibiotics: a simple and

ultrasensitive color test for detection of antibiotics and genotoxic agents. Antimicrob. Agents Chemother. 55, 1204–1210.
Lundström, S.V., Ostman, M., Bengtsson-Palme, J., Rutgersson, C., Thoudal, M., Sircar, T., Blanck, H., Eriksson, K.M., Tysklind, M., Flach, C.F., Larsson, D.G.J., 2016. Minimal selective concentrations of tetracycline in complex aquatic bacterial biofilms. Sci. Total Environ. 553, 587–595.
Lutgring, J., Grass, J., Lonsway, D., Yoo, B., Epson, E., Crumpler, M., Galliher, K., Zahn, M., Evans, E., Jacob, J., 2020. Chlorhexidine MIcs remain stable among antibiotic-resistant bacterial isolates collected from 2005 to 2019 at three US sites. Infect. Control Hospital Epidemiol. 41 s26–s26.
Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., Mu, Q., Alvarez, P., 2015. Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. Water Res. 85, 458–466.
Martínez, J.L., 2008. Antibiotics and antibiotic resistance genes in natural environments. Science 321, 365–367.
Martins, A.F., Vasconcelos, T.G., Henriques, D.M., Frank, C.d.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, 264–269.
Murray, A.K., Stanton, I.C., Wright, J., Zhang, L., Snape, J., Gaze, W.H., 2020. The 'SELection End points in Communities of bacTeria'(SELECT) method: a novel experimental assay to facilitate risk assessment of selection for antimicrobial resistance in the environment. Environ. Health Perspect. 128, 107007.
Murray, A.K., Zhang, L., Yin, X., Zhang, T., Buckling, A., Snape, J., Gaze, W.H., 2018. Novel insights into selection for antibiotic resistance in complex microbial communities. MBio 9, e00969–00918.
Naturvårdsverket. Avloppsreningsverkens förmåga att ta hand om läkemedelsrester och andra farliga ämnen. 2008; Rapport 5794.
Ory, J., Bricheux, G., Togola, A., Bonnet, J.L., Donnadieu-Bernard, F., Nakusi, L., Forestier, C., Traore, O., 2016. Ciprofloxacin residue and antibiotic-resistant biofilm bacteria in hospital effluent. Environ. Pollut. 214, 635–645.
Östman, M., Lindberg, R.H., Fick, J., Björn, E., Tysklind, M., 2017. Screening of biocides, metals and antibiotics in Swedish sewage sludge and wastewater. Water Res. 115, 318–328.
Paulshus, E., Kühn, I., Möllby, R., Colque, P., O'Sullivan, K., Midtvedt, T., Lingaas, E., Holmstad, R., Sørum, H., 2019. Diversity and antibiotic resistance among Escherichia coli populations in hospital and community wastewater compared to wastewater at the receiving urban treatment plant. Water Res. 161, 232–241.
Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-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.
Rodriguez-Mozaz, S., Vaz-Moreira, I., Della Giustina, S.V., Llorca, M., Barceló, D., Schubert, S., Berendonk, T.U., Michael-Kordatou, I., Fatta-Kassinos, D., Martinez, J. L., 2020. Antibiotic residues in final effluents of European wastewater treatment plants and their impact on the aquatic environment. Environ. Int. 140 (105733).
Sahlin, S., Larsson, D.G.J., Ågerstrand, M., 2018. Ciprofloxacin: EQS data overview. The Department of Environmental Science and Analytical Chemistry (ACES) The Department of Environmental Science and Analytical Chemistry (ACES).
Santos, L.H., Gros, M., Rodriguez-Mozaz, S., Delerue-Matos, C., Pena, A., Barceló, D., Montenegro, M.C.B., 2013. Contribution of hospital effluents to the load of pharmaceuticals in urban wastewaters: identification of ecologically relevant pharmaceuticals. Sci. Total Environ. 461, 302–316.
Stanton, I.C., Murray, A.K., Zhang, L., Snape, J., Gaze, W.H., 2020. Evolution of antibiotic resistance at low antibiotic concentrations including selection below the minimal selective concentration. Commun. Biol. 3, 467.
Sörengård, M., Campos-Pereira, H., Ullberg, M., Lai, F.Y., Golovko, O., Ahrens, L., 2019. Mass loads, source apportionment, and risk estimation of organic micropollutants from hospital and municipal wastewater in recipient catchments. Chemosphere 234, 931–941.
Tran, N.H., Reinhard, M., Gin, K.Y.-H., 2018. Occurrence and fate of emerging contaminants in municipal wastewater treatment plants from different geographical regions-a review. Water Res. 133, 182–207.
Varela, A.R., André, S., Nunes, O.C., Manaia, C.M., 2014. Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system. Water Res. 54, 327–336.
Wang, J., Chu, L., Wojnárovits, L., Takács, E., 2020. Occurrence and fate of antibiotics, antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in municipal wastewater treatment plant: An overview. Sci. Total Environ. 140997.
Watkinson, A., Murby, E., Costanzo, S., 2007. Removal of antibiotics in conventional and advanced wastewater treatment: implications for environmental discharge and wastewater recycling. Water Res. 41, 4164–4176.
Watkinson, A., Murby, E., Kolpin, D.W., Costanzo, S., 2009. The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. Sci. Total Environ. 407, 2711–2723.
Wellington, E.M., Boxall, A.B., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D.L., Lee, N.M., Otten, W., 2013. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. Lancet. Infect. Dis. 13, 155–165.
Verlicchi, P., Al Aukidy, M., Galletti, A., Petrovic, M., Barceló, D., 2012. Hospital effluent: investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. Sci. Total Environ. 430, 109–118.
Yang, L.H., Ying, G.G., Su, H.C., Stauber, J.L., Adams, M.S., Binet, M.T., 2008. Growth-inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga pseudokirchneriella subcapitata. Environ. Toxicol. Chem.: Int. J. 27, 1201–1208.
Yeh, P., Tschumi, A.I., Kishony, R.J., 2006. Functional classification of drugs by properties of their pairwise interactions. Nat. Genetics 38, 489–494.
Zorita, S., Mårtensson, L., Mathiasson, L., 2009. Occurrence and removal of pharmaceuticals in a municipal sewage treatment system in the south of Sweden. Sci. Total Environ. 407, 2760–2770.