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Diversity and antibiotic resistance among *Escherichia coli* populations in hospital and community wastewater compared to wastewater at the receiving urban treatment plant

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ABSTRACT

Bacterial diversity and antimicrobial resistance patterns among the indicator organism *Escherichia coli* were monitored in wastewater samples collected over one year from a hospital (HW), a community (CW) and the receiving urban (UW) wastewater treatment plant (WWTP). We compared levels of antibiotic resistance in the different types of wastewater, and identified whether resistant strains were endemic in the wastewater system. If so, implementation of local treatment at certain resistance hotspots (e.g. hospital outlets) could be used to decrease the amount of resistant bacteria in the wastewater. *E. coli* from HW (n = 2644), CW (n = 2525) and UW (n = 2693) were analyzed by biochemical phenotyping (PhenePlate System) and antimicrobial susceptibility testing to nine antibiotics (AREB System). The phenotypic diversities of the total *E. coli* populations were similar for all three sites (Simpson's Diversity index, Di = 0.973), however for individual samples, HW showed low diversities (Median Di = 0.800) and the *E. coli* flora was often dominated by strains that may have originated from the fecal flora of single individuals. The diversities in CW samples was higher (Median Di = 0.936), and UW samples showed similar diversities as the whole collection of isolates (Median Di = 0.971). Resistance to at least one of the nine antibiotics was observed in 45% of the HW isolates, 44% of CW isolates, and 33% of UW isolates. Resistance to gentamicin and chloramphenicol was uncommon (3.2 and 5.3%, respectively), whereas resistance to tetracycline and ampicillin was most common (24% and 31%, respectively). Extended-spectrum beta-lactamase-producing *E. coli* (ESBL-EC) were more common in HW (11.5%) and in CW (6.9%) compared to UW (3.7%). A high diversity (Di = 0.974) was observed among ESBL-EC isolates from UW (n = 99), indicating absence of any clonal structure among these isolates. Common PhP types of ESBL-EC often dominated in each HW sample, but were not identified across different samples, whereas ESBL-EC in CW showed low diversity (Di = 0.857) and were dominated by a specific PhP type that was found across almost all CW samples. The antibiotic resistance rates were highest in hospital wastewater, but surprisingly they were also high in the studied community wastewater, compared to the urban wastewater. The relative contribution of HW seemed low in terms of dissemination of antibiotic resistant bacteria to the WWTP.

1. Introduction

Antimicrobial resistance is an important and rapidly increasing global problem in both human and animal health care. Transfer of antimicrobial resistance between bacteria and development of new resistance mechanisms are inevitable consequences of the continued use of antibiotics. The widespread use of antibiotics in human and veterinary medicine has led to the spread of resistant

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bacteria into many environments (Kümmerer, 2009; Kolář et al., 2001; Gaskins et al., 2002). Resistant bacteria are especially common in hospital environments, from where they may reach the wastewater treatment plants (WWTPs) via hospital wastewater (HW) (Hocquet et al., 2016). Little is known about the further fate of these bacteria, and although some have found no evidence for selection for antibiotic resistance in WWTPs (Flach et al., 2018), others have found that large amounts of resistant bacteria, possibly of hospital origin, remain alive during the wastewater treatment process and are released into recipient waters (Rizzo et al., 2013).

Monitoring antimicrobial resistance through national and international surveillance programs has increased the knowledge of dissemination of resistant bacteria. A number of surveillance programs have been set up, such as the European Antimicrobial Resistance Surveillance Network (EARS-Net) (European Centre for Disease Prevention and Control, 2017), ECO-SENS (Kahlmeter and Poulsen, 2012) and Central Asian and Eastern European Surveillance of Antimicrobial Resistance (CAESAR) (World Health Organization, 2015). Veterinary equivalents are run by the European Food Safety Authority (EFSA) and focus on monitoring antimicrobial resistance in commensal bacteria such as *E. coli* in fecal samples collected from healthy animals. Large numbers of fecal samples from healthy humans are more difficult to obtain, and therefore, less is known about the normal human microbiota and its role as a reservoir of antimicrobial resistant bacteria.

Analyzing untreated wastewater collected from urban WWTPs is an alternate method to sampling hundreds of individuals in the population from which the WWTP receives its wastewater (Kühn et al., 2003). This method can be used as an early warning system for the emergence of new or rare types of antibiotic resistance, as proposed already in the seventies by Linton et al. (1974). Wastewater may work as a favorable niche for resistant bacteria and resistance genes originating from a population that produces the wastewater (Gao et al., 2012; Kümmerer, 2003; Munir et al., 2011; Reinthaler et al., 2013). Increasing resistance rates in urban wastewater (UW) has been found to correspond well to increasing antibiotic resistance rates in the human population (Reinthaler et al., 2013). Identification of the resistance rates in indicator bacteria in wastewater may also serve as a convenient tool to monitor changes in the resistance in the intestinal microbiota of the total human population, e.g. to find out if changes to the antibiotic policy in a region would affect resistance rates of bacteria in human microbiota in that region.

Some Swedish studies have also described a correlation between resistance rates among bacteria in wastewater and in the corresponding human population (Blanch et al., 2006; Kühn et al., 2003). A Swedish clone of *Enterococcus faecium* carrying ampicillin and fluoroquinolone resistance could be followed from its hospital origin (Torell et al., 2003) to its presence in the hospital's wastewater (Iversen et al., 2004). The clone was found further enriched in UW and also found in many samples from receiving waters (Iversen et al., 2004), revealing a likely source for colonization of humans and animals with antibiotic resistant bacteria of hospital origin. In the same study, vancomycin resistant enterococci (VRE) were found in 60% of UW samples in Sweden, and in 36% of HW samples (Iversen et al., 2002), despite claims at the time that Sweden was free of VRE as a consequence of its restrictive antibiotic policy. Later, a clonal group of *E. faecium vanB* with the same resistance pattern as that isolated from HW a few years earlier was found to be the cause of a large proportion of 487 reported healthcare-related VRE in 2007–2009 (Iversen et al., 2002). Another study on >1300 *E. coli* in wastewater in Sweden using phenotyping (PhP) combined with resistance determination revealed high occurrences of resistant bacteria both in UW (34% of all *E. coli*) and in HW (55%) (Kwak et al., 2015). Identifying wastewater outlets that can act as hotspots for antibiotic resistance may be of great importance (Berendonk et al., 2015).

In the present study, we have analyzed the frequencies of antibiotic resistance in the *E. coli* flora in wastewater from three sites connected to a sewage system in Oslo, Norway. The aims were to compare the diversities and the different antibiotic resistance levels in a hospital, a community, and in the total urban wastewater. Hopefully, this could support a future decision on whether implementation of local treatment at certain resistance hotspots could reduce the total load of resistant bacteria in wastewater.

2. Materials and methods

2.1. Sample origins and collection of samples

Three sampling sites were selected specifically to be able to compare hospital effluents to non-hospital effluents (Fig. 1). HW was collected from the main outlet of Oslo University Hospital, Rikshospitalet, a medium sized tertiary care hospital with over 500 hospital beds. Community wastewater (CW) was collected at a wastewater pump station in an area outside Oslo City, selected because its effluents exclusively originated from a residential area with approximately 510 inhabitants, thus providing wastewater with no contributions from health care institutions such as hospitals and nursing homes and excluding any form of agricultural

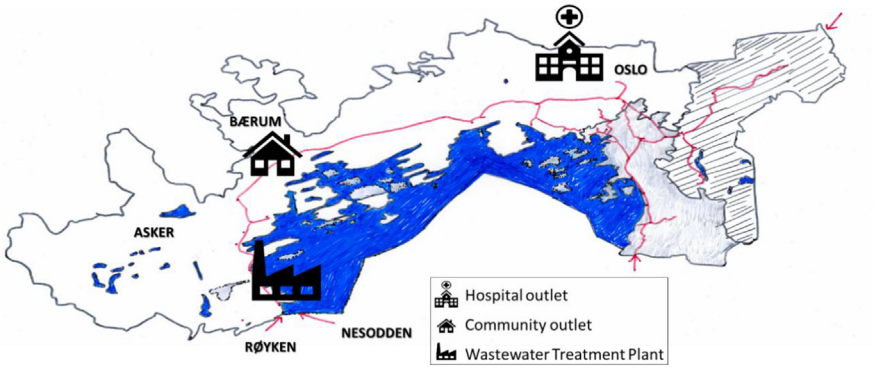

Fig. 1. Wastewater sampling sites and their approximate geographical locations in the wastewater transport infrastructure (red line). The uncolored part of the map indicates the areas from which the main WWTP in larger Oslo city (this study) receives its wastewater. The shaded and hatched parts (right) represent areas served by Oslo's second-largest WWTP (not shown). Wastewater from the hatched area may be rerouted to the main WWTP for extreme precipitation events or production problems. Red arrows indicate surrounding municipalities that only partly deliver wastewater to these WWTPs, including the names of those relevant for the WWTP in this study. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

impact. UW was collected at the inlet of the WWTP Vestfjorden Avløpsselskap (VEAS). This plant treats wastewater from more than 600 000 human inhabitants in the municipalities Oslo, Asker, Bærum, Røyken and Nesodden, including wastewater from the hospital and community sampling sites used in this study. To collect "parallel" wastewater samples in the WWTP compared to those collected from the two prior locations, compensation was made for the estimated travel time of the wastewater from the hospital (6 h) and community (4 h) outlets to the WWTP inlet by sampling at three specific time points: 8 a.m. (HW), 10 a.m. (CW) and 2 p.m. (UW), respectively.

Raw (untreated) wastewater was collected at the same time from three locations every month from June 2016 through May 2017. Three samples were collected monthly, one per day during three consecutive days. Each sample was composited of 24 aliquots of 200 ml, collected at hourly intervals for 24 h, using two Isco 2900 Portable Automatic Water Samplers (HW and UW) and one Isco 3700 Full-Size Portable Sampler (CW) (Teledyne ISCO, Lincoln, Nebraska, USA). All samplers were rinsed with water between daily samples, and rinsed with water, cleaned with 0.1–1% sodium hypochlorite (Klorin™) and bathed in 70% ethanol between monthly sampling occasions.

2.2. Isolation of E. coli

Samples were kept at +4 °C and analyzed within 12 h. Serial dilutions were made in phosphate buffered saline (PBS) and aliquots of 250 µl were plated on 14 cm petri dishes containing a chromogenic medium for *E. coli* (CHROMagar Orientation, CHROMagar Microbiology, Paris, France), preheated to 37 °C to reduce cellular stress, using the Plating Bead method (https://www.zymoresearch.de/rattler-plating-beads). The plates were incubated overnight (16–18 h) at 44 °C to inhibit growth of non-thermotolerant bacteria. Pink to dark red colonies with a surrounding halo on the CHROMagar Orientation plates were regarded as presumptive *E. coli* and further analyzed.

2.3. Analysis of E. coli isolates

2.3.1. Combined phenotyping and resistance determination

After incubation, 80 separately growing *E. coli* colonies (when available) were picked from the CHROMagar plates with sterile toothpicks. The colonies were inoculated into the first columns of ten PhP-RE plates of the PhenePlate system (96 well microtiter plates containing eight sets of 11 dehydrated reagents) (PhPlate AB, Stockholm, Sweden, www.phplate.se) pre-filled with 300 µl PhP suspension medium (PhPlate AB) (Kühn and Möllby, 1993), as described by Colque et al. (2014). Aliquots of 10 µl were transferred from the inoculation column to each well on the corresponding row of the PhP-RE plate and then twice (20 µl) to the first column of ten Antibiotic Resistance Breakpoint (AREB) plates (PhPlate AB), prefilled with 200 µl BBL™ Mueller Hinton II Broth (BD, Le Pont de Claix, France). AREB plates consist of round-bottomed 96 well microtiter plates containing one column for preparing bacterial suspensions, ten columns with dehydrated antibiotics, and a growth control well for each bacterial isolate in the rightmost column. Antibiotics and their final concentrations (in mg/l) were the same as described by Kwak et al. (2015), namely ampicillin (32), cefotaxime (2), ceftazidime (16), chloramphenicol (32), ciprofloxacin (4), gentamicin (16), nalidixic acid (32), cefpodoxime (3), tetracycline (16) and trimethoprim (16), with the modification that ceftazidime was excluded from the analysis. Bacterial suspensions of 10 µl were transferred from the first column of each AREB plate to each well on the corresponding row. The PhP-RE and AREB plates were incubated for 24 (±2) hours at 37 °C and images of each plate were produced using a desktop scanner (HP G4050) (Fig. S1A).

2.3.2. Data analysis

Each PhP-RE plate image was translated into 96 absorbance values by the PhenePlate™ software (PhPlate AB). Each well in the PhP-RE plates was assigned a numerical value based on its color with a gradient ranging from 0 (bright yellow) to 25 (dark blue). Growth in each well in the AREB plates was determined by size and density of the pellets formed in the round bottomed plates, and resistance to each antibiotic was determined by the software as relative growth in its respective well compared to the control well (column 12). Results were read as 0 (susceptible, growth <10% of control well), 1 (intermediate, requiring visual inspection, growth = 10–25% of control well), and 2 (resistant, growth >25% of control well).

The absorbance profiles from the PhP plates were used to cluster the isolates and assign them to PhP types. Isolates with positive fermentation results in the negative control column of the PhP-RE plate (column 2, cellobiose), as well as isolates giving negative results on all tests, were regarded as contaminated or non-*E. coli* and excluded from further analysis.

The PhenePlate™ software was used to cluster the PhP-RE plate data, and the diversity was calculated for each population of *E. coli* as Simpson's diversity index (Di), as described by Kwak et al. (2015). The Mann-Whitney test was used for pairwise comparisons of Di values from the different sample types.

Isolates showing susceptibility to all the 9 antibiotics used were regarded as sensitive, whereas isolates showing resistance to at least one antibiotic or showing intermediate values to two or more antibiotics were regarded as resistant. Isolates showing resistance to cefotaxime and cefpodoxime were regarded as extended spectrum beta-lactamase-producing *E. coli* (ESBL-EC) (Kwak et al., 2015).

The MAR (multiple antibiotic resistance) index is a measure of the total resistance in a population of bacterial isolates (Krumperman, 1983). MAR_{total} indices were calculated for bacterial populations by counting the total number of resistance features divided by the number of all resistance analyses for the isolates in the population. Removal of data for PhP-replicates within individual samples (i.e. multiple isolates with identical PhP-patterns were only counted once) yielded a MAR_{type} index. Comparison between the MAR_{total} and MAR_{type} indices indicates the prevalence of resistant isolates in the sample, as the MAR_{type} index will increase relative to the MAR_{total} when susceptible isolates are disregarded and vice versa.

Phenotyping of 80 isolates per sample resulted in a number of common PhP types (C-types) containing at least 5% of the isolates, and major types (M-types) containing at least 25% of the isolates. Less abundant types were defined as single types (S types).

3. Results

3.1. Sample and population structure

In total 8 640 presumptive *E. coli* isolates from CHROMagar were subject to typing and resistance determination (Table 1). Of these, 778 (9%) could not be confirmed as pure *E. coli* and were excluded from further analysis. All samples contained high numbers of *E. coli*, but concentrations varied much between sampling occasions (Table 2).

The diversities of the total *E. coli* populations were similar for all sites, however, for the 80 isolates analyzed from each individual sample there was a clear difference between the sample sources (Table 2). The composition of *E. coli* in individual samples from HW often consisted of isolates belonging to the same PhP- and

Table 1
Samples and *E. coli* isolates studied.

Number of sampling sites (see Fig. 1)	3
Number of sampling occasions (1 per month)	12
Number of samples[a] per occasion (1 per day)	3
Total number of samples (3 × 12 x 3)	108
Number of isolates analyzed per sample (when available)	80
Total number of isolates subject to PhP typing and resistance determination	8 640

[a] At each sampling occasion, three samples were collected during three consecutive days.

Table 2
Characteristics of 36 samples. CFU denotes the number of *E. coli*-like isolates growing on CHROMagar agar plates. Di: Diversity index.

Wastewater source	CFU/ml (log)			Confirmed *E. coli* isolates analyzed	Median of Di in samples	Total Di per site
	Min	Median	Max			
Hospital	3.5	4.2	4.6	2 644	0.800	0.973
Community	4.7	5.2	5.5	2 525	0.936	0.976
Urban	4.2	4.7	5.2	2 693	0.971	0.974
Total				7 862	0.936	0.973

resistance type, and therefore showed low diversities. *E. coli* from CW samples were more diverse than those from HW samples ($p < 0.001$), whilst *E. coli* in UW samples showed the highest diversity values for individual samples ($p < 0.0001$), similar to those of the whole collection of studied isolates (Fig. 2).

3.2. Antibiotic resistance

In total, 42% of all studied *E. coli* isolates were resistant to at least one of the nine antibiotics used here. The rates of resistance to each individual antibiotic were calculated for each sample type (Fig. 3). HW isolates showed the highest rates of resistance to all included antibiotics, whilst isolates from UW presented the lowest resistance rates (Fig. 3). The MAR_{type} was reduced in comparison to the MAR_{total} for CW (Table 3). This indicates that the high resistance rates seen among CW isolates are to some extent due to the presence of resistant M- or C-types. In contrast, HW and UW displayed relatively higher PhP type specific MAR_{type} indices compared to their overall populations of sampled isolates (Table 3).

3.2.1. Multiple antibiotic resistance

Only 53 of 7 862 (0.7%) isolates were found to be simultaneously resistant to gentamicin and chloramphenicol, out of which 38 were isolated from hospital wastewater, and the majority ($n = 21$) of these had identical phenotypes and resistance patterns and were isolated from the same sample, thus probably being a single clone. Multiple resistance to at least eight of the nine included antibiotics was found in only 73 isolates (0.9%) (Fig. 4). Only ten isolates (seven unique phenotypes in nine samples) were completely resistant to all nine antibiotics, and they were also the only isolates expressing resistance towards both gentamicin and chloramphenicol, i.e. no isolates showing resistance to 8 antibiotics or less were simultaneously resistant to gentamicin and chloramphenicol.

3.2.2. Extended beta-lactamase producing E. coli (ESBL-EC)

ESBL-EC were more common in HW than in CW and UW (Table 4). The ESBL-EC isolated from UW showed the same high diversity as the total *E. coli* population in UW, indicating absence of clonal structures among these isolates. ESBL-EC in HW showed a lower diversity. This was mainly due to a dominance of specific ESBL-EC phenotypes in some samples. In fact, 160 of the 303 ESBL-EC isolated from HW belonged to common PhP types that were not identified in more than one sample. In contrast, in CW, an ESBL-EC with a specific PhP type and with a consistent resistance pattern was found across almost all sampling occasions, resulting in a low diversity for the population of ESBL-EC in these samples (Table 4). This specific PhP-AREB (phenotype and resistance) pattern was rare and almost completely absent in all other samples collected

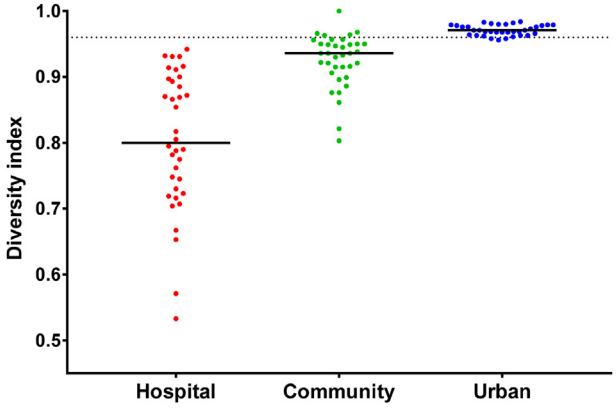

Fig. 2. Diversity for all individual samples. Median values for each site are shown by solid lines. All urban wastewater samples appear to be highly diverse (≥ 0.96, dotted line) throughout the sampling campaigns, whilst hospital samples show large variations.

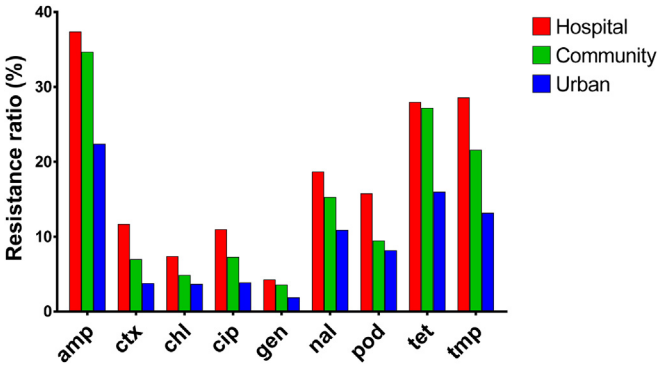

Fig. 3. Rate of resistance to each antibiotic in *E. coli* from each wastewater source over the total sampling period. For all antibiotics, hospital wastewater had the highest rates of resistance, whilst urban wastewater had the lowest rates. amp (ampicillin); ctx (cefotaxime); chl (chloramphenicol); cip (ciprofloxacin); gen (gentamicin); nal (nalidixic acid); pod (cefpodoxime); tet (tetracycline); tmp (trimethoprim).

Table 3
Influence on MAR indices by the presence of multiple isolates with identical PhP-patterns within samples. MAR_{total} index: Calculations were made using resistance data for all *E. coli* isolates obtained from the respective sampling sites. MAR_{type} index: Calculations were made using data from each PhP type only once per sample, irrespective of its prevalence in the respective sample.

	All isolates			Once per PhP type		
	Number of isolates	Resistant isolates (%)	MAR_{total} index	Number of PhP types	Resistant PhP types (%)	MAR_{type} index
HW	2 644	45	0.181	751	51	0.207[a]
CW	2 525	44	0.146	1 024	41	0.133[b]
UW	2 693	33	0.093	1 454	37	0.108[a]
Total	7 862	42	0.140	3 229	41	0.139

[a] Increased MAR index, the population contains more susceptible common types.
[b] Decreased MAR index, the population contains more resistant common types.

throughout the study. Another PhP type, with an identical resistance profile as the aforementioned, was also found in lower numbers (eight isolates), but on multiple occasions in the community site. Such persistence of specific types occurring over time was not observed for the other sampling sites. Most probably, these isolates all belong to specific clones that were endemic to this sampling site for the duration of the study.

3.2.3. Co-occurrence of antibiotic resistances

The correlations between occurrences of resistance against the nine antibiotics for all 7 862 isolates were visualized in a dendrogram (Fig. 5). Resistance to the ESBL-marking antibiotics cefotaxime and cefpodoxime showed the highest correlation (0.82), but also the quinolones ciprofloxacin and nalidixic acid showed high co-occurrences (0.67). Resistance to ampicillin, tetracycline and trimethoprim appeared to be correlated, but to a lesser extent, whereas resistance to gentamicin and chloramphenicol were not correlated to any other resistances.

3.3. Similarities between antibiotic resistant E. coli populations

In order to visualize the similarities between antibiotic resistant *E. coli* populations in different sampling sites, i.e. can we observe the same resistant bacteria in the WWTP as in the sources HW and CW, the combined PhP-AREB data for the resistant isolates were used to calculate population similarity coefficients (Sp) (Kühn et al., 1991) between the *E. coli* populations of the different sampling sites (Table 5). Both HW and CW showed higher similarities to the resistant *E. coli* population in the UW from VEAS WWTP than they do to each other or to the population in non-related UW from Stockholm. It thus appears to exist some influence from both the HW and CW on the resistant *E. coli* population reaching the WWTP.

4. Discussion

We have identified and compared *E. coli* in wastewater from hospital and non-hospital outlets with regard to their relative prevalence of antibiotic resistant *E. coli*. Only a few studies have previously investigated antibiotic resistant bacteria in Norwegian wastewaters (Jørgensen et al., 2017; Schwermer et al., 2018), a country with a relatively low consumption of antibiotics in both the human- and veterinary medical sectors. Also, a relatively low prevalence of antibiotic resistant fecal coliforms and enterococci was found in Norwegian wastewater compared to that of other, southern European countries regarded as high consumers of antibiotics (Pärnänen et al., 2019). In our study, we found that HW contains high numbers of multi-resistant *E. coli* including ESBL-EC compared to community-derived wastewater. We also found that UW has a relatively low prevalence of resistant bacteria compared to the HW and CW investigated in this study.

We have compared resistance rates from the present study in Oslo during 2016–2017 to data from a previous study made in Stockholm during the years 2013–2014 (Kwak et al., 2015). The two studies show very similar resistance rates despite being performed in different countries and years. Norway and Sweden share many cultural factors, including regulations of antibiotic use. The similarity between resistance rates in the hospital samples of the two studies also highlight the usefulness and consistency of the screening method for antibiotic resistance applied, despite lower and more fluctuating diversity levels observed in HW samples from both studies.

In the previous study performed in Sweden it was found that the prevalence of antibiotic resistant *E. coli* in UW and HW seemed to follow the trends of resistance development over time in the urban population and in clinical isolates, respectively (Kwak et al., 2015). It was concluded that analyzing antimicrobial resistance among bacterial isolates from wastewater could be an easy way to monitor antibiotic resistance among fecal bacteria in the society. This method could also be used as an early warning system to detect new, emerging resistances. Although the popularity and potency of molecular technologies have rapidly increased in the last 20 years (Loman and Pallen, 2015), cultivation-based methods remain important tools in research and clinical diagnostics.

An essential aspect in utilizing wastewater as a surveillance tool for the corresponding population of individuals is the question of representability. Only 2.5 μl of urban wastewater were analyzed to yield the required 80 *E. coli* isolates. As a comparison, roughly 290,000 m^3 of wastewater runs through the VEAS WWTP every day. Thus, we are only analyzing one in every 10^{14} bacteria. Nevertheless, results obtained from the UW samples are surprisingly consistent, as seen in Fig. 2, where diversity levels in all individual UW samples matched the discriminatory potential of the PhP system, supporting the applicability of this sample type as a screening tool for the status of resistance in a human population. In contrast, individual hospital samples had lower diversities, possibly reflecting their origin in a small population. The distinct and relatively consistent results for each sample site are probably an indication that small samples of UW can be representative of a large population such as the one observed in this study. PhP typing is also valuable as a quality control of individual wastewater samples, as for instance samples containing clumps of fecal material holding multiple copies of the same strain would be easily recognized by their low diversity.

Site-wise comparisons of the combined PhP-AREB data in the resistant populations of *E. coli* was performed by analyzing the percentage of isolates in a given population whose PhP-AREB profiles could be identified in one or several isolates present in the other (and vice versa). Comparisons between urban wastewater and the hospital outlet and between urban wastewater and the community outlet from this study revealed a higher similarity to UW for both outlets than when comparing the community and hospital outlets to each other or to urban wastewater from a similar

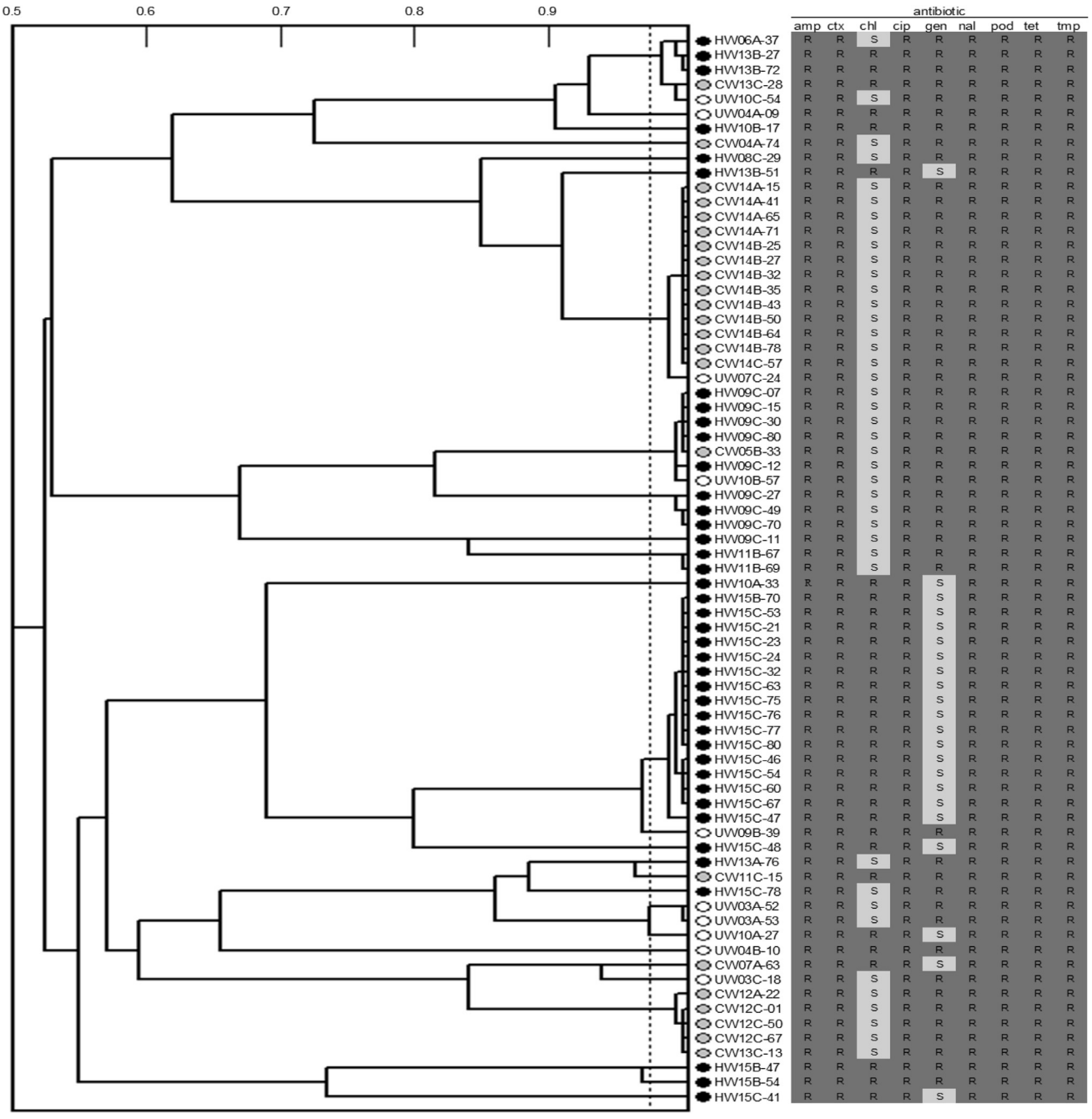

Fig. 4. Clustered PhP typing data showing phenotypic relationship between multi-resistant *E. coli* (≥8 of 9 resistances) from all wastewater samples with their corresponding sampling site origin (hospital - HW: black circles; community - CW: grey circles; urban - UW: white circles) and resistance profiles. All isolates are resistant to all nine antibiotics or all but gentamicin or chloramphenicol. Isolates that have connecting branches to the right of the vertical dashed line (0.96) are closely related and are considered to belong to the same phenotype. R: resistance; S: susceptibility. For explanation of antibiotics abbreviations, see Fig. 3.

Table 4
Prevalence of presumed ESBL-ECs observed in wastewater samples from different sources. Di indicates the diversity of the ESBL-EC isolates.

	Number of *E. coli*	Number (%) of ESBL-EC	Diversity index for ESBL-EC
HW	2 644	303 (11.5)	0.957
CW	2 525	174 (6.9)	0.857
UW	2 693	99 (3.7)	0.974
Total	7 862	576 (7.3)	0.958

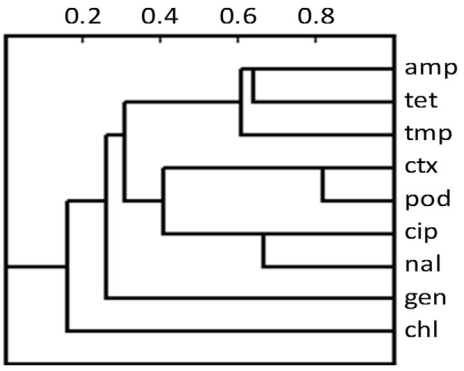

Fig. 5. Co-occurring resistance properties to the nine included antibiotics in the total population of *E. coli* isolates. For explanation of antibiotics abbreviations, see Fig. 3.

study in Sweden (Table 5) (Kwak et al., 2015). This indicates the baseline similarity between independent *E. coli* populations. The higher similarity between UW and the hospital and community outlets studied here, however, indicates that both CW and HW may have some influence on the composition of *E. coli* present in the receiving WWTP. On the other hand, the volumes of wastewater expelled from the hospital and community outlets are similar to one another, but only about 0.025% when compared to the total volume that the WWTP receives (data not shown), greatly limiting the impact of each individual outlet on the diversity and antibiotic resistance of *E. coli* in the WWTP.

An interesting observation was the finding of reoccurring multiresistant PhP types in the CW indicating that the community outlet was constantly colonized by endemic strains of multiresistant *E. coli*. Each sample consisted of 24 pooled aliquots of 200 ml wastewater, thus diluting the potential inhabitants in the sampler tube in roughly 5 L before the sample was brought back for cultivation. This drastically reduces the possibility that the low diversity and repeated observations of identical PhP types with the same resistance patterns during twelve months of sampling occasions in the community site could be artifacts from an improperly cleaned sampler. Sampling equipment was rigorously cleaned between sampling occasions (see section 2.1 Sample origin and collection of samples) to reduce the risk of any carry-over bacteria. Thus, it is more reasonable to hypothesize that some *E. coli* strains are surviving in the wastewater system. In fact, during a four-year study on coliforms and *Aeromonas sp.* in tap water from a drinking water well, a recurring clone of *Aeromonas* was observed throughout the study period, supporting the hypothesis of potential long-time bacterial colonization in these harsh environments (Kühn et al., 1997).

The Di of a bacterial population is valuable in determining if isolates are related. For PhP-RE typing of *E. coli* populations this index was 0.967 for the 2 693 urban wastewater isolates in the present study. In the previous study on 1 325 isolates from urban wastewater in Stockholm, the Di was almost identical (0.965) (Kwak et al., 2015). Several studies have indicated that this Di value is stable in normal *E. coli* populations, and that lower Di values indicate that the studied *E. coli* do not belong to a randomized normal population, but that the population contains many replicates of the same strain, e.g. from the same fecal microbiota (Reyes et al., 2009; Landgren et al., 2005).

Transmission of antibiotic resistant bacteria can be due to spread of resistant bacterial clones in the population or a consequence of horizontal transfer of resistance genes between different bacterial clones or species (Andersson and Diarmaid, 2017). Clonal spread is expected to yield a lower phenotypic diversity among resistant versus susceptible bacteria, whereas similar diversities among resistant and susceptible bacteria would be expected in the case of horizontal transfer of resistance genes. For *E. coli* from the WWTP in our study, Di for all 1 800 susceptible *E. coli* was surprisingly enough lower than for the 893 resistant isolates (Di = 0.960 and 0.972, respectively). Although this difference is small, it indicates that the clonal number is low among the resistant *E. coli* in the WWTP, but higher among the susceptible *E. coli*. Thus, clonal groups of susceptible *E. coli* that do not easily assimilate resistance genes could exist in the urban wastewater. This finding is also supported by previous results obtained in the study by Kwak et al. (2015).

The MAR index can be a useful tool when comparing resistance rates in different bacterial populations. We have calculated the MAR index in two different ways: The MAR_{total} index denotes the value obtained when data for all isolates were included, whereas the MAR_{type} index denotes the value obtained when data from isolates belonging to common types only were included once per individual sample (aligning them with Single types). A higher MAR_{type} index than MAR_{total} index indicates that, even though resistance levels were high (as in the hospital effluents), phenotypes which were more prevalent in the sample (Common and Major types), were in fact less resistant compared to the single types, and as presented in Table 3, the MAR_{type} index in UW was also higher than the MAR_{tot} index. This is an interesting observation, since resistant bacteria have often been considered to be more "successful" than their susceptible counterparts in antibiotic-containing environments such as hospital effluents, which exert a continuous selective pressure towards antibiotic resistant bacteria (Hocquet et al., 2016). On the other hand, resistance is often considered to exert a fitness cost compared to the wild type (Hernando-Amado et al., 2017), a theory that is supported by our findings. In contrast, the difference between MAR_{total} and MAR_{type} indices in the CW population depicts a different situation, in which resistance was more often found among common phenotypes. It seems that some of these strains are endemic to the community outlet, and as some of them were highly resistant ESBL-EC, further investigation is needed in order to determine whether actions should be taken to eliminate such bacteria at the source.

In a study on ESBL-ECs in hospital and urban wastewaters, Gündoğdu et al. observed a dominating phenotype present in all hospital samples, making up 35% of the 198 ESBL isolates analyzed (Gündoğdu et al., 2013). In contrast, we rarely identified recurring PhP types with ESBL properties in different hospital samples in our study, although we did observe reoccurring PhP types of ESBL-EC in the community samples. We also found that the diversity of ESBL-EC in urban wastewater was identical to that of the total population of *E. coli*. This indicates that the presence of ESBL carrying strains is not due to the spread of specific clones, but rather that most *E. coli* types may be capable of harboring ESBL resistance genes, although CTX-M-producing *E. coli* commonly belong to the sequence type ST131 (Bevan et al., 2017). Similar to the study in Stockholm (Kwak

Table 5
Population similarity coefficients (Sp) between resistant bacterial populations in different sampling sites. HW: hospital wastewater; CW: community wastewater; UW: urban wastewater; UWS: urban wastewater Stockholm.

Comparison		Sp
Site	Site	
HW	CW	0.111
HW	UW	0.189
CW	UW	0.233
HW	UWS[a]	0.117
CW	UWS[a]	0.128

[a] *Data from* Kwak et al. (2015).

et al., 2015), the prevalence of ESBL-EC in our study was rather low (11.5% for HW and 3.7% for UW) e.g. compared to the situation in Poland, where 37% HW isolates and 18% UW isolates were ESBL-EC (Korzeniewska et al., 2013). In another study in Spain, ESBL-EC were found to constitute 12% of examined *E. coli* from human and animal wastewater samples (Sabaté et al., 2008). Thus, these studies detected presence of ESBL at several times the frequency reported here. To answer whether hospital outlets should be treated locally, it is therefore important to define risks associated with multiple resistant bacteria in the country-specific wastewater outlets.

Kwak et al. observed increasing resistance rates for *E. coli* in UW during the years 2013–2014 (Kwak et al., 2015). In our study, we did not see increasing resistance rates over time in UW for any of the antibiotics during 2016–2017 (data not shown). This finding correlates well with the Norwegian NORM/NORM-VET 2016 and 2017 reports that noted only slight variations in resistance rates in clinical isolates of *E. coli* from urinary and blood samples between the two years ("NORM/NORM-VET, 2016. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway." 2017; "NORM/NORM-VET, 2017. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway." 2018). As an example, the frequency of ESBL-EC in the NORM/NORM-VET reports increased from 5.8 to 6.6% in blood cultures between the two years but remained unchanged for urinary isolates. We observed a frequency of ESBL-EC in hospital wastewater collected during 2016 and 2017 of nearly twice that of the NORM/NORM-VET reports. However, data in the NORM/NORM-VET reports are collected from a different type of samples. A total of 1471 *E. coli* from all human clinical samples taken between June 1, 2016 and May 31, 2017 at the hospital from where the HW samples in this study originated were subjected to antimicrobial susceptibility testing (AST) (data not shown). Five of the nine antibiotics included in this study were routinely included in the clinical AST-panel, namely ampicillin, cefotaxime, ciprofloxacin, gentamicin and trimethoprim. These 1471 clinical isolates were collected from the same group of patients as those that contributed to the wastewater that was analyzed in this study. Therefore, one could assume that these clinical resistance rates would mirror the rates found in our study. However, *E. coli* isolated from the clinical setting showed higher rates of antibiotic resistance compared to what we observed in the corresponding hospital wastewater. The antimicrobial susceptibility break-points applied on the clinical isolates were two 2-fold dilutions below those used in this study, which may have contributed to the discrepancy observed between these two populations of *E. coli*. It could also be an effect of a dilution prior to our sampling point. Antibiotic resistant bacteria are likely diluted between the hospital outlet and the WWTP inlet by other sources that contain relatively few antibiotic resistant bacteria. In the same way, patients not treated with antibiotics for their disease and employees, whose microbiotas may comprise fewer antibiotic resistant bacteria, could dilute the resistant *E. coli* from the hospital setting. Visitors, staff, and patients with non-infectious illnesses also use hospital lavatories, all of which are groups with lower predisposition toward carriage of antibiotic resistant bacteria. This highlights the importance of identifying high-risk outlets in terms of antibiotic resistant bacteria, and that accommodation specifically designated patients with (antibiotic resistant) infections could be a potential approach in controlling this issue. On the other hand, the relative volumes of such outlets compared to the total volume of urban wastewater are negligible (data not shown).

Selective pressure caused by presence of antibiotics can lead to co-occurrence of antibiotic resistance traits in the form of co- and cross-resistance. In our collection of *E. coli* isolates, we observed predictably high co-occurrences between antibiotics from the same antibiotic classes (cefotaxime and cefpodoxime, and ciprofloxacin and nalidixic acid) (Fig. 5). We also identified a noteworthy co-occurrence between the three unrelated antibiotics ampicillin, tetracycline and trimethoprim, which have completely different mechanisms for how they inhibit bacteria, targeting cell wall synthesis, protein synthesis and nucleic acid synthesis, respectively. Co-occurrence of resistance to the three unrelated antibiotics was most common in HW samples, and least common in UW samples, corresponding well with the frequencies of multiple resistance phenotypes observed in the various types of wastewater. This finding is not new, but emphasizes the impact that the use of antibiotics has on the evolution of resistance, where the use of one antibiotic group can lead to simultaneous selection of resistance against several others. We would likely have found an equally high correlation between resistances to trimethoprim and antibiotics from the sulfa group due to their similar and synergistic mechanisms (Hitchings, 1973), but as this antibiotic is rarely used except in combination with trimethoprim, it was not included in the study. Co-occurrences with chloramphenicol were low in all samples for all antibiotics. Occurrences of resistance features against chloramphenicol and the antibiotics gentamicin, ciprofloxacin, nalidixic acid and the cephalosporins were completely unrelated, and in fact gave rise to weak negative correlations for all but ciprofloxacin. This would indicate that the analyzed material is devoid of any mechanisms of cross-resistance or plasmids carrying resistance genes against chloramphenicol together with any of the other antibiotics included in this study.

Although previous studies have examined non-hospital wastewater outlets, only a few have, to our knowledge, compared occurrences of antibiotic resistant bacteria in hospital and urban wastewaters to specifically non-hospital, residential outlets like the community site investigated here (Bäumlisberger et al., 2015; Brown et al., 2006; Li et al., 2015). The finding that non-hospital wastewater like that of the community outlet studied here has a higher occurrence of resistant bacteria than the average urban wastewater highlight the importance of identifying other potential hotspots for antibiotic resistance contaminants besides the well-recognized hospital outlets.

5. Conclusions

- Measuring levels of antibiotic resistance in *E. coli* from wastewater samples can be representative for the level of antibiotic resistance in the corresponding human population and can be used as an early warning system changes to resistance patterns in the society. Reliable results depend on precise and thorough sampling as well as quality controls to avoid conclusions based on replicate analysis of the same strains.
- *E. coli* in urban wastewater samples were highly diverse and seemed to represent well the *E. coli* flora in the urban population, whereas *E. coli* in samples from hospital and community wastewater were less diverse and were frequently dominated by isolates from either single individuals or that were growing in the wastewater system.
- High levels of resistant *E. coli* in hospital and community wastewater, but lower in the WWTP were found.
- A seemingly endemic strain of multiresistant *E. coli* was found in most community wastewater samples collected during one year.
- A majority of the antibiotic resistant bacteria in WWTPs are likely derived from the presence of such bacteria in the total population of the urban society, as the relative contribution of the studied hospital wastewater was low.
- The levels of antibiotic resistant *E. coli* in hospital wastewater relative to the other sites included here were not sufficient to recommend implementation of local treatment measures.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.05.102.

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