Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Food Safety and Infection Biology

Philosophiae Doctor (PhD)
Thesis 2020:9

Antibiotic resistance in wastewater

Antibiotikaresistens i kloakk

Erik Paulshus

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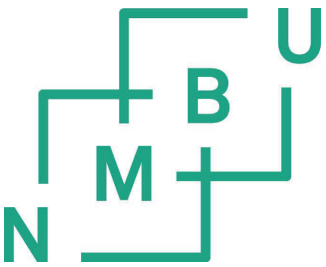
Oslo, 2019



Thesis number 2020:9
ISSN 1894-6402
ISBN 978-82-575-1675-8

“When shit hits the fan.”

Unknown

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Acknowledgments

I would like to acknowledge my colleagues and good friends at Lindern. These years would not have been endurable had it not been for the good talks and inspiring company of all of you.

To my supervisors, Henning, Inger, Roland and Ane: the encouragement and feedback you have provided has meant the world to me. I am so grateful for the genuine interest and dedication you have shown to science, to this project and to me. Although faced with several obstacles along the way, I could always look to you for support and new ideas.

I feel very lucky for all the researchers and other collaborators I have met from Norway, Sweden, USA and Spain. Thank you all for taking part in and supporting my journey through these last few years.

Thanks to my friends for letting me know throughout that there is a world out there after all of this. I truly appreciate it although I've struggled to keep in touch as much as I'd like lately. To my beloved family, and especially my mom and dad, thank you for always believing in me and for gifting me with a curious mind and a great sense of humor (ha ha).

To my dear partner and best friend Runa. I don't know how to repay you for the countless hours you have listened to me frustratedly talk about work and for still tolerating my sense of humor, and I can't promise the latter will change drastically in the future. Although this chapter of our relationship has largely been influenced by my PhD, I am so pleased with how well we have come out of it. Love you to the moon and back!

Abbreviations

AR	Antibiotic resistance/resistant
ARB	Antibiotic resistant bacteria
AREB	Antibiotic resistance in environmental bacteria
ARG	Antibiotic resistance gene
AST	Antibiotic susceptibility testing
CW	Community wastewater
CFU	Colony-forming units
ECOFF	Environmental cutoff
ESBL-EC	Extended spectrum beta lactamase producing *Escherichia coli*
GOI	Gene of interest
HGT	Horizontal gene transfer
HW	Hospital wastewater
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
PCR	Polymerase chain reaction
PhP	PhenePlate
qPCR	Quantitative PCR
UW	Urban wastewater (Wastewater from the studied WWTP)
VRE	Vancomycin-resistant enterococci
WGS	Whole genome sequencing
WWTP	Wastewater treatment plant

List of papers

Paper I

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Erik Paulshus, Inger Kühn, Roland Möllby, Patricia Colque, Kristin O'Sullivan, Tore Midtvedt, Egil Lingaas, Rune Holmstad, Henning Sørum

Water Research 161 (2019) 232-241
DOI: 10.1016/j.watres.2019.05.102

Paper II

Repeated Isolation of Extended-Spectrum-β-Lactamase-Positive *Escherichia coli* Sequence Types 648 and 131 from Community Wastewater Indicates that Sewage Systems Are Important Sources of Emerging Clones of Antibiotic-Resistant Bacteria

Erik Paulshus, Kaisa Thorell, Jessica Guzman-Otazo, Enrique Joffre, Patricia Colque, Inger Kühn, Roland Möllby, Henning Sørum, Åsa Sjöling

Antimicrobial Agents and Chemotherapy Aug 2019, 63 (9) e00823-19
DOI: 10.1128/AAC.00823-19

Paper III

Wastewater concentrations of antibiotics, antibiotic resistance genes and mobile genetic elements in hospital, community and urban wastewaters and associated antibiotic prescription data in Norway

Erik Paulshus, Sara Rodriguez-Mozaz, Hege Salvesen Blix, Saulo Varela Della Giustina, Maggie Ruth Williams, James Tiedje, Syed Hashsham, Inger Kühn, Roland Möllby, Damia Barcelo, Henning Sørum

Submitted to *Water Research X*

Summary

Antibiotic resistance is a characteristic possessed by some bacteria that enables them to withstand the effects of antibiotic treatment. Antibiotic use and abuse may result in development of antibiotic resistant bacteria. After an antibiotic treatment, antibiotics are partially excreted in urine and feces, and wastewater is therefore an important source of both bacteria and antibiotics from healthy and sick individuals. Due to the close contact between large numbers of bacteria and diverse types of antibiotics over prolonged periods, wastewater has the potential to select for and disseminate antibiotic resistance into the environment. Previous studies of hospital wastewater effluents have shown that these outlets discharge more antibiotic resistance factors than the general population.

This study was initiated to map the occurrence of antibiotic resistance in wastewater under Norwegian conditions to identify whether local treatment of hospital wastewater could contribute to limit the dissemination of antibiotic resistance to the environment. Samples of untreated wastewater were collected monthly during one year from a hospital, a community, and from the wastewater treatment plant that treats the wastewater from the Greater Oslo region. The samples were analyzed for the occurrence of antibiotic resistant *E. coli*, 53 different antibiotics, heavy metals, and 144 bacterial genes encoding antibiotic resistance and mechanisms for the transfer of these genes between bacteria.

During this study, higher levels of antibiotics and antibiotic resistant bacteria were found in wastewater from the hospital than from the general population. Broad-spectrum antibiotics were also present at much higher concentrations in the hospital effluents. These results indicate that local treatment of the hospital wastewater system may help limit the spread of resistance to the environment. A cost-benefit analysis should be performed to determine whether localized hospital wastewater treatment may be more effective than large-scale implementation at the wastewater treatment plants.

This study also identified variants of multi-resistant *E. coli* producing ESBL-enzymes that can degrade broad-spectrum β-lactam antibiotics in the wastewater from the studied community. Some of these bacteria were exclusively and repeatedly found in the community wastewater. These belonged to two types of highly infectious *E. coli* (ST131

and ST648) that have been found all over the world. The finding of these bacterial clones in the community wastewater indicates that the dissemination of multi-resistant and pathogenic bacteria through wastewater is not exclusively attributable to hospital effluents. Hence, it is recommended to investigate additional as well as other types of outlets to identify potential high-risk sources for the spread of antibiotic resistance.

High abundances of genes encoding mechanisms for lateral transfer of antibiotic resistance were found in this study, as well as clinically relevant antibiotic resistance genes like *ndm-1* that have only scarcely been reported in association with clinical disease in Norway. These results thus verified a previous study of wastewater from European countries, wherein Norway had the highest levels of lateral transfer genes. Even though Norway has a relatively low prevalence of antibiotic resistance and a moderate consumption of antibiotics due to strict regulation, these results indicate the underlying potential for a substantial mobilization and dissemination in the future.

This study has illuminated important aspects of antibiotic resistance that have previously not been described. The results of this study contribute to a broader knowledge base for decision-making regarding wastewater management in Norway and internationally.

Sammendrag (Summary in Norwegian)

Antibiotikaresistens er en egenskap hos noen bakterier som gjør at de kan stå imot effektene av en antibiotikakur. Både fornuftig bruk og misbruk av antibiotika kan føre til at bakteriene blir mer motstandsdyktige. Etter en antibiotikakur skilles antibiotikumet delvis ut i urin og avføring, og avløpsvann er derfor en viktig kilde til både bakterier og antibiotika fra friske og syke mennesker. På grunn av den tette kontakten mellom store mengder bakterier og ulike typer antibiotika over lengre tid har avløpsvann potensialet for å spre antibiotikaresistens videre ut i miljøet. Tidligere studier av avløpsvann fra sykehus har vist at disse skiller ut større mengder antibiotikaresistens-faktorer enn resten av befolkningen.

Denne studien ble påbegynt for å kartlegge forekomsten av antibiotikaresistens i avløpsvann under norske forhold for å se om lokal rensing av avløpsvann fra sykehus kan bidra til å begrense spredningen av antibiotikaresistens til miljøet. Prøver av avløpsvann ble samlet inn månedlig gjennom ett år fra et sykehus, et boligområde og fra Stor-Oslos hovedrenseanlegg på Slemmestad. Prøvene ble analysert for forekomsten av antibiotikaresistente *E. coli*-bakterier i tillegg til 53 ulike antibiotikastoffer, tungmetaller, og 144 bakteriegener som koder for antibiotikaresistens samt for mekanismer for overføring av disse genene mellom bakterier.

I løpet av denne studien ble det funnet større mengder antibiotika og antibiotikaresistente bakterier i sykehusavløpet enn fra resten av befolkningen. Bredspektrede antibiotika ble også funnet i mye høyere grad i sykehusavløpet. Disse resultatene peker på at lokal behandling av sykehusets avløpssystem kan bidra til å begrense spredningen av resistens til miljøet, men kost-nyttevurderinger behøves for å fastslå om effekten er stor nok til å ikke heller implementere avanserte behandlingstiltak på større skala i renseanlegget.

Denne studien fant også varianter av multi-resistente *E. coli* som produserer ESBL-enzymer med evnen til å bryte ned bredspektrede betalaktam-antibiotika i avløpsvannet fra studiens boligområde. Noen av disse bakteriene ble utelukkende og gjentatte ganger funnet i boligområdets avløpsvann. Disse tilhørte to typer spesielt infeksiøse *E. coli* (ST131 og ST648) som har blitt funnet over hele verden. Funnet av disse bakteriene i

boligområdet tyder på at sykehus ikke er alene om å spre multi-resistente og sykdomsframkallende bakterier via avløpet. Det anbefales derfor å utforske flere og ulike typer avløp for å identifisere potensielle høy-risiko-kilder til spredning av antibiotikaresistens.

I denne studien ble det også funnet store mengder gener som koder for laterale overføringsmekanismer for antibiotikaresistens, i tillegg til klinisk relevante gener, som *ndm-1* som knapt har blitt observert blant kliniske bakterieisolater i Norge. Disse resultatene bekreftet dermed en tidligere studie av Europeiske land som viste at Norge hadde de høyeste forekomstene av horisontale overføringsgener i avløpsvannet. Selv om Norge har en relativt lav forekomst av antibiotikaresistens og moderat forbruk av antibiotika på grunn av streng regulering tyder disse resultatene på at det allikevel eksisterer et underliggende potensial for voldsom mobilisering og spredning av resistens i fremtiden.

Denne studien har belyst viktige aspekter ved antibiotikaresistens som tidligere ikke har vært beskrevet. Funnene i studien bidrar til et bredere beslutningsgrunnlag for videre håndtering av avløpsvann i Norge og ellers i verden.

1. Introduction

1.1 Antibiotics

An antibiotic was previously defined as "a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms"[1]. The original definition by Waksman did not include synthetic derivatives of the drugs or completely synthesized variants (antimicrobial chemotherapeutic agent), whereas later definitions include either "an organic chemical of natural or synthetic origin that inhibits or kills pathogenic bacteria", or "any antimicrobial substance"[2]. In this thesis, the term antibiotic will encompass all natural, semisynthetic or synthetic antimicrobial substances.

1.1.1 History of antibiotics

It is debated whether Fleming was in fact the first to discover antibiotics. The Italian medical scientist Bartolomeo Gosio described in 1893 the antibacterial activity of a substance produced by a *Penicillum* species in corn against *Bacillus anthracis*. Ernest Duchesne, a French military officer, described the antibacterial activity of molds in his thesis in 1897, and arsphenamine (Salvarsan) was discovered by the German physician Paul Ehrlich and colleagues in 1909[2]. The historical significance of penicillin comes from the fact that it was the first antibiotic with minimal toxicity to be effective against deadly infections[3]. Alexander Fleming discovered the activity of penicillin in 1928 and since then, several new antibiotic classes have been introduced to the market (Figure 1). Antibiotic substances are classified by their chemical structure and thus substances of the same class usually have similar antibacterial effects. However, since daptomycin (1986) and linezolid (1987) were discovered, although new drugs are still discovered or made, no new classes of antibiotics have been discovered[4].

The introduction of antibiotics drastically reduced the likelihood that a (today) trivial infection would prove fatal. From World War I to World War II, deaths from bacterial pneumonia declined from 18 percent to less than one percent[5]. The introduction of Salvarsan was a major reason for the decline of syphilis infections in Sweden from 150 of males and 75 of females per 100.000 capita in 1920 to less than 30 per 100.000 capita in mid-1920s, with penicillin proving even more efficient[6]. Between 1900 and 2010, total mortality in the US declined by 54 %, with the percentage of deaths attributable to infectious diseases among the top ten causes in 1900 dropping from 52.7 % to 3.0 % (Figure 2).

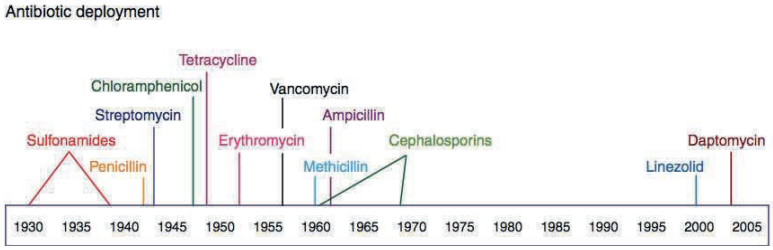

Figure 1. Introduction of some antibiotics on the market. Adapted by permission from Springer Nature: Springer Nature, Nature Chemical Biology, Clatworthy *et al.* (2007) Copyright © 2019.[7]

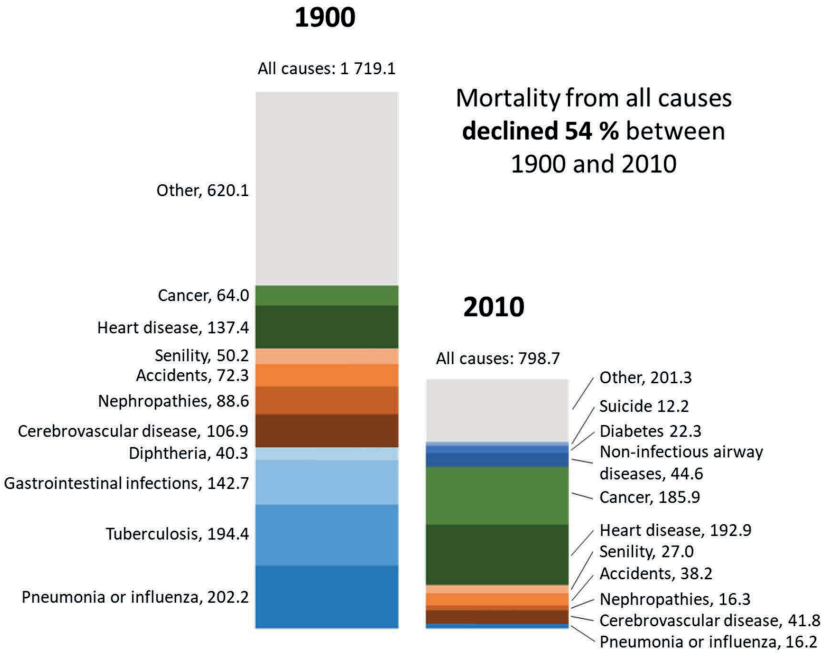

Figure 2. Mortality per 100.000 capita and top 10 causes of death in the USA in 1900 and the corresponding numbers for 2010. Modified from https://www.ncdemography.org/2014/06/16/mortality-and-cause-of-death-1900-v-2010/ [8]

1.1.2 Mechanisms of antibiotics

Antibiotics work by disrupting vital processes in the homeostasis of microorganisms, primarily bacteria, and are characterized by the fact that they do (to a large extent) not affect eukaryote cells, as opposed to e.g. disinfectants (Table 1). They are thus suited for treatment of diseases occurring within the host wherein a disinfectant would most likely cause irreparable effects. The affected bacterial processes are often divided into three major functional groups, based on which synthetic process is targeted: **i)** the cell wall synthesis (β-lactams – penicillin, cephalosporin, carbapenem and monobactam, vancomycins, bacitracin and polymyxins); **ii)** the nucleic acid synthesis (sulfonamides, trimethoprims, quinolones and antimycobacterials); **iii)** the protein synthesis (macrolides, amphenicols, streptogramins, linezolid, clindamycin, tetracyclines and aminoglycosides)[9].

Table 1. Antibiotic classes, their molecular targets in the bacterial cell and the main mechanisms of bacterial resistance to the antibiotic mechanism.[10]

Antibiotic class	**Target**	**Resistance mechanism**
Fluoroquinolones	DNA gyrase	~Enzyme [a]
Polymyxins	Cell membrane	↓permeability
Antimycobacterials	DNA-dependent RNA polymerase	~Enzyme [a]
Macrolides	Ribosomal protein	Concealment [b]
Aminoglycosides	Ribosomal protein	Concealment [b]
Tetracyclines	Ribosomal protein	Protection [c]
Amphenicols	Peptidyltransferase	Inactivation
Sulphonamides	Dihydropteroate synthetase	Replacement [d]
β-lactams	Penicillin-binding proteins (PBP)	↓affinity, ↓permeability, enzymatic degradation

[a] Mutation induces structural alteration
[b] Structural change hides ribosome from the antibiotic's effect
[c] Blockage by protective proteins
[d] Resistant enzyme in new biosynthetic pathway

Antibiotics may be bacteriostatic or bactericidal. Those with a bacteriostatic effect inhibit bacterial cell replication, arresting the bacteria whilst allowing the immune system to enact its effect. Gram-negative bacteria contain lipopolysaccharides (LPS; endotoxin) in their outer membrane that cause severe immunogenic reactions when they are detected by the immune system. Destruction of a large portion of bacteria residing in the blood stream of an already weakened individual could therefore cause a lethal reaction. A bacteriostatic antibiotic that stops or at least dampens the infection without massive release of LPS could then be the preferred choice. Bactericidal antibiotics may be necessary for successful therapy of severe infections like endocarditis, meningitis, or osteomyelitis, although bacteriostatic agents (e.g. chloramphenicol, clindamycin and linezolid) have been effectively used to treat such infections[11]. For the treatment of infections caused by most gram-positive bacteria, a bactericidal antibiotic is the preferred weapon of choice. Combination therapy may potentiate the effects of antibiotics, causing not just additive (1+1=2), but even synergistic (1+1=3) effects. One of the most recognized examples is the combination between sulfonamide and trimethoprim antibiotics, whose effects target two separate, but successive reactions in the nucleic acid synthesis (before and after the folic acid step, respectively). This combination is known for its synergistic effect as the two bacteriostatic drugs together become bactericidal, although the underlying mechanism of this interaction is poorly understood. Furthermore, Ocampo *et al.* demonstrated antagonistic effects between bactericidal and bacteriostatic drugs, most likely because bactericidal drugs are most effective during optimal cell replication, whereas bacteriostatic drugs conversely inhibits this replication[12].

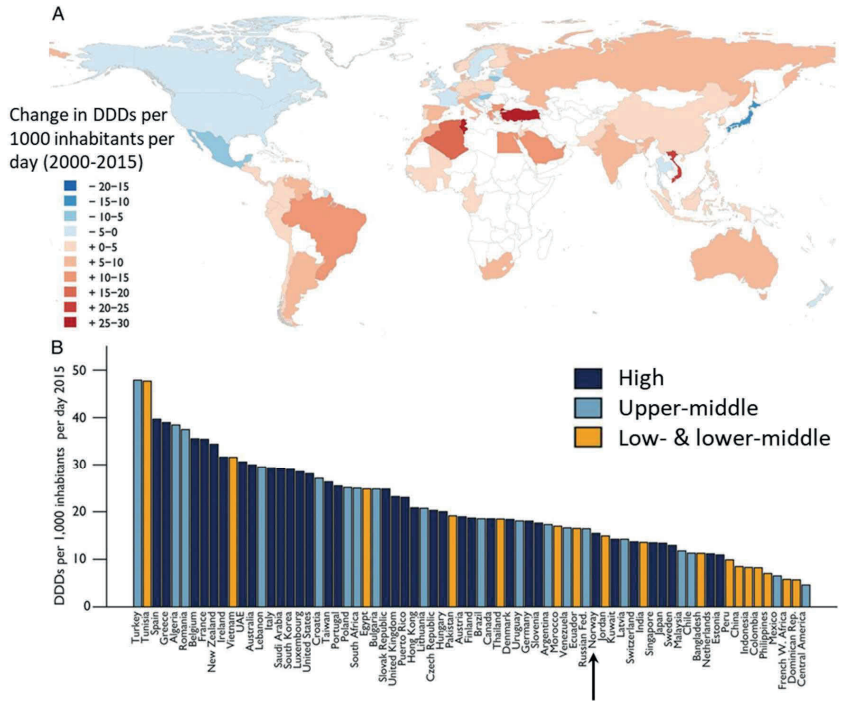

Figure 3. Global consumption of antibiotics as defined daily doses (DDDs) per 1000 inhabitants per day. A) Changes in DDDs/1000/day from 2000 to 2015 per country. B) DDDs/1000/day in 2015 per country, colored by the country's income status. Norway is indicated by an arrow.[13]

1.1.3 Antibiotic consumption

Antibiotic substances (and other pharmaceuticals) are categorized according to the Anatomical Therapeutic Chemical (ATC) classification system where the route of drug administration, disease, pharmacological and chemical subgroups are taken into consideration (e.g. ATC code J01CE01 for benzylpenicillin used in systemic treatment). This system is a tool for monitoring and researching drug utilization, and it is controlled by the WHO Collaborating Centre for Drug Statistics Methodology (WHOCC). Antibiotic consumption is increasing at an alarming rate globally, driven mainly by low income countries (Figure 3). Although low-income countries are still minor consumers compared to high-income countries, the trend is that low-income countries are catching up, and are projected to surpass the latter shortly[13]. Asia, South America and Africa are thus rapidly becoming the highest-consuming continents (Figure 3). Still, antibiotic consumption is relatively low in Norway. In fact, the latest report for 2018 from the Norwegian surveillance system for antibiotic resistance in microbes (NORM) showed

an overall decrease from 2017 in the total sales of antibiotics by 3 %, presented as defined daily doses (DDDs) per 1000 inhabitants per day (Figure 4)[14]. Consumption was reduced for six groups of antibiotics: Tetracyclines, β-lactamase sensitive penicillins, [macrolides, lincosamides, streptogramins] (MLSBs), quinolones, other antibacterials (glycopeptides, colistin, fusidic acid, metronidazol (i.v.), nitrofurantoin, fosfomycin, linezolid, daptomycin and tedizolid) and methenamine (an antibiotic only applicable for urinary tract infections). For β-lactamase resistant penicillins, [cephalosporins, monobactams, carbapenems] and [sulfonamides, trimethoprim] the consumption rates increased from 2017 to 2018 by +7, +3 and +5 %, respectively, whereas no obvious changes were observed for amphenicols, extended-spectrum penicillins, combinations of penicillins or aminoglycosides. The increased consumption observed for the β-lactamase resistant penicillins, the carbapenem group and the sulfonamide/trimethoprim group could be explained by a comparable decrease in consumption rates during the previous year of -7, -8 and -1 %, respectively[15]. The overall consumption of antibiotics similarly decreased between 2016/2017. In June 2015, the Norwegian government launched a new national strategy with the goal of reducing antibiotic consumption in human medicine by 30 % by 2020 compared to 2012[16]. Similar goals were set for food producing terrestrial animals (10 %) and companion animals (30 %) with 2013 as a reference year. The county which has seen the largest decrease in total consumption in primary care is Oslo, with a 30 % reduction since 2012, whereas the total use of antibiotics in Norway has been reduced by 23.7 % since 2012[14]. Achieving the goal of a 30 % reduction by 2020 is thus within reach, at least for some counties.

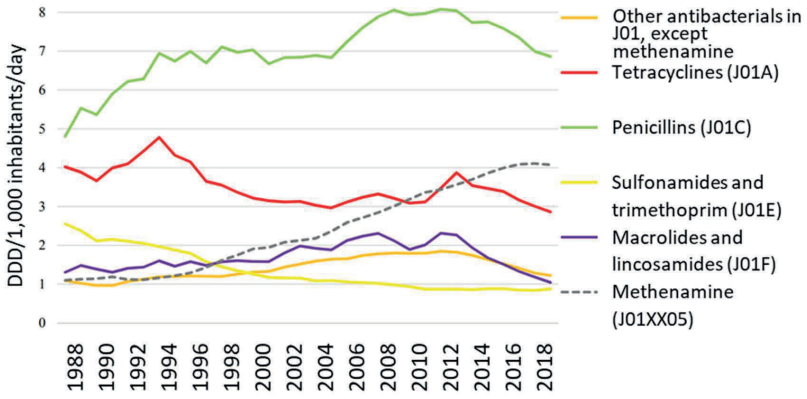

Figure 4. Antibiotic sales for the major groups of antibacterials for systemic use (ATC code J01) in Norway from 1987-2018 (only even years are shown on x-axis).[14]

The total consumption of antibiotics for systemic use expressed as DDDs/1000 inhabitants per day in Europe in 2017 ranged from 11.0-34.1 (average 23.4), with Norway at 14.7 DDDs[17]. Norway is frequently regarded as one of the most careful users of antibiotics, however, the European data places us at 8th place. The ratio of broad-spectrum drugs (penicillins, cephalosporins, macrolides (excluding erythromycin) and fluoroquinolones) versus narrow-spectrum drugs (penicillins, cephalosporins and erythromycin) is another way of describing antibiotic consumption that distinguishes between the types of drugs used whereas the total DDDs do not. The ratios for European countries included in ECDC's report range from 0.1 (Norway) to 22.2 (Malta). Narrow-spectrum drugs are preferably to be used to treat infections, due to the lesser collateral damage caused compared to their broad-spectrum counterparts. However, broad-spectrum drugs may be necessary in treating acute infections where the time delay before antibiotic susceptibility patterns are provided could prove fatal. Antibiotic consumption has been shown to correlate well with observed prevalence of antibiotic resistance (AR) in both hospitals, nursing homes, primary care facilities, communities and on an international level[18–22]. Broad-spectrum antibiotics are commonly used in hospitals, where life and death scenarios are more frequent. In contrast, infections handled by general practitioners in community settings are generally less severe, allowing the practitioner to perform susceptibility testing prior to prescribing an antibiotic, which in turn supports the use of antibiotics with narrower activity spectrums. The increase in AR is assumed to be largely due to the use or overuse of antibiotics in human and animal populations, facilitating the spread of antibiotic resistant bacteria (ARBs) amongst the bacteria in otherwise healthy human and animal environments[23,24].

1.2 Antibiotic resistance

Antibiotics have unknowingly been applied throughout history to combat infectious diseases, though only during the last hundred years have they been purified after their scientific discovery as discussed in section *1.1.1 History of antibiotics*. Bacteria develop antibiotic resistance as a countermeasure to cope with environmental stress. Koch *et al.* (2014) observed that when stressing susceptible *Staphylococcus aureus* with last resort antibiotics in a nutrient-limited, biofilm environment, they adapted into three groups, a susceptible one, an antibiotic producing one and one that had acquired antibiotic resistance properties[25]. Antibiotic resistance is a long-existing means of competition between bacteria and other microorganisms, despite recent (unintentional) efforts by humankind to speed things along. Antibiotic resistance is spread between strains of the

same species, transferred across bacterial species, including between gram-positive and gram-negative bacteria, and between pathogens and non-pathogenic commensals and environmental species[26]. It is a global problem that ignores geographical boundaries or species barriers. The spread of antibiotic resistance constitutes one of the major threats and challenges in both human and veterinary medicine.

1.2.1 Mechanisms of antibiotic resistance

The time it takes for one bacterial cell of *E. coli* to replicate (for one cell to create two daughter cells) is generally between 12-20 minutes at optimal conditions[27], whereas in 1962, *Pseudomonas natriegens* was reported to have the fastest replication to date at a mere 9.8 minutes, given the proper environmental and nutritional circumstances[28]. The rapid replication times of bacteria thus allow them to propagate almost exponentially, limited only by increasingly restricted nutritional and spatial resources as the population grows. In fact, if one were to disregard confinements of space, nutrients and other factors vital to proliferation, a single *E. coli* cell weighing 1 picogram (10^{-12} g), with a replication time of 20 minutes, could grow to the mass of the earth within 48 hours. The adaptability of bacteria is hence far superior to that of eukaryotic organisms. A bacterium can protect itself against the effects of an antibiotic by several means (Figure 5). One such way is to produce enzymes that modify the antibiotic before it can fully perform its function. An example is the β-lactamase class including the extended-spectrum β-lactamases (ESBLs), whose enzymes destroy the amide bond of the β-lactam ring in β-lactam antibiotics by hydrolysis, whereas other modifications may involve only chemically altering the bonds of drugs, reducing their effectiveness without complete inactivation[29]. Other protection mechanisms include reduction of the cell membrane's permeability or production/activation of membrane pumps that actively transport harmful molecules out of the bacteria. These pumps may be substrate-specific or with broad substrate specificity, as frequently seen in multidrug-resistant bacteria. Some bacteria undergo mutational changes or enzymatic alterations to modify the structure of the target molecule to avoid the antibiotic binding to it. This includes protection of the binding site by molecules that effectively dislodge the antibiotic and may even alter the geometry of the binding site to prevent rebinding, as has been shown for both TetM and TetO as a response to tetracycline binding[30,31]. Overproduction of the target molecule as a compensatory mechanism to reduce the antibiotics' effects is another way the bacteria fight back, as well as creating new biochemical pathways altogether[32,33].

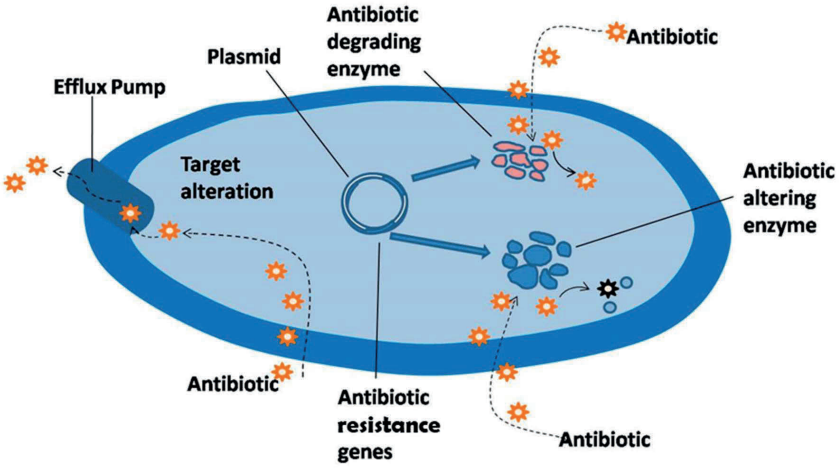

Figure 5. Antibacterial resistance mechanisms. Some mechanisms are not illustrated. Modified from Chandra *et al.* (2017)[34]

1.2.2 Acquisition of antibiotic resistance

Bacterial cells exposed to antibiotic selection pressure may adapt to this pressure by various means. There are two main ways in which bacteria develop resistance to antibiotics; by genetic mutations and by horizontal gene transfer, the latter primarily involving the transfer of plasmids carrying antibiotic resistance genes (ARGs).

Mutations

Utilizing the bacteria's rapid replication cycle, parent cells may give rise to a plethora of non-viable cells that have a lower survivability rate compared to their ancestors, but some may also acquire new properties, enabling them to better cope with the effects of antibiotics through mechanisms described in section *1.2.3. Mechanisms of antibiotic resistance*. Mutational resistance has primary clinical importance in several bacterial species, such as *Mycobacterium tuberculosis* and *Helicobacter pylori*, and for specific antibacterial drugs, particularly synthetic drugs like fluoroquinolones and oxazolidinones. Some bacterial cells may also acquire mutations in genes affecting their DNA repair and replication systems, giving rise to hypermutator strains which drastically increase the frequency of mutations and consequently the chance of successfully producing resistant phenotypes[35].

Horizontal gene transfer

Horizontal gene transfer (HGT) is often deemed the most important method for the spread of antibiotic resistance[35]. Unlike mutational resistance, HGT involves the incorporation of extracellular genetic material, either from other microorganisms, or directly from the environment. HGT is mediated through three distinct processes, namely conjugation (cell-cell), transduction (cell-bacteriophage) and transformation (cell-environment) and allow for more rapid exchange of antibiotic resistance mechanisms, as the mechanisms already exist and need only be transferred to a compatible cell without requiring new generations to be made.

Conjugation, transduction and transformation

The most important form of HGT is undoubtedly conjugation, in which plasmid single-stranded DNA covalently bound to a pilot protein (relaxase) is transferred from one bacterial cell to another by the type IV secretion system[36,37]. Conjugation presents a major challenge in the fight against antibiotic resistance as it provides bacteria with an opportunity to acquire ARGs across different sub-lineages within the same bacterial species, but also across different species of bacteria[36]. Transduction is the transfer of bacterial DNA by bacteriophages (bacterial viruses) as a result of random errors in the packing of new viral particles. This method of transfer is considered an important driver of resistance dissemination in some bacteria, including multi-resistant methicillin-resistant *Staphylococcus aureus* (MRSA)[38] and *E. coli*[39] in wastewater, and an important potential reservoir for ARGs in hospital effluents and the environment[40,41], although a recent study has demonstrated that the impact of bacteriophages is often overestimated in terms of carrying ARGs[42]. Transformation denotes the uptake of extracellular ("free") DNA by bacteria without the need of a donor and it is an important factor as to why destroying bacterial cells is not always a good thing. By killing a relatively harmless but resistant bacterium with an antibiotic to which the bacterium is susceptible, one could facilitate the release and subsequent uptake of DNA segments encoding resistance to the first type of antibiotic into more dangerous bacteria. This is obvious in the intestinal gut during antibiotic treatment, but maybe even more so in the wastewater treatment plants (WWTPs), where highly diverse and high-density bacterial populations combined with the presence of antibiotics may catalyze the transfer of ARGs to even greater levels[43]. Additionally, a primary goal of WWTPs is to destroy organic material, including bacteria, further facilitating the release of potential ARGs.

Mobile genetic elements

Horizontal gene transfer is made possible by the presence of mobile genetic elements (MGEs). The simplest of these elements is the IS (Insertion Sequence) element. These sequences consist of 700 - 2 700 base pairs (bp) and carry inverted repeat (IR) DNA sequences on their ends[44]. The IRs may overlap, resulting in a circularization of the IS element. The IS element may then be excised from the chromosome and can jump to new locations on the bacterial genome by homologous recombination. These jumps may subsequently give rise to new or eliminate prior mutations.

The IS element is the most rudimentary form of transposons (simple transposons) that carry only genes for the transposition itself, along with the IRs. An IS element can evolve by insertion of functional genes like ARGs between the transposase gene and the IR. Composite transposons carry two flanking IS elements that may or may not be identical, as well as additional genes besides their transposition genes. The entire length of the composite transposon is moved with both IS elements simultaneously including protein-coding genes in between and may thus be responsible for the transfer of ARGs.[45]

Integrons are a group of non-mobile genetic elements recognized for their ability to hoard ARGs. These elements carry integrase genes whose role is to acquire and insert so-called gene cassettes into the DNA sequences of the integrons. They may be located on other MGEs, enabling their transfer between bacteria. Integrons may over time accumulate ARGs and thus oftentimes carry several antibiotic resistance genes. The acquisition of a multiple-resistance integron may produce bacteria that can withstand most antibiotic treatments.

Plasmids are the last and maybe most important group of MGEs. These can exist in many forms, but most of them have in common that they are circular stretches of DNA that can be transferred by e.g. the type IV secretion system. Some plasmids already carry genes for transfer systems (conjugative plasmids) while others require existing transfer systems made by other plasmids in the donor cell. Plasmids can carry resistance genes and any number of other MGEs like the IS elements, transposons and integrons. Although the majority of plasmids are relatively small (<10 000 bp) because possessing them is costly, some exceed sizes of over 300 000 bp[46].

Co-, cross- and multi-resistance

Several ARGs may be present simultaneously within one bacterial cell. Simultaneous resistance to multiple antibiotics due to the presence of multiple resistance genes is called co-resistance. The closer the resistance genes are located on a genome (or on MGEs), the higher the risk of co-selecting for resistance when antibiotic pressure is exerted. The closer the genes are on a DNA sequence, the higher the likelihood that when a random recombination event occurs, the genes' relative locations remain unaffected. During a course of antibiotic treatment, any bacteria carrying resistance to that antibiotic will be selected for. However, any co-located resistance genes will be selected for along with the active resistance gene. Some ARGs additionally confer resistance to more than one antibiotic. These mechanisms are known as cross-resistance, and efflux pumps are the main mediators in that they can pump out multiple substances from the bacterial cytoplasm.

1.2.3 To dress for cold weather

To put antibiotic resistance in the simplest terms, one could apply a metaphor such as this: Consider antibacterial agents as cold weather, the more antibiotics, the colder it gets, and subsequently the higher the selection pressure. When the weather is warm, bacteria wear little "clothing", but if it gets too cold, they may perish. There are few threats, but some bacteria are easily offended and produce toxic peptides to inhibit close relatives. Thus, most bacteria wear t-shirts (surface molecules) even during warm weather, but typically regard this as a negligible inconvenience. The threat to the bacterium comes when the weather gets intensely cold. In the presence of high concentrations of antibiotics, only those bacteria that can dress up properly e.g. in a heavy winter coat survives. The heavy winter coat represents an antibiotic resistance mechanism that can be costly (high *fitness cost*) to carry around during regular temperatures, and thus the bacterium prefers to not wear it constantly. Other bacteria may be present when the temperature returns to normal, either because of the introduction of new bacteria or because those that were already present were not affected by the cold. The latter bacteria could be intrinsically resistant if they do not possess the molecules targeted by the antibiotic. If the fitness cost of the resistance mechanism is high enough to put the bacterium at a competitive disadvantage against its new neighbors, it will have to get rid of its winter coat in order to compete with the rest. Plasmids carrying ARGs can be rapidly taken up or expelled, meaning the bacteria may

have no problems in dressing or undressing the winter coat. So far, the resistance has been somewhat manageable by reducing the bacteria's exposure to antibiotics. So, what happens when the weather gets chilly? The antibiotics are present, but the concentrations are below the levels required to effectively treat a patient. Bacteria dressing up to avoid perishing during intense cold may now not need clothing. However, we have all walked outside in a t-shirt during a particularly cold day in August. Being chilly is not life threatening, but we are far more comfortable wearing a light sweater or summer jacket. In a similar manner, bacteria may acquire resistance properties with lower fitness costs, enabling them to stay dressed without a competitive disadvantage in antibiotic-depleted environments (when the weather gets warm again). This latter form of resistance could be much harder to revert to wildtype, as researchers have shown that bacteria may develop no-cost resistance at non-therapeutic levels of antibiotics[47]. Additionally, some bacteria may work as coat stands, holding all the jackets and coats for when the weather gets worse. These persister cells have been demonstrated to represent a minor subpopulation of susceptible bacterial populations. The persister cells are dormant, slow- or non-growing cells that are not antibiotic resistant, but antibiotic tolerant, and thus can survive an ice age of antibiotics. After re-cultivation of the subpopulation of persister cells, the following generation of bacteria form a new population of susceptible bacteria in which a subset of cells again become persister cells.

1.2.4 The problem of antibiotic resistance

Antibiotic resistance is ancient. As long as bacteria have existed, competition for nutrients has been a major struggle in which Darwin's "Survival of the fittest" has played an important role. Bacterial replication is limited in a resource-depleted environment. A microorganism that finds a way to rid the battlefield of its competitors, could have a much better chance of surviving and thriving. Antibiotics are powerful weapons that microorganisms have been using to fight each other for at least 30 000 years[48]. Similarly, affected bacteria have been fighting the effects caused by these antibiotics for as long. Antibiotic resistance is a problem that predates any form of medicine and likely also humans as a species altogether. At the same time, bacteria are vital to our survival in obtaining important nutrients from the food we eat as well to defend us from other harmful bacteria. The problem with antibiotic resistance is that under natural circumstances, this phenomenon co-exists in both harmless and harmful

bacteria, not in itself causing any form of problems for the host. The epidemiology of antibiotic resistance has thus long been problematic. Pathogenic bacteria of clinical importance are only a very small fraction of all the various types of bacteria out there, most of which are completely harmless, but the selection pressure exerted by antibiotics is still just as lethal for the harmless bacteria. Subsequently, an antibiotic course may not only target the disease-causing bacteria but may also at the same time wipe out the healthy intestinal microbiota, and worse, the course applies selection pressure on both harmless and harmful bacteria forcing them to acquire antibiotic resistance genes to fight the antibiotic. These properties may lay dormant in the intestinal microbiota as well as spread to other hosts until another infection strikes and neutralize the life-saving antibiotic treatment. Additionally, environmental levels of resistance have been increasing during the last century, demonstrated by Knapp *et al.* (2010) that analyzed old and new soil samples and found much higher abundances of ARGs targeting several classes of antibiotics in samples collected in 2008 compared to samples collected in the 1970s[49].

1.2.5 Occurrence of antibiotic resistance

Increasing levels of antibiotic resistance is as previously discussed a direct consequence of antibiotic usage. In fact, after the deployment of new antibiotics on the market, resistance was observed shortly thereafter for most antibiotics (Figure 6). Resistance varies drastically between geographical regions, largely due to differences in prescription patterns and usage in hospitals. Countries with established high levels of resistance often see no other choice than to use increasingly powerful antibiotics to combat the bugs that respond by growing tougher, a vicious circle that can be next to impossible to stop. Cassini *et al.* (2019) estimated 33 000 deaths in Europe in 2015 to be attributable to infections with antibiotic resistant bacteria[50]. In the newest report by CDC, updated estimates for 2013 attribute 44 000 deaths in the US to AR infections, a number nearly 50 % higher than the original estimate (23 000) in the previous report from 2013[51,52]. The CDC reports an 18 % decline in AR deaths, but an overall increase of AR infections by 7 % since 2013[52]. Globally, death tolls count upwards of 700 000 deaths per year, with an estimated 10 million annual deaths predicted by 2050 if no action is taken to turn the tide of antimicrobial resistance, according to a report by the UN Ad hoc Interagency Coordinating Group on Antimicrobial Resistance (IACG) published in April 2019[53].

Among reported infectious pathogens from human blood cultures in Norway, *E. coli* is the most abundant and represents 26 % of all (18 343) pathogens isolated from blood cultures in 2018[14]. Resistance to various antibiotics among these isolates vary drastically (range: 0 - 43 %), with the highest occurrences of resistance being to the β-lactams ampicillin (43 %) and amoxicillin-clavulanic acid (25 %), to trimethoprim-sulfamethoxazole (25 %) and to the fluoroquinolone ciprofloxacin (12 %). Resistance levels in *E. coli* isolated from urinary tract infections showed lower resistance levels with corresponding percentages to the blood cultures being 34, 6.7, 20 and 9.3 %, respectively, although it should be mentioned that the breakpoints for some antibiotics, particularly amoxicillin-clavulanic acid, depends on the sample type from which the isolate was cultured. The ECDC's surveillance report of antimicrobial resistance from 2017 reported levels of carbapenem resistance of over 10 % in isolates of *Klebsiella pneumoniae* in several countries, although this resistance feature was rare in *E. coli*[54]. *K. pneumoniae* was also observed with higher resistance levels than *E. coli*, but *Klebsiella spp.* only constitutes 6.8 % of the total of blood culture isolates reported in Norway[14]. Countries reporting high levels of carbapenem resistance were those that also reported higher levels of resistance to other antimicrobial classes[54].

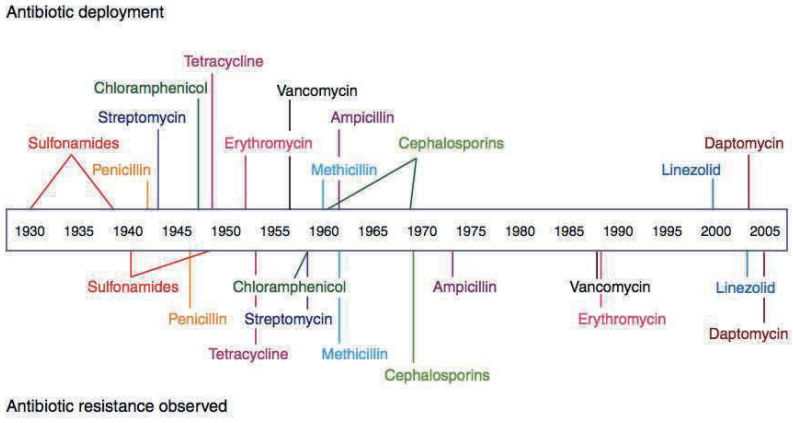

Figure 6. The time span between antibiotic deployment (over the timeline) and the first observation of antibiotic resistance (under the timeline). Reprinted by permission from Springer Nature: Springer Nature, Nature Chemical Biology, Clatworthy *et al.* (2007) Copyright © 2019.[7]

1.2.6 Clinically relevant antibiotic resistance genes

Some ARGs are more relevant than others. Antibiotic resistance first becomes problematic when antibiotic treatment of an infectious disease is rendered mute by its presence. Some ARGs are more abundant than others, whilst others affect a broader spectrum of antibiotics (see cross-resistance). For those ARGs that are present on mobile genetic elements, the risk of a potential pathogen acquiring the ARG is much higher. The ARG may be present in a wide variety of bacterial species, increasing the likelihood that the resistance property is retained in the microbiota of the host until an antibiotic course creates an environment in which the pathogen is compensated sufficiently for the cost of harboring the ARG. Several ARGs may be present on a single MGE, in which selection for one will also select for the other(s) (see co-resistance). ARGs that target antibiotics reserved as last resort drugs are also considered clinically important (Table 2). These emerging ARGs can be rare on a global basis but growing rates of trading and travel across borders is increasing the global dissemination of antimicrobial resistance. Synthesized antibiotics like ciprofloxacin were during the initial years after their deployment believed to be inherently resilient to bacterial adaptation towards resistance as these drugs did not occur in nature. This was invalidated as resistance mechanisms have rapidly developed to all drugs as they became available.

Colistin

The most frequent variant of colistin resistance is *mcr-1* (mobilized colistin resistance-1), with the ninth *mcr*-gene recently described[55]. Colistin is rarely used in Norway, with only 0.006 DDDs/1000 inhabitants/day being used in 2016 and 2018[14]. The rate of resistance to colistin was not presented for human clinical isolates in the NORM report. No colistin resistance genes were detected in clinical isolates of *E. coli* from the poultry production during 2015-2018. Plasmid-encoded *mcr-1* was detected in an isolate cultured from imported leafy herbs that also displayed resistance to cephalosporins ($bla_{CTX\text{-}M\text{-}65}$). Two isolates out of 18 analyzed isolates *Salmonella* spp. from various animals were positive for colistin, but confirmation by whole genome sequencing was not yet performed at the time the report was published[14].

Table 2. Notifiable antibiotic resistant bacteria or antibiotic resistance genes in animals in Norway, according to the regulation on disease in animals from 2014, including their categorization. Antibiotic resistant bacteria and genes were implemented as notifiable diseases in on June 1st, 2019. Disease categories A, B and C are defined by Norwegian law and regulate which measures that should be implemented when a disease is detected, ranging from most critical (A) to least critical (C).

Resistance to antibiotic	**Family/species**	**Gene(s)**	**Disease category**
Colistin	*Enterobacteriaceae*	*mcr-1, mcr-2*	C
Extended-spectrum β-lactamases/AmpC	*Enterobacteriaceae*	*TEM*, *SHV*, *CTX-M* [a]	C
Fluoroquinolone	*Enterobacteriaceae*, Gram negative bacteria	*gyrA, parC, qnr, aac(6')-Ib-cr, qepA* [b]	C
Carbapenem	*Enterobacteriaceae*	*ndm-1, KPC*	C
Methicillin	Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA)	*mecA*	B [c], C
	MRSA	*mecA*	C
	S. pseudintermedius (MRSP)	*mecA*	C
Linezolid	*Enterococcus faecium, E. faecalis*	*Rp1C*, *Rp1D*, *cfr*, *cfr(B), cfr(B) variants, optrA, poxtA, DEGs* [d]	C
Vancomycin	*E. faecium, E. faecalis* (VRE)	*vanA, vanB*	C

[a] *bla*-types of these, such as bla_{SHV-1} and $bla_{CTX-M-15}$.
[b] As reported by Hooper and Jacoby (2015)[56].
[c] LA-MRSA is considered a category B disease when detected in cattle, goat, sheep or swine.
[d] As reported by Bender *et al.* (2018)[57].

Extended-spectrum β-lactamases

ESBL-producing members of the family *Enterobacteriaceae* are a growing concern globally. *E. coli* carrying CTX-M enzymes are the species most commonly associated with globally disseminated ESBLs. CTX-M-15 is the dominating CTX-M variant worldwide, followed by CTX-M-14, mainly found in South-East Asia, and CTX-M-27 that is rapidly emerging globally, especially in Japan and Europe. Among ESBL-producing *E. coli*, the population structure is dominated globally by the high-risk clone named ST131 and clade C, a subtype of this sequence type (ST), is one of the most influential global antibiotic resistance clones having emerged during the 2000's. Other important ESBL-producing *E. coli* STs include ST405, ST38, ST648, ST410 and ST1193[58]. Among *E. coli* isolated from blood cultures in Norway in 2018, 6.5 % were ESBL-producing *E. coli* (ESBL-EC), unchanged from 2017 (6.6 %)[14]. According to ECDC, 15 % of *E. coli* were resistant to third-generation cephalosporins in 2017, whereof 87 % were ESBL positive, meaning that approximately 13 % of the total *E. coli* reported in European countries were ESBL positive[54].

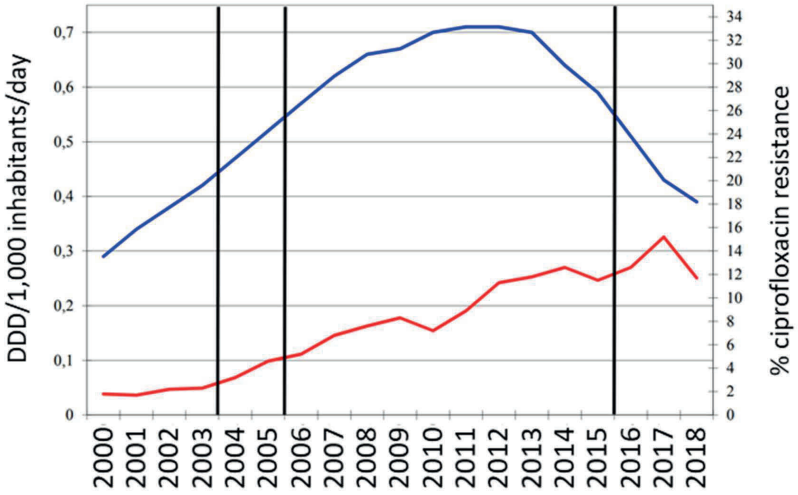

Figure 7. Ciprofloxacin consumption (blue) and resistance rates (red) among *E. coli* from blood cultures. The vertical bars represent changes in minimum inhibitory concentration (MIC) breakpoint values used for susceptibility testing.[14]

Quinolones

Resistance to quinolones in *E. coli* is most often mediated by a mutation in amino acid Ser83 in the DNA gyrase gene *gyrA*. Although ciprofloxacin usage has been reduced since 2013, ciprofloxacin resistance rates among *E. coli* have increased relatively steadily since 2000 (Figure 7). Among human blood cultures collected in 2018, 11.7 % of isolated *E. coli* were resistant to ciprofloxacin[14]. The resistance rates of ciprofloxacin in wild and domestic animals during 2006-2016 were reported by Kaspersen *et al.* (2018) and ranged from 0.0 % in horses, reindeer and sheep, to 3.6 % in broilers[59]. Fluoroquinolone resistant *E. coli* were detected in 12 of 194 leafy greens/leafy herbs samples in 2018[14].

Carbapenems

The most prevalent genotypes for carbapenem resistance in Norway have changed from KPC around 2008 to NDM and OXA-48-like variants in the last couple of years (Figure 8). Carbapenem resistance is a dreaded resistance phenotype type but is fortunately rare in Norway. Of 54 patients carrying carbapenem resistance in 2018, 44 were due to import (travelling and acquisition abroad), five were acquired in Norway and for the remaining five there were no data on travel status. The fact that isolates carrying this type of resistance are still counted by the number and not as rate of resistance is a good thing, but nevertheless the numbers are increasing at an alarming rate each year. Also, only 12 of the 54 patients were presented with an infection associated with the resistant isolate, whereas the remainder of the isolates was discovered from random screening samples, indicating that the actual number may be higher.

Methicillin

MRSA is developed by a mutation in the *mecA* gene or less frequently in *mecC*. Norway has had a strict policy to combat the emergence of Livestock associated (LA) MRSA pathogens. LA-MRSA was detected for the first time in Norwegian swine in 2011 (in a slaughter house), and in multiple herds in relation to two outbreaks in 2013/2014. Since then, Norway has so far eradicated LA-MRSA from Norwegian swine herds, as no herds were positive in the large screening program in 2018, including 716 herds of different production types. MRSA in humans has remained unchanged during the last two years, although the prevalence increased yearly during 2007-2016[14]. The main goal in Norwegian healthcare institutions is to prevent endemic establishment of MRSA. The

number of infections that occur within Norway remains relatively unchanged during the last ten years, whereas the number of infections abroad has been increasing steadily during the same period. The isolates causing infections are relatively diverse, with the top five genotypes representing 6.3, 6.3, 6.9, 7.2 and 8.7 % of the total population of human MRSA isolates, respectively.

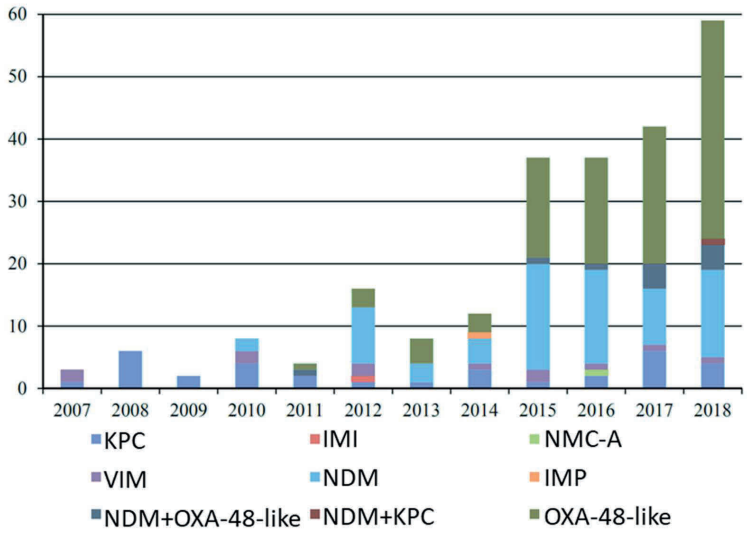

Figure 8. Carbapenemase-producing bacterial species of the order Enterobacterales from human clinical samples collected in Norway.[14]

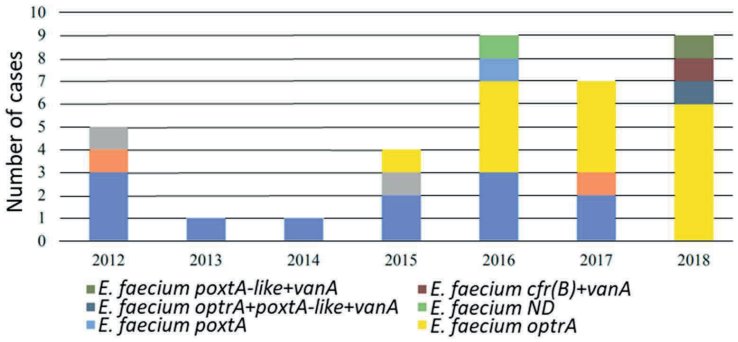

Figure 9. Number of linezolid-resistant *Enterococcus* and their resistance mechanisms. ND = Not determined genotype.[14]

Linezolid

Linezolid is one of the most recently introduced antibiotics and is reserved for the use in complicated gram-positive infections. Several resistance mechanisms are described (Figure 9). As a result of recent implementation and strict regulations on usage, we may not yet see the full consequences in terms of resistant bacteria, as the number of isolates registered from humans in Norway is currently under 10 per year (Figure 9).

Vancomycin

Vancomycin resistance is most often caused by the resistance genes *vanA* and *vanB*. Vancomycin resistance in Norway spiked in 2011 despite few observations in 2010 and none prior to 2010 (Figure 10). During the last few years, the number of patients colonized with vancomycin-resistant enterococci (VRE) has again begun to rise, similar to a third of the European countries in the same time span, where vancomycin resistance on average was found in 15 % of enterococci in 2017 (up from 10 % in 2014)[54]. Among Norwegian blood culture isolates, 2.5 % of *Enterococcus* spp. were resistant to vancomycin whereas only 0.5 % were positive for either *vanA* or *vanB*. *Enterococcus faecalis* were isolated three times more often compared to *E. faecium*, but all VRE belonged to the latter species. Avoparcin, used routinely as a growth promoter in Norwegian poultry production from 1986 until it was banned in 1995 induces cross-resistance to vancomycin and has been shown to have induced an extensive reservoir of VRE in the Norwegian broiler production, which persisted for many years after the ban was implemented. For the first time since monitoring started in 2006, no VRE were detected in Norwegian poultry in 2019, although narasin was used actively until that practice was discontinued in 2015 due to public demand[14].

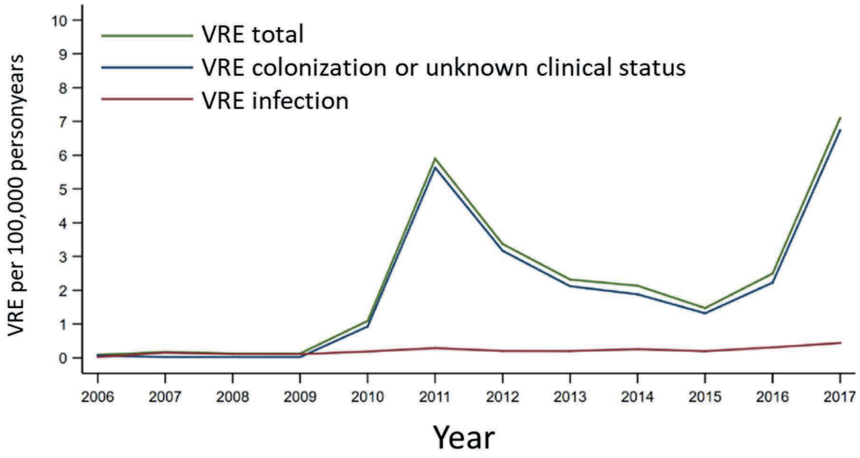

Figure 10. Cases of human VRE in Norway during the last decade.[60]

1.3 Surveillance of antibiotic resistance

Surveilling the occurrence of antibiotic resistance is important to enable efficient measures to be implemented. Surveillance is performed through various monitoring programs, using several different, but preferably comparable methods for antimicrobial susceptibility testing. Antibiotics have been demonstrated to exert selection pressures even at low (µg/l) antibiotic concentrations below the minimum inhibitory concentrations (MIC)[61], although some researchers have estimated even lower thresholds in the ng/l range[62].

1.3.1 Monitoring programs

The NORM/NORM-VET report has been mentioned frequently through the previous sections of this introduction regarding antibiotics and antibiotic resistance in Norway. Monitoring programs both in veterinary and human medicine are required to collect data in order to identify trends in the development of antibiotic resistance. Surveillance of human and animal health in Norway is performed through several monitoring programs, but those that monitor antibiotics and antibiotic resistance are listed in Table 3.

Table 3. Monitoring programs in veterinary and human medicine in Norway related to antibiotics and antibiotic resistance.

Monitoring program	Medicine
Norwegian Surveillance System for Communicable Diseases (MSIS)	Human
The Norwegian Surveillance System for Antibiotic Use and Healthcare-Associated Infections (NOIS)	Human
Norwegian Surveillance System for Antimicrobial Drug Resistance (NORM/NORM-VET)	Human/veterinary
MRSA in pigs	Veterinary

1.3.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing has two major goals. One is to determine whether any form of acquired resistance is present in a natural population of bacteria. The other aims to determine whether an antibiotic course at therapeutic concentration will have any effect on a clinical infection. The MIC of an antibiotic is the lowest concentration in which no bacterial growth is visible. Figure 11 shows the top end of the normal distribution curve of the wildtype population which is designated as the environmental cutoff (ECOFF), whereof isolates below this value are considered to not carry any form of resistance to the specific antibiotic. The clinical MIC is shown further to the right on the x-axis, at a higher concentration and represents the concentration that needs to be exceeded for successful clinical therapy (the MIC). The area between the ECOFF and the clinical MIC represents a grey zone of uncertain (intermediate) resistance. Note that some isolates are placed outside the wild-type distribution but are still regarded as susceptible for the purpose of clinical treatment. The cut-offs for clinical susceptibility and resistance vary per antibiotic and microbial species. Antibiotic susceptibility testing (AST) is performed by several methods. Common phenotypic methods are the disc diffusion, broth dilution and test strip, but molecular methods are rapidly gaining a foothold as pipelines. Particularly whole-genome sequencing (WGS) is being developed to interpret the concurrent presence of multiple ARGs into estimations of MIC values.

Phenotypic antimicrobial susceptibility testing

Phenotypic antibiotic susceptibility testing includes the traditional methods for analyzing the resistance profile of a bacterium and involve techniques where the results are visible to the naked eye.

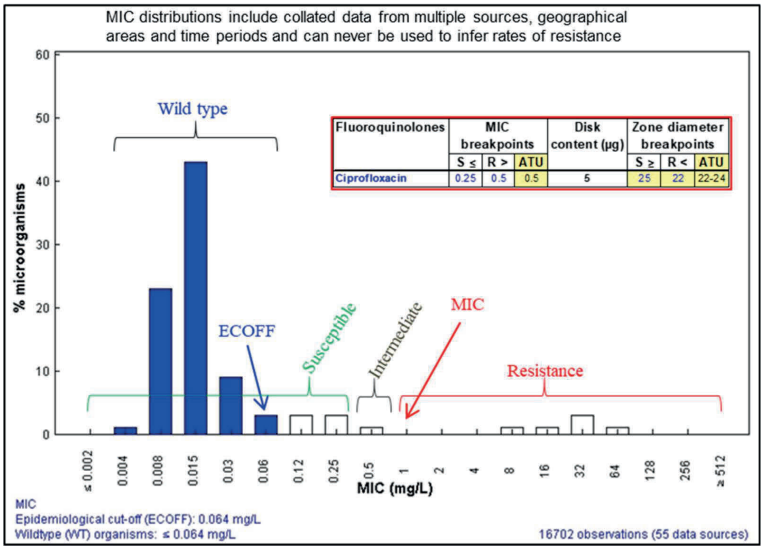

Figure 11. Histogram of MIC values of *Escherichia coli* (n = 16 702) for the antibiotic ciprofloxacin submitted to the EUCAST database. The blue bars represent wild-type isolates, with the right-most of these being the ECOFF value (0.064 mg/l; listed as 0.06 on the x-axis). Note that some isolates were found to have MICs of 0.12 - 0.5 mg/l but were not considered wildtype as they deviated from the normal distribution curve. The table (red box) lists the cut-off values for reporting results by microbiological laboratories for different AST methods. Modified from: www.eucast.org.

Broth dilution

The broth dilution method involves a series of two-fold dilutions of antibiotics in broth in microtiter plates (or glass tubes). Bacteria are inoculated into each well and the lowest antibiotic concentration in which no visual growth can be observed is designated as the bacteria's MIC. The broth dilution method is considered the golden standard.

Disc diffusion

The disc diffusion method involves growing bacteria on standardized agar plates such as Mueller Hinton agar plates whilst simultaneously challenging the bacteria by applying pre-fabricated discs containing either one antibiotic or a combination (e.g. trimethoprim/sulfamethoxazole). The discs' antibiotics will gradually diffuse into the agar, creating a gradient of concentrations that decreases further from the disc. Bacteria harboring ARGs or other mechanisms that enable them to survive the antibiotic's effect

will grow closer to the disc than those that do not possess thus mechanisms. The width of the non-growth zone around the disc is inversely correlated with the MIC value of the cultivated bacterial isolate. A wide zone indicates that the bacterium is susceptible, with a middle ground indicating intermediate resistance. Whereas antibiotic concentrations can be made very precisely in the broth dilution method, since the antibiotic substance is homogenously diluted, for the disc diffusion test, conditions such as temperature may disrupt the level of diffusion, although this usually has no practical implications.

Test strip

A third method of AST is the test strip, which employs a paper strip pre-coated with antibiotics at gradually decreasing concentrations. The strip is placed on top of a recently inoculated Mueller Hinton agar plate and the MIC is decided by where the growth of the bacteria begins. This method is in a way a hybrid of the previously described methods, as the growth of bacteria on agar plates infused with a gradient of diffused antibiotics is combined with the twofold dilution employed in the broth dilution method. The test strip is primarily used for its rapid application and easy interpretation.

Genotypic antimicrobial susceptibility testing

More advanced approaches to AST involves the analysis of DNA sequences associated with AR, including antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs). These approaches include qualitative polymerase chain reactions (PCRs), quantitative PCRs (qPCRs), WGS and metagenome sequencing.

PCR

PCRs utilize the DNA polymerase and a set of primers which are small strands of DNA, complementary and preferably unique to the gene of interest (GOI). The GOI, if present in the sample, acts as a single stranded DNA template for the primers to bind (anneal) to. Through subsequent duplication steps, enough strands of identical DNA are made to be visualized to the naked eye on an electrophoresis gel, or to be used for downstream applications such as sequencing.

Quantitative PCR

A qPCR is similar to the PCR, but in addition to revealing the presence of a GOI it provides a specific number for how many doubling cycles was required to reach a specified threshold of fluorescence. The number of doubling cycles is directly linked to the number of DNA templates that were present in the original sample and can be used to calculate the abundance of the GOI. The abundance is commonly normalized to house-keeping genes (e.g. 16S *rRNA*) in the sample to enable comparison between samples that may contain different amounts of bacteria.

Whole genome sequencing

Whereas PCR and qPCR only read a single sentence or paragraph of the bacterial genome, whole genome sequencing (WGS) reads the entire book from start to finish (or at least as much as possible, see contigs). When the entire genome is sequenced, specialized programs can search the "book" for specific sequences that are recognized as antibiotic resistance genes (or virulence genes, house-keeping genes and others). WGS offers very rapid analysis of the presence of a practically unlimited number of (known) ARGs, although it is not always able to determine whether a gene is functional. WGS also enables the comparison of minor differences in the detected sequences, which is useful when mapping the geographical or temporal spread of resistance as a part of outbreak investigations. Additionally, WGS data can be stored for future analyses that are looking for sequences that are not yet known or listed in online databases.

Metagenomics

Metagenome sequencing is like WGS in that "all" DNA sequences present are read irrespective of their relevance, and subsequently analyzed to determine whether the pool of sequences contain something important. In metagenome sequencing however, the goal is not (yet) to completely assemble the genome of a single organism, but rather to assemble as many parts of genomes as possible from a pool of organisms. This can be done e.g. by sequencing all 16S rRNA sequences in the sample and using these sequences to determine the occurrence and distribution between bacterial phyla (a higher-ranking group of the taxonomic ladder than species and family). If AST is the preferred application of the sequencing data, the 16S rRNA primers can be replaced by primers that target ARGs of interest. Metagenomic shotgun sequencing is a subtype of

metagenomic sequencing where all DNA strands are fragmented to small, random segments that are sequenced and reassembled without the limitation of 16S rRNA targeting only bacteria etc.

Proteins and DNA expression

Presence of an ARG does not always translate to unsuccessful antibiotic treatment. Bacteria may carry the blueprints (genes) for mechanisms that would make them impervious to the effects of an antibiotic, but other factors may also be influencing their utilization of said blueprints. If the bacteria do not "build" (express) the mechanism, they are not able to use it to prevent the antibiotic's effects. Reverse transcriptase qPCR (RT-qPCR) analyses the bacterial messenger RNA (mRNA) to interpret the level of expression of a known gene by an isolate or from a mixed sample. By reversing the process of protein production, the mRNA is reverted to DNA by a reverse transcriptase enzyme. The resulting DNA sequence can be analyzed in a traditional qPCR to measure the level of mRNA present in the original sample. Proteomics is comparable to metagenomics, but for completed proteins rather than gene sequences. In proteomics, mass spectrometry is utilized to obtain protein composition, structure and concentration. Proteomics has the benefit of revealing the true translation of mRNA into proteins, whereas RT-qPCR bases protein expression on an assumption that the level of transcription and translation is completely correlated. The rapid degradation of mRNA (1-3 minutes) may also mask the actual level of expression.

1.4 Antibiotic resistance in wastewater

1.4.1 Wastewater

The logistical challenge of providing clean water and sanitation systems has been present since the dawn of civilization. Centralization and urbanization with insufficient infrastructure, water resources and sanitation systems brought with it infectious diseases. The dilution of wastewater effluents from human settlements in natural bodies of waters like rivers or lakes was only a feasible solution until the population densities outgrew the waters' capacities. Treatment processes for purification of drinking water and wastewater for agriculture has developed drastically in recent history. Today, outbreaks of infectious diseases through wastewater and in drinking water in Norway are rare. A few noticeable outbreaks have happened during recent years. In 2004, an outbreak of giardiasis in Bergen was traced to drinking water, likely caused by leaking wastewater pipes and/or insufficient water treatment[63]. An outbreak of campylobacteriosis in drinking water in Røros city was identified in 2007[64], and further confirmed after *E. coli* were discovered in the drinking water and linked to contamination of the ground water source during a similar event in 2009. Recently, *Campylobacter jejuni* was found to be the cause of a large outbreak in a drinking water reservoir at Askøy outside Bergen, resulting in two deaths and over two thousand cases of gastroenteritis. The importance of adequate wastewater management to prevent such event is therefore clear.

1.4.2 The occurrence and impact of antibiotic resistance in wastewater

Antibiotics and their metabolites are excreted into wastewater with urine and feces[65–67]. Additionally, wastewater may contain a substantial amount of genes coding for antibiotic resistance that may reach the surrounding coastal ecosystem[68,69]. Thus, the high number of bacteria in wastewater combined with the selection pressure from antibiotic residues makes wastewater a major route for the transmission of antibiotic resistance between bacteria that has been recognized for several decades[70–73]. Several recent studies have highlighted the impact of treated wastewater discharges from WWTPs on AR abundances in recipient surface waters, indicating that conventional wastewater treatment may not be enough to fully manage the problem[74,75]. Additionally, several human disease-related ARGs have an environmental origin, highlighting the circular nature of the water cycle[76]. Risk of ARB spread in low-income countries is

probably higher than in developed countries, but quantitative data to support this are scarce[77]. Untreated wastewater reaching the environment due to excess volumes from heavy rainfall events or insufficiently treated WWTP effluents could lead to an ecological build-up of environmental contaminants that could be hard to remove once established. Higher relative abundances of ARGs were found in rivers downstream of WWTP outlets compared to upstream of the same WWTPs in several studies, with differences being observed as far as 20 km downstream in one study[78,79]. Receiving surface waters are also affected by discharges from WWTPs[74,75]. Heavy metals in insufficiently treated wastewater may also select for ARGs within the WWTP and ultimately contribute to their release into the environment[80].

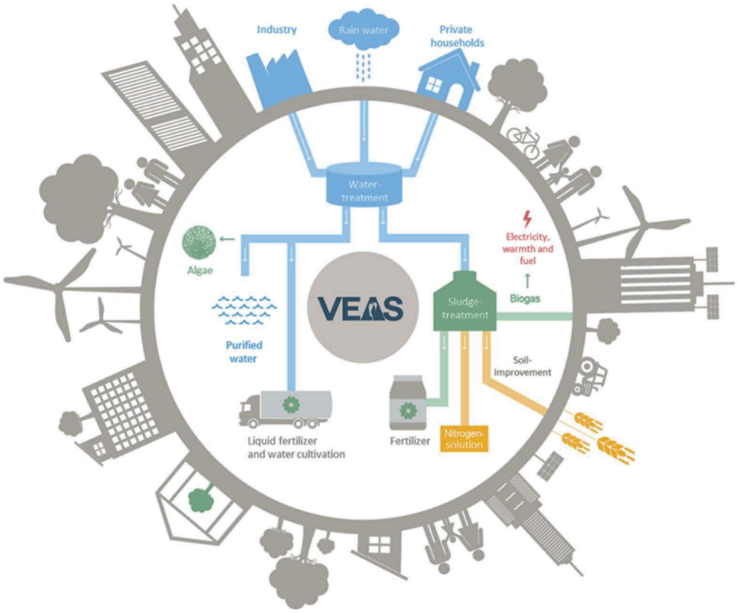

Figure 12. The WWTP investigated in this study, VEAS, has a goal of a circular economy in which the reuse of byproducts from wastewater treatment can cover the costs of the wastewater treatment.[81]

The main objective of wastewater treatment has traditionally been to remove harmful pathogens, but the utilization of nutrients from treated wastewater (nitrogen, biogas etc.) has become increasingly important in creating a circular economy in which the

byproducts of the treatment process can help pay for the costs of the same treatment (Figure 12). Wastewater treatment is divided into several steps. Primary treatment involves the sedimentation of solid waste within the water. Firstly, large particles that are unable to pass through subsequent steps of the complete treatment process are removed by filtration. The sedimentation produces wastewater sludge that can contain nearly 50 % of suspended solids within the wastewater. This step has negligible effects on the rates of antibiotic resistance, although a reduction in the total abundance of resistant bacteria may occur due to the removal of particles that may physically transport the resistant bacteria. Secondary wastewater treatment can include biofiltration (further sedimentation) and aeration or oxidation ponds (oxidation) to further purify the wastewater. The role of tertiary treatment is to remove organic material from the water, such as phosphates and nitrates that could contaminate the ecosystem, as well as to extract valuable resources from the wastewater. Activated carbon and sand filters are some commonly used materials to assist in this process. Quaternary treatments are not employed by all WWTPs. These are additional treatment steps with the aim of sterilizing the wastewater, free of any disease-causing agents (bacteria, viruses, parasites etc.) including antibiotic resistance. Several options are available for this type of supplementary treatment, including UV-light irradiation, ozone-, H_2O_2- and chlorine treatment. The point of the quaternary treatment step(s) is to remove residual bacteria that were part of the previous treatment steps' as well as to remove potential harmful DNA. The first study on the effects of advanced treatments on ARBs in wastewater was performed in the 70s[82]. Because most of the advanced technological solutions available are expensive, it is a need to identify the main sources of antibiotics and of antibiotic resistance and prioritize the implementation of advanced treatment processes in these sites. Good examples of priority effluents are those that may have exceptionally high prevalence of resistance, such as the effluents of health care facilities or antibiotic production plants. Beside the high prevalence, hospital effluents contribute to the spread of antibiotic resistance genes emerging in the clinical ambient (e.g. *ndm-1*). With the discharge of these effluents into urban WWTPs, the contamination of the environment by such emerging resistance determinants is likely to occur. In a Swedish study, a widespread ampicillin-fluoroquinolone multiple resistant Swedish clone of *Enterococcus faecium* of hospital origin[83] could be directly traced to hospital wastewater (HW) where it was found in 50 % of all samples[84]. This clone seemed to be enriched in wastewater effluents, and was also found in many samples from recipient surface water[84,85], suggesting that recipient water containing bacteria from wastewater

may be a source for colonization of humans and animals with antibiotic-resistant bacteria of hospital origin. That wastewater, especially HW, can harbor and transmit antibiotic resistant bacteria has also been supported by other studies[23,86–92]. Local treatment to reduce the environmental burden of hospital effluents has been implemented in some countries, e.g. at Herlev Hospital in Denmark. Such localized wastewater treatment will also reduce the impact of runoff scenarios during heavy rain events, where contaminated wastewater directly reaches the aquatic environments. Because of continuing migration of humans to the region of Oslo, the region will face infrastructural challenges during the coming decades, with increasing requirements for improvements[93].

1.4.3 Surveillance of antibiotic resistance in wastewater

Water quality is an important aspect of wastewater treatment and daily sampling is performed in most if not all WWTPs to analyze the concentrations of organic and potentially hazardous material. Resistance rates among clinical isolates are prioritized, which is well and good when the treatment of a patient is the goal. However, understanding the whole picture is essential in limiting the local and global dissemination of antibiotic resistance. The environmental pollution with wastewater cannot be ignored as the environment undoubtedly returns the favor. Sampling wastewater can be used to create an early warning system for the emergence of new or rare bacterial antibiotic resistance in the target population[94]. The concept of using samples from hospital (HW) and urban wastewater (UW) for studying antibiotic resistance among bacteria in the corresponding human population was proposed already in the 70s by Linton *et al.* (1974)[95]. They concluded that the bacterial flora of the normal healthy population seemed to be the greatest contributors to antibiotic resistant bacteria in UW, and that HW showed much higher rates of antibiotic resistant bacteria. In a large European study on enterococci from various environments, the bacterial population structure in wastewater samples was shown to resemble that of a large population of fecal samples from individual humans and could thus be regarded as pooled fecal samples[96]. No national monitoring programs are currently in place in Norway to surveil the prevalence of resistance in wastewater. However, the Global Sewage Surveillance Project has recently been initiated by an international research consortium with the goal of annually investigating the occurrences of antibiotic resistance in wastewater globally[97]. Some studies have also included the status of Norwegian wastewaters during recent years[98–101].

2. Aims of study

Main objective

The present study was initiated to determine whether the impact of hospitals on the prevalence of antibiotic resistance in urban wastewater may benefit from specific measures directed at the hospital wastewater.

Sub-objectives

1. To investigate the levels of antibiotic resistant (AR) *Escherichia coli* in institutional (hospital) and community (non-hospital) wastewater.

   Achieved by

   - Cultivation, phenotyping and resistance determination of *E. coli* in wastewater samples collected from representative wastewater outlets and the WWTP inlet (Paper I).

2. To investigate the potential for dissemination of AR from hospital and community wastewater.

   Achieved by

   - Searching for presence of multi-resistant (pathogenic) *E. coli* clones in different types of wastewater (Paper I and II).
   - Identifying the levels of antibiotic resistance genes (ARGs) and antibiotics in different types of wastewater (Paper III).

3. To recommend whether to enforce local treatment of wastewater effluents from high-risk wastewater outlets.

   Achieved by

   - Comparing risk factors (ARBs, ARGs, antibiotics) between hospital, community and urban wastewater (Paper I, II and III).

3. Summaries of individual papers

Paper I

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

Erik Paulshus, Inger Kühn, Roland Möllby, Patricia Colque, Kristin O'Sullivan, Tore Midtvedt, Egil Lingaas, Rune Holmstad, Henning Sørum

Water Research 161 (2019) 232–241

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 number 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 β-lactamase-producing *E. coli* (ESBL-EC) were more common in HW (12 %) 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 HW, but surprisingly they were also high in the studied CW, compared to the UW. The relative contribution of HW seemed low in terms of dissemination of antibiotic resistant bacteria to the WWTP.

Paper II

Repeated isolation of extended-spectrum-β-lactamase-positive *Escherichia coli* sequence types 648 and 131 from community wastewater indicates that sewage systems are important sources of emerging clones of antibiotic-resistant bacteria

Erik Paulshus, Kaisa Thorell, Jessica Guzman-Otazo, Enrique Joffre, Patricia Colque, Inger Kühn, Roland Möllby, Henning Sørum, Åsa Sjöling

Antimicrobial Agents and Chemotherapy 63(9) (2019)

In this study, water samples were collected from a wastewater pump station in a Norwegian suburban community over a period of 15 months. A total of 45 daily samples were cultured and analyzed for the presence of *Escherichia coli*. Eighty *E. coli*-like colonies were collected from each daily sample and then phenotyped and analyzed for antibiotic resistance using the PhenePlate-AREB system. During the sampling period, two unique *E. coli* phenotypes with resistance to cefotaxime and cefpodoxime indicating carriage of extended-spectrum β-lactamases (ESBL) were observed repeatedly. Whole-genome sequencing of 15 representative isolates from the two phenotypes identified these as two distinct clones belonging to the two globally spread *E. coli* multilocus sequence types (STs) ST131 and ST648 and carrying $bla_{CTX\text{-}M\text{-}15}$. The number of ESBL-positive *E. coli* strains in the community wastewater pump station was 314 of 3,123 (10%) analyzed *E. coli* strains. Of the ESBL-positive isolates, 37% belonged to ST648, and 7% belonged to ST131. Repeated findings of CTX-M-15-positive ST648 and ST131 over time indicate that these STs are resident in the analyzed wastewater systems and/or circulate abundantly in the community.

Paper III

Wastewater concentrations of antibiotics, antibiotic resistance genes and mobile genetic elements in hospital, community and urban wastewaters and associated antibiotic prescription data in Norway

Erik Paulshus, Sara Rodriguez-Mozaz, Hege Salvesen Blix, Saulo Varela Della Giustina, Maggie Ruth Williams, James Tiedje, Syed Hashsham, Inger Kühn, Roland Möllby, Damia Barcelo, Henning Sørum

Submitted to *Water Research X*

Antibiotic resistance is an ever-present threat to global public and animal health. Selection pressures from antibiotic use and abuse are a constant risk for the development of antibiotic resistant (AR) bacteria. Wastewater, specifically from hospitals and wastewater treatment plants (WWTP) are important sources of environmental pollution with AR properties. However, much is still unknown regarding the possible impact of wastewater from different sources on the potential for AR development. In this study, samples of hospital (HW), community (CW) and urban (UW, WWTP) wastewater were collected monthly for one year. Wastewater samples were analyzed for 53 unique antibiotic compounds/metabolites, the abundance of 144 AR genes and genes associated with horizontal gene transfer. Antibiotic prescription data for the equivalent human populations were also included. Thirteen antibiotic compounds/metabolites were detected in HW and CW, and ten in UW. Several emerging and clinically important AR genes (e.g. *ndm-1*, bla_{IMP}, bla_{VIM}) were frequently detected in HW. The high abundance of mobile genetic elements (MGEs) like insertion sequence elements (IS elements) in all three wastewater types indicates that the wastewater contains bacterial genetic systems with a potential to develop and disseminate multi-drug resistance. The levels of antibiotics, ARGs and HGT-associated genes were highest in hospital wastewater, and they were higher in the community wastewater than in the urban wastewater from the WWTP, despite similar or lower levels of antibiotic consumption. In fact, average concentrations of several antibiotics in HW (cefalexin, metronidazole, ofloxacin and tetracycline) and in CW (cefotaxime) were higher than the predicted concentrations based on water usage and antibiotic consumption (with a 100 % ingestion-elimination efficiency). Hopefully, this study can assist in creating a platform for the continued research and political decision-making regarding AR.

4. Discussion

4.1 General discussion

In paper I, we analyzed the concentrations of *E. coli* in hospital, community and urban wastewater. We found that HW contained the lowest concentrations of *E. coli* compared to the community and urban sites. The median levels of *E. coli* for HW were ~15 900 colony-forming units (CFU)/ml, whereas corresponding numbers for CW and UW were ~159 500 CFU/ml and ~50 100 CFU/ml, respectively. During our initial phase of the study, we had estimated similar volumes to be discharges from the hospital as the community. We reported more accurate volumes of discharged wastewater in paper III, namely 240 m^3/day, 50 m^3/day and 290 000 m^3/day for HW, CW and UW, respectively. In fact, Brechét *et al.* (2014) estimated that water consumption in a hospital could reach 700 L per hospital bed per day[102]. The higher volume in HW compared to CW explains a large portion of the discrepancy between the concentrations of bacteria in the two sites. The number of all discharged bacteria could now be estimated for HW (3.8×10^{12} CFU/day) and CW (8.0×10^{12} CFU/day). Although resistance rates were lower in CW compared to HW, the rates were still much higher than in UW, whereas we had expected rates in CW to be lower than in UW. The higher density and total number of bacteria observed in the CW might explain the high levels of resistance observed in those samples. Investigation of other community areas could possibly elucidate this question. In the review by Hocquet *et al.* (2016) on ARBs in hospital wastewater systems, the authors summarized relevant studies from 2000-2015 involving *E. coli* and/or ESBLs in wastewaters from hospitals and WWTPs influents (denoted "community effluents")[77]. The CFU values of *E. coli* from these studies ranged from 7.4×10^4 CFU/ml to 3.9×10^6 CFU/ml for WWTPs. Corresponding numbers for HW were 3.7×10^4 to 5.4×10^6. However, in one of the listed studies, by Chagas *et al.* (2011), the researchers had found only 7.4×10^3 CFU/ml, and additionally, that number represented total coliforms, suggesting that the CFU/ml values for *E. coli* were probably even lower[103]. Nevertheless, the concentrations of *E. coli* in HW and UW from our study are relatively similar to those reported previously. Although samples collected in a WWTP should represent well the urban population of a large city supplying the WWTP, our results from CW demonstrate that the inter-variation between local outlets can make or break the outcome of a study.

The question of whether the effluents of hospital and community sites from this study affect the influent of the WWTP, or to what extent, does not have a concise answer. The median number of *E. coli* entering the WWTP was $1.5x10^{16}$ CFU per day during the study period (Paper I). Of these, 0.025 % and 0.053 % could be attributed to the HW and CW outlets, respectively. Although the percentage of contribution from the two outlets might have been higher if we had measured total coliforms or total CFUs, there is no reason to expect that we would have landed far from the current results. The potential for dissemination of antibiotic resistant *E. coli* into the environment during extreme flood events such as the one in Oslo in August 2016 (www.yr.no) is a constant threat. Masters *et al.* (2017) found that 41 % of *E. coli* isolates from a metropolitan river were identical to WWTP isolates following an extreme flood in 2011[104]. The study concluded that "it is crucial to adapt better strategies for management of WWTPs in preparation for such events". An argument for local treatment of hospital wastewater is to limit the impact of direct discharge into the environment, when untreated wastewater may reach the environment without first entering the WWTP, such as during heavy rainfall. Stark *et al.* (2016) demonstrated that untreated wastewater could have detrimental effects on the ecosystem[105]. A malfunction in a secondary treatment plant in Davis, Antarctica, in 2005 lead to the release of wastewater not fully treated into the environment. The presence of the fecal indicator bacteria *E. coli* containing *int1* in environmental samples and bivalves as well as increased levels of histopathological abnormalities and increased nitrogen uptake in fish species in the area indicated that the ecosystem was severely impacted by the discharged wastewater effluents. Alexander *et al.* (2015) showed that the abundance of ARGs in wastewater sampled from a combined sewer overflow was higher than those in treated WWTP effluents[106]. They thus demonstrated that heavy rainfall may lead to increased discharge of ARGs and dissemination of AR to the environment. VEAS WWTP participated in the initiation of this study with the foresight that Oslo's population number would continue to rise. Adverse effects from bad weather will continue to prove a threat, particularly if the WWTP's capacity is saturated from the growing populace. Global warming may also increase the frequency of adverse weather events in the future. The implementation of local treatment of high-risk outlets such as HW could have negligible effects on the bacterial communities of WWTP inlets. Nevertheless, it could be an important critical control point to avoid potential devastating effects on the surrounding environments during future floods.

In paper I, we described different population types of ESBL in HW and CW, respectively. In the previous studies summarized by Hocquet *et al.* (2016), drastically different rates of ESBL-EC or ESBL enterobacteria (ESBL-E) were reported[77]. Percentages of ESBL-EC among *E. coli* in WWTPs were reported at 0.1 % in France[102] and 2.3 % in Sweden[92], lining up with our results of 3.7 %. In HW, we found 12 % of the *E. coli* to be ESBL-EC whereas they constituted between 3.8 % in Ireland[107] to 14 % in Sweden[92]. In fact, Kwak *et al.* (2015) found similar resistance rates as compared to the present study for most antibiotics in HW and in UW, further supporting the results of the present study (Figure 13)[92]. Some studies reported the percentages of ESBLs among enterobacteria, and these appeared to be much higher than those observed this study for *E. coli* in both UW: 18 % in Poland[108], and HW: 39 % in Brazil[103]. The ESBL population in our study varied greatly with respect to PhP types in HW, with new PhP types appearing each month. Close to no PhP types among the multi-resistant ESBL population were isolated from two consecutive months. These results were a stark contrast to those previously reported by Gündŏgdu *et al.* (2013), where the researchers found a dominating phenotype to be present in all samples and make up 35 % of the analyzed ESBL-EC[109]. In contrast, the ESBL population of CW was dominated by a few PhP types, of which the sequence types (ST) ST131 and ST648 were frequently isolated throughout the study period (Paper I and II). These STs have been found in many different environments and are frequently associated with extraintestinal infections. In a study from the Czech Republic, Jamborova *et al.* (2018) analyzed 169 *E. coli* ST131 isolates from humans (nosocomial and community), wastewater, wild birds and dogs[110]. They found that 60 % of the analyzed isolates carried $bla_{CTX\text{-}M\text{-}15}$, whilst 25 % carried $bla_{CTX\text{-}M\text{-}27}$. These results support our findings that indicate that these community isolates may be prevalent in the human community population and/or the wastewater system. The $bla_{CTX\text{-}M\text{-}15}$ gene was found in all ST131 and ST648 isolates from our study, further highlighting the importance of these STs globally as causes of infections with multi-resistant clones. Although the PhP-types of the ST131 and ST648 in this study were almost exclusively found in the CW, we cannot eliminate the possibility that ST131 and ST648 are also abundant in wastewater from the hospital outlet. By comparing whole genome sequences of isolates from the ST131 and ST648 as well as ST38 and ST405, demonstrated that ST131 carried more pathogenicity factors[111]. Isolates belonging to the three other STs carried fewer uropathogenic *E. coli* (UPEC) specific genes as well as fewer other virulence genes compared to the ST131. They also

documented that ST131 isolates were markedly different from non-ST131 in terms of core genome, reinforcing the clonal diversification of ST131. These results point towards the possibility that a specific sub-lineage of ST131 with a unique PhP profile exists in the investigated CW, comparable to the one we described for ST648 in paper II.

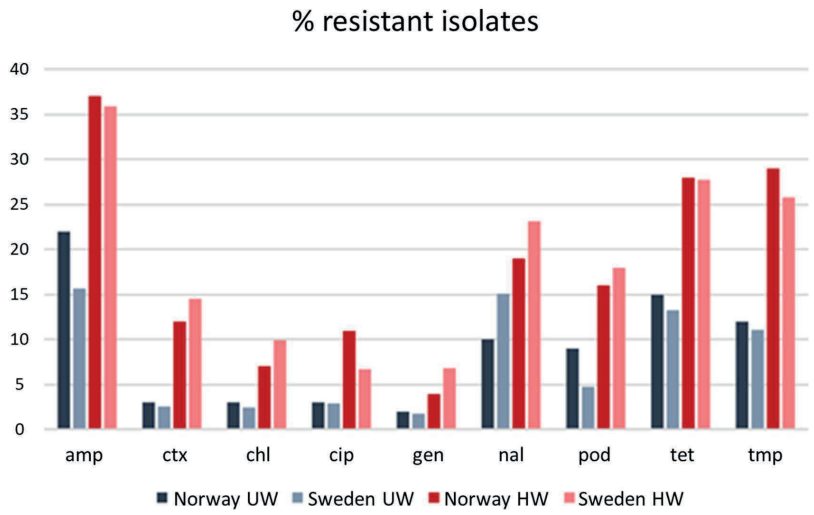

Figure 13. Antibiotic resistance rates in urban and hospital wastewater from Norway (Paper I) and Stockholm, Sweden[92].

The community area in this study housed approximately 510 inhabitants. The large numbers of reoccurring PhP types of ESBL *E. coli* and the generally low diversity levels in community wastewater (CW) suggests that the isolates we screened in paper I were either; **i**: shared between a large portion of the population producing that wastewater, or **ii**: dominant clones of *E. coli* reside in the wastewater system. Unpublished research from the Karolinska Institutet using the PhP methodology showed that healthy individuals commonly had one to three dominating PhP types in their intestinal microbiota. The researchers found an average of 4.1 PhP types per individual among a total of 841 *E. coli*, supporting the finding of the low diversity in CW from the present study (manuscript in preparation). Although I was unable to further address this issue during my project, a possible way forward could be to cultivate biofilm from the pump station walls where the wastewater had been sampled after sterilizing the surface. In

paper II, we described the capacity for biofilm formation in representative isolates from the ST131 and ST648 groups. The four ST131 isolates were classified as high biofilm producers while the ST648 were divided between high (n=4), medium (n=3) and low (n=4) producers. The significant increase in biofilm production observed in several isolates when cultured at 28 °C versus 37 °C indicated that the biofilm production is indeed associated with environmental habitation (Paper II). These finding supported our hypothesis that these isolates could be able to survive in the pump station over prolonged periods. Also, Farkas *et al.* found that the correlation between *intI* and *qac* cassettes (mediating resistance to quaternary ammonium compounds) in drinking water treatment plants was higher in biofilms compared to planktonic cells[112], a further indication that biofilm formation is a potential catalyst for dissemination of AR. A different approach would be to sample the human population from which the wastewater originated. The first might be the most difficult to perform from a technical point of view, whereas the latter might be faced with confidentiality issues, restricting the collection of fecal samples from volunteers. Either way, we would potentially obtain valuable information regarding the underlying causes for the microbiological status of the community wastewater.

By comparing the multiple antibiotic resistance (MAR) indices for the total populations of *E. coli* per sample type (MAR_{total}) to the PhP type-adjusted MAR indices (MAR_{type}) in paper I, we made an interesting observation. The MAR_{type} indices were higher than the corresponding MAR_{total} indices in HW and UW, undermining our initial hypothesis that multi-resistant clones were more likely to dominate the wastewater environments with high selection pressures. This was not the case for CW, where the MAR_{type} was indeed lower than the MAR_{total}, demonstrating an abundance of resistant isolates over susceptible ones. In paper II, we observed the presence of several metal resistance genes including copper after WGS of the representatives from ST131 and ST648. We then reported that all ST131 and ST648 isolates were tolerant to heavy metals at concentrations equivalent to those of the susceptibility control strains. The concentrations measured in CW were however >1000-fold lower than the MIC levels for copper and zinc, indicating no selection pressure from heavy metals that would explain the continuous presence of these clones in the CW. Additionally, the isolate from the first sampling campaign analyzed by WGS (ST648-BE01A-01) carried several resistance genes not found in other ST648 during succeeding sampling campaigns. The subsequent isolates carrying fewer resistance genes suggested little to no selection

pressure from antibiotics or heavy metals in CW. This agreed with the antibiotic concentrations in the community outlet, further suggesting no selection pressure was present to explain the finding of the ST131 and ST648 (Paper III). On the other hand, previous studies have reported *bla*$_{CTX\text{-}M\text{-}15}$ to confer no fitness cost to the carrier and that it may in fact provide a competitive advantage[113,114]. The resistance rates in CW positioned in between the rates observed for HW and UW, causing further confusion. A possible explanation is that the multi-resistant clones found in CW are indeed endemic to the human population in that community, or as previously discussed, the bacteria could be growing in the pump station. The latter was the inspiration of several ideas regarding potential explanations for this phenomenon. I attempted to collect wastewater from the manhole cover just upstream from the pump station. One isolate among the 720 isolates analyzed did indeed match the PhP type of the ST648. However, because the sampling was performed with the same equipment, albeit rigidly rinsed between collection of the individual samples, the single matching isolate was disregarded as possible contamination. We would have expected to find numerous isolates matching the PhP types upstream of the pump station to explain the repeated isolation of ESBLs in the CW. Our study reveals the possible role non-hospital wastewater outlets may have in the dissemination of multi-resistant bacteria. However, the representability of the community outlet as a proxy for non-hospital outlets in general needs further investigation.

The last monthly sample showed much higher rates of resistance than the rest of the HW samples. No change was detectable in the UW wastewater samples (Paper I). This follows the development of resistance in clinical isolates from blood cultures reported by the Norwegian NORM/NORM-VET reports from the corresponding period[15,115]. Norway has enforced a strict antibiotic usage policy to combat antibiotic resistance during recent years. Rates of resistance in other European countries were relatively constant, and for some even decreased as reported by the ECDC in 2018[54].

Transmission of antibiotic resistant bacteria can primarily be mediated through clonal expansion of already resistant strains, or by HGT. Whereas HGT does not alter the bacterial community composition directly, clonal expansion should result in a reduced diversity. This may partly explain the low Di values observed in CW (Paper I). Indeed, the isolates belonging to ST648 clustered closely together despite being collected during a period of over a year (Paper II). The *bla*$_{CTX\text{-}M\text{-}15}$ genes were conserved between the

ST648 of this study, as would be expected due to clonal expansion. However, the conserved CTX-M-15 plasmid identified in the ST648 isolates of this study has been found also in the feces of wildlife in the United States[116] and in isolates from river water in India[117]. Thus, HGT has probably been involved at some point to produce the ESBL *E. coli* that have since been established in the community and/or wastewater pump station investigated here. The levels of β-lactam antibiotics in the CW fails to explain the rates of ESBLs observed from this site (Paper III). Indeed, among the three wastewater sampling sites the community site had the lowest overall concentrations of β-lactam antibiotics. As such, we would have expected higher numbers of ESBLs also in UW.

In wastewater collected in 2011, Brechét *et al.* (2014) detected ST131 among ESBL-EC isolated from "community wastewater" (wastewater not directly connected to hospitals, but including some industrial waste and stormwater), HW, UW, river water and patients, but not in the treated WWTP effluent[102]. ESBL-EC ST648 was also isolated from patients, but in none of the environmental samples. The ESBL enzymes were predominantly of the CTX-M variety (88%), including CTX-M-1 (50%) and CTX-M-15 (25%). SHV-12 was the only variant of *bla*$_{SHV}$ that was detected, but it made up only 5% of the ESBL enzymes. Of the seven ST131 detected in environmental samples, only one produced CTX-M-15, whilst the rest produced CTX-M-1 (n=3), -27 (n=2) and -14 (n=1). The four ST131 detected from clinical samples, however, all produced CTX-M-15[102]. The ST648 were one of only two exceptions linking CC/ST to specific sampling sites, as it was not found in any environmental samples, whereas there was a high degree of genetic diversity in the rest of the ESBL-EC population[102]. Korzeniewska and Harnisz (2013) detected *bla*$_{CTX\text{-}M\text{-}1}$ and several others CTX-M variants, but no *bla*$_{CTX\text{-}M\text{-}15}$ in their ESBL-phenotype positive isolates from urban wastewater and environmental recipients[118]. They also detected TEM and OXA variants mediating both ESBLs and non-ESBL beta-lactamases and SHV variants mediating ESBLs. It is quite evident that *E. coli* has the ability of substantial diversification. In a recent review by Peirano and Pitout (2019) where the authors summarized recent updates on ESBL research, CTX-M-15 and ST131 were the globally dominating ESBL enzymes and sequence types, respectively[58]. Thus, the presence of a multi-resistant ST131 harboring *bla*$_{CTX\text{-}M\text{-}15}$ observed in community wastewater in this study is a worrying observation.

I did not search specifically for ST131 in the present study. However, based on the number of isolates exhibiting phenotypic ESBL resistance belonging to the PhP type described in Paper II, an estimated 7 % of the ESBL-EC population in CW belonged to ST131 during 2016-2017. This particular PhP type was (almost) completely absent from HW and UW samples (Paper I), indicating that the ST131 may have several PhP type profiles, or more unlikely, that the hospital samples included here contained very low numbers to no ST131 isolates. A Canadian study detected a decrease in the prevalence of virulent *E. coli* including the B2 phylogroup during WWTP treatment[119], indicating that the ST131 is underrepresented in the environment and may be poorly adapted to survival here[77]. Our observation of a continuously reoccurring clone of ESBL-EC ST131 contrasts this hypothesis as it appears to be thriving in the community wastewater pump station, unless it is being persistently released into the environment from (presumably) healthy human carriers (Paper II). Brechét *et al.* (2014) also requested further investigation into the contrasting prevalence of ST131 in the environment (rare) to humans (abundant)[102]. In their study, they reported ST131 to constitute 3 % of the ESBL-EC in UW/HW and 10 % of those from inpatients[102]. Increasing frequencies of ESBL-EC among *E. coli* were reported from Canada between 2000-2010, in which the rates of ESBL-EC increased from 0.3 % in 2000 to 14 %, whereof 78 % of the ESBL-EC belonged to ST131 in 2010[120]. In an Austrian study, Zarfel *et al.* (2013) demonstrated that UTI-related ESBL-EC more often carried CTX-M-15 whereas CTX-M-1 predominated among isolates from wastewater sludge[121]. This suggests that the detection of CTX-M-15 indicates the simultaneous presence of ST131 among an ESBL-EC population, as this sequence type may be more frequently cultured from clinical samples[102]. The normalized abundances of ARGs targeted by the primer pairs “CTX-M” and “CTX-M-1,3,15” were comparable between hospital and community wastewater samples, whereas lower abundances were observed in samples of urban origin (Paper III).

Primer pair hits for bla_{VIM} and bla_{NDM} were observed in almost all hospital samples during our sampling campaign (Paper III). bla_{NDM} has been found in a few clinical isolates in Norway during the last decade[122], but not to an extent that can explain the frequent detection of the gene in hospital wastewater from this study (Paper III). *Pseudomonas aeruginosa* have been described to be frequent carriers of bla_{VIM} (and ciprofloxacin resistance) and have been isolated on several occasions[77]. Studies on VRE are also describing higher occurrences in hospital wastewaters of vancomycin resistance

compared to urban wastewaters[77], coinciding with our findings, although vancomycin resistance genes were scarcely observed, and some were not detected at all (Paper III).

The number of contaminants of emerging concern (CECs) registered in the chemical abstracts service (CAS) increases by 4 000 per day and has increased from less than 20 million inorganic and organic substances in 2000 to over 130 million in 2017[123,124]. While metabolism can reach 90 % for some compounds[65], and some antibiotics may have half-lives of just a few hours, some antibiotics conversely are resistant to natural degradation[125]. Thus, some antibiotics may be released unmetabolized into the environment, although we detected no oxytetracycline in any of the samples from this study (Paper III). Wastewater is frequently a topic of interest among the Norwegian population[126]. In a news article in 2017, Marine biologist Professor Ketil Hylland at the Norwegian University of Oslo requested that local treatment of HW should be enforced to avoid collateral environmental pollution of the Norwegian coast line[127]. The comment was made as a response to an earlier article reporting that untreated wastewater from 1.5 million Norwegians including hospitals (~30 % of the Norwegian population) was discharged directly into the ocean by municipalities that did not meet the demands for wastewater treatment (Figure 14)[128]. In contrast, the highest concentrations of ciprofloxacin observed in HW in this study were half that of a study of Vietnamese rural HW where researchers measured ciprofloxacin at concentrations of up to 40 µg/l[129]. Concentrations as high as 600 µg/l ciprofloxacin were detected in HW from the Netherlands, and also at 100 µg/l in household toilet samples from the same study[130].

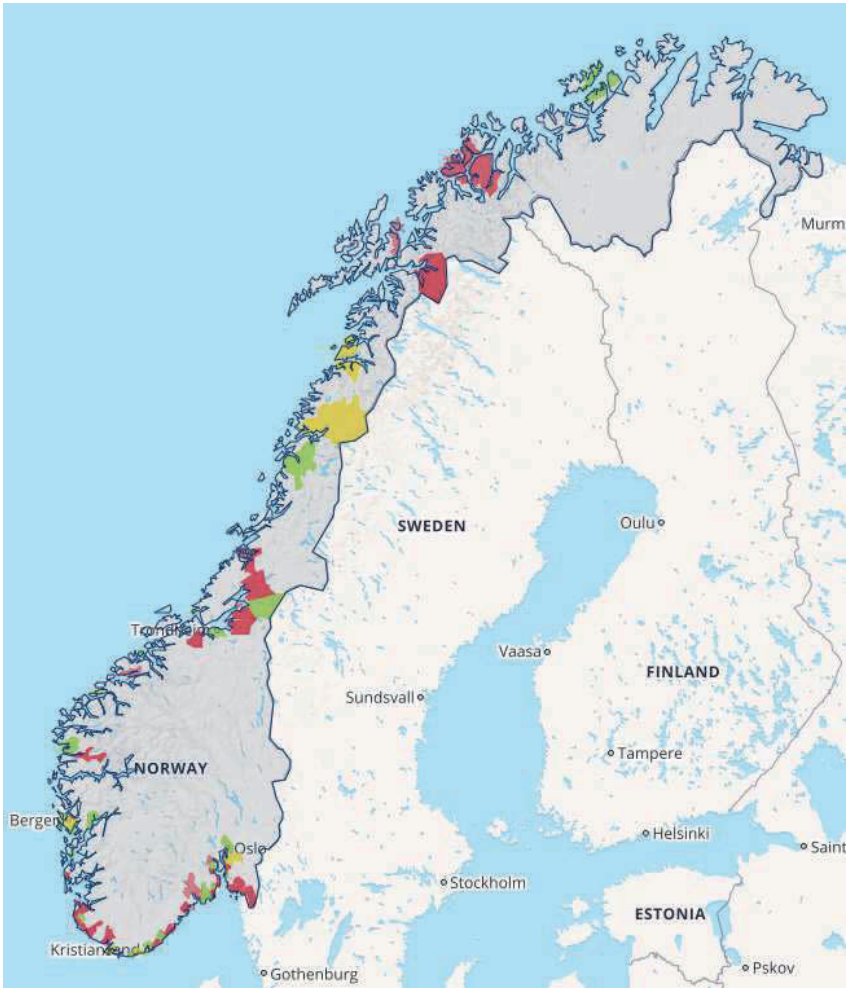

Figure 14. Map presenting data for the catchment areas of the 240 largest WWTPs in Norway that have high population densities and that discharge their treated effluents into the ocean. Data are from Statistics Norway (SSB). Inland municipalities are not shown. Areas that partially or completely break regulations for treatment are highlighted (red). Missing data (yellow) and areas that meet the requirements (green) are also shown.[128]

Caucci *et al.* (2016) observed higher relative abundances of ARGs during autumn and winter season, agreeing with the higher rates of antibiotic prescriptions during these seasons[131]. Similarly, we observed a significant drop for all antibiotics during the summer months, most noticeable in CW, but there were no apparent seasonal variations in the normalized abundances of ARGs (Paper III). It is important to note, however, that antibiotic concentrations in wastewater are direct consequences of antibiotic consumption where an increase of intake should swiftly increase the levels in wastewater, whereas effects on the resistome of a human population requires more time. In paper III, we compared the monthly antibiotic concentrations in the wastewater per antibiotic class to the antibiotic prescription data (expressed as DDDs) for the corresponding human populations of the wastewater outlets and inlet. Three positive correlations (correlation coefficient, $r > 0.50$) were found in HW: MLSBs ($r = 0.83$), trimethoprim ($r = 0.68$) and sulfonamides ($r = 0.56$). For β-lactams, fluoroquinolones and tetracyclines in HW, and for all antibiotics in CW and UW, correlation coefficients were all < 0.50. Paper III thus indicates that the usefulness of information about antibiotic prescription data is limited at best in prediction the resulting effects on wastewater concentrations. However, it is worth noting that prescription data were based on total monthly values, whereas concentration measurements only covered three days per month. Additionally, the community wastewater originated from a population size only 1:240 that of the entire municipality to which the prescription data applied.

No samples in this study were positive for mecA, a gene typically associated with staphylococci on mucosal and skin surfaces, similarly to a broader European study utilizing the same highly parallel qPCR array[100], although other studies have detected mecA in wastewater effluents and even drinking water effluents[132–134]. MRSA in wastewaters may reflect carriage, and not necessarily an enhanced risk for acquisition from the environment[77].

Other potential hot spots for antimicrobial resistance may be related to livestock production, pharmaceutical plants or other types of factories/facilities. Pharmaceutical production facility effluents may be a potential target for measures, as studies have indicated higher abundances of ARGs in sludge from such facilities compared to traditional WWTP sludge[135]. Noyes *et al.* (2016) showed that livestock production is a potential hot spot for dissemination of antibiotic resistance in a study on effluents of dairy and beef production using a metagenomics approach where tetracycline resistance

genes were predominant (particularly *tetQ* and *tetW* sequences)[136]. The implementation of advanced quaternary wastewater treatment technologies has recently been estimated to cost only about $20 (4-5 Big Macs) per person per year[123]. Several researchers have reported increased levels of some resistance factors during wastewater treatment, supporting the implementation of advanced treatment in the WWTP rather than in selected hotspots[102,112,137–143].

Advanced wastewater treatment may be essential to achieve sufficient removal of antibiotic resistance factors before treated effluents are discharged into the environment. Several alternatives for advanced wastewater treatment exist. One such alternative is ozone treatment, one of the most rapid treatment options for ARBs and ARGs[144], although it faces limitations in targeting intracellular ARGs with doses commonly used for elimination of micro-pollutants[145]. This fact could cause problems with pathogens exhibiting low susceptibility to ozone like *Pseudomonas aeruginosa*, whereas others including *Enterococcus spp.* are highly susceptible to ozone treatment[146]. *E. coli*, including some antibiotic resistant phenotypes also appear to be susceptible to ozone treatment[147]. Another alternative of advanced treatment is chlorination, which has been demonstrated to reduce abundances several ARG, although six of the 282 included ARGs were simultaneously enriched[148]. On the other hand, one study demonstrated poor efficiency on ARB inactivation from chlorination treatment (< 90 %)[144], and those bacteria that survive chlorination may also possess chlorine resistance genes that could co-select for ARGs[149]. Applying advanced treatment technologies may also have disadvantages, as chlorination has been reported to produce carcinogens[150]. Ultraviolet radiation is a non-chemical process of wastewater disinfection. A study using a combination of UV surface exposure and H_2O_2 reported a reduction in total coliforms including antibiotic resistant *E. coli*, although several ARGs (*blaTEM*, *qnrS* and *tetW*) were still present after 4 hours of treatment[151]. Zhang *et al.* (2016) demonstrated promising results when exposing artificial wastewater containing sulfonamide antibiotics to UV light[152]. In a pilot experiment with equipment specialized for the complete exposure of all material to UV-C light (up to 3kJ/cm^2), we found similar results in regard to reduction of *E. coli* as those reported by Ferro and colleagues[151]. We also found a considerable reduction of several antibiotics including ciprofloxacin (1-2 log reduction), viral particles (adenovirus, norovirus and bacteriophages) and DNA degradation (strand breakage), indicating that treatment with UV light may be a promising alternative (data not published). However, although UV light is used

extensively in water treatment facilities, wastewater is more challenging because of its high particle density. Also, UV radiation has been demonstrated to produce byproducts that may have comparable effects as the original compounds[153]. In summary, several options for advanced treatment of wastewater are available, all of which have been shown in some studies to be effective against the levels of antibiotic resistant bacteria, antibiotic resistance genes or antibiotics in wastewater. However, other studies have found the absence of effect, or even enrichment of resistance during treatment. No wastewater treatment technologies exist to date that specifically target resistant microbes over susceptible ones, or antibiotic resistance genes over other genes. Treatment of wastewater will on its own probably not be enough to completely deal with the rising tide of antibiotic resistance. This problem requires supplementary measures as part of a larger public health strategy, including reducing the overall antibiotic consumption through public and medical professional awareness campaigns, discovery of new drug alternatives and stricter regulations regarding management of antibiotic-contaminated wastes. A better understanding of the main transmission routes for antibiotic resistance to the environment, including the characterization of so far unrecognized sources of wastewater carrying high levels of antibiotic resistance will be important.

4.2 Methodological considerations

Selection of sampling sites

The underlying goal of the study was to answer the question of whether hospitals' wastewater effluents not just contribute to dissemination of antibiotic resistance, but to such an extent that they should be required to purify or destroy these effluents before they reach the main wastewater system. In order to answer this question, three sites were selected for sampling of wastewater. The first, Oslo University Hospital, Rikshospitalet ("*National Hospital*"), was selected as a representative hospital outlet. The hospital has one outlet to which all wastewater is transported, making for an optimal location for sample collection, although the arrangement of the sampling site required some practical adaptation before tolerable sample quality was achieved. The hospital is a highly specialized referral hospital, potentially reducing its suitability as a general representative of Norwegian hospitals. Nevertheless, the project's goal was to identify whether hospitals required supplementary treatment of wastewater effluent exceeding that of the urban WWTP. In this way, using a specialized hospital that might have a relatively higher occurrence of diseases including antibiotic resistance compared to a general practice hospital would be advantageous rather than a drawback. Because 90 % of antibiotics in Norway are prescribed in the primary care, a community outlet was selected as a "negative" control for comparison with the levels of antibiotic resistance of the hospital wastewater. The community site investigated in this study was chosen for several reasons. Firstly, it received no input of wastewater effluents from hospitals, nursing homes or similar institutional facilities. Secondly, it was sealed off from surface runoffs, limiting the confounding factor of heavy rainfalls. The results from this particular community outlet raised several new questions, including the possibility that other wastewater outlets not previously considered important in terms of antibiotic resistance could be potential hotspots for both multi-resistant and pathogenic bacteria like *E. coli*. Whether or not the community and/or the pump station are unique in this regard may require a new PhD project. Studies that have examined hospital wastewater or other sources of wastewater often also include the WWTP that receives the wastewater from these sources. There are two large WWTPs treating wastewater from Oslo and surrounding municipalities, namely VEAS (~70 %) and BEVAS (~30 %) (Figure 1 in paper I). The VEAS plant receives the wastewater from the hospital and community sites through the same wastewater pipeline and was thus the preferred

choice. Whereas the community wastewater was the negative control for the hospital wastewater within this study, the WWTP was an external control to better be able to compare the results of the study to other studies on antibiotic resistance in wastewater. The panel of ARGs and MGEs (n = 254) included in the recent study by Pärnanen *et al.* (2019) was broader than that of the current study[100]. Nevertheless, the same antibiotic classes were represented in both studies, and both studies sampled wastewater from the same time periods and from the same Norwegian WWTP. We included a delay between the exact start of sampling for each site (Paper I). This was done to compensate for the estimated transport time of the wastewater to the WWTP of 6 hours (HW) and 4 hours (CW). However, the 24-hour continuous sampling most likely alleviated any potential effects of mismatches between hourly aliquots. Similarly, the inaccuracy of a travel time estimate through a 20-40 km tunnel is probably significantly wider than one hour.

Sampling procedure

We wanted to avoid bias possibly introduced by intact fecal particles in samples collected by the traditional grab sampling, where one sample is essentially collected from one aliquot of wastewater. Thus, sampling was performed using automated samplers collecting aliquots of 200 ml over approximately 24 seconds every hour. The aliquots were afterwards pooled and vigorously (exhaustingly) mixed in a 10L plastic container for maximal homogenization. Low diversity levels for hospital and community samples were observed throughout the study, although urban wastewater samples, processed in the exact same manner, were much more diverse in terms of microbial contents. The latter indicates that the low diversity levels observed in paper I is likely due to the short distance between the toilet bowls and the sampling site, or a consequence of a smaller population supplying the wastewater. Several arguments point to the former, as individual 200 ml aliquots were isolated at two occasions containing a red-tinted liquid. This was probably a result of hemorrhagic gastroenteropathy in a patient whose visit to the toilet lined up perfectly with the 24 second window of that hour (Figure 15A). A further argument is that in the samples with the lowest diversity indices, over half of the 80 isolates expressed the same biochemical phenotype. Because 80 isolates were selected from 24 separately sampled aliquots, and average Joe defecates usually no more than twice per day, the bacterial composition should have been much more diverse from the pooled wastewater sample (3.3 isolates per aliquot on average). Thus, it seems that the no matter how forcefully the pooled wastewater is

shaken, the wastewater still requires a 20-40 km trip in order to be completely homogenized. Remnants of toilet paper and/or biofilm could regularly be observed sticking to the hard-to-reach inside of the 5 m long plastic tube used to collect samples from CW (Figure 15B). The risk of cross-contamination between daily and monthly sampling occasions was taken seriously, and thus the sampling equipment was rigorously cleaned as described in paper I. The results obtained from UW showed consistently high diversity indices however, indicating that this was likely not the sole reason for the low diversity indices observed in HW and CW, despite the inferior access to water to clean the equipment post-sampling.

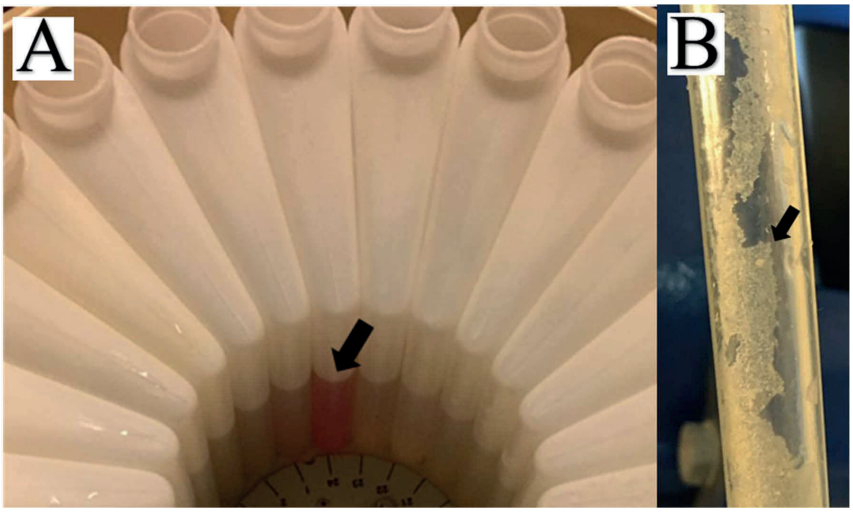

Figure 15. A) Individual 200 ml aliquots of hospital wastewater are collected in separate plastic bottles. The twenty-forth aliquot (arrow) was probably collected immediately after a patient with hemorrhagic gastroenteritis (or hemorrhoids) flushed the toilet. B) Plastic tube used for wastewater sampling. A combination of toilet paper remnants and/or bacterial biofilm growth can be seen on the interior surface of the tube (arrow).

Pilot campaigns 01-03

The three initial sampling campaigns (01-03) were run during sampling optimization. Hospital samples proved quite troublesome because of the high velocity / low flow of the wastewater pipeline. We therefore concluded to extend the sampling by another three months to compensate for the initial suboptimal samples. In short, the sampler struggled with the collection of correct amounts of wastewater per aliquot in the 24-hour composite samples because of air that would regularly enter the tube as it was too light to stay suspended below the surface. The solution was to acquire a specialized sampler head, a heavy metal piece built for exactly those circumstances, from a specialized supplier of laboratory equipment (Dipl.ing. Houm AS). The new piece coincidentally relieved the need for a hose clamp to keep it in place. The hose clamp used during the pilot sampling campaigns would catch floating debris, mainly toilet paper, further reducing the efficiency of the sampler. ARGs were only analyzed for the last 12 months, whereas data for month 02 and 03 were obtained as the samples were shipped before the decision to exclude the first three campaigns was made, but those data should be interpreted with caution (Paper III). Analysis of ARBs performed in paper I disregarded sampling months 01-03 completely, but for paper II, we included isolates collected during this period also. Paper II asked the question of whether the PhP types of these isolates were present in any given sample, and although the quality of these samples was poor, the presence of the first ST648 (BE01A-01) was likely unrelated to any quality issues. This isolate, the whole basis of the second paper, was coincidentally also the very first *E. coli* picked from the CHROMagar plates from the community wastewater in March 2016. The lower diversity in the initial samples highlights the importance of proper sampling protocols, which could have saved a few months of work, although it was an educational process.

Analysis of *E. coli*, antibiotics and ARGs

E. coli was selected as model organism as it is well described in the literature and has the capacity to acquire numerous resistance mechanisms including multi-resistance phenotypes. The cultivation and subsequent biochemical phenotyping and simplified broth dilution resistance determination used in paper I was selected because of previous experience with the method by the Swedish collaborators. Alternatives that could have replaced this methodology include selective cultivation with and without supplemented antibiotics, and metagenome sequencing. The PhP typing is a relatively rapid method of

screening hundreds or thousands of bacterial isolates, with the advantage that it allows the simultaneous analysis of multiple resistance phenotypes from each bacterial isolate. Selective cultivation can in a shorter time provide results on resistance proportions in bacterial populations, but with lower resolution compared to the PhP typing for the reason mentioned above. The resolution of metagenome sequencing is superior for its particular area, but the resulting sequence data often lacks the association between bacterial species, resistance properties and occurrence of multiple resistance within individual organisms. Finally, experience with the chosen method between members of the Swedish collaborator was also a deciding factor.

A pilot study was run during autumn 2015, before I started in the position as a PhD student. In the pilot, 24 isolates (3 test plates x 8 isolates/plate) were cultivated per sample. The resulting analysis indicated that this number was too low. Because the exact number needed was uncertain, 80 isolates per sample was deemed fitting, as ten plates (+ controls) would be needed per sample. The total monthly analysis thus included 720 isolates (3 days x 3 sites x 80 isolates/sample). To avoid bias in selection of large colonies over small ones, all isolates that were sufficiently isolated from neighboring bacteria were picked for further analysis by PhP and AREB from one CHROMagar plate (Figure 16C). A potential confounder here was the time-consuming setup of the assays, possibly allowing for bacterial growth within the same time-frame, which could skew the results one way or another. There are robots capable of performing such protocols on a large scale, however, no such robots were available for this project. Also, these robots be better suited for the transferal of relatively soluble liquids. Mucoid bacteria may be problematic when running automated robots that may be less capable of distinguishing between alternating viscosities during preprogrammed operations. Although we found that the ST131 from community wastewater in this study were excellent biofilm producers and excessive capsular production has been suggested to inhibit the capacity for biofilm formation[154], clones belonging to ST131 are also known uropathogens, a trait which is frequently associated with bacterial colonies with mucoid appearances after cultivation on agar plates (Figure 16). Thus, the manual analysis of with the PhP/AREB methodology was arguably a superior choice for this particular study.

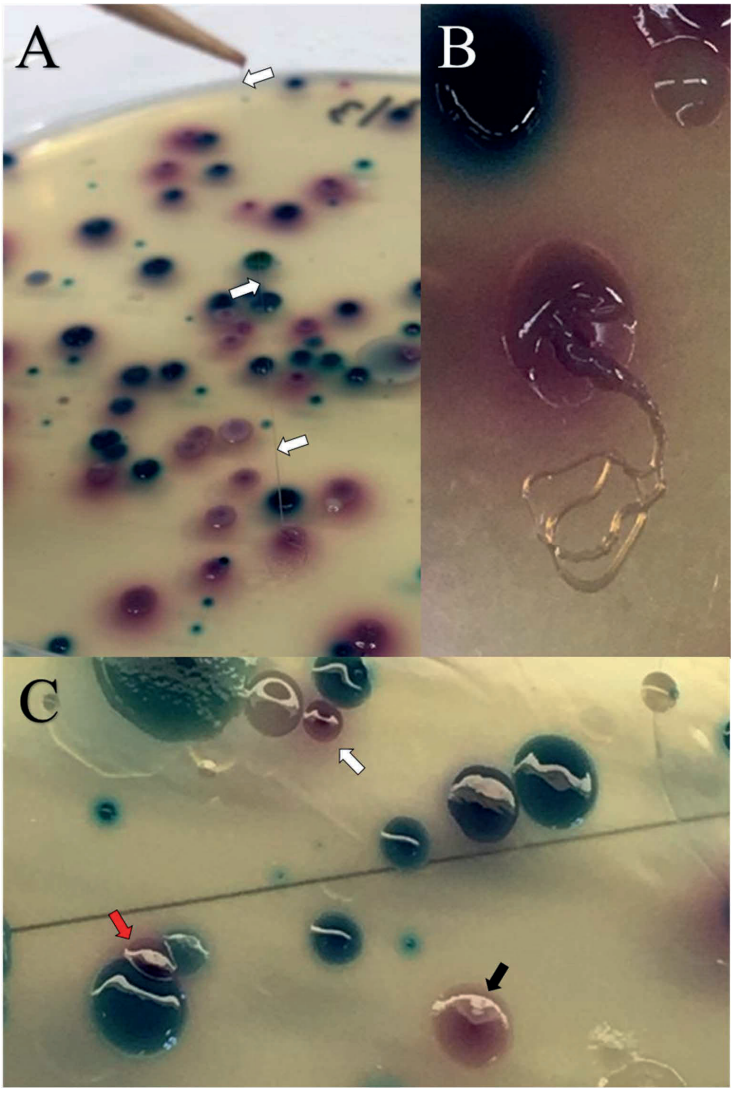

Figure 16. *E. coli* from hospital wastewater on CHROMagar agar plates growing with extensively mucoid colonies. A) A particularly mucoid colony has been picked up with a sterile tooth pick and stretched (white arrows) a good distance from the agar surface. B) Close up image of the same colony. The lower half of the colony is dislocated from contact with the tooth pick. C) Three colonies of *E. coli* (dark red) are visible. One colony is well isolated (black arrow). The second colony involves a risk of contamination (white arrow) of surrounding colonies. The third colony is guaranteed to involve contamination (red arrow) of surrounding colonies.

Imaging of PhP and AREB microtiter plates was performed using a HP Deskjet G4050 desktop scanner with the same software settings as for the one used by the research collaborators at Karolinska Institutet in Stockholm, Sweden. Although a desktop scanner is not necessarily deemed the most accurate laboratory instrument, we obtained surprisingly consistent imaging results throughout the study, with susceptible and resistant control strains being clustered together with a Di index of >0.96 overall. Previous experiences at Karolinska Institutet have generated similar results (data not shown). An appearance of "half-moon" artefacts in the AREB plates occurred intermittently during regular use of the scanner (Figure 17A). The problem appeared at random times and rescanning of the plates without moving the scanner would produce normal images without further problems (Figure 17B). A component located just beneath the glass plate of the scanner was suspected to be responsible for the phenomenon, however the underlying cause was never verified nor eliminated. A little patience, however, resulted in acceptable images of all plates, and all images without half-moons were of equal and good quality. Also, the problem was detected before the end of the third sampling campaign, avoiding any interference with the results from the remaining twelve months from which Paper I was assembled. Intraassay results appear to be reliable, but one should be cautious in comparing results from different laboratories despite using the same methodology. Nevertheless, the total resistance rates observed for *E. coli* in both HW and UW were relatively consistent with those observed for equivalent samples from HW and UW a few years earlier from Stockholm, Sweden, a country sharing geographical and cultural properties with Norway[92].

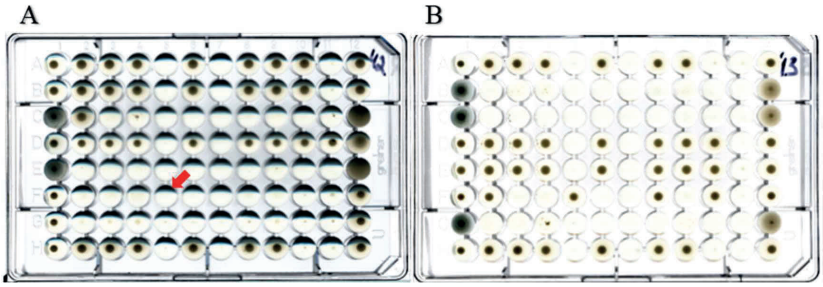

Figure 17. AREB microtiter plates used for phenotypic resistance determination. A) An AREB plate exhibiting the half-moon artefacts (red arrow) of unknown origin. B) An AREB plate exhibiting satisfactory quality for subsequent automated analysis of resistance profiles with the PhP Software. See Paper I for further description.

Antibiotics included in the AREB plates were predetermined by PhPlate AB, the manufacturer of the plates, based on previous experiences from own research and relevant antibiotic classes. Originally, these plates contain ten antibiotics, but we elected to disregard the columns containing the ESBL indicator antibiotic ceftazidime (CAZ). A couple of arguments supported this decision. Firstly, we observed a high rate of carpet growth-like plaque forming in wells containing CAZ without the simultaneous presence of a bacterial pellet. The growth was suspected to be a biofilm induced bacterial stress from the antibiotic, but nonetheless made evaluation of pellet growth difficult. The analysis software was not able to separate between these dark carpets of growth, which to the naked eye clearly contained no pelleted bacterial cells. The second and closing argument was that the AREB plates also contained the ESBL indicator antibiotics cefotaxime and cefpodoxime, exhibiting much lower rates of pellet-less biofilm production. As most isolates exhibiting phenotypic ESBL resistance have previously been demonstrated to carry one or several genes encoding ESBLs[92], the inclusion of CAZ could result in an overestimation of ESBL resistance in all wastewater sample types and thus it was excluded from the study.

By including just one isolate per PhP type in the analysis of the multiple antibiotic resistance (MAR_{type}) in paper I, we might be able to partially compensate for the undesirable effects of low-quality samples. Because the phenotypic diversity in one fecal particle is lower compared to the diversity in two unrelated fecal particles combined, we might reduce the bias that a singular fecal particle brings by removing reoccurring PhP types before analysis. The MAR_{type} index simultaneously reduces the resolution of the data, as fewer data points are included. The consequences of relying on the MAR_{type} index is that we might unintentionally remove many isolates that do in fact occur naturally at high frequencies in the wastewater. Multiple individually sampled batches of wastewater collected in parallel or right after one another may give an indication of which scenario is the most likely one. In my study, we isolated the same PhP types of both the ST131 and ST648 from multiple samples throughout a full year. The finding indicated that large numbers of identical isolates dominate in the CW, whereas the same phenomenon is not observed in HW or UW. Most likely, the MAR_{type} index can at best be applied as a supplementary analysis to elaborate on the microbial population responsible for the value of the MAR_{total} index.

Antibiotic resistance genes were analyzed by a highly parallel qPCR assay in an established research group at the Michigan State University. The genes selected for analysis were based on an initial screening of the pooled sample material from all campaigns for 384 resistance genes, separated by sampling site. Based on this initial screening, 144 resistance genes were selected for more in-depth analysis with the following criteria: i) genes of emerging/clinical threat (e.g. *ndm-1*, *mcr-1*, *mcr-2*, *mecA*, *van*) irrespective of their detection during the initial screening; ii) the genes most frequently detected in all sample types. Although all 384 genes in the full array are included due to their varying international significance, increasing the resolution of the study by analyzing a higher number of samples required the exclusion of several genes to not exceed the project's budget.

A total of 53 antibiotics and their metabolites were analyzed in paper III. Paper III was organized as a mostly descriptive study, presenting the concentrations of antibiotics and their seasonal variation for each of the three sample types. The prospect of comparing the wastewater concentrations of antibiotics to the prescription registers for the corresponding populations (hospital/municipalities) to see whether any correlations between antibiotic consumption and subsequent release of antibiotics into the wastewater effluents could be identified was interesting. The data sets of antibiotic concentrations and prescriptions were not particularly well suited for comparative because of few overlapping compounds between the two data sets. Immediately, this is an indication that there are few if any correlations between the data. Those data that are only present in the prescription register could be non-existent in the panel of antibiotics analyzed in the wastewater, or they could be metabolized before excretion, or not excreted whatsoever. In contrast, antibiotics only found in the data set from measurements of wastewater concentrations are most surely interesting findings, albeit hard to explain. Antibiotic prescription in Norway is strictly regulated, and the presence in wastewater of an antibiotic that is not sold in Norway must imply one of two things: i) the antibiotic has been bought abroad before subsequently being brought into the country, or ii) the methodology is flawed. The latter seems unlikely due to the expertise of the research group that performed the analysis. The prospect that Norfloxacin could have been bought abroad is not impossible, or even unlikely, but the concentrations found in the three consecutive samples of community wastewater, if true, are indicative of a larger release of antibiotics into the wastewater than just from the use of a single individual or family. Increasing public awareness regarding the risks and consequences of antibiotic use and abuse could thus be an important prioritization in the future.

5. Main conclusions

- The diversity values of the *E. coli* community in samples from urban wastewater were consistently at levels representative of a large human population.
- Hospital wastewater contained higher rates of antibiotic resistant strains of *E. coli* including ESBL-producing and multi-resistant strains compared to community and urban wastewater.
- Several antibiotic resistance genes of emerging/clinical relevance were frequently detected in hospital wastewater.
- High levels of mobile genetic elements in untreated wastewater from different sources may indicate an untapped potential for dissemination of antibiotic resistance in the future.
- The relative contribution of hospital wastewater to the dissemination of antibiotic resistant bacteria to the wastewater treatment plant was low.
- Community wastewater may be a previously unrecognized source of antibiotic resistant bacteria including globally disseminated, multi-resistant, pathogenic clones of *E. coli*, specifically ESBL-producing strains belonging to sequence types ST131 and ST648.
- Wastewater pump stations receiving non-hospital wastewater may play a role as bioreactors in enriching antibiotic resistant bacteria.
- Investigation of further wastewater outlets as potential hot-spots for the dissemination of antibiotic resistance is warranted.

6. References

1. Waksman SA. What is an antibiotic or an antibiotic substance? *Mycologia*. 1947;39(5):565-569.
2. Mohr KI. History of antibiotics research. In: Stadler M, Dersch P, eds. *Current Topics in Microbiology and Immunology*. Vol 398. Springer, Cham; 2016:237-272. doi:10.1007/82_2016_499
3. Kuriyama T, Karasawa T, Williams DW. Antimicrobial chemotherapy: Significance to healthcare. In: Percival SL, Williams DW, Randle J, Cooper T, eds. *Biofilms in Infection Prevention and Control: A Healthcare Handbook*. Elsevier; 2014:209-244. doi:10.1016/B978-0-12-397043-5.00013-X
4. Durand GA, Raoult D, Dubourg G. Antibiotic discovery: history, methods and perspectives. *Int J Antimicrob Agents*. 2019;53(4):371-382. doi:10.1016/j.ijantimicag.2018.11.010
5. Markel H. The real story behind penicillin. *PBS NewsHour*. https://www.pbs.org/newshour/health/the-real-story-behind-the-worlds-first-antibiotic. Published 2013. Accessed October 31, 2019.
6. Danielsson D. Gonorrhoea and syphilis in Sweden - past and present. *Scand J Infect Dis Suppl*. 1990;69(69):69-76.
7. Clatworthy AE, Pierson E, Hung DT. Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol*. 2007;3(9):541-548. doi:10.1038/nchembio.2007.24
8. Tippett R. Mortality and cause of death, 1900 v. 2010. *Carolina Demography*. https://www.ncdemography.org/2014/06/16/mortality-and-cause-of-death-1900-v-2010/. Published 2014. Accessed November 10, 2019.
9. Kapoor G, Saigal S, Elongavan A. Action and resistance mechanisms of antibiotics: A guide for clinicians. *J Anaesthesiol Clin Pharmacol*. 2017;33(3):300-305. doi:10.4103/joacp.JOACP_349_15
10. Quinn PJ, Markey BK, Leonard FC, FitzPatrick ES, Fanning S, Hartigan PJ. Antibacterial resistance. In: *Veterinary Microbiology and Microbial Disease*. 2nd ed. Chichester, England: Wiley-Blackwell; 2011:159.
11. Pankey GA, Sabath LD. Clinical relevance of bacteriostatic versus bactericidal nechanisms of action in the treatment of gram-positive bacterial infections. *Clin Infect Dis*. 2004;38(6):864-870. doi:10.1086/381972
12. Ocampo PS, Lázár V, Papp B, et al. Antagonism between bacteriostatic and bactericidal antibiotics is prevalent. *Antimicrob Agents Chemother*. 2014;58(8):4573-4582. doi:10.1128/AAC.02463-14
13. Klein EY, Van Boeckel TP, Martinez EM, et al. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc Natl Acad Sci U S A*. 2018;115(15):E3463-E3470. doi:10.1073/pnas.1717295115
14. *NORM/NORM-VET 2018. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway*. Tromsø / Oslo; 2019.
15. *NORM/NORM-VET 2017. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway*. Tromsø / Oslo; 2018.
16. Ministry of Health and Care Services. *National Strategy against Antibiotic Resistance 2015-2020*. Oslo; 2015.

17. European Centre for Disease Prevention and Control. *Antimicrobial Consumption, Annual Epidemiological Report for 2017*. Stockholm; 2018.

18. Fridkin SK, Edwards JR, Courval JM, et al. The effect of vancomycin and third-generation cephalosporins on prevalence of vancomycin-resistant enterococci in 126 U.S. adult intensive care units. *Ann Intern Med*. 2001;135(3):175-183. doi:10.7326/0003-4819-135-3-200108070-00009

19. Daneman N, Bronskill SE, Gruneir A, et al. Variability in antibiotic use across nursing homes and the risk of antibiotic-related adverse outcomes for individual residents. *JAMA Intern Med*. 2015;175(8):1331-1339. doi:10.1001/jamainternmed.2015.2770

20. Costelloe C, Metcalfe C, Lovering A, Mant D, Hay AD. Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: Systematic review and meta-analysis. *BMJ*. 2010;340(7756):1120. doi:10.1136/bmj.c2096

21. Steinke D, Davey P. Association between antibiotic resistance and community prescribing: A critical review of bias and confounding in published studies. *Clin Infect Dis*. 2001;33(s3):S193-S205. doi:10.1086/321848

22. Goossens H, Ferech M, Vander Stichele R, Elseviers M. Outpatient antibiotic use in Europe and association with resistance: A cross-national database study. *Lancet*. 2005;365(9459):579-587. doi:10.1016/S0140-6736(05)70799-6

23. Chang X, Meyer MT, Liu X, et al. Determination of antibiotics in sewage from hospitals, nursery and slaughter house, wastewater treatment plant and source water in Chongqing region of Three Gorge Reservoir in China. *Environ Pollut*. 2010;158(5):1444-1450. doi:10.1016/j.envpol.2009.12.034

24. Kim S, Aga DS. Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *J Toxicol Environ Health B Crit Rev*. 2007;10(8):559-573. doi:10.1080/15287390600975137

25. Koch G, Yepes A, Förstner KU, et al. Evolution of resistance to a last-resort antibiotic in *Staphylococcus aureus* via bacterial competition. *Cell*. 2014;158(5):1060-1071. doi:10.1016/j.cell.2014.06.046

26. Courvalin P. Transfer of antibiotic resistance genes between gram-positive and gram- negative bacteria. *Antimicrob Agents Chemother*. 1994;38(7):1447-1451. doi:10.1128/AAC.38.7.1447

27. Hansen FG, Atlung T, Braun RE, Wright A, Hughes P, Kohiyama M. Initiator (DnaA) protein concentration as a function of growth rate in *Escherichia coli* and *Salmonella typhimurium*. *J Bacteriol*. 1991;173(16):5194-5199. doi:10.1128/jb.173.16.5194-5199.1991

28. Eagon RG. *Pseudomonas natriegens*, a marine bacterium with a generation time of less than 10 minutes. *J Bacteriol*. 1962;83:736-737.

29. Munita JM, Arias CA. Mechanisms of antibiotic resistance. In: Kudva IT, Cornick NA, Plummer PJ, et al., eds. *Virulence Mechanisms of Bacterial Pathogens*. Vol 23. 5th ed. Washington, DC: American Society for Microbiology; 2016:1-45. doi:10.1128/microbiolspec.VMBF-0016-2015

30. Dönhöfer A, Franckenberg S, Wickles S, Berninghausen O, Beckmann R, Wilson DN. Structural basis for TetM-mediated tetracycline resistance. *Proc Natl Acad Sci U S A*. 2012;109(42):16900-16905. doi:10.1073/pnas.1208037109

31. Li W, Atkinson GC, Thakor NS, et al. Mechanism of tetracycline resistance by ribosomal protection protein Tet(O). *Nat Commun*. 2013;4(1):1477. doi:10.1038/ncomms2470

32. Hamilton-Miller JMT. Reversal of activity of trimethoprim against Gram-positive cocci by thymidine, thymine and 'folates.' *J Antimicrob Chemother*. 1988;22(1):35-39. doi:10.1093/jac/22.1.35

33. Zervos MJ, Schaberg DR. Reversal of the in vitro susceptibility of enterococci to trimethoprim-sulfamethoxazole by folinic acid. *Antimicrob Agents Chemother*. 1985;28(3):446-448. doi:10.1128/AAC.28.3.446

34. Chandra H, Bishnoi P, Yadav A, Patni B, Mishra AP, Nautiyal AR. Antimicrobial resistance and the alternative resources with special emphasis on plant-based antimicrobials - A review. *Plants*. 2017;6(2):457-462. doi:10.3390/plants6020016

35. Woodford N, Ellington MJ. The emergence of antibiotic resistance by mutation. *Clin Microbiol Infect*. 2007;13(1):5-18. doi:10.1111/j.1469-0691.2006.01492.x

36. Llosa M, Gomis-Rüth FX, Coll M, De la Cruz F. Bacterial conjugation: A two-step mechanism for DNA transport. *Mol Microbiol*. 2002;45(1):1-8. doi:10.1046/j.1365-2958.2002.03014.x

37. Trokter M, Waksman G. Translocation through the conjugative type IV secretion system requires unfolding of its protein substrate. *J Bacteriol*. 2018;200(6):e00615-17. doi:10.1128/JB.00615-17

38. Varga M, Kuntová L, Pantůček R, Mašlaňová I, Růžičková V, Doškař J. Efficient transfer of antibiotic resistance plasmids by transduction within methicillin-resistant *Staphylococcus aureus* USA300 clone. *FEMS Microbiol Lett*. 2012;332(2):146-152. doi:10.1111/j.1574-6968.2012.02589.x

39. Gunathilaka GU, Tahlan V, Mafiz AI, Polur M, Zhang Y. Phages in urban wastewater have the potential to disseminate antibiotic resistance. *Int J Antimicrob Agents*. 2017;50(5):678-683. doi:10.1016/j.ijantimicag.2017.08.013

40. Subirats J, Sànchez-Melsió A, Borrego CM, Balcázar JL, Simonet P. Metagenomic analysis reveals that bacteriophages are reservoirs of antibiotic resistance genes. *Int J Antimicrob Agents*. 2016;48(2):163-167. doi:10.1016/j.ijantimicag.2016.04.028

41. Calero-Cáceres W, Muniesa M. Persistence of naturally occurring antibiotic resistance genes in the bacteria and bacteriophage fractions of wastewater. *Water Res*. 2016;95:11-18. doi:10.1016/j.watres.2016.03.006

42. Enault F, Briet A, Bouteille L, Roux S, Sullivan MB, Petit M-A. Phages rarely encode antibiotic resistance genes: a cautionary tale for virome analyses. *ISME J*. 2017;11(1):237-247. doi:10.1038/ismej.2016.90

43. Rizzo L, Manaia C, Merlin C, et al. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Sci Total Environ*. 2013;447:345-360. doi:10.1016/j.scitotenv.2013.01.032

44. Mahillon J, Chandler M. Insertion sequences. *Microbiol Mol Biol Rev*. 1998;62(3):725-774.

45. Vandecraen J, Chandler M, Aertsen A, Van Houdt R. The impact of insertion sequences on bacterial genome plasticity and adaptability. *Crit Rev Microbiol*.

2017;43(6):709-730. doi:10.1080/1040841X.2017.1303661

46. Wang S, Zhou K, Xiao S, et al. A multidrug resistance plasmid pIMP26, carrying *blaIMP-26*, *fosA5*, *blaDHA-1*, and *qnrB4* in *Enterobacter cloacae*. *Sci Rep*. 2019;9(1):10212. doi:10.1038/s41598-019-46777-6

47. Westhoff S, Van Leeuwe TM, Qachach O, Zhang Z, Van Wezel GP, Rozen DE. The evolution of no-cost resistance at sub-MIC concentrations of streptomycin in *Streptomyces coelicolor*. *ISME J*. 2017;11(5):1168-1178. doi:10.1038/ismej.2016.194

48. D'Costa VM, King CE, Kalan L, et al. Antibiotic resistance is ancient. *Nature*. 2011;477(7365):457-461. doi:10.1038/nature10388

49. Knapp CW, Dolfing J, Ehlert PAI, Graham DW. Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ Sci Technol*. 2010;44(2):580-587. doi:10.1021/es901221x

50. Cassini A, Högberg LD, Plachouras D, et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infect Dis*. 2019;19(1):56-66. doi:10.1016/S1473-3099(18)30605-4

51. CDC. *Antibiotic Resistance Threats in the United States, 2013*. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2013.

52. CDC. *Antibiotic Resistance Threats in the United States, 2019*. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019.

53. IACG. *No Time to Wait: Securing the Future from Drug-Resistant Infections*. United Nations; 2019.

54. European Centre for Disease Prevention and Control. *Surveillance of Antimicrobial Resistance in Europe – Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2017*. Stockholm: ECDC; 2018.

55. Carroll LM, Gaballa A, Guldimann C, Sullivan G, Henderson LO, Wiedmann M. Identification of novel mobilized colistin resistance gene *mcr-9* in a multidrug-resistant, colistin-susceptible *Salmonella enterica* serotype Typhimurium isolate. *MBio*. 2019;10(3). doi:10.1128/mBio.00853-19

56. Hooper DC, Jacoby GA. Mechanisms of drug resistance: Quinolone resistance. *Ann N Y Acad Sci*. 2015;1354(1):12-31. doi:10.1111/nyas.12830

57. Bender JK, Cattoir V, Hegstad K, et al. Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: Towards a common nomenclature. *Drug Resist Updat*. 2018;40:25-39. doi:10.1016/j.drup.2018.10.002

58. Peirano G, Pitout JDD. Extended-spectrum β-lactamase-producing *Enterobacteriaceae*: Update on molecular epidemiology and treatment options. *Drugs*. 2019;79(14):1529-1541. doi:10.1007/s40265-019-01180-3

59. Kaspersen H, Urdahl AM, Simm R, Slettemeås JS, Lagesen K, Norström M. Occurrence of quinolone resistant *E. coli* originating from different animal species in Norway. *Vet Microbiol*. 2018;217:25-31. doi:10.1016/j.vetmic.2018.02.022

60. Elstrøm P, Astrup E, Hegstad K, Samuelsen Ø, Enger H, Kacelnik O. The fight to

keep resistance at bay, epidemiology of carbapenemase producing organisms (CPOs), vancomycin resistant enterococci (VRE) and methicillin resistant *Staphylococcus aureus* (MRSA) in Norway, 2006 - 2017. *PLoS One*. 2019;14(2):e0211741. doi:10.1371/journal.pone.0211741

61. Gullberg E, Cao S, Berg OG, et al. Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog*. 2011;7(7). doi:10.1371/journal.ppat.1002158

62. Bengtsson-Palme J, Larsson DGJ. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. *Environ Int*. 2016;86:140-149. doi:10.1016/j.envint.2015.10.015

63. Nygård K, Schimmer B, Søbstad Ø, et al. A large community outbreak of waterborne giardiasis- delayed detection in a non-endemic urban area. *BMC Public Health*. 2006;6(1):141. doi:10.1186/1471-2458-6-141

64. NIPH. *Utbrudd Av Diarésykdom i Røros Kommune, Mai 2007 [Norwegian]*. Oslo; 2007.

65. Kümmerer K. Antibiotics in the aquatic environment - A review - Part II. *Chemosphere*. 2009;75(4):435-441. doi:10.1016/j.chemosphere.2008.12.006

66. Le Corre KS, Ort C, Kateley D, Allen B, Escher BI, Keller J. Consumption-based approach for assessing the contribution of hospitals towards the load of pharmaceutical residues in municipal wastewater. *Environ Int*. 2012;45(1):99-111. doi:10.1016/j.envint.2012.03.008

67. Leung HW, Minh TB, Murphy MB, et al. Distribution, fate and risk assessment of antibiotics in sewage treatment plants in Hong Kong, South China. *Environ Int*. 2012;42(1):1-9. doi:10.1016/j.envint.2011.03.004

68. Uyaguari MI, Fichot EB, Scott GI, Norman RS. Characterization and quantitation of a novel β-lactamase gene found in a wastewater treatment facility and the surrounding coastal ecosystem. *Appl Environ Microbiol*. 2011;77(23):8226-8233. doi:10.1128/AEM.02732-10

69. Zhu Y-G, Zhao Y, Li B, et al. Continental-scale pollution of estuaries with antibiotic resistance genes. *Nat Microbiol*. 2017;2(4):16270. doi:10.1038/nmicrobiol.2016.270

70. Kümmerer K. Introduction: Pharmaceuticals in the Environment. In: Kümmerer K, ed. *Pharmaceuticals in the Environment*. Berlin, Heidelberg: Springer; 2001:1-8. doi:10.1007/978-3-662-04634-0_1

71. Kümmerer K, Henninger A. Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clin Microbiol Infect*. 2003;9(12):1203-1214. doi:10.1111/j.1469-0691.2003.00739.x

72. Zuccato E, Castiglioni S, Bagnati R, Melis M, Fanelli R. Source, occurrence and fate of antibiotics in the Italian aquatic environment. *J Hazard Mater*. 2010;179(1-3):1042-1048. doi:10.1016/j.jhazmat.2010.03.110

73. Munir M, Wong K, Xagoraraki I. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res*. 2011;45(2):681-693. doi:10.1016/j.watres.2010.08.033

74. Hembach N, Schmid F, Alexander J, Hiller C, Rogall ET, Schwartz T. Occurrence of the *mcr-1* colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal

wastewater treatment plants in Germany. *Front Microbiol*. 2017;8(1282). doi:10.3389/fmicb.2017.01282

75. Jäger T, Hembach N, Elpers C, et al. Reduction of antibiotic resistant bacteria during conventional and advanced wastewater treatment, and the disseminated loads released to the environment. *Front Microbiol*. 2018;9(2599). doi:10.3389/fmicb.2018.02599

76. Wright GD. Antibiotic resistance in the environment: A link to the clinic? *Curr Opin Microbiol*. 2010;13(5):589-594. doi:10.1016/j.mib.2010.08.005

77. Hocquet D, Muller A, Bertrand X. What happens in hospitals does not stay in hospitals: antibiotic-resistant bacteria in hospital wastewater systems. *J Hosp Infect*. 2016;93(4):395-402. doi:10.1016/j.jhin.2016.01.010

78. Proia L, Von Schiller D, Sànchez-Melsió A, et al. Occurrence and persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers. *Environ Pollut*. 2016;210(452):121-128. doi:10.1016/j.envpol.2015.11.035

79. Sabri NA, Schmitt H, Van der Zaan B, et al. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. *J Environ Chem Eng*. 2018. doi:10.1016/j.jece.2018.03.004

80. Di Cesare A, Eckert EM, D'Urso S, et al. Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. *Water Res*. 2016;94:208-214. doi:10.1016/j.watres.2016.02.049

81. VEAS (Vestfjorden Avløpsselskap). https://www.facebook.com/veasnorge/. Published 2019. Accessed November 13, 2019.

82. Grabow WOK, van Zyl M, Prozesky OW. Behaviour in conventional sewage purification processes of coliform bacteria with transferable or non-transferable drug-resistance. *Water Res*. 1976;10(8):717-723. doi:10.1016/0043-1354(76)90010-5

83. Torell E, Kühn I, Olsson-Liljequist B, et al. Clonality among ampicillin-resistant *Enterococcus faecium* isolates in Sweden and relationship with ciprofloxacin resistance. *Clin Microbiol Infect*. 2003;9(10):1011-1019. doi:10.1046/j.1469-0691.2003.00701.x

84. Iversen A, Kühn I, Rahman M, et al. Evidence for transmission between humans and the environment of a nosocomial strain of *Enterococcus faecium*. *Environ Microbiol*. 2004;6(1):55-59. doi:10.1046/j.1462-2920.2003.00534.x

85. Templeton MR, Oddy F, Leung WK, Rogers M. Chlorine and UV disinfection of ampicillinresistant and trimethoprim-resistant *Escherichia coli*. *Can J Civ Eng*. 2009;36(5):889-894. doi:10.1139/L09-040

86. Guardabassi L, Brønnum PT, Danø R, Forslund A, Dalsgaard A. Dissemination of vancomycin-resistant enterococci harboring *vanA* through disposal of waste derived from industrial production of vancomycin. *Microb Drug Resist*. 2002;8(4):401-406. doi:10.1089/10766290260469688

87. Novais C, Coque TM, Boerlin P, et al. Vancomycin-resistant *Enterococcus faecium* clone in swine, Europe. *Emerg Infect Dis*. 2005;11(12):1985-1987. doi:10.3201/eid1112.050822

88. Torres C, Reguera JA, Sanmartin MJ, Pérez-Díaz JC, Baquero F. *vanA*-mediated vancomycin-resistant *Enterococcus* spp. in sewage. *J Antimicrob Chemother*. 1994;33(3):553-561. doi:10.1093/jac/33.3.553

89. Iversen A, Kühn I, Franklin A, Möllby R. High prevalence of vancomycin-resistant enterococci in Swedish sewage. *Appl Environ Microbiol*. 2002;68(6):2838-2842. doi:10.1128/AEM.68.6.2838-2842.2002

90. Diwan V, Tamhankar AJ, Khandal RK, et al. Antibiotics and antibiotic-resistant bacteria in waters associated with a hospital in Ujjain, India. *BMC Public Health*. 2010;10:414. doi:10.1186/1471-2458-10-414

91. Colque P, Fernandez H, Möllby R, et al. Antibiotic resistance in environmental *Escherichia coli* - A simple screening method for simultaneous typing and resistance determination. *J Water Health*. 2014;12(4):692-701. doi:10.2166/wh.2014.216

92. Kwak YK, Colque P, Byfors S, Giske CG, Möllby R, Kühn I. Surveillance of antimicrobial resistance among *Escherichia coli* in wastewater in Stockholm during 1 year: Does it reflect the resistance trends in the society? *Int J Antimicrob Agents*. 2015;45(1):25-32. doi:10.1016/j.ijantimicag.2014.09.016

93. Ministry of Climate and Environment. *Et Norge Uten Miljøgifter (NOU 2010:9) [Norwegian]*. Oslo; 2010.

94. Jarnheimer PA, Ottoson J, Lindberg R, et al. Fluoroquinolone antibiotics in a hospital sewage line; occurrence, distribution and impact on bacterial resistance. *Scand J Infect Dis*. 2004;36(10):752-755. doi:10.1080/00365540410021027

95. Linton KB, Richmond MH, Bevan R, Gillespie WA. Antibiotic resistance and R factors in coliform bacilli isolated from hospital and domestic sewage. *J Med Microbiol*. 1974;7(1):91-103. doi:10.1099/00222615-7-1-91

96. Kühn I, Iversen A, Burman LG, et al. Comparison of enterococcal populations in animals, humans, and the environment - A European study. *Int J Food Microbiol*. 2003;88(2-3):133-145. doi:10.1016/S0168-1605(03)00176-4

97. Hendriksen RS, Munk P, Njage P, et al. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nat Commun*. 2019;10:1124. doi:10.1038/s41467-019-08853-3

98. Jørgensen SB, Søraas A V., Arnesen LS, Leegaard TM, Sundsfjord A, Jenum PA. A comparison of extended spectrum β-lactamase producing *Escherichia coli* from clinical, recreational water and wastewater samples associated in time and location. Singer AC, ed. *PLoS One*. 2017;12(10):e0186576. doi:10.1371/journal.pone.0186576

99. Schwermer CU, Krzeminski P, Wennberg AC, Vogelsang C, Uhl W. Removal of antibiotic resistant *E. coli* in two Norwegian wastewater treatment plants and by nano- and ultra-filtration processes. *Water Sci Technol*. 2018;77(4):1115-1126. doi:10.2166/wst.2017.642

100. Pärnänen KMM, Narciso-Da-Rocha C, Kneis D, et al. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Sci Adv*. 2019;5(3):eaau9124. doi:10.1126/sciadv.aau9124

101. Cacace D, Fatta-Kassinos D, Manaia CM, et al. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings. *Water Res*. 2019;162:320-330. doi:10.1016/j.watres.2019.06.039

102. Bréchet C, Plantin J, Sauget M, et al. Wastewater treatment plants release large amounts of extended-spectrum β-lactamase-producing *Escherichia coli* into the environment. *Clin Infect Dis*. 2014;58(12):1658-1665. doi:10.1093/cid/ciu190

103. Chagas TPG, Seki LM, Cury JC, et al. Multiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital wastewater in Rio de Janeiro, Brazil. *J Appl Microbiol*. 2011;111(3):572-581. doi:10.1111/j.1365-2672.2011.05072.x

104. Masters NM, Wiegand A, Thompson JM, Vollmerhausen TL, Hatje E, Katouli M. Assessing the population dynamics of *Escherichia coli* in a metropolitan river after an extreme flood event. *J Water Health*. 2017;15(2):196-208. doi:10.2166/wh.2016.285

105. Stark JS, Corbett PA, Dunshea G, et al. The environmental impact of sewage and wastewater outfalls in Antarctica: An example from Davis station, East Antarctica. *Water Res*. 2016;105:602-614. doi:10.1016/j.watres.2016.09.026

106. Alexander J, Bollmann A, Seitz W, Schwartz T. Microbiological characterization of aquatic microbiomes targeting taxonomical marker genes and antibiotic resistance genes of opportunistic bacteria. *Sci Total Environ*. 2015;512-513:316-325. doi:10.1016/j.scitotenv.2015.01.046

107. Galvin S, Boyle F, Hickey P, Vellinga A, Morris D, Cormican M. Enumeration and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent from municipal, hospital, and secondary treatment facility sources. *Appl Environ Microbiol*. 2010;76(14):4772-4779. doi:10.1128/AEM.02898-09

108. Korzeniewska E, Korzeniewska A, Harnisz M. Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. *Ecotoxicol Environ Saf*. 2013;91:96-102. doi:10.1016/j.ecoenv.2013.01.014

109. Gündoğdu A, Jennison A V, Smith H V, Stratton H, Katouli M. Extended-spectrum β-lactamase producing *Escherichia coli* in hospital wastewaters and sewage treatment plants in Queensland, Australia. *Can J Microbiol*. 2013;59(11):737-745. doi:10.1139/cjm-2013-0515

110. Jamborova I, Johnston BD, Papousek I, et al. Extensive genetic commonality among wildlife, wastewater, community, and nosocomial isolates of *Escherichia coli* sequence type 131 (H30R1 and H30Rx Subclones) that carry *blaCTX-M-27* or *blaCTX-M-15*. *Antimicrob Agents Chemother*. 2018;62(10). doi:10.1128/AAC.00519-18

111. Shaik S, Ranjan A, Tiwari SK, et al. Comparative genomic analysis of globally dominant ST131 clone with other epidemiologically successful extraintestinal pathogenic *Escherichia coli* (ExPEC) lineages. *MBio*. 2017;8(5). doi:10.1128/mBio.01596-17

112. Farkas A, Crăciunaş C, Chiriac C, Szekeres E, Coman C, Butiuc-Keul A. Exploring the role of coliform bacteria in class 1 integron carriage and biofilm formation during drinking water treatment. *Microb Ecol*. 2016;72(4):773-782. doi:10.1007/s00248-016-0758-0

113. Ranjan A, Scholz J, Semmler T, et al. ESBL-plasmid carriage in *E. coli* enhances in vitro bacterial competition fitness and serum resistance in some strains of pandemic sequence types without overall fitness cost. *Gut Pathog*. 2018;10(1):24. doi:10.1186/s13099-018-0243-z

114. Schaufler K, Semmler T, Pickard DJ, et al. Carriage of extended-spectrum beta-lactamase-plasmids does not reduce fitness but enhances virulence in some strains of pandemic *E. coli* lineages. *Front Microbiol*. 2016;7:336. doi:10.3389/fmicb.2016.00336

115. *NORM/NORM-VET 2016. Usage of Antimicrobial Agents and Occurrence of*

Antimicrobial Resistance in Norway. Tromsø / Oslo; 2017.

116. Colavecchio A, Jeukens J, Freschi L, et al. Complete genome sequences of two phage-like plasmids carrying the CTX-M-15 extended-spectrum β-lactamase gene. *Genome Announc*. 2017;5(19). doi:10.1128/genomeA.00102-17

117. Akiba M, Sekizuka T, Yamashita A, et al. Distribution and relationships of antimicrobial resistance determinants among extended-spectrum-cephalosporin-resistant or carbapenem-resistant *Escherichia coli* isolates from rivers and sewage treatment plants in India. *Antimicrob Agents Chemother*. 2016;60(5):2972-2980. doi:10.1128/AAC.01950-15

118. Korzeniewska E, Harnisz M. Extended-spectrum beta-lactamase (ESBL)-positive *Enterobacteriaceae* in municipal sewage and their emission to the environment. *J Environ Manage*. 2013;128:904-911. doi:10.1016/j.jenvman.2013.06.051

119. Frigon D, Biswal BK, Mazza A, Masson L, Gehr R. Biological and physicochemical wastewater treatment processes reduce the prevalence of virulent *Escherichia coli*. *Appl Environ Microbiol*. 2013;79(3):835-844. doi:10.1128/AEM.02789-12

120. Peirano G, Van Der Bij AK, Gregson DB, Pitout JDD. Molecular epidemiology over an 11-year period (2000 to 2010) of extended-spectrum β-lactamase-producing *Escherichia coli* causing bacteremia in a centralized Canadian region. *J Clin Microbiol*. 2012;50(2):294-299. doi:10.1128/JCM.06025-11

121. Zarfel G, Galler H, Feierl G, et al. Comparison of extended-spectrum-β-lactamase (ESBL) carrying *Escherichia coli* from sewage sludge and human urinary tract infection. *Environ Pollut*. 2013;173:192-199. doi:10.1016/j.envpol.2012.09.019

122. Samuelsen Ø, Overballe-Petersen S, Bjørnholt JV, et al. Molecular and epidemiological characterization of carbapenemase-producing *Enterobacteriaceae* in Norway, 2007 to 2014. *PLoS One*. 2017;12(11). doi:10.1371/journal.pone.0187832

123. Kreuzinger N, Murphy D, Greene E, Water JPI Knowledge Hub on Contaminants of Emerging Concern. *JPI Water Draft Report, 2019*.

124. Dulio V, van Bavel B, Brorström-Lundén E, et al. Emerging pollutants in the EU: 10 years of NORMAN in support of environmental policies and regulations. *Environ Sci Eur*. 2018;30(1):5. doi:10.1186/s12302-018-0135-3

125. Wang R, Feng F, Chai Y, et al. Screening and quantitation of residual antibiotics in two different swine wastewater treatment systems during warm and cold seasons. *Sci Total Environ*. 2019;660:1542-1554. doi:10.1016/j.scitotenv.2019.01.127

126. Johansen BH, Røiseland SF. MDG vil ta kloakkutslippene til Stortinget. *NRK*. April 25, 2017.

127. Johansen BH. Sykehusene bør rense sitt eget avløpsvann. *NRK*. April 26, 2017.

128. Johansen AD, Johansen BH. Den usynlige kloakken. *NRK*. April 24, 2017.

129. Lien LTQ, Hoa NQ, Chuc NTK, et al. Antibiotics in wastewater of a rural and an urban hospital before and after wastewater treatment, and the relationship with antibiotic use-a one year study from Vietnam. *Int J Environ Res Public Health*. 2016;13(6). doi:10.3390/ijerph13060588

130. Hiller CX, Hübner U, Fajnorova S, Schwartz T, Drewes JE. Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater

treatment processes: A review. *Sci Total Environ*. 2019;685:596-608. doi:10.1016/j.scitotenv.2019.05.315

131. Caucci S, Karkman A, Cacace D, et al. Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. *FEMS Microbiol Ecol*. 2016;92(5). doi:10.1093/femsec/fiw060

132. Wan MT, Chou CC. Spreading of β-lactam resistance gene (*mecA*) and methicillin-resistant *Staphylococcus aureus* through municipal and swine slaughterhouse wastewaters. *Water Res*. 2014;64:288-295. doi:10.1016/j.watres.2014.07.014

133. Gómez P, Lozano C, Benito D, et al. Characterization of staphylococci in urban wastewater treatment plants in Spain, with detection of methicillin resistant *Staphylococcus aureus* ST398. *Environ Pollut*. 2016;212:71-76. doi:10.1016/j.envpol.2016.01.038

134. Fernando DM, Tun HM, Poole J, et al. Detection of antibiotic resistance genes in source and drinking water samples from a first nations community in Canada. *Appl Environ Microbiol*. 2016;82(15):4767-4775. doi:10.1128/AEM.00798-16

135. Tao W, Zhang XX, Zhao F, et al. High levels of antibiotic resistance genes and their correlations with bacterial community and mobile genetic elements in pharmaceutical wastewater treatment bioreactors. *PLoS One*. 2016;11(6):1-17. doi:10.1371/journal.pone.0156854

136. Noyes NR, Yang X, Linke LM, et al. Characterization of the resistome in manure, soil and wastewater from dairy and beef production systems. *Sci Rep*. 2016;6. doi:10.1038/srep24645

137. Ferreira Da Silva M, Vaz-Moreira I, Gonzalez-Pajuelo M, Nunes OC, Manaia CM. Antimicrobial resistance patterns in *Enterobacteriaceae* isolated from an urban wastewater treatment plant. *FEMS Microbiol Ecol*. 2007;60(1):166-176. doi:10.1111/j.1574-6941.2006.00268.x

138. Karkman A, Johnson TA, Lyra C, et al. High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. *FEMS Microbiol Ecol*. 2016;92(3). doi:10.1093/femsec/fiw014

139. Wu Y, Cui E, Zuo Y, Cheng W, Rensing C, Chen H. Influence of two-phase anaerobic digestion on fate of selected antibiotic resistance genes and class I integrons in municipal wastewater sludge. *Bioresour Technol*. 2016;211:414-421. doi:10.1016/j.biortech.2016.03.086

140. Makowska N, Koczura R, Mokracka J. Class 1 integrase, sulfonamide and tetracycline resistance genes in wastewater treatment plant and surface water. *Chemosphere*. 2016;144:1665-1673. doi:10.1016/j.chemosphere.2015.10.044

141. Ju F, Li B, Ma L, Wang Y, Huang D, Zhang T. Antibiotic resistance genes and human bacterial pathogens: Co-occurrence, removal, and enrichment in municipal sewage sludge digesters. *Water Res*. 2016;91:1-10. doi:10.1016/j.watres.2015.11.071

142. Zhang CM, Xu LM, Wang XC, Zhuang K, Liu QQ. Effects of ultraviolet disinfection on antibiotic-resistant *Escherichia coli* from wastewater: inactivation, antibiotic resistance profiles and antibiotic resistance genes. *J Appl Microbiol*. 2017;123:295-306. doi:10.1111/jam.13480

143. Rafraf ID, Lekunberri I, Sànchez-Melsió A, Aouni M, Borrego CM, Balcázar JL.

Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia. *Environ Pollut*. 2016;219:353-358. doi:10.1016/j.envpol.2016.10.062

144. Pak G, Salcedo DE, Lee H, et al. Comparison of antibiotic resistance removal efficiencies using ozone disinfection under different pH and suspended solids and humic substance concentrations. *Environ Sci Technol*. 2016;50(14):7590-7600. doi:10.1021/acs.est.6b01340

145. Czekalski N, Imminger S, Salhi E, et al. Inactivation of antibiotic resistant bacteria and resistance genes by ozone: From laboratory experiments to full-scale wastewater treatment. *Environ Sci Technol*. 2016;50(21):11862-11871. doi:10.1021/acs.est.6b02640

146. Alexander J, Knopp G, Dötsch A, Wieland A, Schwartz T. Ozone treatment of conditioned wastewater selects antibiotic resistance genes, opportunistic bacteria, and induce strong population shifts. *Sci Total Environ*. 2016;559:103-112. doi:10.1016/j.scitotenv.2016.03.154

147. Michael-Kordatou I, Andreou R, Iacovou M, et al. On the capacity of ozonation to remove antimicrobial compounds, resistant bacteria and toxicity from urban wastewater effluents. *J Hazard Mater*. 2017;323:414-425. doi:10.1016/j.jhazmat.2016.02.023

148. Lin W, Zhang M, Zhang S, Yu X. Can chlorination co-select antibiotic-resistance genes? *Chemosphere*. 2016;156:412-419. doi:10.1016/j.chemosphere.2016.04.139

149. Khan S, Beattie TK, Knapp CW. Relationship between antibiotic- and disinfectant-resistance profiles in bacteria harvested from tap water. *Chemosphere*. 2016;152:132-141. doi:10.1016/j.chemosphere.2016.02.086

150. Chen XC, Luo Q, Chen H, Wei Z, Wang ZJ, Xu KW. Occurrence and distribution of volatile organic compounds in conventional and advanced drinking water treatment processes. *Huanjing Kexue/Environmental Sci*. 2013;34(12):4642-4648.

151. Ferro G, Guarino F, Castiglione S, Rizzo L. Antibiotic resistance spread potential in urban wastewater effluents disinfected by UV/H2O2 process. *Sci Total Environ*. 2016;560-561:29-35. doi:10.1016/j.scitotenv.2016.04.047

152. Zhang R, Yang Y, Huang CH, Zhao L, Sun P. Kinetics and modeling of sulfonamide antibiotic degradation in wastewater and human urine by UV/H 2 O 2 and UV/PDS. *Water Res*. 2016;103:283-292. doi:10.1016/j.watres.2016.07.037

153. Weng SC, Yang JY, Li YH, Blatchley ER. UV-induced effects on toxicity of model disinfection byproducts. *Sci Total Environ*. 2017;599-600:94-97. doi:10.1016/j.scitotenv.2017.04.198

154. Wang H, Wilksch JJ, Strugnell RA, Gee ML. Role of capsular polysaccharides in biofilm formation: An AFM nanomechanics study. *ACS Appl Mater Interfaces*. 2015;7(23):13007-13013. doi:10.1021/acsami.5b03041

7. Scientific papers I – III

Paper I

Water Research 161 (2019) 232–241

Contents lists available at ScienceDirect

Water Research

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

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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ARTICLE INFO

Article history:
Received 28 February 2019
Received in revised form
27 May 2019
Accepted 29 May 2019
Available online 30 May 2019

Keywords:
PhP typing
AREB
Indicator bacteria
Extended spectrum beta-lactamase
Surveillance
Wastewater transport infrastructure

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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https://doi.org/10.1016/j.watres.2019.05.102

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

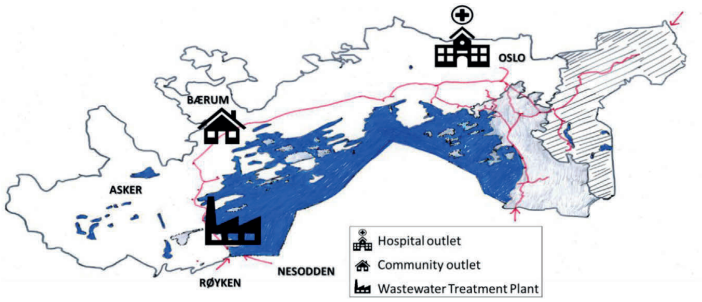

Fig. 1. Wastewater sampling sites and their approximate geographical locations in the wastewater transport infrastructure (red line). The uncolored part of the map indicates the areas from which the main WWTP in larger Oslo city (this study) receives its wastewater. The shaded and hatched parts (right) 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

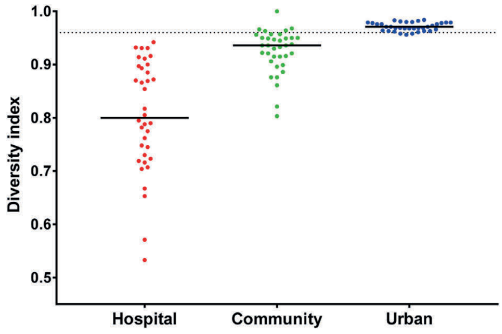

Fig. 2. Diversity for all individual samples. Median values for each site are shown by solid lines. All urban wastewater samples appear to be highly diverse (≥ 0.96, dotted line) throughout the sampling campaigns, whilst hospital samples show large variations.

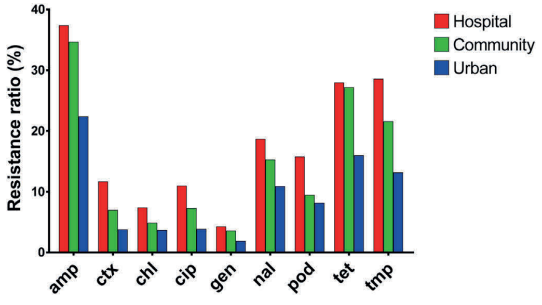

Fig. 3. Rate of resistance to each antibiotic in *E. coli* from each wastewater source over the total sampling period. For all antibiotics, hospital wastewater had the highest rates of resistance, whilst urban wastewater had the lowest rates. amp (ampicillin); ctx (cefotaxime); chl (chloramphenicol); 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

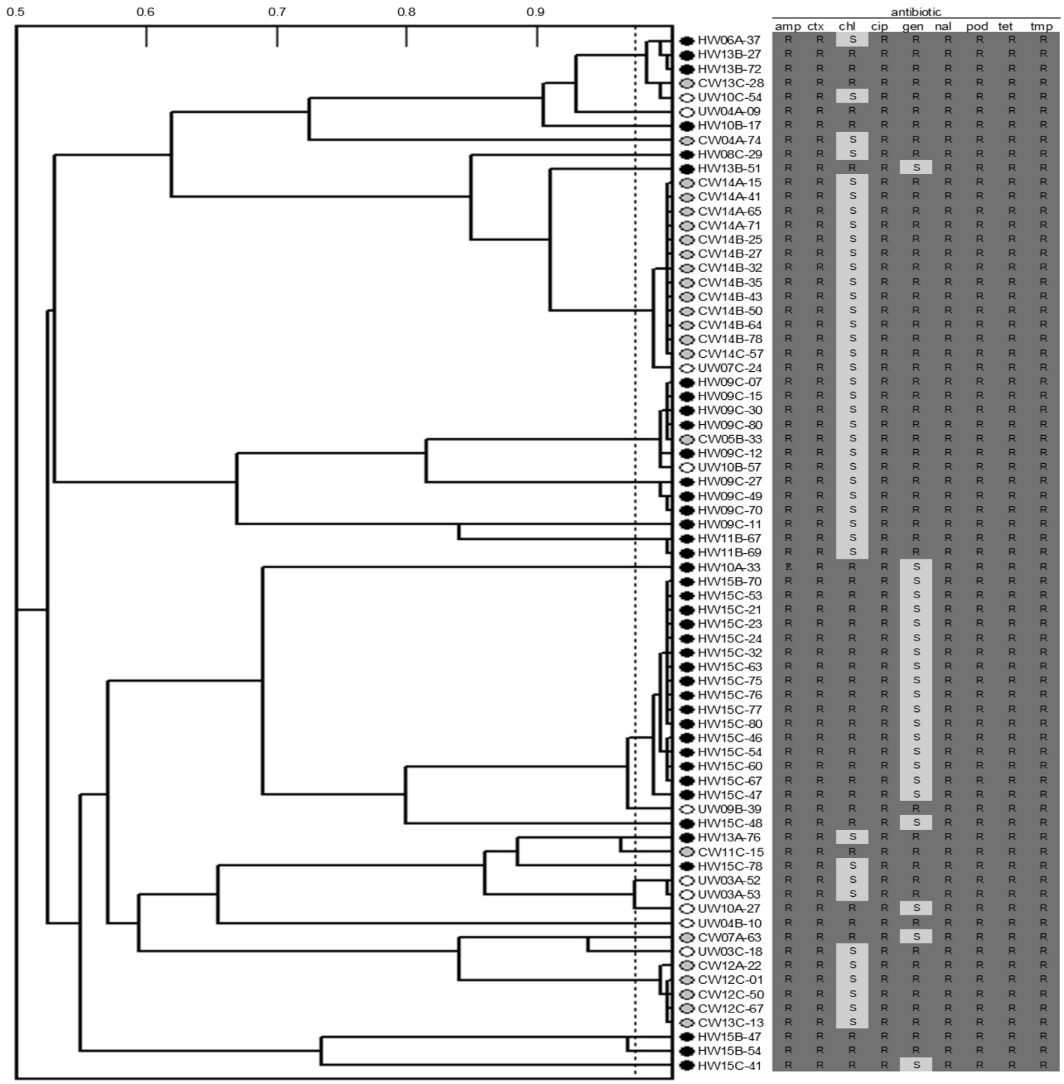

Fig. 4. Clustered PhP typing data showing phenotypic relationship between multi-resistant *E. coli* (≥8 of 9 resistances) from all wastewater samples with their corresponding sampling site origin (hospital - HW: black circles; community - CW: grey circles; urban - UW: white circles) and resistance profiles. 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

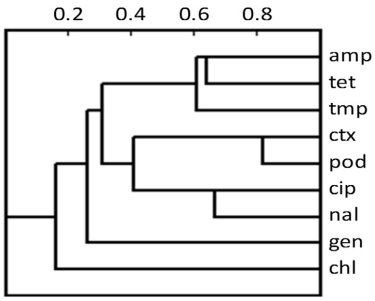

Fig. 5. Co-occurring resistance properties to the nine included antibiotics in the total population of *E. coli* isolates. For explanation of antibiotics abbreviations, see Fig. 3.

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.

Acknowledgements

Thanks to the hospital staff, Bærum municipality and VEAS for access to sampling sites and help with sampling. This study was supported by the Norwegian regional research fund RFFHSTAD (Regionalt Forskningsfond Hovedstaden), project number 245686, the Norwegian wastewater treatment plant Vestfjorden Avløpsselskap (VEAS), and the Norwegian University of Life Sciences (NMBU).

Appendix A. Supplementary data

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

References

Andersson, D.I., Diarmaid, H., 2017. Selection and transmission of antibiotic-resistant bacteria. Microbiol. Spectr. 5 (4), 1–17. https://doi.org/10.1128/microbiolspec.MTBP-0013-2016.

Bäumlisberger, M., Youssar, L., Schilhabel, M.B., Jonas, D., 2015. Influence of a non-hospital medical care facility on antimicrobial resistance in wastewater. PLoS ONE 10 (3), 1–16. https://doi.org/10.1371/journal.pone.0122635.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.N., Kreuzinger, N., Houvinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439.

Bevan, E.R., Jones, A.M., Hawkey, P.M., 2017. Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype. J. Antimicrob. Chemother. 72, 2145–2155. https://doi.org/10.1093/jac/dkx146.

Blanch, A.R., Belanche-Muñoz, L., Bonjoch, X., Ebdon, J., Gantzer, C., Lucena, F., Ottoson, J., Kourtis, C., Iversen, A., Kühn, I., Mocé, L., Muniesa, M., Schwartzbrod, J., Skraber, S., Papageorgiou, G.T., Taylor, H., Wallis, J., Jofre, J., 2006. Integrated analysis of established and novel microbial and chemical methods for microbial source tracking. Appl. Environ. Microb. 72 (9), 5915–5926. https://doi.org/10.1128/AEM.02453-05.

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. https://doi.org/10.1016/j.scitotenv.2005.10.007.

Colque, P., Fernandez, H., Möllby, R., Otth, L., Tiodolf, M., Wilson, M., Kühn, I., 2014. Antibiotic resistance in environmental *Escherichia coli* - a simple screening method for simultaneous typing and resistance determination. J. Water Health 12 (4), 692–701. https://doi.org/10.2166/wh.2014.216.

European Centre for Disease Prevention and Control, 2017. Antimicrobial Resistance Surveillance in Europe 2016. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). ECDC, Stockholm. https://doi.org/10.2900/296939.

Flach, C.F., Genheden, M., Fick, J., Larsson, J.D.G., 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. https://doi.org/10.1021/acs.est.8b03354.

Gao, P., Munir, M., Xagoraraki, I., 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci. Total Environ. 421–422, 173–183. https://doi.org/10.1016/j.scitotenv.2012.01.061.

Gaskins, H.R., Collier, C.T., Anderson, D.B., 2002. Antibiotics as growth promotants: mode of action. Anim. Biotechnol. 13 (1), 29–42. https://doi.org/10.1081/ABIO-120005768.

Gündoğdu, A., Jennison, A.V., Smith, H.V., Stratton, H., Katouli, M., 2013. Extended-spectrum β-lactamase producing *Escherichia coli* in hospital wastewaters and sewage treatment plants in Queensland, Australia. Can. J. Microbiol. 59 (11), 737–745. https://doi.org/10.1139/cjm-2013-0515.

Hernando-Amado, S., Sanz-García, F., Blanco, P., Martínez, J.L., 2017. Fitness costs associated with the acquisition of antibiotic resistance. Essays Biochem. 61 (1), 37–48. https://doi.org/10.1042/ebc20160057.

Hitchings, G.H., 1973. Mechanism of Action of Trimethoprim-Sulfamethoxazole—I. J. Infect. Dis. 128 (Suppl.), S433–436. https://doi.org/10.1093/infdis/128.Supplement_3.S433.

Hocquet, D., Muller, A., Bertrand, X., 2016. What happens in hospitals does not stay in hospitals: antibiotic-resistant bacteria in hospital wastewater systems. J. Hosp. Infect. 93, 395–402. https://doi.org/10.1016/j.jhin.2016.01.010.

Iversen, A., Kühn, I., Franklin, A., Möllby, R., 2002. High prevalence of vancomycin-resistant Enterococci in Swedish sewage. Appl. Environ. Microb. 68 (6), 2838–2842. https://doi.org/10.1128/AEM.68.6.2838-2842.2002.

Iversen, A., Kühn, I., Rahman, M., Franklin, A., Burman, L.G., Olsson-Liljequist, B., Torell, E., Möllby, R., 2004. Evidence for transmission between humans and the environment of a nosocomial strain of Enterococcus faecium. Environ. Microbiol. 6 (1), 55–59. https://doi.org/10.1046/j.1462-2920.2003.00534.x.

Jørgensen, S.B., Søraas, A.V., Arnesen, L.S., Leegaard, T.M., Sundsfjord, A., Jenum, P.A., 2017. A comparison of extended spectrum β-lactamase producing *Escherichia coli* from clinical, recreational water and wastewater samples associated in time and location. PLoS ONE 12 (10), 1–15. https://doi.org/10.1371/journal.pone.0186576.

Kahlmeter, G., Poulsen, H.O., 2012. Antimicrobial susceptibility of *Escherichia coli* from community-acquired urinary tract infections in Europe: The ECO·SENS study revisited. Int. J. Antimicrob. Agents 39 (1), 45–51. https://doi.org/10.1016/j.ijantimicag.2011.09.013.

Kolář, M., Urbánek, K., Látal, T., 2001. Antibiotic selective pressure and development of bacterial resistance. Int. J. Antimicrob. Agents 17, 357–363. https://doi.org/10.1016/S0924-8579(01)00317-X.

Korzeniewska, E., Korzeniewska, A., Harnisz, M., 2013. Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. Ecotoxicol. Environ. Saf. 91, 96–102. https://doi.org/10.1016/j.ecoenv.2013.01.014.

Krumperman, P.H., 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Appl. Environ. Microb. 46 (1), 165–170. https://doi.org/10.1007/s11356-014-3887-3.

Kühn, I., Allestam, G., Stenström, T.A., Möllby, R., 1991. Biochemical fingerprinting of water coliform bacteria, a new method for measuring phenotypic diversity and for comparing different bacterial populations. Appl. Environ. Microb. 57 (11), 3171–3177.

Kühn, I., Huys, G., Coopman, R., Kersters, K., Janssen, P., 1997. A 4-year study of the diversity and persistence of coliforms and *Aeromonas* in the water of a Swedish drinking water well. Can. J. Microbiol. 43, 9–16. https://doi.org/10.1139/m97-002.

Kühn, I., Iversen, A., Burman, L.G., Olsson-Liljequist, B., Franklin, A., Finn, M., Aarestrup, F., Seyfarth, A.M., Blanch, A.R., Vilanova, X., Taylor, H., Caplin, J., Moreno, M.A., Dominguez, L., Herrero, I.A., Möllby, R., 2003. Comparison of enterococcal populations in animals, humans, and the environment - a European study. Int. J. Food Microbiol. 88, 133–145. https://doi.org/10.1016/S0168-1605(03)00176-4.

Kühn, I., Möllby, R., 1993. The PhP RS system. A simple microplate method for studying coliform bacterial populations. J. Microbiol. Methods 17 (4), 255–259. https://doi.org/10.1016/0167-7012(93)90054-L.

Kümmerer, K., Henninger, A., 2003. Promoting resistance by the emission of antibiotics from hospitals and households into effluent. Clin. Microbiol. Infect. 9 (12), 1203–1214. https://doi.org/10.1111/j.1469-0691.2003.00739.x.

Kümmerer, K., 2009. Antibiotics in the aquatic environment - A review - Part I. Chemosphere 75, 417–434. https://doi.org/10.1016/j.chemosphere.2008.11.086.

Kwak, Y.K., Colque, P., Byfors, S., Giske, C.G., Möllby, R., Kühn, I., 2015. Surveillance of antimicrobial resistance among Escherichia coli in wastewater in Stockholm during 1 year: does it reflect the resistance trends in the society? Int. J. Antimicrob. Agents 45 (1), 25–32. https://doi.org/10.1016/j.ijantimicag.2014.09.016.

Landgren, M., Odén, H., Kühn, I., Österlund, A., Kahlmeter, G., 2005. Diversity among 2481 Escherichia coli from women with community-acquired lower urinary tract infections in 17 countries. J Antimicrob. Chemother. 55 (6), 928–937. https://doi.org/10.1093/jac/dki122.

Li, J., Cheng, W., Xu, L., Strong, P.J., Chen, H., 2015. Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system. Environ. Sci. Pollut. Res 22, 4587–4596. https://doi.org/10.1007/s11356-014-3665-2.

Linton, K.B., Richmond, M.H., Bevan, R., Gillespie, W.A., 1974. Antibiotic resistance and R factors in coliform bacilli isolated from hospital and domestic sewage. J. Med. Microbiol. 7 (1), 91–103. https://doi.org/10.1099/00222615-7-1-91.

Loman, N.J., Pallen, M.J., 2015. Twenty years of bacterial genome sequencing. Nat. Rev. Microbiol. 13, 787–794. https://doi.org/10.1038/nrmicro3565.

Munir, M., Wong, K., Xagoraraki, I., 2011. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Res. 45 (2), 681–693. https://doi.org/10.1016/j.watres.2010.08.033.

NORM/NORM-VET 2016, 2017. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo. ISSN:1890-9965. https://www.vetinst.no/overvaking/antibiotikaresistens-norm-vet.

NORM/NORM-VET 2017, 2018. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo. ISSN:1890-9965. https://www.vetinst.no/overvaking/antibiotikaresistens-norm-vet.

Pärnänen, K.M.M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V.D., Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5 (3), 1–10. https://doi.org/10.1126/sciadv.aau9124.

Reinthaler, F.F., Galler, H., Feierl, G., Haas, D., Leitner, E., Mascher, F., Melkes, A., Posch, J., Pertschy, B., Winter, I., Himmel, W., Marth, E., Zarfel, G., 2013. Resistance patterns of Escherichia coli isolated from sewage sludge in comparison with those isolated from human patients in 2000 and 2009. J. Water Health 11 (1), 13–20. https://doi.org/10.2166/wh.2012.207.

Reyes, D., Vilchez, S., Paniagua, M., Colque, P., Weintraub, A., Möllby, R., Kühn, I., 2009. Diversity of intestinal Escherichia coli populations in Nicaraguan children with and without diarrhoea. J. Med. Microbiol. 58, 1593–1600. https://doi.org/10.1099/jmm.0.012724-0.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-

Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032.

Sabaté, M., Prats, G., Moreno, E., Ballesté, E., Blanch, A.R., Andreu, A., 2008. Virulence and antimicrobial resistance profiles among Escherichia coli strains isolated from human and animal wastewater. Res. Microbiol. 159, 288–293. https://doi.org/10.1016/j.resmic.2008.02.001.

Torell, E., Kühn, I., Olsson-Liljequist, B., Hæggman, S., Hoffman, B.M., Lindahl, C., Burman, L.G., 2003. Clonality among ampicillin-resistant Enterococcus faecium isolates in Sweden and relationship with ciprofloxacin resistance. Clin. Microbiol. Infect. 9 (10), 1011–1019. https://doi.org/10.1046/j.1469-0691.2003.00701.x.

Schwermer, C.U., Krzeminski, P., Wennberg, A.C., Vogelsang, C., Uhl, W., 2018. Removal of antibiotic resistant *E. Coli* in two Norwegian wastewater treatment plants and by nano- and ultra-filtration processes. Water Sci. Technol. 77 (4), 1115–1126. https://doi.org/10.2166/wst.2017.642.

World Health Organization, 2015. Central Asian and Eastern European Surveillance of Antimicrobial Resistance. CAESAR Manual 2. http://www.euro.who.int/__data/assets/pdf_file/0005/293369/CAESAR-V2-Surveillance-Antimicrobial-Resistance-2015-en.pdf?ua=1.

Paper II

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

MECHANISMS OF RESISTANCE

Repeated Isolation of Extended-Spectrum-β-Lactamase-Positive *Escherichia coli* Sequence Types 648 and 131 from Community Wastewater Indicates that Sewage Systems Are Important Sources of Emerging Clones of Antibiotic-Resistant Bacteria

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ABSTRACT Antibiotic resistance in bacteria is an emerging problem globally. Resistant bacteria are found in human and animal microbiota, as well as in the environment. Wastewater receives bacteria from all these sources and thus can provide a measurement of abundance and diversity of antibiotic-resistant bacteria circulating in communities. In this study, water samples were collected from a wastewater pump station in a Norwegian suburban community over a period of 15 months. A total of 45 daily samples were cultured and analyzed for the presence of *Escherichia coli*. Eighty *E. coli*-like colonies were collected from each daily sample and then phenotyped and analyzed for antibiotic resistance using the PhenePlate-AREB system. During the sampling period, two unique *E. coli* phenotypes with resistance to cefotaxime and cefpodoxime indicating carriage of extended-spectrum β-lactamases (ESBL) were observed repeatedly. Whole-genome sequencing of 15 representative isolates from the two phenotypes identified these as two distinct clones belonging to the two globally spread *E. coli* multilocus sequence types (STs) ST131 and ST648 and carrying $bla_{\text{CTX-M-15}}$. The number of ESBL-positive *E. coli* strains in the community wastewater pump station was 314 of 3,123 (10%) analyzed *E. coli* strains. Of the ESBL-positive isolates, 37% belonged to ST648, and 7% belonged to ST131. Repeated findings of CTX-M-15-positive ST648 and ST131 over time indicate that these STs are resident in the analyzed wastewater systems and/or circulate abundantly in the community.

KEYWORDS ESBL *E. coli*, Norway, ST131, ST648, antibiotic resistance, antibiotic surveillance, persistence, sewage, wastewater, whole-genome sequencing

Microbial infectious diseases are a leading cause of morbidity and mortality worldwide. In the past few decades, the incidence of antibiotic resistance among pathogenic bacteria has increased, so surveillance of microbial pathogens and antibiotic-resistant bacteria in clinical settings, communities, and the environment is important (1). Wastewater systems (WWSs) receive a continuous discharge of fecal and urinary waste from human and animal communities (2). Moreover, wastewater treatment plants and sewage are considered reservoirs for bacteria harboring antibiotic resistance genes (ARGs), and might provide a perfect scenario for horizontal gene transfer of ARGs since they represent a unique mixture of antibiotics, disinfectants, metals, resistant bacteria, and fecal microbiota (3). Therefore, WWSs might represent a

Citation Paulshus E, Thorell K, Guzman-Otazo J, Joffre E, Colque P, Kühn I, Möllby R, Sørum H, Sjöling Å. 2019. Repeated isolation of extended-spectrum-β-lactamase-positive *Escherichia coli* sequence types 648 and 131 from community wastewater indicates that sewage systems are important sources of emerging clones of antibiotic-resistant bacteria. Antimicrob Agents Chemother 63:e00823-19. https://doi.org/10.1128/AAC.00823-19.

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Received 24 April 2019
Returned for modification 16 May 2019
Accepted 20 June 2019
Accepted manuscript posted online 24 June 2019
Published 23 August 2019

suitable interface for the surveillance of infectious bacteria and the presence of bacteria carrying ARGs. In addition, if properly collected, WWS samples provide a reliable representation of the large variability of bacteria circulating in large populations connected to the wastewater treatment plant or in individual sewage (4–6). Several studies have suggested an association between the presence of enteric pathogens in WWSs and active disease in the community (2, 7).

Nonpathogenic and commensal *Escherichia coli* are part of the normal intestinal microbiota of humans and animals. Some strains of *E. coli* carrying virulence genes are able to cause symptoms and disease in the host. Pathogenic *E. coli* are classified in two main groups: intestinal pathogenic *E. coli* and extraintestinal pathogenic *E. coli* (ExPEC) (8). *E. coli* strains have been largely recognized for their ability to both harbor and disseminate ARGs to other *Enterobacteriaceae* when colonizing humans and animals, as well for as their ability to survive in different kinds of environments (9).

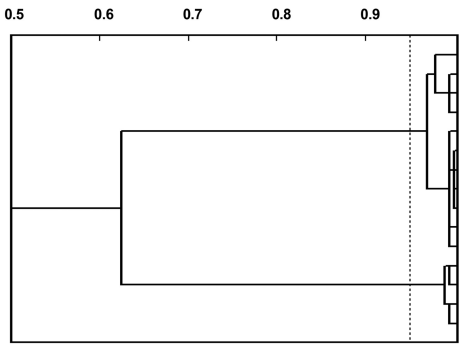
Infection by ExPEC strains can produce disease in humans ranging from urinary tract infections (UTIs) to septicemia (8). The emergence of antibiotic resistance, especially *E. coli* strains producing extended-spectrum β-lactamases (ESBLs), has complicated the medical treatment of ExPEC infections (10). ESBLs consist of a group of enzymes that can hydrolyze broad-spectrum cephalosporins and monobactams but remain susceptible to β-lactamase inhibitors (11, 12). ESBL-producing *E. coli* sequence type 131 (ST131) is a globally distributed uropathogenic *E. coli* (UPEC) lineage with higher virulence capacity and antibiotic resistance than other ExPEC clones, which might explain its high occurrence and persistence in urinary and bloodstream infections (10, 13). The global dissemination of *E. coli* ST131 has been largely related to the pandemic emergence of the CTX-M-15 group of ESBL enzymes (14). The CTX-M-encoding genes probably originate from the chromosomes of various species of the *Kluyvera* genus that were mobilized into enterobacterial plasmids (12). Five different groups of CTX-M and more than 80 different variants have been described. The most commonly found variants are CTX-M-2, CTX-M-3, CTX-M-14, and CTX-M-15 (12). Other ExPEC sequence types, such as ST38, ST405, and ST648, have also been associated with sepsis and UTIs and the global dissemination of different CTX-M variants (10). *E. coli* ST131 and ST648 producing CTX-M have been reported worldwide, not only in human infections but also in animal samples (13). During the last several decades, a pandemic spread of enzymes belonging to the CTX-M group has been reported. As a consequence, CTX-M-producing enterobacteria, including *E. coli*, are causing highly antibiotic-resistant infections that are difficult to treat (11, 12).

During a larger study of *E. coli* isolated from the wastewater of an urban hospital, a suburban community pump station, and a treatment plant in Norway, ESBL carriage was indicated in 7.3% of the isolates by resistance to cefotaxime and cefpodoxime (15). The aim of this study was to further characterize a subpopulation of these ESBL-producing *E. coli* strains isolated repeatedly from the suburban community wastewater pump station.

RESULTS

Two ESBL *E. coli* PhP types were repeatedly found in wastewater from an urban community in Norway. A total of 3,123 *E. coli* isolates from the wastewater pump station were analyzed by biochemical fingerprinting (the PhenePlate [PhP] system) and antibiotic resistance screening; 314 of these isolates (10%) exhibited phenotypic resistance to cefotaxime and cefpodoxime, indicating ESBL gene carriage. Of these isolates, several belonged to diverse PhP types that were usually observed in only one sample, and several isolates were also resistant to additional antibiotics. However, two distinct PhP types of ESBL-producing *E. coli* were repeatedly identified in wastewater samples throughout the sampling period. One PhP type was identified in 115 isolates, and the other was identified in 22 isolates.

During the first and second months of sampling (March and April 2016), nine isolates with the first PhP pattern were isolated that were resistant to ampicillin, cefotaxime, cefpodoxime, trimethoprim, tetracycline, gentamicin, ciprofloxacin, and



	PhP type	antibiotics								
		amp	ctx	chl	cip	gen	nal	pod	tet	tmp
BE01A-01	1	R	R	S	R	R	R	R	R	R
BE06A-17	1	R	R	S	R	S	R	R	S	S
BE08B-12	1	R	R	S	R	S	R	R	S	S
BE07A-66	1	R	R	S	R	S	R	R	S	S
BE04C-09	1	R	R	S	R	S	R	R	S	S
BE10A-06	1	R	R	S	R	S	R	R	S	S
BE12B-58	1	R	R	S	R	S	R	R	S	S
BE11A-16	1	R	R	S	R	S	R	R	S	S
BE14B-06	1	R	R	S	R	S	R	R	S	S
BE05A-26	1	R	R	S	R	S	R	R	S	S
BE09B-57	1	R	R	S	R	S	R	R	S	S
BE05A-16	2	R	R	S	R	S	R	R	S	S
BE05A-31	2	S	S	S	R	S	R	S	S	S
BE06B-35	2	R	R	S	R	S	R	R	S	S
BE11B-40	2	R	R	S	R	S	R	R	S	S

FIG 1 PhP (dendrogram and PhP type) and AREB typing for 15 isolates selected for whole-genome sequencing. Abbreviations: amp, ampicillin; ctx, cefotaxime; chl, chloramphenicol; cip, ciprofloxacin; gen, gentamicin; nal, nalidixic acid; pod, cefpodoxime; tet, tetracycline; tmp, trimethoprim; R, resistant; S, sensitive.

nalidixic acid. One additional isolate from March 2017 was resistant to all tested antibiotics, i.e., it was also resistant to chloramphenicol, and one isolate from April 2017 was resistant to all tested antibiotics except chloramphenicol and trimethoprim. The remaining 104 isolates of this PhP type had the same resistance pattern to ampicillin, cefotaxime, cefpodoxime, ciprofloxacin, and nalidixic acid. These isolates were found during all months but were particularly abundant in March 2016. After having identified this specific PhP type, we went back to the original data and identified eight additional non-ESBL members of this PhP type. Hence, in total, 3.9% ($n = 123$) of all *E. coli* isolates belonged to this PhP type, and 93% of these were suspected to be ESBL positive.

The other PhP type was originally isolated as putatively ESBL positive in 22 isolates. Reanalysis identified the same PhP type in a total of 74 isolates from the pump station wastewater; of these, 30% ($n = 22$) were ESBL positive, and half of the ESBL-positive isolates were resistant to all tested antibiotics except chloramphenicol.

Whole-genome sequencing of the two clonal groups identified them as ST648 and ST131. To further study the ESBL clones, a subset of 11 isolates from the first PhP type collected over the whole sampling period, including one multiresistant isolate from March 2016, were cultured, and DNA was extracted. Four isolates from the other PhP type, including one non-ESBL isolate, were also included (Fig. 1). Whole-genome sequencing was performed using an Illumina MiSeq to sequence the genomes of the 15 selected isolates. The genomes were sequenced to a coverage of >100× and assembled. The 15 genomes were analyzed using the CGE MLST finder, and the first PhP type was found to belong to *E. coli* ST648, while the four isolates of the other PhP type belonged to the uropathogenic *E. coli* clone ST131 (Fig. 1).

To determine the phylogenetic relationship of the 15 Norwegian isolates to other ST648 and ST131 isolated globally, a reference set of ST648 and ST131 genomes (13) and one ST648 isolate from a river in Bolivia (16) were downloaded from the Sequence Read Archive (SRA). A representative set of *E. coli* reference genomes (17), spanning the different Clermont *E. coli* phylogroups (18), was also included in the data set (see Table S1 in the supplemental material). The sequence reads were assembled and annotated using the same settings used for the Norwegian isolates. A phylogenetic tree based on the core genome determined by Roary was constructed (Fig. 2). The phylogenetic tree revealed that the Norwegian ST648 isolates formed a separate monophyletic cluster compared to the global set of isolates. The Norwegian isolates clustered together with an isolate from environmental water in the United States isolated in 2006 and an isolate collected from human feces in Lebanon in 2013. This clade separated from the rest of the ST648 isolates included in the phylogenetic analysis (Fig. 2).

The ST131 isolates clustered at two locations in the phylogenetic tree, indicating that three isolates (ST131-BE05A-16, ST131-BE06B-35, and ST131-BE11B-40) belonged

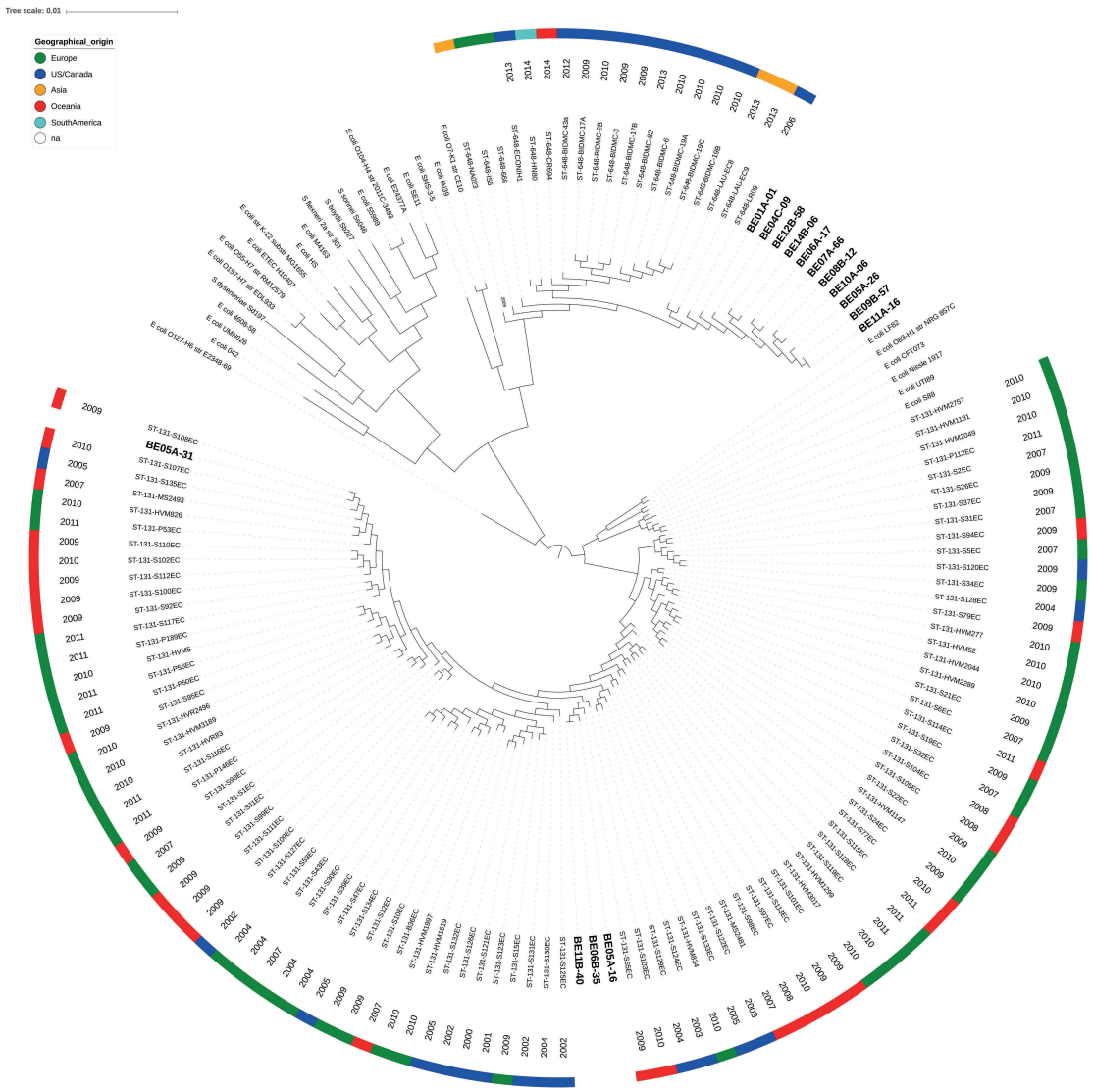


FIG 2 Core genome phylogenetic tree of ST648 and ST131 isolates compared to reference strains. Isolates are labeled by origin and year of isolation. Norwegian ST648 and ST131 isolates are indicated in bold text.

to the previously described ST131 clade A cluster (19), while the fourth, non-ESBL isolate (ST131-BE05A-31), which was resistant only to ciprofloxacin and nalidixic acid (Fig. 1), also belonged to ST131 clade A but clustered closely with isolates S108EC and S107EC isolated in Australia in 2009 and 2010, respectively, that were classified as CTX-M-27 carriers (20). None of the ST131 isolates belonged to the most virulent subtype ST131 clade C H30R.

***In silico* plasmid analysis revealed several plasmids in the genomes.** The genomes were searched for plasmid content and resistance genes using PlasmidFinder (21) and ResFinder (22), respectively. PlasmidFinder analysis of isolate ST648-BE01A-01, as well as of the four ST131 isolates, detected IncF (Table 1). The IncF plasmids

TABLE 1 Isolate identification, plasmid profiles and antibiotic resistance genes for 15 selected isolates

Isolate[a]	Plasmid(s)	pMLST	Resistance gene(s)
ST648-BE01A-01	Col8282	IncF[F1:A1:B1]	$bla_{CTX-M-15}$, *aac(3)IId*, *ARR-3*, *aadA5*, *tet*(34), *aac(6′)-Ib-cr*, *mph*(A), *sul1*, *dfrA17*, *tet*(*B*), *catB3*, *mdf*(A), bla_{OXA-1}, *ant(3″)*, bla_{TEM-1B}, *catA1*
ST648-BE04C-09	Col8282		$bla_{CTX-M-15}$, *mdf*(A), *tet*(34)
ST648-BE05A-26			$bla_{CTX-M-15}$, *mdf*(A), *tet*(34)
ST648-BE06A-17	Col8282		$bla_{CTX-M-15}$, *mdf*(A), *tet*(34)
ST648-BE07A-66	Col8282		$bla_{CTX-M-15}$, *mdf*(A), *tet*(34)
ST648-BE08B-12	Col8282		$bla_{CTX-M-15}$, *mdf*(A), *tet*(34)
ST648-BE09B-57	Col8282		$bla_{CTX-M-15}$, *mdf*(A), *tet*(34)
ST648-BE10A-06	Col8282		$bla_{CTX-M-15}$, *mdf*(A), *tet*(34)
ST648-BE11A-16	Col8282		$bla_{CTX-M-15}$, *mdf*(A), *tet*(34)
ST648-BE12B-58	Col8282		$bla_{CTX-M-15}$, *mdf*(A), *tet*(34)
ST648-BE14B-06	Col8282		$bla_{CTX-M-15}$, *mdf*(A), *tet*(34)
ST131-BE05A-16	Col(BS512), col156, Col(MG828)	IncF[F1:A1:B16]	*catB3*, *mdf*(A), *aac(6′)-Ib-cr*, bla_{OXA-1}, $bla_{CTX-M-15}$
ST131-BE05A-31	Col8282, col156, Col(MG828)	IncF[F1:A2:B20]	*mdf*(A)
ST131-BE06B-35	Col(BS512), col156, Col(MG828)	IncF[F1:A1:B16]	*catB3*, *mdf*(A), *aac(6′)-Ib-cr*, bla_{OXA-1}, $bla_{CTX-M-15}$
ST131-BE11B-40	Col(BS512), col156, Col(MG828)	IncF[F1:A1:B16]	*catB3*, *mdf*(A), *aac(6′)-Ib-cr*, bla_{OXA-1}, $bla_{CTX-M-15}$

[a]Isolate names indicate the location (BE), month (01-15, representing each consecutive month from March 2016 to May 2017), and day (A, B, and C) in which they were isolated, as well as colony number.

appeared to have been lost in all ST648 isolated after the first group of multiresistant isolates represented by isolate ST648-BE01A-01. Plasmid MLST (pMLST) analyses identified IncF[F1:A1:B1] in ST648-BE01A-01, while IncF[F1:A1:B16] was present in the three ST131 strains that clustered together, and IncF[F1:A2:B20] was present in the non-ESBL ST131-BE05A-31. All ST648 isolates except ST648-BE05A-26 harbored the small plasmid Col8282 and additional smaller plasmids (Table 1). ST131-H30R strains have previously been reported to harbor IncF plasmids, as well as Col156 and Col(MG828) (23). To verify plasmid profiles in all isolates used for the phylogenetic comparison, we analyzed all genomes using PlasmidFinder. IncF, Col8282, Col156, Col(BS512), and Col(MG828) were found in a large number of the isolates. IncN and IncH12 were found in ST648 isolates, while IncF dominated in ST131 (Table S2).

The plasmid(s) harboring $bla_{CTX-M-15}$ are conserved within, but different between, the ST648 and ST131 clones. Resistance gene content was analyzed *in silico* using ResFinder. All Norwegian isolates except the non-ESBL ST131-BE05A-31 carried $bla_{CTX-M-15}$, which corroborated the phenotypic analysis using the AREB plates and confirmed that these isolates are ESBL carriers. In ST648-BE01A-01, 15 *in silico* resistance genes, in addition to $bla_{CTX-M-15}$, were identified using the analysis tools (Table 1). In addition, this isolate was phenotypically resistant to ciprofloxacin and nalidixic acid, indicating additional chromosomal mutations that were not searched for. Since this strain carried an IncF plasmid, apparently lost in the subsequent Norwegian ST648 isolates, these genes might be present on IncF plasmid(s). Manual analyses of contigs revealed that *mph*(A), *sul1*, *aadA5*, *dfrA17*, and *ant(3″)-Ia* resided on the same contig, and *AAR-3*, *catB3*, bla_{OXA-1}, and *aac(6′)-Ib-cr* were located together on another contig. The $bla_{CTX-M-15}$ gene was located in a separate 112,056-bp contig in isolate ST648-BE01A-01. Manual BLAST analysis of the contig revealed that $bla_{CTX-M-15}$ is located together with repFIB. The sequence was found to be 99% identical to the 112,210-bp circularized plasmid AnCo1, previously described in *E. coli* strain 243 isolated from feces of wildlife in Colorado (24), and 99% identical to plasmid pV234-a, a 112,009-bp plasmid isolated from river water in India (25). The contig was also 96% identical to the 109,071-bp plasmid AnCo2, identified in *E. coli* strain 244 from the same study as AnCo1 (24). AnCo1, pV234-a, and AnCo2 all harbor $bla_{CTX-M-15}$. BLAST analysis of the shorter contigs harboring the remaining resistance genes in ST648-BE01A-01 indicated all genes to be plasmid borne. The content of the two contigs containing either (i) *mph*(A), *sul1*, *aadA5*, *dfrA17*, and *ant(3″)-Ia* or (ii) *AAR-3*, *catB3*, bla_{OXA-1}, and *aac(6′)-Ib-cr* was found on several plasmids in GenBank with conserved gene order and intergenic sequences.

Since $bla_{CTX\text{-}M\text{-}15}$ was found in all ST648 and three of the ST131 isolates, we next analyzed the genetic content of all $bla_{CTX\text{-}M\text{-}15}$ contigs. The genetic location of $bla_{CTX\text{-}M\text{-}15}$ was identical in all ST648 isolates, as well as identical to the location in the previously reported plasmids AnCo1, AnCo2, and pV234-a. Hence, the ST648 isolates harbor a $bla_{CTX\text{-}M\text{-}15}$ plasmid that is evolutionarily conserved and appear to be compatible with the chromosomal background of ST648. Several hypothetical genes, as well as the aerobic cobaltochelatase subunits *cobS* and *cobT*, glutaredoxin, and tRNA genes, flank the $bla_{CTX\text{-}M\text{-}15}$ gene in the plasmid. The ST131 isolates had a smaller contig with a length of 6,169 bp containing the $bla_{CTX\text{-}M\text{-}15}$ gene. The contig had 100% homology to larger plasmids isolated from other *E. coli* ST131 isolates, including an ST131 O25b:H4, as well as to plasmids from *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., *Salmonella enterica*, and *Shigella sonnei*. In the ST131 isolates, $bla_{CTX\text{-}M\text{-}15}$ was located next to a gene encoding the cupin fold-metalloprotein WbuC and surrounded by a Tn*3* family transposase and an IS*Ec9* transposase commonly associated with CTX-M-15.

The ST648 and ST131 isolates are tolerant to Cu^{2+} and Zn^{2+}, and the MIC exceeds the concentrations in wastewater. The genomes of the 15 sequenced isolates indicated the presence of copper resistance genes, as well as resistance to other heavy metals. We measured the concentrations of copper, zinc, nickel, and chromium ions in all three wastewater sites included in the larger study (15), but the levels were comparable and not higher in the wastewater pump station, and no seasonal variation was observed. The concentrations of copper and zinc in the community wastewater averaged 1.51 μM (extremes, 0.85 to 2.36 μM) and 2.41 μM (extremes, 1.32 to 3.67 μM), respectively, and were about 50-fold higher than those of nickel and chromium. The MICs for the 15 isolates for tolerance toward copper and zinc were determined using agar plates supplemented with copper and zinc sulfate salts. The MICs for the control strains were 16 mM for Cu^{2+} and 4 mM for Zn^{2+}, while the MICs for the 15 isolates were similar for all and measured 16 mM for Cu^{2+} and 8 mM for Zn^{2+}. The metal tolerance did not increase in the isolates collected later in the study. These results indicate that ST648 and ST131 in this study are tolerant to copper at a level comparable to the control strains and, while slightly more tolerant to zinc than the controls, the levels in the wastewater are probably not high enough to confer any selective pressure that explains the repeated findings of these STs in the wastewater pump station.

ST131 isolates produce more biofilm than ST648 and display red, dry, and rough biofilm morphotypes. To assess their ability to form biofilms, the ST131 and ST648 isolates were analyzed by crystal violet staining after incubation at 28 and 37°C. At 28°C, all four ST131 isolates and four of eleven ST648 isolates were classified as high biofilm producers, three ST648 isolates were classified as moderate biofilm producers, and four isolates, including the multiresistant ST648-BE01A-01, were classified as low producers (Fig. 3A). Most isolates showed less biofilm formation at 37°C than at 28°C, and a significant reduction was observed for three ST131 isolates and for four of the ST648 isolates (Fig. 3A).

A similar pattern of biofilm formation was observed in the production of extracellular polymeric substances (EPS) among ST131 and ST648 isolates, with a significant induction of EPS production at 28°C (Fig. 3B). In particular, ST131 isolates showed enhanced EPS production at 28°C compared to ST648 isolates (Fig. 3B; see also Fig. S1 in the supplemental material).

Evaluation of the production of fimbriae, extracellular polymeric substances, and cellulose by the red, dry, and rough (rdar) morphotype characterization indicated that among all isolates grown at 28°C for 48 h on LB plates without salt (LB_{ws}) containing Congo red, the ST131 isolates had a more conserved and fully formed rdar morphotype expressing both cellulose (pdar morphotype), and curli fimbriae (bdar morphotype) (Fig. 4A). On the contrary, the ST648 isolates were smaller and displayed a greater diversity of morphotypes. For example, six of the ST648 isolates (ST648-BE01A-01, ST648-BE04C-09, ST648-BE05A-26, ST648-BE09B-57, ST648-BE10A-06, and ST648-BE11A-16) expressed the smooth-and-white (saw) morphotype without expression of neither cellulose nor curli (Fig. 4B) while the remaining five isolates (ST648-BE06A-17,

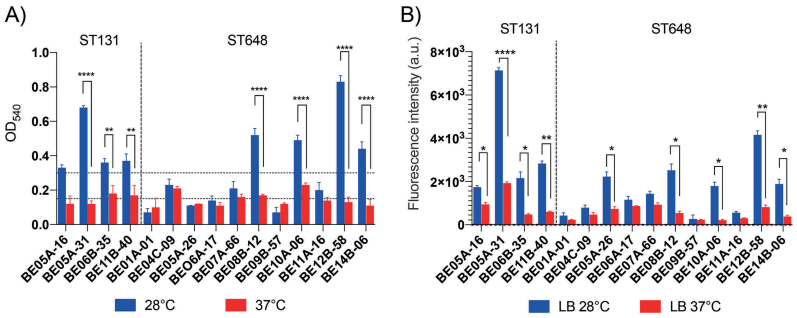


FIG 3 Biofilm formation analysis by *E. coli* ST131 and ST648. Crystal violet (CV) (A) and FITC-CoA (B) staining of biofilm adherent to polystyrene microplates plates on after incubation for 48 h at 28 or 37°C at the liquid-air interface. For CV staining, retained CV in adherent bacteria is equivalent to the production of biofilm. For FITC-ConA staining, the fluorescence intensity (arbitrary units [a.u.]) represents the EPS production. Horizontal dotted lines indicate low/medium/high biofilm production. Error bars indicate the standard errors of the mean. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.5$.

ST648-BE07A-66, ST648-BE08B-12, ST648-BE12B-58, and ST648-BE14B-06) expressed the bdar morphotype (Fig. 4C). Altogether, our data indicated that 28°C favored biofilm formation, in particular in ST131 isolates.

DISCUSSION

During the last 10 to 15 years, a global epidemic of ESBL-producing enterobacteria conferred by members of the CTX-M enzymes has emerged (12, 26). CTX-M-encoding genes are probably originally mobilized from the chromosomes of various species of the *Kluyvera* genus to plasmids that are well adapted particularly to *E. coli* (12).

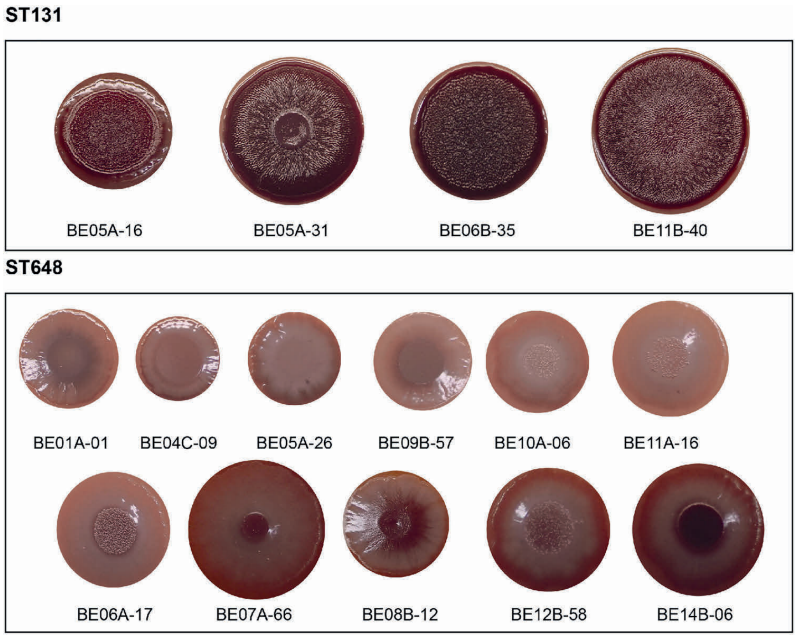


FIG 4 Biofilm phenotypes of Norwegian ST131 and ST648 *E. coli* isolates. The figure shows rdar morphotype formation of ST131 and ST648 isolates after 48 h of incubation at 28°C on Congo red LB without salt plates.

Consequently, ESBL-positive *E. coli* have increased over time. Carriage of CTX-M enzymes, particularly the CTX-M-15 variant, seems to be associated with specific strains of *E. coli*. Much of the emergent increase in CTX-M-15 is attributed to *E. coli* ST131, a pandemic clone that causes ExPEC UTIs and is widely known for worldwide dissemination of multidrug resistance and CTX-M-15 (19). ST131 has emerged as a major human health threat in the last decade, and belongs to a group of so-called high-risk clones, with preference for CTX-M carriage. This group is an important cause of community- and hospital-acquired infections, such as UTIs, surgical-site infections, bloodstream infections, and sepsis, and in addition to ST131 this group includes ST131, ST405, ST410, ST38, ST393, ST69, and ST648 (12, 19, 27, 28). Several of these STs have been identified in animals, birds, and environmental waters and on fresh produce, in addition to humans, indicating a facilitated spread across different niches (28–30). Several STs, including ST648 and ST131, have also been found in environmental water and wastewater (16, 31), supporting the findings of high numbers of ST648 and ST131 in wastewater in this study.

We found that ST131 and ST648 together constituted 44% of all ESBL-positive *E. coli* in the wastewater pump station. Although the relatively high prevalence of these two sequence types in ESBL-positive isolates is supported by several other studies (32, 33), and ST131 was frequent in a study of clinical, recreational-water, and wastewater samples in the same catchment area as our study (34), one might argue that our findings of ESBL-positive uropathogenic *E. coli* detected locally in the pump station wastewater is due to fecal matter from one or few persons in the community. To avoid bias caused by grab sampling, wherein one sample reflects a single moment at the time of sampling, continuous sampling over several hours is recommended (35, 36). In this study, a sampling device was used over 24 h for each daily sample (15). Using this sampling technique, the first detection of multiresistant ST648, including isolate ST648-BE01A-01, was during the first and third days of sampling in March and the third day in April. The remaining ST648 isolates with CTX-M-15 and resistance to nalidixic acid and ciprofloxacin were found during all months. The results might suggest that the wastewater is receiving a constant influx of ST648 from the community or, alternatively, that ST648 and ST131 reside locally in the pipes of the wastewater system. Considering the first explanation, the wastewater systems in regular communities may be more relevant to use as sites of monitoring the spread and establishment of globally emerging multidrug-resistant (MDR) bacterial clones related to the intestinal microbiota than so far acknowledged. The focus has primarily been toward establishment in the hospitals and the spread of such clones from hospitals. The alternative explanation indicates that it is possible that wastewater systems constitute a considerable source of resistant bacteria and of resistance genes to be spread into society.

Analysis of metal tolerance in comparison to the levels of copper and zinc in the wastewater did not indicate that the levels of metal ions confer any selective force onto the ST648 and ST131 clones. To analyze their ability to form biofilms, all sequenced isolates were tested using crystal violet analysis and Congo red agar plates. The ST131 isolates were better biofilm producers than the ST648 isolates, which varied in their biofilm-producing capabilities. In accordance with other studies, we could not determine a clear correlation with biofilm production and virulence markers (37).

Uropathogenic *E. coli* (UPEC) are responsible for 50 and 95% of nosocomial and community UTIs, respectively (10). The intestinal tract in humans has been considered a reservoir of UPEC strains able to infect the urinary tract and to produce severe infections (38, 39). During acute UTIs, infected individuals often have $>10^5$ CFU/ml urine (40). Thus, UPEC infections could increase bacterial numbers in urine on an individual and on a community level, which subsequently end up in wastewater treatment plants and the environment. Indeed, UPEC, as well as other types of *E. coli*, are frequently found in wastewater systems and environmental waters (15, 16, 34, 35).

We found ESBL-positive *E. coli* ST648 in wastewater, as well as ST131 isolates at lower numbers over several months. ST131 isolates were less frequently ESBL positive compared to ST648. The ST648 clone was first isolated and described in 2008 (41).

Although ST648 can cause disease in humans and was first isolated from human urine, the sequence type is also able to cause disease in animals, since it has been isolated from household animals with cystitis and from wild and domesticated animals (42, 43). ST648 has since its discovery been described to be able to carry resistance genes to recent antibiotics, including bla_{NDM-1}, bla_{NDM-5} (44), and *fosA* (45). Our data indicate a higher prevalence of CTX-M-15 in ST648 than in ST131 and ST648 isolates dominated the ESBL-positive *E. coli* identified in this study. This is corroborated by other studies reporting either similar or higher frequencies of CTX-M-15 carriage in ST648 than in ST131 (32, 43, 46). Interestingly, CTX-M-15 plasmids have been suggested to confer advantage and increase the fitness of its bacterial hosts during infection (47, 48). Further studies are needed to elucidate whether CTX-M-15 plasmids add additional advantageous traits in an ST648 genetic background. However, the conserved CTX-M-15 plasmid identified in the Norwegian ST648 isolates has been found also in the feces of wildlife in the United States (24) and in isolates from river water in India (25). Therefore, the impact of environmental fitness by this plasmid should be determined.

In conclusion, by using PhP plate analysis and confirming observed clones by whole-genome sequencing, we repeatedly identified uropathogenic *E. coli* belonging to the virulent types ST648 and ST131 and carrying $bla_{CTX-M-15}$, as well as several additional resistance genes, in a suburban community wastewater catchment pump station. Additional sampling 2 years later could not identify the PhP types corresponding to these sequence types at the same pump station, which may suggest a transient accumulation of apparently clonal isolates in 2016 and 2017. Monitoring of MDR bacteria in wastewater systems in communities might thus identify emerging clones and/or fluctuations in the bacterial population carrying ARGs and could serve as early warning systems.

MATERIALS AND METHODS

Isolation of strains and PhP analysis. Water samples from a wastewater pump station in a suburban community in Norway were collected over 15 months from March 2016 to May 2017. Sampling was performed as described by Paulshus et al. (15). Briefly, daily samples were collected 3 days in a row each month. Each daily sample was composited of 24 aliquots of 200 ml, collected at hourly intervals for 24 h, using an Isco 3700 full-size portable sampler (Teledyne ISCO, Lincoln, NE). The sampler was rinsed with water between each daily sample and then rinsed with water, cleaned with 0.1 to 1% sodium hypochlorite (Klorin), and bathed in 70% ethanol between monthly sampling occasions. A total of 45 samples were collected over the 15 months. The samples were numbered for the month of collection and labeled A, B, and C for each of the three consecutive sampling days. The daily samples were plated in serial dilutions onto CHROMagar orientation (CHROMagar, Paris, France) agar plates. Up to 80 *E. coli*-like colonies were collected from each daily sample and subjected to PhenePlate (PhP) typing (PhPlate AB) (5, 35). The PhP system quantifies fermentation patterns for each isolate by assigning numerical values to each carbohydrate source (in 96-plate wells) as a discrete value on a scale from completely negative (25) to completely positive (0), respectively. Each of the 11-cipher profiles per isolate was then compared to all other isolates in the sample and was grouped into PhP types. Isolates within the same PhP type share >97.5% identity in their PhP profiles. Isolates that have identical PhP types are most likely of a common origin or have very similar genomes, i.e., they are clones.

The colonies were simultaneously analyzed for resistance to nine antibiotics with the AREB microplates (5, 35). In total, 3,123 isolates of *E. coli* were investigated, and 15 isolates were selected to represent isolates from the two observed clonal groups collected over time during different months and with antibiotic resistance patterns of interest. These representative isolates were further characterized in the present study.

DNA extraction and library preparation. The 15 selected isolates were grown on LB agar overnight at 37°C. One colony per isolate was picked and used for DNA extraction. The colony was washed with 200 μl of MilliQ water, and bacterial lysis was immediately initiated by treatment with 1 mg/ml lysozyme and proteinase K in lysis buffer overnight. DNA was extracted by using a DNeasy blood and tissue extraction kit (Qiagen), as recommended by the manufacturer, and eluted in 200 μl of MilliQ water. The DNA concentration was measured using Qubit, and 50 ng of DNA was used for library preparation. Sequencing libraries were prepared by using a TruSeq Nano DNA high-throughput library prep kit (Illumina, San Diego, CA) with a mean fragment length of 900 bp. Libraries were sequenced using the MiSeq platform (v.3) chemistry and 2 × 300 bp, generating a coverage of >100× for each isolate.

Bioinformatics analysis. The sequence data were processed using the BACTpipe assembly and annotation pipeline v.2.6.1 (49), where reads were assessed for species classification using mash-screen (50), quality trimming and filtering was performed using bbduk (51), and *de novo* assembly and quality assessment was performed using SPAdes (52) within the Shovill pipeline v.1.0.0 (https://github.com/tseemann/shovill); the draft genomes were then annotated using prokka (53), and annotation and assembly statistics were collected using MultiQC (54). Sequence typing and plasmid MLST was performed

using CGE pipeline v1.1 (55). After obtaining the sequence typing results, a reference set of ST648 and ST131 genomes was downloaded for comparison, together with a representative set of *E. coli* reference genomes from the different Clermont groups (17; see also Table S1 in the supplemental material). Sequences downloaded from the Sequence Read Archive (SRA) were assembled and annotated using the same pipeline as described above. Upon annotation, a core genome was constructed using the Roary pangenome pipeline (56), and a phylogenetic tree was computed using FastTree (57). The tree was visualized and annotated using iTol (58).

Heavy metal concentrations in wastewater. Concentrations of the heavy metals copper (Cu) and zinc (Zn), as well as nickel (Ni) and chromium (Cr), were determined for all wastewater samples by using Eurofins method NS EN ISO 17294-2 (Eurofins Environment Testing AS, Moss, Norway). The limits of quantification were 2.0 μg/liter for zinc and 0.5 μg/liter for the other heavy metals.

Metal tolerance and MIC determination. Isolates were cultured and tested for tolerance to $ZnSO_4{\cdot}7H_2O$ and $CuSO_4{\cdot}5H_2O$ by the agar dilution MIC determination method for metals (59), with some minor modifications. Briefly, Mueller-Hinton medium was used to prepare series of 2-fold dilutions of $ZnSO_4{\cdot}7H_2O$ (from 0.5 to 16 mM) and $CuSO_4{\cdot}5H_2O$ (from 0.5 to 32 mM). The pH of the medium was adjusted to 5.5 and 7.0, respectively. Plates were inoculated with spots of 2 μl of bacterial suspensions adjusted to a 0.5 McFarland standard. Plates were incubated for 48 h at 37°C to detect bacterial growth. MICs were established as the minimum concentrations of metal salt where bacterial growth was not present (60). Tolerance to metals was evaluated comparing the MIC values obtained from tested isolates with MIC values from the susceptibility controls. The *E. coli* strains ATCC 25922 and CV601 were used as controls, and determination tests were conducted in triplicate.

Crystal violet biofilm assays. Biofilm assays were performed on microtiter plates as described previously (61). Overnight cultures were diluted 1:100 in fresh LB medium without salt. Aliquots (150 μl) of the diluted samples were incubated in the wells of a flat-bottomed 96-well polystyrene microtiter plate (Corning) for 48 h at 28 or 37°C. Each cell culture (100 μl) was transferred into another flat-bottom 96-well polystyrene microtiter plate and spectrophotometrically measured at 600 nm to assess planktonic bacterial growth. After the planktonic bacteria were discarded from the initial microtiter plate, bacterial cells bound to the wells were gently washed twice with 200 μl of phosphate-buffered saline (PBS), air dried, and then stained with 200 μl of 0.1% (wt/vol) crystal violet for 15 min. After incubation, the stained plates were washed four times with distilled water to remove excess crystal violet, and the stained biofilms were solubilized with 200 μl of 80% ethanol. The absorbance (540 nm) was measured with a SpectraMax i3x spectrophotometer (Molecular Devices). For each experiment, the absorbance of the blank control with crystal violet was subtracted from the experimental sample. Three replicates were used in each experiment, and three independent experiments were performed. Biofilm production was classified as low, medium, or high as previously described (62).

Semiquantitative analysis of extracellular polymeric substances. For quantification of extracellular polymeric substances (EPS), assays were performed in Corning 96-well black polystyrene microplates with clear, flat bottoms, as described previously (63). After washing steps following overnight incubation, the wells were treated with fixative (3% paraformaldehyde and 0.25% glutaraldehyde in 0.01 M PBS) for 15 min. The wells were subsequently washed and stained with concanavalin A (ConA) conjugated to fluorescein isothiocyanate (FITC; 25 g/ml) for 15 min at room temperature. Each well was subsequently washed in PBS, and the fluorescence at 488 nm was measured with a SpectraMax i3x spectrophotometer plate reader (Molecular Devices). The analysis was repeated in three independent experiments.

Biofilm morphotype formation assay. Portions (5 μl) of an overnight culture were centrifuged, and the pellets were suspended in PBS to an optical density at 600 nm of 5. The concentrated cultures were spotted onto LB without salt (LB_{ws}) agar plates supplemented with Congo red (40 μg/ml) and Coomassie brilliant blue (20 μg/ml). Plates were incubated at 28°C for 48 h and photographed.

Statistical analysis. Statistical significance for the biofilm analysis was determined using two-way analysis of variance, with a P value of $\leq$0.05 considered significant.

Data availability. The sequenced genomes have been deposited at GenBank under accession numbers SPIT00000000, SPIU00000000, SPIV00000000, SPIW00000000, SPIX00000000, SPIY00000000, SPIZ00000000, SPJA00000000, SPJB00000000, SPJC00000000, SPJD00000000, SPJE00000000, SPJF00000000, SPJG00000000, and SPJH00000000.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00823-19.

SUPPLEMENTAL FILE 1, PDF file, 2.6 MB.

ACKNOWLEDGMENTS

This study was supported by the Norwegian regional research fund RFFHSTAD (Regionalt Forskningsfond Hovedstaden), project 245686; the Norwegian wastewater treatment plant Vestfjorden Avløpsselskap (VEAS); and the Norwegian University of Life Sciences (NMBU). Support was also obtained from Swedish Research Links Dnr 2017-05423, and sequencing was performed with partial support from the Center for Translational Microbiome Research, Karolinska Institutet, a collaboration between Karo-

linska Institutet, Science for Life Laboratory and Ferring Pharmaceuticals, and bioinformatic support from Fredrik Boulund.

R.M., P.C., and I.K. are shareholders of PhPlate Microplate Techniques AB.

REFERENCES

1. Pepper IL, Brooks JP, Gerba CP. 2018. Antibiotic-resistant bacteria in municipal wastes: is there reason for concern? Environ Sci Technol 52:3949–3959. https://doi.org/10.1021/acs.est.7b04360.
2. Yan T, O'Brien P, Shelton JM, Whelen AC, Pagaling E. 2018. Municipal wastewater as a microbial surveillance platform for enteric diseases: a case study for *Salmonella* and salmonellosis. Environ Sci Technol 52:4869–4877. https://doi.org/10.1021/acs.est.8b00163.
3. Karkman A, Do TT, Walsh F, Virta M. 2018. Antibiotic resistance genes in waste water. Trends Microbiol 26:220–228. https://doi.org/10.1016/j.tim.2017.09.005.
4. Blanch AR, Belanche-Munoz L, Bonjoch X, Ebdon J, Gantzer C, Lucena F, Ottoson J, Kourtis C, Iversen A, Kuhn I, Moce L, Muniesa M, Schwartzbrod J, Skraber S, Papageorgiou GT, Taylor H, Wallis J, Jofre J. 2006. Integrated analysis of established and novel microbial and chemical methods for microbial source tracking. Appl Environ Microbiol 71 72:5915–5926. https://doi.org/10.1128/AEM.02453-05.
5. Colque Navarro P, Fernandez H, Möllby R, Otth L, Tiodolf M, Wilson M, Kühn I. 2014. Antibiotic resistance in environmental *Escherichia coli*: a simple screening method for simultaneous typing and resistance determination. J Water Health 12:692–701. https://doi.org/10.2166/wh.2014.216.
6. Newton RJ, McLellan SL, Dila DK, Vineis JH, Morrison HG, Eren AM, Sogin ML. 2015. Sewage reflects the microbiomes of human populations. mBio 6:e02574-14. https://doi.org/10.1128/mBio.02574-14.
7. Yang K, Pagaling E, Yan T. 2014. Estimating the prevalence of potential enteropathogenic *Escherichia coli* and intimin gene diversity in a human community by monitoring sanitary sewage. Appl Environ Microbiol 80:119–127. https://doi.org/10.1128/AEM.02747-13.
8. Kaper JB, Nataro JP, Mobley HL. 2004. Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140. https://doi.org/10.1038/nrmicro818.
9. Biswal BK, Khairallah R, Bibi K, Mazza A, Gehr R, Masson L, Frigon D. 2014. Impact of UV and peracetic acid disinfection on the prevalence of virulence and antimicrobial resistance genes in uropathogenic *Escherichia coli* in wastewater effluents. Appl Environ Microbiol 80:3656–3666. https://doi.org/10.1128/AEM.00418-14.
10. Pitout JD. 2012. Extraintestinal pathogenic *Escherichia coli*: a combination of virulence with antibiotic resistance. Front Microbiol 3:9. https://doi.org/10.3389/fmicb.2012.00009.
11. Cantón R, Coque TM. 2006. The CTX-M β-lactamase pandemic. Curr Opin Microbiol 9:466–475. https://doi.org/10.1016/j.mib.2006.08.011.
12. Canton R, Gonzalez-Alba JM, Galan JC. 2012. CTX-M enzymes: origin and diffusion. Front Microbiol 3:110. https://doi.org/10.3389/fmicb.2012.00110.
13. Shaik S, Ranjan A, Tiwari SK, Hussain A, Nandanwar N, Kumar N, Jadhav S, Semmler T, Baddam R, Islam MA, Alam M, Wieler LH, Watanabe H, Ahmed N. 2017. Comparative genomic analysis of globally dominant ST131 clone with other epidemiologically successful extraintestinal pathogenic *Escherichia coli* (ExPEC) lineages. mBio 8:e01596-17. https://doi.org/10.1128/mBio.01596-17.
14. Schembri MA, Zakour NL, Phan MD, Forde BM, Stanton-Cook M, Beatson SA. 2015. Molecular characterization of the multidrug resistant *Escherichia coli* ST131 clone. Pathogens 4:422–430. https://doi.org/10.3390/pathogens4030422.
15. 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. https://doi.org/10.1016/j.watres.2019.05.102.
16. Guzman-Otazo J, Gonzales-Siles L, Poma V, Bengtsson-Palme J, Thorell K, Flach C-F, Iñiguez V, Sjöling Å. 2019. Diarrheal bacterial pathogens and multi-resistant enterobacteria are frequent in the Choqueyapu River in La Paz, Bolivia. PLoS One 14:e0210735. https://doi.org/10.1371/journal.pone.0210735.
17. Cimdins A, Simm R, Li F, Lüthje P, Thorell K, Sjöling Å, Brauner A, Römling U. 2017. Alterations of c-di-GMP turnover proteins modulate rdar biofilm formation in commensal and uropathogenic *Escherichia coli*. Microbiologyopen 6:e00508. https://doi.org/10.1002/mbo3.508.
18. Clermont O, Bonacorsi S, Bingen E. 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 66:4555–4558. https://doi.org/10.1128/AEM.66.10.4555-4558.2000.
19. Petty NK, Ben Zakour NL, Stanton-Cook M, Skippington E, Totsika M, Forde BM, Phan MD, Gomes Moriel D, Peters KM, Davies M, Rogers BA, Dougan G, Rodriguez- Baño J, Pascual A, Pitout JD, Upton M, Paterson DL, Walsh TR, Schembri MA, Beatson SA. 2014. Global dissemination of a multidrug-resistant *Escherichia coli* clone. Proc Natl Acad Sci U S A 111:5694–5699. https://doi.org/10.1073/pnas.1322678111.
20. Matsumura Y, Pitout JDD, Gomi R, Matsuda T, Noguchi T, Yamamoto M, Peirano G, deVinney R, Bradford PA, Motyl MR, Tanaka M, Nagao M, Takakura S, Ichiyama S. 2016. Global *Escherichia coli* sequence type 131 clade with $bla_{CTX\text{-}M\text{-}27}$ gene. Emerg Infect Dis 22:1900–1907. https://doi.org/10.3201/eid2211.160519.
21. Carattoli A, Zankari E, Garcia-Fernandez A, Voldby Larsen M, Lund O, Villa L, Møller Aarestrup F, Hasman H. 2014. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 58:3895–3903. https://doi.org/10.1128/AAC.02412-14.
22. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640–2644. https://doi.org/10.1093/jac/dks261.
23. Johnson TJ, Aziz M, Liu CM, Sokurenko E, Kisiela DI, Paul S, Andersen P, Johnson JR, Price LB. 2016. Complete genome sequence of a CTX-M-15-producing *Escherichia coli* strain from the H30Rx subclone of sequence type 131 from a patient with recurrent urinary tract infections, closely related to a lethal urosepsis isolate from the patient's sister. Genome Announc 4:e00334-16. https://doi.org/10.1128/genomeA.00334-16.
24. Colaveccio A, Jeukens J, Freschi L, Rheault JGE, Kukavica-Ibrulj I, Levesque RC, LeJeune J, Goodridge L. 2017. Complete genome sequences of two phage-like plasmids carrying the CTX-M-15 extended-spectrum β-lactamase gene. Genome Announc 5:e00102-17. https://doi.org/10.1128/genomeA.00102-17.
25. Akiba M, Sekizuka T, Yamashita A, Kuroda M, Fujii Y, Murata M, Lee K, Joshua DI, Balakrishna K, Bairy I, Subramanian K, Krishnan P, Munuswamy N, Sinha RK, Iwata T, Kusumoto M, Guruge KS. 2016. Distribution and relationships of antimicrobial resistance determinants among extended-spectrum-cephalosporin-resistant or carbapenem-resistant *Escherichia coli* isolates from rivers and sewage treatment plants in India. Antimicrob Agents Chemother 60:2972–2980. https://doi.org/10.1128/AAC.01950-15.
26. Woerther PL, Burdet C, Chachaty E, Andremont A. 2013. Trends in human fecal carriage of extended-spectrum beta-lactamases in the community: toward the globalization of CTX-M. Clin Microbiol Rev 26:744–758. https://doi.org/10.1128/CMR.00023-13.
27. Naseer U, Sundsfjord A. 2011. The CTX-M conundrum: dissemination of plasmids and *Escherichia coli* clones. Microb Drug Resist 17:83–97. https://doi.org/10.1089/mdr.2010.0132.
28. Ortega-Paredes D, Barba P, Mena-López S, Espinel N, Zurita J. 2018. *Escherichia coli* hyperepidemic clone ST410-A harboring $bla_{CTX\text{-}M\text{-}15}$ isolated from fresh vegetables in a municipal market in Quito-Ecuador. Int J Food Microbiol 280:41–45. https://doi.org/10.1016/j.ijfoodmicro.2018.04.037.
29. Guenther S, Ewers C, Wieler LH. 2011. Extended-spectrum beta-lactamases producing *Escherichia coli* in wildlife, yet another form of environmental pollution? Front Microbiol 2:246. https://doi.org/10.3389/fmicb.2011.00246.
30. Mathers AJ, Peirano G, Pitout JD. 2015. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant *Enterobacteriaceae*. Clin Microbiol Rev 8:565–591. https://doi.org/10.1128/CMR.00116-14.
31. Zahra R, Javeed S, Malala B, Babenko D, Toleman MA. 2018. Analysis of *Escherichia coli* STs and resistance mechanisms in sewage from Islam-

abad, Pakistan, indicates a difference in *E. coli* carriage types between South Asia and Europe. J Antimicrob Chemother https://doi.org/10.1093/jac/dky109.
32. Musicha P, Feasey NA, Cain AK, Kallonen T, Chaguza C, Peno C, Khonga M, Thompson S, Gray KJ, Mather AE, Heyderman RS, Everett DB, Thomson NR, Msefula CL. 2017. Genomic landscape of extended-spectrum β-lactamase resistance in *Escherichia coli* from an urban African setting. J Antimicrob Chemother 72:1602–1609. https://doi.org/10.1093/jac/dkx058.
33. Campos ACC, Andrade NL, Ferdous M, Chlebowicz MA, Santos CC, Correal JCD, Lo-Ten-Foe JR, Rosa ACP, Damasco PV, Friedrich AW, Rossen J. 2018. Comprehensive molecular characterization of *Escherichia coli* isolates from urine samples of hospitalized patients in Rio de Janeiro, Brazil. Front Microbial 16:243. https://doi.org/10.3389/fmicb.2018.00243.
34. Jørgensen SB, Søraas AV, Arnesen LS, Leegaard TM, Sundsfjord A, Jenum PA. 2017. A comparison of extended spectrum β-lactamase-producing *Escherichia coli* from clinical, recreational water, and wastewater samples associated in time and location. PLoS One 12:e0186576. https://doi.org/10.1371/journal.pone.0186576.
35. Kwak YK, Colque P, Byfors S, Giske CG, Möllby R, Kuhn I. 2015. Surveillance of antimicrobial resistance among *Escherichia coli* in wastewater in Stockholm during 1 year: does it reflect the resistance trends in the society? Int J Antimicrob Agents 45:25–32. https://doi.org/10.1016/j.ijantimicag.2014.09.016.
36. Diwan V, Stålsby Lundborg C, Tamhankar AJ. 2013. Seasonal and temporal variation in release of antibiotics in hospital wastewater: estimation using continuous and grab sampling. PLoS One 8:e68715. https://doi.org/10.1371/journal.pone.0068715.
37. Novais Â, Vuotto C, Pires J, Montenegro C, Donelli G, Coque TM, Peixe L. 2013. Diversity and biofilm production ability among isolates of *Escherichia coli* phylogroup D belonging to ST69, ST393, and ST405 clonal groups. BMC Microbiol 13:144. https://doi.org/10.1186/1471-2180-13-144.
38. Foxman B. 2010. The epidemiology of urinary tract infection. Nat Rev Urol 7:653–660. https://doi.org/10.1038/nrurol.2010.190.
39. Vimont S, Boyd A, Bleibtreu A, Bens M, Goujon J-M, Garry L, Clermont O, Denamur E, Arlet G, Vandewalle A. 2012. The CTX-M-15-producing *Escherichia coli* clone O25b: H4-ST131 has high intestine colonization and urinary tract infection abilities. PLoS One 7:e46547. https://doi.org/10.1371/journal.pone.0046547.
40. Wilson ML, Gaido L. 2004. Laboratory diagnosis of urinary tract infections in adult patients. Clin Infect Dis 38:1150–1158. https://doi.org/10.1086/383029.
41. Nicolas-Chanoine MH, Blanco J, Leflon-Guibout V, Demarty R, Alonso MP, Canica MM, Park YJ, Lavigne JP, Pitout J, Johnson JR. 2008. Intercontinental emergence of *Escherichia coli* clone O25:H4-ST131-producing CTX-M-15. J Antimicrob Chemother 61:273–281. https://doi.org/10.1093/jac/dkm464.
42. Huber H, Zweifel C, Wittenbrink MM, Stephan R. 2013. ESBL-producing uropathogenic *Escherichia coli* isolated from dogs and cats in Switzerland. Vet Microbiol 162:992–996. https://doi.org/10.1016/j.vetmic.2012.10.029.
43. Ewers C, Bethe A, Stamm I, Grobbel M, Kopp PA, Guerra B, Stubbe M, Doi Y, Zong Z, Kola A, Schaufler K, Semmler T, Fruth A, Wieler LH, Guenther S. 2014. CTX-M-15-D-ST648 *Escherichia coli* from companion animals and horses: another pandemic clone combining multiresistance and extraintestinal virulence? J Antimicrob Chemother 69:1224–1230. https://doi.org/10.1093/jac/dkt516.
44. Hornsey M, Phee L, Wareham DW. 2011. A novel variant, NDM-5, of the new Delhi metallo-β-lactamase in a multidrug-resistant *Escherichia coli* ST648 isolate recovered from a patient in the United Kingdom. Antimicrob Agents Chemother 55:5952–5954. https://doi.org/10.1128/AAC.05108-11.
45. Dantas-Palmeira J, Ferreira H, Madec JY, Haenni M. 2018. Pandemic *Escherichia coli* ST648 isolate harbouring *fosA3* and $bla_{CTX-M-8}$ on an Inc1/ST113 plasmid: a new successful combination for the spread of fosfomycin resistance? J Glob Antimicrob Resist 15:254–255. https://doi.org/10.1016/j.jgar.2018.10.025.
46. Peirano G, van der Bij AK, Gregson DB, Pitout JDD. 2012. Molecular epidemiology over an 11-year period (2000 to 2010) of extended-spectrum-β-lactamase-producing *Escherichia coli* causing bacteremia in a centralized Canadian region. J Clin Microbiol 50:294–299. https://doi.org/10.1128/JCM.06025-11.
47. Schaufler K, Semmler T, Pickard DJ, de Toro M, de la Cruz F, Wieler LH, Ewers C, Guenther S. 2016. Carriage of extended-spectrum β-lactamase-plasmids does not reduce fitness but enhances virulence in some strains of pandemic *Escherichia coli* lineages. Front Microbiol 7:336. https://doi.org/10.3389/fmicb.2016.00336.
48. Ranjan A, Scholz J, Semmler T, Wieler LH, Ewers C, Muller S, Pickard DJ, Schierack P, Tedin K, Ahmed N, Schaufler K, Guenther S. 2018. ESBL-plasmid carriage in *Escherichia coli* enhances *in vitro* bacteria competition fitness and serum resistance in some strains of pandemic sequence types without overall fitness cost. Gut Pathog 10:24. https://doi.org/10.1186/s13099-018-0243-z.
49. BACTpipe. 2017. BACTpipe: bacterial whole-genome sequence assembly and annotation pipeline v.2.6.1 https://github.com/ctmrbio/BACTpipe.
50. Ondov BD, Treangen TJ, Melsted P, Mallonee AB, Bergman NH, Koren S, Phillippy AM. 2016. Mash: fast genome and metagenome distance estimation using MinHash. Genome Biol 17:132. https://doi.org/10.1186/s13059-016-0997-x.
51. Bushnell B. 2016. BBMap short read aligner. University of California, Berkeley, CA. http://sourceforge.net/projects/bbmap.
52. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.
53. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
54. Ewels P, Magnusson M, Lundin S, Kaller M. 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32:3047–3048. https://doi.org/10.1093/bioinformatics/btw354.
55. Thomsen MCF, Ahrenfeldt J, Cisneros JLB, Jurtz V, Larsen MV, Hasman H, Aarestrup FM, Lund O. 2016. A bacterial analysis platform: an integrated system for analyzing bacterial whole genome sequencing data for clinical diagnostics and surveillance. PLoS One 11:e0157718. https://doi.org/10.1371/journal.pone.0157718.
56. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MT, Fookes M, Falush D, Keane JA, Parkhill J. 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 31:3691–3693. https://doi.org/10.1093/bioinformatics/btv421.
57. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2: approximately maximum-likelihood trees for large alignments. PLoS One 5:e9490. https://doi.org/10.1371/journal.pone.0009490.
58. Letunic I, Bork P. 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res 44:W242–W245. https://doi.org/10.1093/nar/gkw290.
59. Aarestrup FM, Hasman H. 2004. Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride and antimicrobial substances used for disinfection. Vet Microbiol 100:83–89. https://doi.org/10.1016/j.vetmic.2004.01.013.
60. Wiegand I, Hilpert K, Hancock RE. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration of antimicrobial substances. Nat Protoc 3:163–175. https://doi.org/10.1038/nprot.2007.521.
61. Joffré E, Nicklasson M, Alvares S, Xiao X, Sun L, Zhu B, Sjöling Å. 2019. The bile salt glycocholate induces global changes in gene and protein expression and activates virulence in enterotoxigenic *Escherichia coli*. Sci Rep 9:108. https://doi.org/10.1038/s41598-018-36414-z.
62. Stepanović S, Cirković I, Ranin L, Svabić-Vlahović M. 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett Appl Microbiol 38:428–432. https://doi.org/10.1111/j.1472-765X.2004.01513.x.
63. Nickerson KP, Chanin RB, Sistrunk JR, Rasko DA, Fink PJ, Barry EM, Nataro JP, Faherty CS. 2017. Analysis of *Shigella flexneri* resistance, biofilm formation, and transcriptional profile in response to bile salts. Infect Immun 85:e01067-16. https://doi.org/10.1128/IAI.01067-16.

Paper III

191029 EP

Wastewater concentrations of antibiotics, antibiotic resistance genes and mobile genetic elements in hospital, community and urban wastewaters and associated antibiotic prescription data in Norway

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Abstract

Antibiotic resistance is an ever-present threat to global public and animal health. Selection pressures from antibiotic use and abuse are a constant risk for the development of antibiotic resistant (AR) bacteria. Wastewater, specifically from hospitals and wastewater treatment plants (WWTP) are important sources of environmental pollution with AR properties. However, much is still unknown regarding the possible impact of wastewater from different sources on the potential for AR development. In this study, samples of hospital (HW), community (CW) and urban (UW, WWTP) wastewater were collected monthly for one year. Wastewater samples were analyzed for 53 unique antibiotic compounds/metabolites, the abundance of 144 AR genes and genes associated with horizontal gene transfer. Antibiotic prescription data for the equivalent human populations were also included. Thirteen antibiotic compounds/metabolites were detected in HW and CW, and ten in UW.

Several emerging and clinically important AR genes (e.g. *ndm-1*, bla_{IMP}, bla_{VIM}) were frequently detected in HW. The high abundance of mobile genetic elements (MGEs) like insertion sequence elements (IS elements) in all three wastewater types indicates that the wastewater contains bacterial genetic systems with a potential to develop and disseminate multi-drug resistance. The levels of antibiotics, ARGs and HGT-associated genes were highest in hospital wastewater, and they were higher in the community wastewater than in the urban wastewater from the WWTP, despite similar or lower levels of antibiotic consumption. In fact, average concentrations of several antibiotics in HW (cefalexin, metronidazole, ofloxacin and tetracycline) and in CW (cefotaxime) were higher than the predicted concentrations based on water usage and antibiotic consumption (with a 100 % ingestion-elimination efficiency). Hopefully, this study can assist in creating a platform for the continued research and political decision-making regarding AR.

Keywords: ARG, MGE, WWTP, sewage, consumption, antibiotic resistance dissemination

1. Introduction

WHO considers antibiotic resistance (AR) to be one of the biggest threats to global public health. Selective pressure exerted by antibiotics and other compounds holding antimicrobial properties, like heavy metals, coerce microbes to develop antibiotic resistance via the evolution of novel mechanisms or by horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs) located on plasmids or other mobile genetic elements (MGEs) (Bengtsson-Palme et al., 2018). Antibiotic use and abuse in human, animal and environmental sectors and the spread of AR determinants within and between these sectors including global dissemination are all drivers of AR (McEwen and Collignon, 2018). Consumed antibiotics are excreted by humans and animals in both unmetabolized and metabolized forms, but unfinished courses of antibiotics may additionally be directly discarded e.g. in toilets (Mispagel and Gray, 2005). Wastewater is consequently regarded as one of the primary sources of

environmental pollution of antibiotics and ARGs (Berendonk et al., 2015; Manaia et al., 2018; Pazda et al., 2019). Recent studies have investigated the presence of antibiotic resistance genes and antibiotics in wastewater from hospitals and wastewater treatment plants (WWTPs) and their impact on recipient surface waters (Hembach et al., 2017; Jäger et al., 2018; Pärnänen et al., 2019; Rodriguez-Mozaz et al., 2015). Because only a small proportion of the overall wastewater volume that enters the WWTPs are contributed by hospitals, the relative contribution to AR properties in the WWTP environment by non-hospital sources appears to be largely unexplored (Paulshus et al., 2019a). Hospital wastewater (HW) is commonly regarded as a high-impact source of antibiotic resistance factors, which may benefit from the implementation of local wastewater treatment to reduce the impact of such effluents on AR dissemination. Conventional wastewater treatment could have negligible effects on, and may even enrich the relative levels of antibiotic resistance during the treatment process, highlighting the importance of directly addressing this issue (Hiller et al., 2019; Hocquet et al., 2016; Rizzo et al., 2013). Cost-benefit analyses are required to determine whether local wastewater treatment could be efficient in reducing the overall AR disseminated through the subsequent WWTP. Genes encoding antibiotic resistance and virulence are often linked (Zhang et al., 2015), and virulent enteropathogens have previously been detected in Norwegian wastewater systems, including within smaller communities (Ørmen et al., 2019). However, few studies have been completed targeting the prevalence of antibiotic resistance properties in wastewater in Norway. One study investigated ESBL-producing *E. coli* (ESBL-EC) in wastewater and their association to clinical ESBL-EC and ESBL-EC isolated from recreational waters (Jørgensen et al., 2017), whilst another explored the removal efficiencies of different filtration processes on AR *E. coli* in two Norwegian WWTPs. Recently, the abundance of ARGs in wastewater samples from Norway and seven other countries in Europe were investigated (Pärnänen et al., 2019), and a global metagenomic surveillance study was employed to evaluate the bacterial resistome in untreated wastewater from 60 countries from all over the world (Hendriksen et al., 2019). However, the environmental dissemination of AR is a complex problem requiring a comprehensive overview. In this descriptive and longitudinal study, we present the results from a highly synchronized quantitative analysis of antibiotic concentrations and normalized abundances of ARGs and genes responsible for HGT in wastewater samples from a

hospital and a community outlet, and their common WWTP, as well as the associated antibiotic prescription data. Hopefully, this study can assist in creating a platform for the continued research and political decision-making regarding AR.

2. Materials and methods

2.1. Sampling

Raw wastewater was collected monthly over 15 months from three sites: a hospital outlet (HW); a community outlet (CW); the inlet of a wastewater treatment plant (WWTP) (UW). The WWTP treats wastewater from the majority of Norway's capital and some surrounding municipalities, including both outlets included in the study. The two wastewater outlets discharge approximately 240 m^3 (hospital) and 50 m^3 (community) wastewater per day whilst the WWTP inlet receives a daily average of 290.000 m^3. Sampling was performed as described by Paulshus et al. (2019). Briefly, three samples were collected for three consecutive days per site each month, each sample collected by mixing 24 aliquots of 200 ml collected at hourly intervals and brought back on ice for analysis.

2.2. Quantification of antibiotic concentrations

Samples were analyzed in triplicate for the determination of fifty-three different antibiotics (Table S1), according to the protocol previously described by Gros et al. (2013). Briefly, for each sample, 50 ml water was filtered simultaneously through 2.7 and 0.45 µm pore-size membranes by vacuum filtration. Water samples were adjusted to pH 3 with HCl (1.0M) and EDTA (4%, v/v). Samples were further loaded into Oasis HLB cartridges (60 mg, 3 ml) (Waters Corp., Milford, MA, USA) for pre-concentration of analytes and stored at -20°C until extraction. Extracts were reconstituted and analyzed according to Gros et al. (2013). In brief, chromatographic separation was performed with an ultra-performance liquid chromatography (UPLC) system (Waters Corp.) holding with a quaternary pump system using an Acquity BEH T3 column (50mm 2.1 mm i.d., 1.7 mm particle size) (Waters Corp.). The UPLC system was connected to a triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems; Foster City, CA, USA) with a Turbo V ion spray source. Analysis was performed in positive ionization mode in a multiple reaction monitoring (MRM) mode. Accurate

quantification was achieved by determination of total recoveries (n = 3) for each water matrix (hospital effluent, community effluent and urban influent wastewater). Internal calibration with isotopically labeled standards was used to calculate concentrations. Average concentrations for each month were calculated by averaging the concentrations of each of the three daily samples for each respective month.

Concentrations of antibiotics predicted to cause no effects on the selection toward antibiotic resistance (Bengtsson-Palme and Larsson, 2016) were compared to concentrations observed in wastewater from the present study to evaluate the potential for development of antibiotic resistant bacteria in wastewater with hospital, community and urban origin, respectively.

2.3. DNA extraction

For each sample, 25 ml wastewater was filtered on DNA filter membranes that were stored at -80°C for subsequent DNA extraction. Extraction was performed using the DNeasy PowerWater Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. DNA concentrations were measured with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and samples with < 10 ng/ml were re-extracted from reserve DNA filters, prolonging protocol steps (centrifugation time, setting time of solutions before centrifugation, extra centrifugation to remove residual methanol) to the suppliers suggested extensions to ensure acceptable eluate concentrations. Eluted DNA was stored at -20°C until analysis.

2.4. Quantification of ARGs

Quantification of DNA was performed in triplicate with a SmartChip Real-Time PCR System (Takara Bio Inc., Shiga, Japan). Initial screening was performed with the complete panel of 384 antibiotic resistance genes and genes associated with HGT (Stedtfeld et al., 2018), by pooling DNA from all replicates for each sample type and analyzing the three resulting composite samples. The results from the initial screening were used to select a subset of 144 genes (Table S2) for more detailed analysis of ARG abundance in each individual wastewater sample collected during the duration of the study. The

subset of 144 genes were selected based on the following criteria: i) genes of emerging/clinical threat (e.g. *ndm-1*, *mcr-1*, *mcr-2*, *mecA*, *van*) irrespective of their detection during the initial screening; ii) the genes most frequently detected in all sample types. Copy numbers (CNs) were calculated by converting Ct values using the formula $10^{((28\text{-}Ct)/3.333)}$, and normalized CNs of ARGs and HGT genes were calculated by dividing each CN by the CN for the 16S *rRNA* gene in the corresponding sample.

2.5. Antibiotic prescription data

Prescription sales data for community and urban wastewater were acquired from The Norwegian Institute of Public Health's prescription register. The data were grouped per municipality for those municipalities from which the samples were collected. Data on antibiotic use in the hospital included in the study was collected from the Norwegian hospital pharmacies drug statistics database (SLS). For each antibiotic product and concentration, total sales numbers were calculated in grams by multiplying the number of prescriptions sold in defined daily doses (DDDs) with the gram content of each DDD. Data was obtained for three groups based on the origin of the wastewater samples; I: Oslo University Hospital Rikshospitalet (Hospital); II: Bærum municipality (Community); III: Oslo, Bærum, Asker, Røyken and Nesodden municipalities (Urban). Further analyses included only those antibiotics that were listed in the prescription register and in addition analyzed in the wastewater samples. The wastewater concentrations of the metabolites of Metronidazole (Metronidazole OH) and Sulfadiazine (N-acetylsulfadiazine) were merged with their parent compounds before comparison to the prescription data.

Antibiotic sales numbers were converted to estimated wastewater concentrations (ngL^{-1}) with average wastewater volumes of 240 m^3/day (hospital), 54 m^3/day (community) and 290 000 m^3/day (urban) for hospital, community and urban wastewater, respectively. Community prescription data were divided by a factor of 240 to compensate for the disproportionate sizes of the populations in the municipality (120 000 individuals) to which the community (500 individuals) supplying the wastewater belonged.

3. Results

3.1. Antibiotic concentrations in wastewater

The levels of 53 antibiotic compounds, including some metabolites, were measured quantitatively in wastewater samples collected from a hospital outlet, a community outlet and the inlet to their common WWTP. Fifteen antibiotics including one metabolite (metronidazole OH) were detected in at least one wastewater sample (Table S1). Several antibiotics were only detected in one (Norfloxacin) or two (Cephalexin, clarithromycin, metronidazole, metronidazole OH, penicillin V and sulfapyridine) of the sample types, whereas eight antibiotics were found in all sample types (Figure 1). Thirteen unique compounds were detected in hospital as well as in community wastewater, whilst ten compounds were detected in urban samples. The highest concentrations were observed in hospital wastewater for most antibiotics. Cephalexin and metronidazole were nearly exclusively found in hospital wastewater. Phenoxymethylpenicillin (penicillin V) was found at quantifiable levels in only a few community wastewater samples and was thus the only detected antibiotic that was not present in hospital wastewater. Norfloxacin, a quinolone antibiotic that has not been sold in Norway since 2001, was detected in three community samples during the spring of 2017, where it was found at high concentrations. Conversely, another quinolone, ciprofloxacin was the only antibiotic to be detected in all sample types during all sampling months. Average monthly concentrations of ciprofloxacin ranged from 87 to 7 700 ng/l. Tetracycline was found in all but two community wastewater samples and four urban wastewater samples. A noticeable drop in concentrations was observed for all detected compounds during the summer months June, July and August, although the drop was not as pronounced in urban wastewater. The highest levels of antibiotic concentrations in hospital wastewater were observed for metronidazole, tetracycline and sulfamethoxazole. High levels of antibiotic concentrations in community wastewater were observed only transiently with the exceptions of ciprofloxacin and tetracycline, whereas antibiotic concentrations in urban wastewater were consistently low. PNEC (Bengtsson-Palme and Larsson, 2016) levels were exceeded consistently in most hospital samples for most detected antibiotics. Conversely, PNEC levels were only occasionally surpassed in community wastewater for some of the detected antibiotics, and rarely in urban

wastewater with exception of ciprofloxacin and cefotaxime. A heatmap of average monthly antibiotic concentrations for each month, sample type and antibiotic compound is presented in Figure 1.

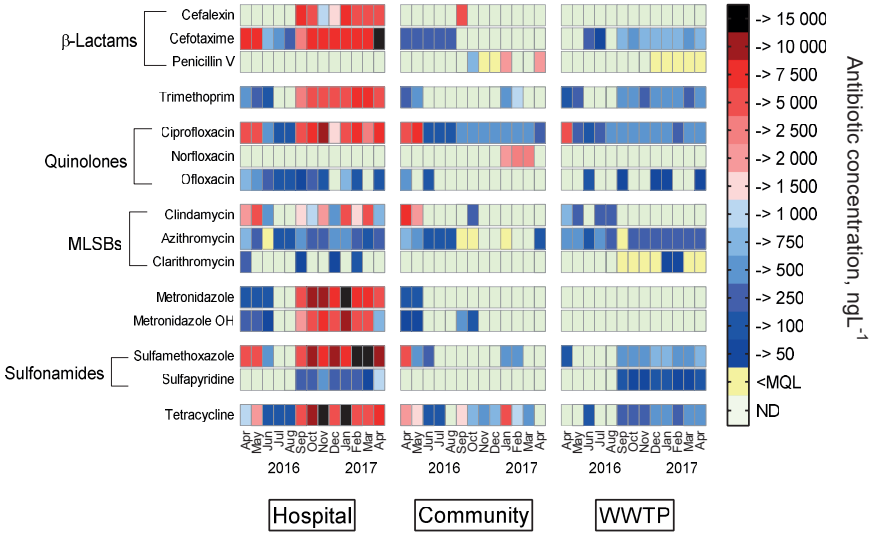

Figure 1. Antibiotic concentrations measured monthly from April 2016 to April 2017 in hospital wastewater, community wastewater and urban wastewater. Each box represents the average concentration of three 24-hour samples collected consecutively over three days. Antibiotics that were not detected in any of the samples were excluded from the figure (see supplementary table S1). ND: Not detected (<MDL, minimum detection limit); <MQL: detected, but below the minimum quantification limit. For samples in which one or two of the daily replicates were positive, but <MQL, the <MQL values were assigned values of (MQL+MDL)/2 to enable the calculation of averages for these samples, whereas <MDL were assigned values of 0.

3.2. ARG abundance

After an initial screening of a larger, 384 gene array (Stedtfeld et al., 2018), a subset of 144 genes, including one 16S *rRNA* gene, 103 ARGs and 40 genes responsible for HGT, were selected for analysis (Table S1), representing the following antibiotic classes: Aminoglycosides (17 genes); amphenicols (7); β-lactamases (21); fluoroquinolones (4); macrolides/lincosamides/streptogramin B (MLSBs, 7); polymyxins (colistin, 2); rifamycins (rifampicin, 2); sulphonamides (2); tetracyclines

(10); trimethoprims (6); glycopeptides (vancomycin, 24), and additionally integrases, transposases and IS elements (Figure S1). Nine genes were not detected in any samples, namely *mecA*, *bla-SME*, *KPC*, *blaIMI*, *arsA*, *mcr-2*, *vanSE*, *vanG* and *vanXA*, although some genes were detected in only one sample, including *mcr-1* and several vancomycin resistance genes. Multiple genes were much more common in hospital wastewater in terms of relative abundance, compared to wastewater of community and urban origin. Some of these that were also found in relative abundances close to one tenth of the 16S *rRNA* gene numbers, including *aacC2*, *aac(6')-Ib*, *aadB*, *ISAba3*, *int1*, *cefa_qacEdelta*, *IS21-ISAs29*, *IS6100*, some *tnpA* variants, *strB*, *sul1* and *tet39*. Of the genes that were included despite poor detection rates during the initial screening, but were considered clinically relevant (primarily carbapenem, vancomycin and colistin resistance genes), bla_{NDM-1}, bla_{VIM}, bla_{imp}, *vanB*, *vanWB*, *vanXB*, *vanRB*, *vanYB*, *vanC2/C3* and *vanSB* were detected in most hospital samples, with bla_{imp} and *vanTG* also being detected in most community and urban samples. In fact, a number of genes were observed at higher relative abundances in community wastewater than hospital wastewater, namely *blaCMY*, *blaOXY-1*, *bla-ACT*, *qnrD*, *oqxA*, *intI2*, *pcoA*, *copA*, *tcrB*, *terW*, *IS26*, *IS3*, *IS256*, *ISEcp1*, *IS200*, *Tn5403*, *IncN rep*, some *tnpA* variants, *erm(B)*, *erm(Q)*, *tetM* and *vanTG*. Urban gene copy counts were more similar to community samples, and no genes were most abundant in urban samples compared to the other two sample types, with the exception of *ISAba3* in a few samples. In summary, aminoglycoside, rifampicin, sulphonamide and vancomycin resistance genes were much more abundant in hospital wastewater, whereas HGT genes were somewhat more common in community wastewater.

New Delhi metallo-beta-lactamase (NDM) was detected at relatively high levels in all but two hospital samples as well as two and five samples from community and urban wastewater. bla_{VIM} was detected in most hospital samples and one community sample, albeit at lower levels than *NDM-1*, whereas *KPC* was not observed in any samples.

Normalized abundance of erythromycin resistance genes (*erm(B)*, *erm(Q)*) were higher in community wastewater than hospital wastewater, and particularly during late winter-early spring of 2017.

229 Quinolone encoding *qnr* resistance genes were also more abundant in community wastewater than
230 hospital wastewater.

231 Table 1. A non-exhaustive list of BLAST hits for different *tnpA* primer pairs (Assay IDs 201-207) included in the present
232 study. The wastewater type containing the highest normalized abundances of the corresponding primer pair are highlighted
233 where applicable. For information on primer sequences, the reader is referred to Stedtfeld et al. (2018). For detailed
234 information on the normalized abundances, see supplementary Figure S1. CW: community wastewater, HW: hospital
235 wastewater.

Assay ID	BLAST[a] hit // Bacterial species (sample type)	Highest abundance
201	*Escherichia coli* (sewage), *Klebsiella pneumoniae* (human), *E. coli* (rectal swap)	CW
202	*E. coli* (sewage), *Acinetobacter baumanii* (sputum), *E. coli* (human), *K. pneumoniae* (human)	HW
203	*Acinetobacter pittii* (human), *A. pittii* (human), *Acinetobacter defluvii* (sewage), *K. pneumoniae* (human), *Aeromonas hydrophila* (sewage), *E. coli* (pig)	HW
204	*E. coli* (raw milk cheese), *K. pneumoniae* (human), *E. coli* (pig)	
205	*K. pneumoniae* (human), *K. pneumoniae* (human), *E. coli* (human), *E. coli* (human stool)	CW
206	*Enterococcus faecium* (human), *Enterococcus sp.* (human), *Enterococcus sp.* (human), *Enterococcus durans* (chicken)	CW
207	*E. faecium* (human), *Enterococcus sp.* (human), *Enterococcus sp.* (human), *Lactococcus lactis* (kimchi, Korean national dish), *E. faecium* (feces)	CW

236 [a] https://blast.ncbi.nlm.nih.gov/Blast.cgi

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Integrase class 1 (*int1*), sulfonamide resistance (*sul1*) and *qacEdelta* were observed at high abundances in hospital wastewater and were strictly following each other. Similarly, these three genes were abundant in all community and most urban wastewater samples, albeit with slightly lower numbers.

Seven primer pairs were included that targeted the transposase genes of *tnpA*. Some of these were more abundant in hospital wastewater samples, whereas others were most abundant in community and urban wastewater samples. The seven different *tnpA* primer pairs were analyzed by a BLAST search in order to elucidate this observation and the first few unique results for each primer set are presented in Table 1.

Noticeable correlation coefficients between ARG abundances and antibiotic concentrations grouped by antibiotic class were only observed for tetracycline in community and urban wastewater (Table 2).

Table 2. Correlation coefficients between monthly averages of antibiotic concentrations and normalized abundances of ARGs in wastewater. Both data sets were grouped by antibiotic class before analysis.

	ARG		
Antibiotics	**Hospital**	**Community**	**Urban**
β-lactams	0.05	-0.02	-0.39
Fluoroquinolones	-0.37	-0.44	-0.23
MLSBs	-0.07	-0.44	0.37
Sulfonamides	0.30	0.08	-0.28
Tetracyclines	0.09	**0.87**	**0.73**
Trimethoprims	-0.24	0.32	0.37

3.3. Antibiotic prescriptions

Antibiotic prescription data representative of each sampling site was collected for the months that sampling was performed. Antibiotics that were not analyzed for in wastewater were excluded from the prescription data. Nineteen antibiotics overlapping the wastewater analysis and prescription data were

included for analysis (Table 3). The consumption of β-lactam antibiotics differed drastically between the hospital (high use of broad-spectrum β-lactams) and the municipalities associated with the community (high use of narrow-spectrum β-lactams) (Figure 2). Consumption data for erythromycin presented fluctuations comparable to those observed for erythromycin resistance genes. The total consumption of erythromycin in the community associated municipality was nearly identical to the hospital's consumption, and 10 % of the WWTP associated municipalities (including the community associated one). The WWTP associated municipalities houses about 650 000 individuals, whereas the community associated municipality houses about 120 000 individuals (~18 %).

Table 3. Average concentrations of antibiotics in wastewater and predicted concentrations calculated from water usage and antibiotic prescription sales in grams. SD, standard deviation; ND, not detected; Penicillin G, Benzylpenicillin; Penicillin V, Phenoxymethylpenicillin.

	Measured concentration (+/-SD), ng/l			Predicted concentration (+/-SD), ng/l		
Antibiotic	Hospital	Community	Urban	Hospital	Community	Urban
Amoxicillin	ND	ND	ND	5746 (2135)	11296 (1988)	3444 (522)
Azithromycin	196 (165)	101 (202)	168 (112)	1605 (682)	998 (213)	291 (55)
Penicillin G	ND	ND	ND	58106 (14574)	1 (5)	6 (8)
Cefalexin	2074 (2250)	352 (1268)	ND	ND	820 (236)	297 (40)
Cefotaxime	4895 (3114)	67 (92)	351 (279)	223132 (39502)	44 (54)	37 (9)
Cefuroxime	ND	ND	ND	ND	6 (21)	1 (1)
Ciprofloxacin	3259 (2435)	1000 (1772)	489 (765)	12469 (4172)	3192 (290)	939 (98)
Clarithromycin	22 (46)	ND	3 (7)	718 (928)	952 (952)	232 (37)
Clindamycin	1299 (1044)	554 (1518)	86 (200)	20876 (5211)	2471 (392)	728 (61)
Doxycycline	ND	ND	ND	1198 (336)	1376 (248)	461 (72)
Metronidazole	7080 (7210)	55 (123)	ND	5940 (2279)	2493 (379)	1023 (115)
Ofloxacin	178 (212)	33 (103)	12 (20)	ND	59 (41)	15 (4)
Oxytetracycline	ND	ND	ND	ND	ND	1 (1)
Penicillin V	ND	286 (579)	ND	10257 (3771)	51318 (6266)	17001 (2118)
Spiramycin	ND	ND	ND	ND	26 (23)	14 (5)
Sulfadiazine	ND	ND	ND	ND	ND	ND
Sulfamethoxazole	5694 (4057)	469 (1292)	310 (266)	42974 (13446)	4338 (382)	1365 (110)
Tetracycline	4552 (4511)	730 (776)	137 (118)	703 (1227)	8502 (978)	1135 (178)
Trimethoprim	2636 (2251)	150 (293)	203 (154)	9959 (2597)	1974 (177)	582 (49)

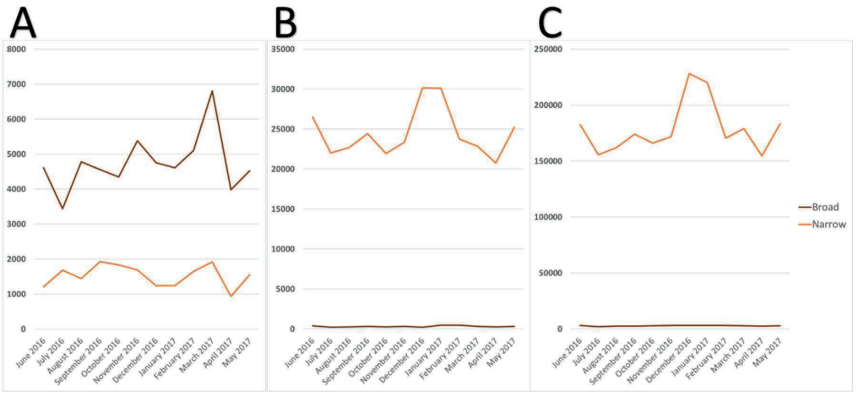

Figure 2. Monthly antibiotic consumption in total grams of broad-spectrum (cefalexin, cefotaxime and cefuroxime) and narrow-spectrum (amoxicillin, benzylpenicillin and phenoxymethylpenicillin) β-lactam during the same period as the wastewater samples in this were sampled. A) Consumption of β-lactam antibiotic in the hospital providing the hospital wastewater samples for this study. B) Consumption of β-lactam antibiotic in the municipality of 120 000 inhabitants to which the community of 500 inhabitants providing community wastewater samples to this study belongs. C) Consumption of β-lactam antibiotics in the five municipalities that supply wastewater to the WWTP from which samples of urban wastewater in this study was collected.

4. Discussion

Norway has a strict regulation of antibiotic use, with a prioritization of usage of narrow-spectrum alternatives if possible. In 1997-2002, Norway was the European country with the lowest overall antibiotic consumption and the highest ratio of narrow- to broad-spectrum β-lactams (Goossens et al., 2005). Although Norway is currently not the country in Europe with the lowest total overall antibiotic consumption (European Centre for Disease Prevention and Control, 2018a), the focus on prioritizing narrow-spectrum antibiotics is most likely a contributing factor to the low rate of antibiotic resistant bacteria in Norway (European Centre for Disease Prevention and Control, 2018b). Environmental monitoring of antibiotics for comparison to prescription data is however difficult because of various degrees of degradation of different classes of antibiotics (Dolliver et al., 2008).

Norfloxacin was detected in community wastewater samples during three months. This drug is not marketed in Norway, although it is hard to exclude that the drug could be privately imported. The relatively high concentrations of ~2 000 ng/l indicate that the finding is highly unlikely to be due to a single individual or family using norfloxacin off-record to treat an illness without consulting their general physician. An alternative hypothesis suggests that a hoarded supply of antibiotics have been discarded directly into a toilet. This explanation is also questionable because the observations of norfloxacin in the community wastewater samples were made during two out of three, 3/3 and 2/3 days during the three months from which the antibiotic was observed, respectively.

The overall reduction in wastewater concentrations of antibiotics and antibiotic resistance genes are probably associated with vacationing with a reduced number of medical appointments and overall wastewater production as a consequence. The implementation of local treatment of wastewater in high-risk outlets such as hospital wastewater has previously been recommended as a means to reduce the environmental pollution with antibiotic resistance properties. Our results may indicate a possibility for the implementation of seasonal wastewater treatment during periods when the risks for the dissemination of antibiotic resistant (and pathogenic) bacteria are particularly high. These periods could include identified and ongoing outbreaks of infectious diseases as well as during extreme water precipitation events, where the risk of spreading untreated effluents through storm drains as the WWTP capacity is saturated.

However, antibiotic resistance is not solely present in hospital wastewaters, as several recent studies have reported the levels of antibiotic resistance in wastewater from residential areas and WWTP inlets and outlets, demonstrating that antibiotic resistance properties not only occurred in HW, but also in residential wastewater and the WWTPs (Li et al., 2015; Paulshus et al., 2019b, 2019a; Rizzo et al., 2013; Sigala and Unc, 2012).

No samples surpassed the PNEC threshold for sulfamethoxazole although the values here are much higher (16 000 ng/l) than those seen for other the antibiotics included here (64-4000ng/l). Sulfapyridine did not have a specified PNEC-value. The MIC values of sulphonamides in susceptible

311 bacteria are often above 5 000 ng/ml or 5 000 000 ng/l which is well above the levels detected in the
312 wastewater in this study (Davies and Mackenzie, 1994; Niemann et al., 2019).

313 Zhang et al. (2019, 2016) showed that miniscule concentrations of tetracycline and sulfamethoxazole
314 in wastewater sludge could substantially reduce the diversity of the microbial population and
315 simultaneously increase the number of ARGs. They also observed that the abundance of *tetA*, *tetC*,
316 *tetG*, *tetK*, *tetM* and *sul1* correlated significantly with the abundance of *intl1*. Another study on
317 treatment of activated sludge reported that the total bacterial numbers decreased whilst the frequency
318 of ARBs and relative abundance of ARGs (sulfonamides and tetracyclines) increased (Makowska et
319 al., 2016). Karkman et al. (2016) detected all transposases and two thirds of the ARGs included in the
320 assay in a study on UW from a WWTP over four different seasons. They observed reductions to most
321 ARGs and HGT genes during WWTP treatment, whereas Tn*25* transposase and clinical class 1
322 integrons were enriched. Similarly, antibiotics may be resistant to degradation due to physicochemical
323 and biological characteristics (Dolliver et al., 2008). Hence, it is imperative to find supplementary
324 solutions that may deal with the problem of AR dissemination before the problem reaches the
325 wastewater treatment plant.

326 Despite only being reported in a few number of clinical isolates (Samuelsen et al., 2017), NDM-1 was
327 detected in nearly all hospital wastewater samples and additionally in several community and urban
328 wastewater samples. NDM-1 was first described in Norway in 2011 (Samuelsen et al., 2011) and was
329 recently reported in a European-wide study (David et al., 2019). The presence of NDM-1 genes in
330 most hospital wastewater samples is worrying and may indicate that the gene is prevalent to a larger
331 extent in bacteria not directly related to disease, thus remaining largely undetected.

332 No samples in this study were positive for *mecA*, a gene typically associated with staphylococci on
333 mucosal and skin surfaces, similar to a European study utilizing the same highly parallel qPCR array
334 (Pärnänen et al., 2019), although other studies have detected *mecA* in wastewater effluents and even
335 drinking water effluents (Fernando et al., 2016; Gómez et al., 2016; Wan and Chou, 2014).

Seven primer pairs (201-207, Table S2) were included to cover a broad array of *tnpA* genes. Primer pairs 202 and 203 detected highest normalized copy numbers in HW, whilst primer pairs 201 and 205-207 all detected higher levels in CW than HW. In fact, higher relative counts were found for genes detected by primer pairs 205-207 in UW compared to HW. These results suggest that different wastewater environments may harbor varying levels of specific variants of transposases. Pärnänen et al. (2019) reported some of the highest relative concentrations of transposases in Norway compared to other European countries, in contrast to ARGs, for which Norway had the lowest prevalence of the countries included. The study did not however compare various sources of wastewater, nor report the distribution of the different types of transposases, but it did show a relative reduction in transposases during wastewater treatment.

The finding of co-fluctuating *int1* and *sul1* genes observed in all wastewater sample types in this study supports the quality of the study. The finding is likely a consequence of co-localization of these gene cassettes on class 1 integrons (Gillings, 2017). The class 1 integron-integrase gene has previously also been suggested as a proxy for anthropogenic pollution partway due to its association with genes conferring resistance to antibiotics, heavy metals and disinfectants (Gillings et al., 2015).

Class 1 integrons contain in addition to the *int1* gene both the *sul1* gene and the truncated *qacEdelta* on the same genetic structure. This fits with an equal distribution of these three genes in the present study. Here, it is clear that class 1 integrons are more common in hospital sewage compared to the community sewage and the level of class 1 integrons is lowest in the urban sewage.

Gene cassettes carried by class 1 integrons are typically causing aminoglycoside resistance and trimethoprim resistance (Domingues et al., 2015). In this study it seems that several aminoglycoside resistance cassettes are probably commonly carried on the class 1 integrons. In particular the *aadB* resistance gene is highly common in the hospital sewage, which may indicate that a major part of the class 1 integrons are carrying the *aadB* cassette. It is also probable that many of the class 1 integrons carry the trimethoprim resistance cassettes (*dfrA*'s)

Among the detected antibiotic resistance genes in this study both *strB* and *sul2* has the same distribution with a high level of occurrence in all three sampling sites but still with some higher occurrence in the hospital samples. These genes are normally carried by the RSF1010 type of plasmids. These plasmids have been found to be common in the environmental microbiota like orchard soil (Okubo et al., 2019; Sundin and Bender, 1996). It is possible that the RSF1010-like plasmids may be enriched in the hospital environment through patient gut microbiota or in the hospital sewage based on the common occurrence in the environment.

Tetracycline resistance is often caused by tetracycline pumps carried on transferable plasmids (Schnappinger and Hillen, 1996). In this study it is evident that *tetA* and *tet E* are common at all three sampling sites throughout the year while *tetB* is less common. The *tet E* gene has been connected to aquatic environments and linked to both *Aeromonas* spp and vibrio-like bacteria (Duman et al., 2019; Sørum et al., 1992; Zhang et al., 2009). *tetQ*, *tetW*, *tetO* and *tetX* are highly common at all sampling sites indicating that they occur in the environment in general. These tetracycline genes are often connected with certain groups of bacteria, including typical intestinal bacteria like *Campylobacter* spp. and anaerobic bacteria (Ohashi and Fujisawa, 2017; Wu et al., 2010).

The macrolide resistance genes *ermB* and *ermQ* are highly represented at all three sampling sites and in particular highest in the winter period at the community sampling site. These results coincide with increased prescription of macrolides to control mycoplasma infections in the airways of human patients in the same time of the year. These patients are typically patients that normally are healthy and contracts the mycoplasma infections through the epidemiological spread of the mycoplasma bacteria through kindergartens, public schools and work places. These patients are not normally seriously ill, but they have longstanding infections often resulting in the need of macrolides to control the infection (Ferrer et al., 2015).

Plasmid borne *qnr* genes are often linked to aquatic environments (Cattoir et al., 2008), but have been introduced into many pathogenic bacteria the last two decades. In this study it is seen that in particular *qnrS2* is highly common at all three sampling sites. As Cattoir et al. (2008) suggest that *qnrS2* are originating from aquatic bacteria this may indicate a natural background contribution of *qnrS2* in

these sampling sites. In the summer period it seems that the community wastewater has an increased level of *qnrD* and *oqxA* which is interesting and may be connected to the increased temperature in that period.

In the study by Pärnänen et al. (2019) it was reported that the samples analyzed from the same wastewater treatment plant as sampled in this study contained a high level of transposases relative to the antibiotic resistance genes detected. The same trend was seen in this study, which may indicate that there is a natural background of transposases that might be part of the HGT reservoir that is activated when antibiotics are used more intensively in human and animal medicine with increased level of antibiotic resistance as the result.

IS*26* is found to occur to a high level in the community sewage and also to a lesser extent in the hospital sampling site. IS26 are often linked to development of transposons with multiple antibiotic resistance genes and sometimes linked to virulence genes in the Enterobacteriaceae family (Reid et al., 2015). It is of particular interest that this group of IS elements is highly common in the community sewage.

The same tendency as with IS*26* is the case with IS*256* that is mostly linked to staphylococci and antibiotic resistance. IS*3* was also found to occur at a high level in the autumn in the community site. IS*3* represents a large family of IS-elements and these results indicate that there is a higher ability to develop drug resistance transposons in the community sewage in the winter period. This could be related to an increased use of antibiotics in the community in the winter season.

IS6100 was found at high levels in the hospital sewage and this IS element is recognized for its ability to mobilize various drug resistance and other genes that allow bacteria to degrade toxic compounds. IS6100 are often found to be involved in lateral transfer of genes related to resistance and survival of bacteria in harsh environments (Nagata et al., 2019).

These results underline the importance of prudent use of antibiotics in the society and may also be of value in creating a platform for the continued surveillance, research and political decision making regarding the global health-threat of antibiotic resistance.

5. Conclusions

- Wastewater analyzed in this study contained diverse bacterial genetic systems that can potentially be used to develop and disseminate multi-drug resistance.

- Hospital wastewater is an important source of emerging and clinically relevant antibiotic resistance genes.

- Non-hospital wastewater outlets including those originating exclusively from private homes may also be potential hot-spots for antibiotic resistance.

- Antibiotic consumption was a poor predictor for the antibiotic concentrations found in wastewater.

- Investigation of other wastewater outlets as potential sources of environmental pollution with antibiotic resistance is recommended.

- Local treatment of hospital wastewater may reduce, but not completely eliminate the problem of antibiotic resistance dissemination through wastewater treatment plants

Acknowledgement

Thanks Morten Lindbæk for valuable input and discussions on the antibiotic consumption in Norway. Thanks also to the hospital staff, Bærum municipality and VEAS for access to sampling sites and help with sampling. This study was supported by the Norwegian regional research fund RFFHSTAD (Regionalt Forskningsfond Hovedstaden), project number 245686, the Norwegian wastewater treatment plant Vestfjorden Avløpsselskap (VEAS), and the Norwegian University of Life Sciences (NMBU).

The study was also supported in part by grants from the MSU Center for Health Impacts of Agriculture (CHIA) and the Superfund Research Program (P42ES04911) from the National Institute for Environmental Health Sciences.

This work was financed by the Water JPI through the national funding agencies supporting the consortium WaterJPI/0001/2013 STARE—"Stopping Antibiotic Resistance Evolution"

Sara Rodriguez-Mozaz acknowledges her Ramón y Cajal fellowship (RyC-2014-16707) from the Spanish Ministry of Economy, Industry and Competitiveness.

References

Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2018. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol. Rev. https://doi.org/10.1093/femsre/fux053

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. https://doi.org/10.1016/j.envint.2015.10.015

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: The environmental framework. Nat. Rev. Microbiol. https://doi.org/10.1038/nrmicro3439

Cattoir, V., Poirel, L., Aubert, C., Soussy, C.J., Nordmann, P., 2008. Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental Aeromonas spp. Emerg. Infect. Dis. 14, 231–237. https://doi.org/10.3201/eid1402.070677

David, S., Reuter, S., Harris, S.R., Glasner, C., Feltwell, T., Argimon, S., Abudahab, K., Goater, R., Giani, T., Errico, G., Aspbury, M., Sjunnebo, S., Feil, E.J., Rossolini, G.M., Aanensen, D.M., Grundmann, H., 2019. Epidemic of carbapenem-resistant Klebsiella pneumoniae in Europe is driven by nosocomial spread. Nat. Microbiol. https://doi.org/10.1038/s41564-019-0492-8

Davies, A.M., Mackenzie, N.M., 1994. Pharmacokinetics of baquiloprim and sulphadimidine in pigs after intramuscular administration. Res. Vet. Sci. 57, 69–74. https://doi.org/10.1016/0034-5288(94)90084-1

Dolliver, H., Gupta, S., Noll, S., 2008. Antibiotic degradation during manure composting. J. Environ. Qual. 37, 1245–1253. https://doi.org/10.2134/jeq2007.0399

Domingues, S., Da Silva, G.J., Nielsen, K.M., 2015. Global dissemination patterns of common gene cassette arrays in class 1 integrons. Microbiol. (United Kingdom) 161, 1313–1337. https://doi.org/10.1099/mic.0.000099

Duman, M., Saticioglu, I.B., Altun, S., 2019. Molecular characterization and antimicrobial resistance profile of

fecal contaminants and spoilage bacteria that emerge in rainbow trout (Oncorhynchus mykiss) farms. Biosci. Microbiota, Food Heal. 38, 41–48. https://doi.org/10.12938/bmfh.18-007

European Centre for Disease Prevention and Control, 2018a. Antimicrobial consumption, Annual epidemiological report for 2017. Stockholm.

European Centre for Disease Prevention and Control, 2018b. Surveillance of antimicrobial resistance in Europe – Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2017. Stockholm: ECDC.

Fernando, D.M., Tun, H.M., Poole, J., Patidar, R., Li, R., Mi, R., Amarawansha, G.E.A., Dilantha Fernando, W.G., Khafipour, E., Farenhorst, A., Kumara, A., 2016. Detection of antibiotic resistance genes in source and drinking water samples from a first nations community in Canada. Appl. Environ. Microbiol. 82, 4767–4775. https://doi.org/10.1128/AEM.00798-16

Ferrer, P., Sabaté, M., Ballarín, E., Fortuny, J., Rottenkolber, M., Schmiedl, S., Laporte, J.-R., Ibáñez, L., 2015. Sales of macrolides, lincosamides, streptogramins, and amoxicillin/clavulanate in the in- and outpatient setting in 10 European countries, 2007–2010. Springerplus 4, 612. https://doi.org/10.1186/s40064-015-1398-4

Gillings, M.R., 2017. Class 1 integrons as invasive species. Curr. Opin. Microbiol. https://doi.org/10.1016/j.mib.2017.03.002

Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. https://doi.org/10.1038/ismej.2014.226

Gómez, P., Lozano, C., Benito, D., Estepa, V., Tenorio, C., Zarazaga, M., Torres, C., 2016. Characterization of staphylococci in urban wastewater treatment plants in Spain, with detection of methicillin resistant *Staphylococcus aureus* ST398. Environ. Pollut. 212, 71–76. https://doi.org/10.1016/j.envpol.2016.01.038

Goossens, H., Ferech, M., Vander Stichele, R., Elseviers, M., 2005. Outpatient antibiotic use in Europe and association with resistance: A cross-national database study. Lancet 365, 579–587. https://doi.org/10.1016/S0140-6736(05)70799-6

Gros, M., Rodríguez-Mozaz, S., Barceló, D., 2013. Rapid analysis of multiclass antibiotic residues and some of

their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. J. Chromatogr. A 1292, 173–188. https://doi.org/10.1016/j.chroma.2012.12.072

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E.T., Schwartz, T., 2017. Occurrence of the *mcr-1* colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.01282

Hendriksen, R.S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S.K., Kjeldgaard, J., Kaas, R.S., Clausen, P.T.L.C., Vogt, J.K., Leekitcharoenphon, P., van de Schans, M.G.M., Zuidema, T., de Roda Husman, A.M., Rasmussen, S., Petersen, B., Amid, C., Cochrane, G., Sicheritz-Ponten, T., Schmitt, H., Alvarez, J.R.M., Aidara-Kane, A., Pamp, S.J., Lund, O., Hald, T., Woolhouse, M., Koopmans, M.P., Vigre, H., Petersen, T.N., Aarestrup, F.M., 2019. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nat. Commun. 10, 1124. https://doi.org/10.1038/s41467-019-08853-3

Hiller, C.X., Hübner, U., Fajnorova, S., Schwartz, T., Drewes, J.E., 2019. Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater treatment processes: A review. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2019.05.315

Hocquet, D., Muller, A., Bertrand, X., 2016. What happens in hospitals does not stay in hospitals: antibiotic-resistant bacteria in hospital wastewater systems. J. Hosp. Infect. 93, 395–402. https://doi.org/10.1016/j.jhin.2016.01.010

Jäger, T., Hembach, N., Elpers, C., Wieland, A., Alexander, J., Hiller, C., Krauter, G., Schwartz, T., 2018. Reduction of antibiotic resistant bacteria during conventional and advanced wastewater treatment, and the disseminated loads released to the environment. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.02599

Jørgensen, S.B., Søraas, A. V., Arnesen, L.S., Leegaard, T.M., Sundsfjord, A., Jenum, P.A., 2017. A comparison of extended spectrum β-lactamase producing *Escherichia coli* from clinical, recreational water and wastewater samples associated in time and location. PLoS One 12, e0186576. https://doi.org/10.1371/journal.pone.0186576

528 Karkman, A., Johnson, T.A., Lyra, C., Stedtfeld, R.D., Tamminen, M., Tiedje, J.M., Virta, M., 2016. High-
529 throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. FEMS
530 Microbiol. Ecol. 92. https://doi.org/10.1093/femsec/fiw014

531 Li, J., Cheng, W., Xu, L., Strong, P.J., Chen, H., 2015. Antibiotic-resistant genes and antibiotic-resistant
532 bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant
533 system. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-014-3665-2

534 Makowska, N., Koczura, R., Mokracka, J., 2016. Class 1 integrase, sulfonamide and tetracycline resistance
535 genes in wastewater treatment plant and surface water. Chemosphere 144, 1665–1673.
536 https://doi.org/10.1016/j.chemosphere.2015.10.044

537 Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G.,
538 Iakovides, I.C., Zammit, I., Kampouris, I., Vaz-Moreira, I., Nunes, O.C., 2018. Antibiotic resistance in
539 wastewater treatment plants: Tackling the black box. Environ. Int.
540 https://doi.org/10.1016/j.envint.2018.03.044

541 McEwen, S.A., Collignon, P.J., 2018. Antimicrobial resistance: A one health perspective. Microbiol. Spectr. 6.
542 https://doi.org/doi:10.1128/microbiolspec.ARBA-0009-2017

543 Mispagel, H., Gray, J.T., 2005. Antibiotic Resistance from Wastewater Oxidation Ponds. Water Environ. Res.
544 77, 2996–3002. https://doi.org/10.2175/106143005x73875

545 Nagata, Y., Kato, H., Ohtsubo, Y., Tsuda, M., 2019. Lessons from the genomes of lindane-degrading
546 sphingomonads. Environ. Microbiol. Rep. https://doi.org/10.1111/1758-2229.12762

547 Niemann, L., Feudi, C., Eichhorn, I., Hanke, D., Müller, P., Brauns, J., Nathaus, R., Schäkel, F., Höltig, D.,
548 Wendt, M., Kadlec, K., Schwarz, S., 2019. Plasmid-located dfrA14 gene in Pasteurella multocida isolates
549 from three different pig-producing farms in Germany. Vet. Microbiol. 230, 235–240.
550 https://doi.org/10.1016/j.vetmic.2019.01.016

551 Ohashi, Y., Fujisawa, T., 2017. Detection of antibiotic resistance genes in the feces of young adult Japanese.
552 Biosci. Microbiota, Food Heal. 36, 151–154. https://doi.org/10.12938/bmfh.17-004

553 Okubo, T., Ae, R., Noda, J., Iizuka, Y., Usui, M., Tamura, Y., 2019. Detection of the sul2–strA–strB gene
554 cluster in an ice core from Dome Fuji Station, East Antarctica. J. Glob. Antimicrob. Resist. 17, 72–78.

https://doi.org/10.1016/j.jgar.2018.11.005

Ørmen, Ø., Aalberg, K., Madslien, E.H., 2019. Multiplex polymerase chain reaction detection of enteropathogens in sewage in Norway. Acta Vet. Scand. https://doi.org/10.1186/s13028-019-0445-5

Pärnänen, K.M.M., Narciso-Da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V. Della, Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5, eaau9124. https://doi.org/10.1126/sciadv.aau9124

Paulshus, E., Kühn, I., Möllby, R., Colque, P., O'Sullivan, K., Midtvedt, T., Lingaas, E., Holmstad, R., Sørum, H., 2019a. 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. https://doi.org/10.1016/j.watres.2019.05.102

Paulshus, E., Thorell, K., Guzman-Otazo, J., Joffre, E., Colque, P., Kühn, I., Möllby, R., Sørum, H., Sjöling, Å., 2019b. Repeated Isolation of Extended-Spectrum-β-Lactamase-Positive Escherichia coli Sequence Types 648 and 131 from Community Wastewater Indicates that Sewage Systems Are Important Sources of Emerging Clones of Antibiotic-Resistant Bacteria . Antimicrob. Agents Chemother. 63. https://doi.org/10.1128/aac.00823-19

Pazda, M., Kumirska, J., Stepnowski, P., Mulkiewicz, E., 2019. Antibiotic resistance genes identified in wastewater treatment plant systems – A review. Sci. Total Environ. 697, 134023. https://doi.org/10.1016/j.scitotenv.2019.134023

Reid, C.J., Chowdhury, P.R., Djordjevic, S.P., 2015. Tn6026 and Tn6029 are found in complex resistance regions mobilised by diverse plasmids and chromosomal islands in multiple antibiotic resistant Enterobacteriaceae. Plasmid 80, 127–137. https://doi.org/10.1016/j.plasmid.2015.04.005

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M.,

583 Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and
584 urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242.
585 https://doi.org/10.1016/j.watres.2014.11.021

586 Samuelsen, Ø., Overballe-Petersen, S., Bjørnholt, J.V., Brisse, S., Doumith, M., Woodford, N., Hopkins, K.L.,
587 Aasnæs, B., Haldorsen, B., Sundsfjord, A., 2017. Molecular and epidemiological characterization of
588 carbapenemase-producing *Enterobacteriaceae* in Norway, 2007 to 2014. PLoS One 12.
589 https://doi.org/10.1371/journal.pone.0187832

590 Samuelsen, Ø., Thilesen, C.M., Heggelund, L., Vada, A.N., Kümmel, A., Sundsfjord, A., 2011. Identification of
591 NDM-1-producing Enterobacteriaceae in Norway. J. Antimicrob. Chemother.
592 https://doi.org/10.1093/jac/dkq483

593 Schnappinger, D., Hillen, W., 1996. Tetracyclines: Antibiotic action, uptake, and resistance mechanisms. Arch.
594 Microbiol. https://doi.org/10.1007/s002030050339

595 Sigala, J., Unc, A., 2012. A PCR-DGGE approach to evaluate the impact of wastewater source on the antibiotic
596 resistance diversity in treated wastewater effluent. Water Sci. Technol. 65, 1323–1331.
597 https://doi.org/10.2166/wst.2012.024

598 Sørum, H., Roberts, M.C., Crosa, J.H., 1992. Identification and cloning of a tetracycline resistance gene from
599 the fish pathogen Vibrio salmonicida. Antimicrob. Agents Chemother. 36, 611–615.
600 https://doi.org/10.1128/AAC.36.3.611

601 Stedtfeld, R.D., Guo, X., Stedtfeld, T.M., Sheng, H., Williams, M.R., Hauschild, K., Gunturu, S., Tift, L.,
602 Wang, F., Howe, A., Chai, B., Yin, D., Cole, J.R., Tiedje, J.M., Hashsham, S.A., 2018. Primer set 2.0 for
603 highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. FEMS
604 Microbiol. Ecol. 94. https://doi.org/10.1093/femsec/fiy130

605 Sundin, G.W., Bender, C.L., 1996. Dissemination of the strA-strB streptomycin-resistance genes among
606 commensal and pathogenic bacteria from humans, animals, and plants. Mol. Ecol.
607 https://doi.org/10.1111/j.1365-294X.1996.tb00299.x

608 Wan, M.T., Chou, C.C., 2014. Spreading of β-lactam resistance gene (*mecA*) and methicillin-resistant
609 *Staphylococcus aureus* through municipal and swine slaughterhouse wastewaters. Water Res. 64, 288–
610 295. https://doi.org/10.1016/j.watres.2014.07.014

611 Wu, N., Qiao, M., Zhang, B., Cheng, W. Da, Zhu, Y.G., 2010. Abundance and diversity of tetracycline
612 resistance genes in soils adjacent to representative swine feedlots in China. Environ. Sci. Technol. 44,
613 6933–6939. https://doi.org/10.1021/es1007802

614 Zhang, L., Levy, K., Trueba, G., Cevallos, W., Trostle, J., Foxman, B., Marrs, C.F., Eisenberg, J.N.S., 2015.
615 Effects of Selection Pressure and Genetic Association on the Relationship between Antibiotic Resistance
616 and Virulence in Escherichia coli. Antimicrob. Agents Chemother. https://doi.org/10.1128/aac.01094-15

617 Zhang, M.Q., Yuan, L., Li, Z.H., Zhang, H.C., Sheng, G.P., 2019. Tetracycline exposure shifted microbial
618 communities and enriched antibiotic resistance genes in the aerobic granular sludge. Environ. Int. 130.
619 https://doi.org/10.1016/j.envint.2019.06.012

620 Zhang, T., Zhang, M., Zhang, X., Fang, H.H., 2009. Tetracycline resistance genes and tetracycline resistant
621 lactose-fermenting Enterobacteriaceae in activated sludge of sewage treatment plants. Environ. Sci.
622 Technol. 43, 3455–3460. https://doi.org/10.1021/es803309m

623 Zhang, Y., Geng, J., Ma, H., Ren, H., Xu, K., Ding, L., 2016. Characterization of microbial community and
624 antibiotic resistance genes in activated sludge under tetracycline and sulfamethoxazole selection pressure.
625 Sci. Total Environ. 571, 479–486. https://doi.org/10.1016/j.scitotenv.2016.07.014

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Supplementary material

Wastewater concentrations of antibiotics, antibiotic resistance genes and mobile genetic elements in hospital, community and urban wastewaters and associated antibiotic prescription data in Norway

Figure S1.

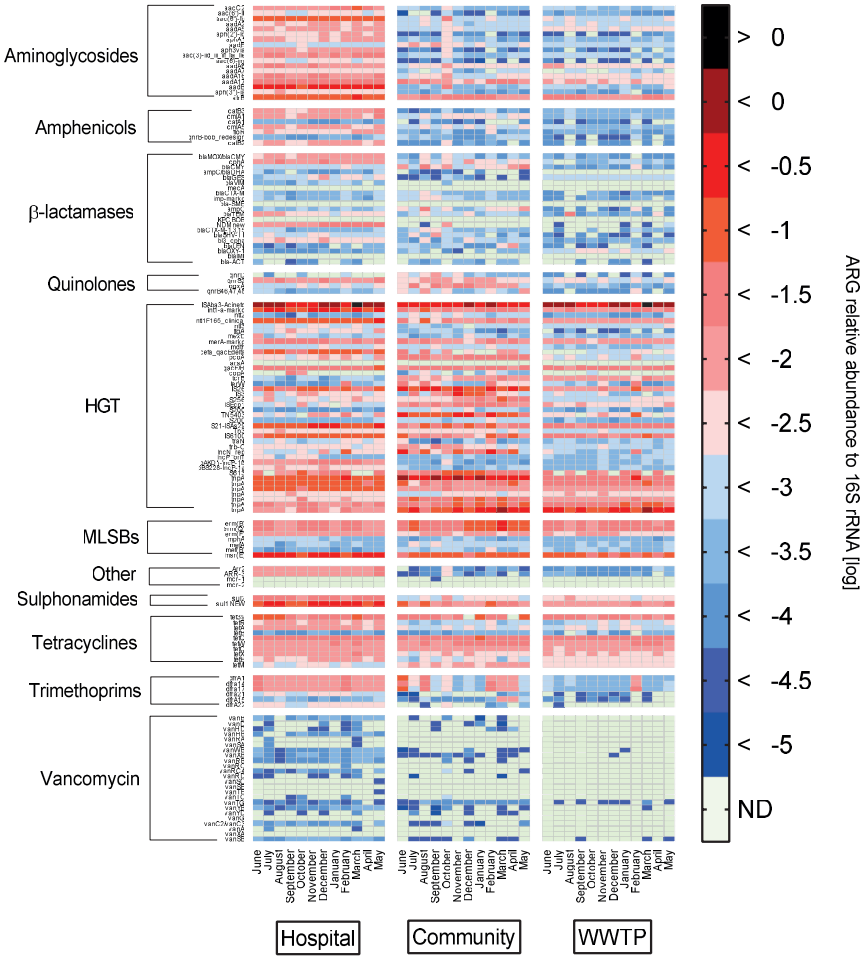

Figure S1. Abundances of antibiotic resistance genes normalized to 16S rRNA genes in hospital, community and urban wastewater during June 2016 to May 2017. Each rectangle represents the normalized abundance value for each ARG/HGT-mediating gene (rows) and each sample/month (columns). The genes are colored according to their transformed normalized abundance. The color legend on the right is segmented by logarithmic intervals of $\sqrt{10}$ ($<10^{-5}$, 10^{-5}-$10^{-4.5}$, $10^{-4.5}$-10^{-4} etc.). ND: Not detected.

Table S1.

Table S1. Antibiotics analyzed in wastewater samples. Antibiotics that were detected in ≥1 sample(s) are highlighted. MDL: Minimum detection limit (a.k.a. LOD – Limit of detection); MQL: Minimum quantification limit (a.k.a. LOQ: Limit of quantification).

Class	Compound	MDL[a]	MQL[a]	Recoveries[a]
Cephalosporins	Cefalexin	7.0	23.3	125 %
	Cefapirin	13.7	45.5	45 %
	Cefotaxime	6.1	21.6	76 %
	Cefazolin	47.9	30.5	70 %
	Ceftiofur	7.3	24.4	31 %
	Cefuroxime	12.9	43.0	142 %
Dihydrofolate reductase inhibitors	Trimethoprim	9.5	35.2	65 %
Fluoroquinolones	Cinoxacin	8.8	29.5	62 %
	Ciprofloxacin	16.5	54.9	157 %
	Danofloxacin	8.4	26.3	31 %
	Enrofloxacin	59.6	52.4	45 %
	Marbofloxacin	15.0	48.2	96 %
	Norfloxacin	6.7	21.1	47 %
	Ofloxacin	8.7	30.8	120 %
	Orbifloxacin	7.0	22.2	88 %
Lincosamides	Clindamycin	7.8	24.4	77 %
	Lincomycin	6.1	19.0	67 %
Macrolides	Azithromycin	16.6	49.3	74 %
	Clarithromycin	9.2	29.5	26 %
	Roxythromycin	12.3	39.6	85 %
	Spiramycin	10.7	74.5	76 %
	Tilmicosin	12.5	32.8	80 %
	Tylosin	8.8	27.5	72 %
Nitroimidazole antibiotics	Metronidazole	16.4	53.0	125 %
	Metronidazole OH	10.5	36.4	128 %
Penicillins	Amoxicillin	10.8	34.2	68 %
	Ampicillin	20.2	63.7	73 %
	Oxacillin	7.5	23.6	43 %
	Penicillin G	47.0	57.5	68 %
	Penicillin V	11.6	36.7	72 %
Quinolones	Flumequine	9.1	28.6	34 %
	Nalidixic Acid	15.6	50.6	59 %
	Oxolinic Acid	12.0	32.5	115 %
	Pipemidic Acid	27.3	86.3	58 %
Sulfonamides	N-Acetylsulfadiazine	11.2	35.7	73 %
	N-Acetylsulfamerazine	28.5	58.8	202 %
	N-Acetylsulfamethazine	6.6	20.8	73 %
	Sulfabenzamide	9.2	28.9	89 %
	Sulfadiazine	8.2	25.9	39 %
	Sulfadimethoxine	10.3	32.3	68 %
	Sulfamerazine	11.3	35.8	99 %
	Sulfamethizole	8.4	26.7	61 %

	Sulfamethoxazole	9.6	30.9	45 %
	Sulfamethoxypiridazine	11.1	36.0	58 %
	Sulfanitran	18.0	58.7	43 %
	Sulfapyridine	11.5	30.7	63 %
	Sulfathiazole	9.4	29.7	63 %
	Sulfisomidin	10.7	33.9	78 %
	Sulfisoxazole	7.1	22.5	138 %
Tetracyclines	Chlorotetracycline	19.1	61.8	86 %
	Doxycycline	26.5	77.0	123 %
	Oxytetracycline	84.5	41.8	120 %
	Tetracycline	13.2	42.8	68 %

[a] Averages of individually calculated MDL/MQL/recoveries for each antibiotic.

Table S2.

Table S2. Assay ID and gene targets of the 144 ARGs and HGT-mediating genes included in the present study. For further information, the authors refer to the methodological description by Stedtfeld et al. (2018).[1]

Assay ID	Gene	Function	Gene Category
1	16S old 1	16S rRNA	16S rRNA
3	aacC2	deactivate	Aminoglycoside
8	aac(6')-II	deactivate	Aminoglycoside
26	IS613	MGE	Transposase
34	blaMOX/blaCMY	deactivate	Beta Lactam
46	cphA	deactivate	Beta Lactam
51	catB3	catB3	Amphenicol
58	dfrA1	protection	other
95	aac(6')-Ib	deactivate	Aminoglycoside
97	aadA2	deactivate	Aminoglycoside
98	aadA5	deactivate	Aminoglycoside
104	aph(2')-Id	deactivate	Aminoglycoside
108	blaCMY	deactivate	beta Lactam
112	ampC/blaDHA	deactivate	Beta Lactam
120	blaGES	deactivate	Beta Lactam
127	cmlA1	cmlA1	Amphenicol
130	catA1	catA1	Amphenicol
133	sul2	protection	Sulfonamide
147	blaVIM	deactivate	Beta Lactam
155	mecA	protection	Beta Lactam
162	blaCTX-M	deactivate	Beta Lactam
170	aphA1	deactivate	Aminoglycoside
174	aadE	deactivate	Aminoglycoside
177	strB	protection	Sulfonamide
180	tetA	efflux	Tetracycline
181	tetB	efflux	Tetracycline
185	tetQ	protection	Tetracycline
191	tetW	protection	Tetracycline
192	tetO	protection	Tetracycline
196	tetX	deactivate	Tetracycline
201	tnpA	MGE	Transposase
202	tnpA	MGE	Transposase
203	tnpA	MGE	Transposase
204	tnpA	MGE	Transposase
205	tnpA	MGE	Transposase
206	tnpA	MGE	Transposase

207	tnpA	MGE	Transposase
211	VanB	VanB	Vancomycin
213	vanD	protection	Vancomycin
214	vanHD	protection	Vancomycin
215	vanHB	protection	Vancomycin
216	vanRA	protection	Vancomycin
218	vanSA	protection	Vancomycin
220	vanWB	protection	Vancomycin
223	vanXB	protection	Vancomycin
243	ttgA	efflux	MDR
246	mexE	efflux	MDR
291	tetE	efflux	Tetracycline
306	vanRB	protection	Vancomycin
307	vanRC	protection	Vancomycin
308	vanRC4	protection	Vancomycin
309	vanRD	protection	Vancomycin
311	vanSC	protection	Vancomycin
313	vanSE	protection	Vancomycin
314	vanTE	protection	Vancomycin
315	vanTC	protection	Vancomycin
316	vanTG	protection	Vancomycin
317	vanYB	protection	Vancomycin
318	vanYD	protection	Vancomycin
324	imp-marko	deactivate	Beta Lactam
328-n2	qnrB-bob_redesign	efflux	Amphenicol
331	merA-marko	unknown	MDR
336	int1-a-marko	MGE	Integrase
338	intI2	MGE	Integrase
340	IncN_rep	MGE	plasmid incompatibility
342	IncP_oriT	MGE	plasmid incompatibility
359	intI1F165_clinical	MGE	Integrase
362-n2	NDM new	deactivate	Beta Lactam
363	sul1 NEW	protection	Sulfonamide
371	ISAba3-Acineto	MGE	Insertional sequence
375	cmlA5	efflux	Amphenicol
380	pAKD1-IncP-1β	MGE	plasmid replication
381	pBS228-IncP-1α	MGE	plasmid replication
403	aph3via	aph3via	Aminoglycoside
410	aac(3)-iid_iii_iif_iia_iie	aac(3)-iid_iii_iif_iia_iie	Aminoglycoside
417	aac(6)-im	aac(6)-im	Aminoglycoside
424	aadA6	aadA6	Aminoglycoside

425	aadA7	aadA7	Aminoglycoside
427	aadA16	aadA16	Aminoglycoside
428	aadA17	aadA17	Aminoglycoside
429	aadB	aadB	Aminoglycoside
435	aph(3'')-ia	aph(3'')-ia	Aminoglycoside
505	tet39	tet39	tetracycline
508	tetR	tetR	tetracycline
600	dfra14	dfra14	trimethoprim
601	dfra17	dfra17	trimethoprim
603	dfra21	dfra21	trimethoprim
608	dfrA15	dfrA15	trimethoprim
610	dfrA22	dfrA22	trimethoprim
703	Arr2	Arr2	other
704	mcr-1	mcr-1	other
804	erm(B)	erm(B)	MLSB
809	erm(Q)	erm(Q)	MLSB
812	mphA	deactivate	MLSB
817	erm(F)	erm(F)	MLSB
820	mef(B)	mef(B)	MLSB
824	msr(E)	msr(E)	MLSB
904	catB2	catB2	Phenicol
913	floR	floR	Amphenicol
1002	vanG	protection	Vancomycin
1003	vanC2/vanC3	protection	Vancomycin
1108	blaCTX-M-1,3,15	blaCTX-M-1,3,15	Beta lactamase
1110	blaSHV-11	blaSHV-11	Beta lactamase
1113	bl3_cpha	bl3_cpha	Beta lactamase
1116	blaLEN	bla_LEN	Beta lactamase
1118	blaOXY-1	deactivate	Beta lactamase
1119	bla-SME	deactivate	Beta Lactam
1129	blaIMI	bla_IMI	Beta lactamase
1300	mdth	mdth	MDR-chromosomal
1301	cefa_qacEdelta	cefa_qacEdelta	MDR-mobile
1303	pcoA	pcoA	MDR-mobile
1305	arsA	arsA	MDR-mobile
1308	qacF/H	qacF/H	MDR-mobile
1505	ampC	deactivate	Beta Lactam
1511	mefA	efflux	MLSB
1512	blaTEM	deactivate	Beta Lactam
1513	tetM	protection	Tetracycline
1514	vanA	protection	Vancomycin

1515	vanXA	protection	Vancomycin
1521	vanSB	protection	Vancomycin
1522	intl3	MGE	Integrase
1523	KPC BOB	deactivate	Beta Lactam
1525	traN	traN	plasmid
1527	trb-C		plasmid
1529	copA	copA	MDR-mobile
1542	ARR-3	ARR-3	other
1543	mcr-2	mcr-2	other
1544	bla-ACT	bla-ACT	Beta lactamase
1546	IS26	IS26	MGE
1547	IS3	IS3	MGE
1548	IS256	IS256	MGE
1550	ISEcp1	ISEcp1	MGE
1551	IS200	IS200	MGE
1556	TN5403		MGE
1557	IS200		MGE
1558	IS21-ISAs29		MGE
1559	Tn3		MGE
1561	IS6100		MGE
1571	tcrB		MDR-mobile
1572	terW		MDR-mobile
1574	qnrD		fluoroquinolone
1576	qnrS2		fluoroquinolone
1577	oqxA		fluoroquinolone
1579	qnrB46,47,48		fluoroquinolone

References

1. Stedtfeld RD, Guo X, Stedtfeld TM, et al. Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. *FEMS Microbiol Ecol*. 2018;94(9). doi:10.1093/femsec/fiy130

ISBN: 978-82-575-1675-8
ISSN: 1894-6402

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