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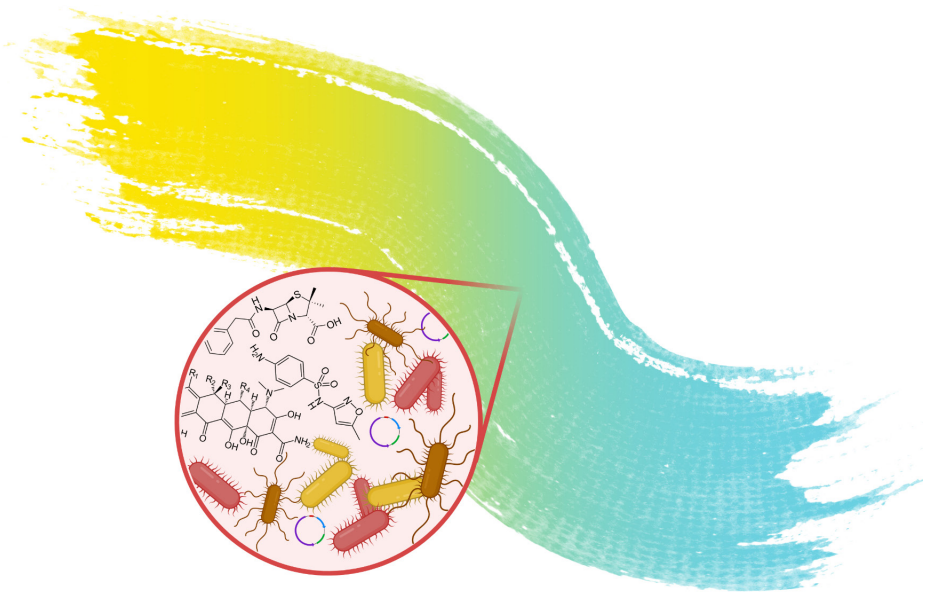
Antimicrobial resistance in on-site sewage facilities

Environmental impact on receiving waters and mitigation strategies

Valentina Ugolini



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Abstract

The environment, partly as a recipient of wastewater discharges, is a major reservoir for the proliferation and transmission of antimicrobial resistance (AMR) – a growing global health threat driven by the overuse and misuse of antimicrobial chemicals. This thesis investigated the role of decentralized, on-site sewage facilities (OSSF) in the environmental dissemination of AMR. First, I developed a robust, microbiologically sensitive analytical method for quantifying antimicrobial chemicals from sources (influent and effluent wastewater) to recipients (groundwater, surface water), aimed at supporting (inter)national AMR monitoring efforts. Next, I reviewed the global literature to identify OSSF as overlooked contributors to environmental AMR, highlighting a critical need for quantitative data on AMR determinants and their co-occurrences with antimicrobials chemicals. I also prioritized antimicrobial chemicals of concern in OSSF settings, based on a meta-analysis of their AMR selection risk, ecological risk, and environmental hazard. Then, to characterize and quantify the dissemination of AMR contaminants from source to recipient, I conducted an extensive field study in a Swedish OSSF and its associated groundwater, revealing that the OSSF insufficiently removed AMR contaminants. In contrast to AMR determinants, antimicrobial chemicals exhibited higher temporal variation. Strong correlations between AMR determinants and chemical contaminants suggest interactions between these factors in the AMR dissemination process. Finally, I evaluated biochar as an eco-friendly material for mitigating AMR contaminants. Biochars with high specific surface area efficiently removed chemical contaminants, while those with greater external surface area, rather than microporous structures, better mitigated AMR determinants. This led to a combination of biochars for improving the overall mitigation. This thesis advances the understanding of the role of OSSF in the environmental dimension of AMR and provides critical insights that can support their monitoring, regulation, and mitigation efforts needed to combat AMR for a sustainable future.

Keywords: (waste)water extraction; pharmaceuticals; antimicrobial resistance genes; groundwater; surface water; effluent wastewater; water treatment; biochar

Antimikrobiell resistens i decentraliserade avloppsanläggningar: Miljöpåverkan på recipient samt begränsningsstrategier

Abstract

Miljön, delvis som mottagare av avloppsvattenutsläpp, är en viktig reservoar för spridning och överföring av antimikrobiell resistens (AMR) - ett växande hot mot den globala hälsan som drivs av överanvändning och felaktig användning av antimikrobiella kemikalier. I den här avhandlingen undersöktes vilken roll decentraliserade avloppsanläggningar (OSSF) har för spridningen av AMR i miljön. Först utvecklade jag en robust, mikrobiologiskt känslig analysmetod för att kvantifiera antimikrobiella kemikalier från källa (inkommande och utgående avloppsvatten) till recipient (grundvatten, ytvatten), i syfte att stödja (inter)nationella AMR-övervakningsinsatser. Därefter granskade jag den internationella litteraturen för att identifiera OSSF som förbisedda källor till AMR i miljön, vilket belyser ett kritiskt behov av kvantitativa data om resistensfaktorer och deras förekomst tillsammans med antimikrobiella kemikalier. Jag prioriterade antimikrobiella kemikalier som är problematiska i OSSF-miljöer, baserat på en metaanalys av deras selektionsrisk för AMR, ekologiska risk och miljöfara. För att karakterisera och kvantifiera spridningen av AMR-föroreningar från källa till recipient genomförde jag sedan en omfattande fältstudie i en svensk OSSF och dess tillhörande grundvatten, vilket visade att OSSF inte avlägsnade AMR-föroreningar i tillräcklig utsträckning. I motsats till resistensfaktorerna uppvisade antimikrobiella kemikalier en högre temporal variation. Starka korrelationer mellan resistensfaktorer och kemiska föroreningar tyder på att de samverkar i processen för spridning av AMR. Slutligen utvärderade jag biokol som miljövänliga material för att begränsa AMR-föroreningar. Biokol med hög specifik ytarea avlägsnade effektivt kemiska föroreningar, medan de med större yttre ytarea, snarare än mikroporösa strukturer, bättre minskade förekomsten av resistensfaktorer. Detta ledde till en kombination av olika biokol för att förbättra den totala minskningen. Denna avhandling ökar förståelsen för OSSF:s roll i miljödimensionen av AMR och ger viktiga insikter som kan användas för att stödja övervakning, reglering och de begränsningsinsatser som behövs för att bekämpa AMR, för en hållbar framtid.

Keywords: (Avlopps)vattenutvinning; läkemedel; gener för antimikrobiell resistens; grundvatten; ytvatten; avloppsvatten; vattenrening; biokol

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Ugolini V.**, Lai F.Y. (2024). Novel, alternative analytical methodology for determination of antimicrobial chemicals in aquatic environments and public use assessment: Extraction sorbent, microbiological sensitivity, stability, and applicability. *Analytica Chimica Acta*, 1286(342029). https://doi.org/10.1016/j.aca.2023.342029

II. **Ugolini V.**, Khan U.A., Löffler P., Spilsbury F., Lai F.Y. (2025). Insight into on-site sewage facilities as an overlooked contributor to antimicrobial resistance: Environmental impacts and existing mitigation strategies. (Under Review)

III. **Ugolini V.**, Flores Quintana H., Subirats Medina J., Löffler P., Ulinder P., Ahrens L., Rapp P., Dunge C., Lai F.Y. An overlooked dissemination of antimicrobial resistance from on-site sewage facilities to groundwater environment: Temporal co-occurring and associated dynamics between antimicrobial agents, high-use chemicals and microbial contaminants. (Manuscript)

IV. **Ugolini V.**, Löffler P., Celma A., Wiberg K., Ahrens L., Sigmund G., Lai F.Y. Biochar efficiency in mitigating antimicrobial resistance contaminants: Implications for water treatment in on-site sewage facilities. (Manuscript)

Paper I is published open access.

The contribution of Valentina Ugolini to the papers included in this thesis was as follows:

I. Contributed to the planning of the study design, conducted the experiments, performed laboratory work and data analysis. Wrote the original manuscript and performed editing and revision.

II. Contributed to the planning of the study design, compiled the literature, conducted the data retrieval and analysis. Wrote the original manuscript and performed editing and revision.

III. Contributed to the planning of the study design and conducted sampling campaigns. Performed sample extraction and HPLC/MS-MS analysis for chemicals and *in-situ* filtration for HT-qPCR analysis and data analysis. Wrote the original manuscript and performed editing and revision.

IV. Contributed to the planning of the study design, conducted the experiments, performed laboratory work and data analysis. Wrote the original manuscript and performed editing and revision.

Additional publications

In addition, the author has contributed to the following papers, which are not included in the thesis:

I. Alygizakis N., Ng K., Čirka L., Berendonk T., Cerqueira F., Cytryn E., Deviller G., Fortunato G., Iakovides I., Kampouris I., Michael-Kordatou I., Lai F.Y., Lundy L., Manaia C.M., Marano R.B.M., Paulus G.K., Piña B., Radu E., Rizzo L., Ślipko K., Kreuzinger N., Thomaidis N.S., **Ugolini V.**, Vaz-Moreira I., Slobodnik J., Fatta-Kassinos D. (2024). Making waves: The NORMAN antibiotic resistant bacteria and resistance genes database (NORMAN ARB&ARG)–An invitation for collaboration to tackle antibiotic resistance. *Water research,* 257(121689). https://doi.org/10.1016/j.watres.2024.121689

Abbreviations

AMR	Antimicrobial Resistance
ARG	Antimicrobial Resistance Gene
GW	Groundwater
HT-qPCR	High Throughput Quantitative Polymerase Chain Reaction
IDL	Instrumental Detection Limit
IQL	Instrumental Quantification Limit
LC-MS	Liquid Chromatography – Mass Spectrometry
MDL	Method Detection Limit
MDR	Multidrug resistance
MGE	Mobile Genetic Element
MLSB	Macrolide-Lincosamide-StreptomycinB
MQL	Method Quantification Limit
OSSF	On-Site Sewage Facilities
SPE	Solid Phase Extraction
PE	Population equivalent

1. Introduction

1.1 Concerns over antimicrobial resistance (AMR) and its emergence in the environment

Antimicrobial resistance (AMR) is a phenomenon that *"occurs when bacteria, viruses, fungi and parasites no longer respond to antimicrobial medicines"* (World Health Organization (WHO), 2023). The high production rates and the poorly regulated use of antimicrobial chemicals in clinical settings, along with their occasional application in preventive practices, highly contributes to the development and transmission of AMR (Andleeb et al., 2020). In 2019 alone, AMR was responsible for nearly 5 million deaths worldwide, representing a major burden on global health (Murray et al., 2022). Early on, AMR has been mainly focused on clinical or veterinary settings. In recent years, the role of the environment in contributing to AMR development and dissemination is increasingly recognized (Larsson and Flach, 2022). In 2017, this emerging concern was emphasized by the United Nations Environment Programme (UNEP) (Lai et al., 2021; UNEP, 2017). In 2022, UNEP joined the former tripartite of the World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the World Organization for Animal Health (WOAH). This resulted in a quadripartite alliance focused on addressing AMR through a *One Health* perspective, linking human, animal, plant and environmental health (UNEP, 2022).

The environment is the final collector of anthropogenic activities, accumulating a diverse mixture of pollutants, including antimicrobial chemicals and antimicrobial resistance genes (section 1.1.1 and 1.1.3), which circulate through soil, water and air (Martak et al., 2024). Given their widespread presence and the growing recognition of the risk they pose to the environment, they are considered to be contaminants of emerging concern (CECs) (Richardson and Kimura, 2020). Identifying their key sources of pollution, transmission mechanisms and dissemination pathways is crucial for effective AMR mitigation.

Municipal effluent wastewater is one of the primary sources through which AMR contaminants enter the environment (Sambaza and Naicker, 2023). It contains antimicrobial chemicals that are often excreted unchanged after

consumption, retaining their microbiologically active properties. These chemicals contribute to resistance selection processes within the microbial community (section 1.1.2), promoting AMR development. Additionally, wastewater contains antimicrobial resistant bacteria, antibiotic resistance genes (ARG), and genes involved in ARG mobilization, such as mobile genetic elements (MGE) and integrons, further facilitating AMR transmission. After the discharge of effluent wastewater, any antimicrobial chemicals and AMR-related genes not effectively removed during treatment reach the aquatic environment, contributing to AMR development and dissemination.

In the following sections, the nature of AMR contaminants and how antimicrobial resistance develops is explained in more detail.

1.1.1 Antimicrobial chemicals and their mode of action

Certain microorganisms (e.g., *Aspergillus* spp, *Streptomyces* spp) naturally produce antimicrobial chemicals to gain competitive advantages for nutrients and ecological niches. Since the discovery of their therapeutic potential in treating bacterial, fungal, parasitic or viral infections, they have been increasingly used in our society (Klein et al., 2021). Today, antimicrobial chemicals are produced through natural fermentation, semi-synthetic or synthetic processes (Elander, 2003), enabling the development of a diverse range of agents with various mechanisms of action and target organisms. These include antibacterials, antifungals, antiparasitics and antivirals that can exhibit a wide spectrum of activity. They function either through killing (e.g., microbiocidal activity) or inhibiting the growth of the target organism by blocking cell reproduction (e.g., microbiostatic activity) (Rayasam et al., 2023). Antibacterials function through several mechanisms: i) *cell wall synthesis* by either inhibiting the synthesis of peptidoglycan (β-lactams) or binding to precursors of peptidoglycan (e.g., glycopeptides), ii) *protein synthesis* by interfering with 30S (e.g., aminoglycosides, tetracyclines) or 50S (e.g., macrolides, lincosamides, streptogramins) ribosomal subunits, iii) *cell membrane integrity*, specifically for gram-negative bacteria, by interacting with phospholipids and increasing membrane permeability (e.g., polymixins), iv) *nucleic acid synthesis* by inhibiting essential enzymes for DNA replication as DNA gyrase (e.g., fluoroquinolones) and RNA polymerase (e.g., rifampins) or v) *folic acid synthesis* by inhibiting the

production of metabolic intermediates (e.g., sulfonamides, trimethoprim) (Rayasam et al., 2023). Antifungals act similarly on cell membrane integrity by binding to ergosterol (e.g., amphotericin) and inhibiting ergosterol synthesis (e.g., azoles) or inhibiting cell wall synthesis (e.g., echinocandins) (Lee et al., 2023). Antiparasitics, such as antimalarial drugs (e.g., chloroquine), act by causing an accumulation of free hematin, a byproduct of hemoglobin degradation, which intoxicates the parasite (Zhou et al., 2020). Antivirals hinder the development of viruses by interfering with replication (e.g., acyclovir).

1.1.2 AMR development and selection mechanisms

The natural occurrence of antimicrobial chemicals has driven evolutionary processes that led to the development of AMR mechanisms, encoded by genes constituting the *resistome* (Gillings, 2013). Within the resistome, phenotypically expressed genes can be classified as *intrinsic* or *acquired*, while non-phenotypically expressed genes can be *silent* or *proto* (Perry et al., 2014). Intrinsic resistance genes are present in all organisms within the same taxa, as they are embedded in the main genome, and they are passed through vertical gene transfer (VGT) to future generations. Acquired resistance genes originate from other organisms through horizontal gene transfer (HGT) over the course of evolution and subsequently inherited via VGT. Silent or proto resistance genes remain unexpressed, making the organisms susceptible to antimicrobial chemicals. However, their activation can be *induced* by natural mutation or under selective pressure (e.g., antimicrobial chemicals), posing a potential risk for future AMR development. Moreover, microbial communities can shift towards resistance when exposed to external selective pressures. These pressures eliminate susceptible bacteria, allowing resistant strains to thrive and become dominant. Under these conditions, random genetic mutation and HGT are accelerated, promoting AMR development. Of the HGT mechanisms (i.e., conjugation, transduction ad transformation), conjugation is the most common (Tao et al., 2022), and implies the actions of mobile genetic elements (MGE) to facilitate intracellular DNA mobility. These includes transposons, insertion sequences (IS) and integrons, responsible for mobilization within genomes and plasmids which actively move between cells (Partridge et al., 2018).

In the environment, AMR often develops in non-pathogenic bacteria, resulting in *environmental resistance* (Perry et al., 2014). Therefore, the environment acts as reservoir for the resistome, with a potential of transferring the resistance back to pathogenic bacteria, resulting in *clinical resistance* (Perry et al., 2014). This can ultimately pose a threat to human or animal health. Larsson and Flach (2022) identified four key requirements for this transfer: 1) the ability of a gene to move within the genome facilitated by insertion sequences or integrons; 2) the relocation of the gene to a mobile elements, such as a plasmid; 3) the direct or indirect transfer of the resistant gene to a pathogen via HGT; and 4) human or animal uptake of the resistant pathogen.

Traditionally, antimicrobial chemicals have been known to exert a selective pressure when exceeding *minimum inhibitory concentrations (MIC)*, causing microbial growth inhibition and stress response (Gullberg et al., 2011). Recently, this selective pressure has been observed even at sub-MIC levels (Stanton et al., 2020), meaning that AMR mechanisms are triggered even in the absence of growth inhibition. This leads to the introduction of key concepts: *minimal selective concentration (MSC)* as the lowest selective concentration at which the resistance strain dominates over the susceptible (Gullberg et al., 2014, 2011; Stanton et al., 2020); and *predicted-no-effect concentration (PNEC)* for AMR selection as a threshold below which antimicrobial chemicals are unlikely to drive resistance (Bengtsson-Palme and Larsson, 2016). Comparing measured environmental concentrations (MECs) of antimicrobial chemicals with these thresholds allows to assess the degree of selective pressure imposed on microbial communities.

Beyond antimicrobial chemicals, other substances able to trigger resistance mechanisms exist. Biocides (e.g., triclosan and triclocarban; Halden et al., 2017) and metals (Baker-Austin et al., 2006) are involved in co-selection processes, but increasing evidence suggests that also other chemicals (e.g., non-antibiotic pharmaceuticals) may have an important role in AMR development (Murray et al., 2024).

1.1.3 Mechanisms of AMR

Resistance to antimicrobial chemicals occurs through four main mechanisms: (i) limiting uptake by decreasing cell permeability to prevent

antimicrobial entry; (ii) inactivation (e.g., addition of an acetyl group) or degradation (e.g., hydrolysis); (iii) target alteration or protection; and (iv) extrusion via efflux pumps (Reygaert, 2018). Limiting uptake is a common intrinsic resistance mechanism, typical for gram-negative bacteria. Efflux-pumps are also typically intrinsic, but they can also be acquired (Gillings, 2013). Efflux-pumps have different levels of specificity, from high, such as ATP binding cassette (ABC) transporters (e.g., *mefA* encoding gene for macrolides specific efflux pump) to low, such as resistance-nodulation-division (RND) transporters (e.g., *tolC* encoding gene for multidrug efflux pump targeting several antibacterial families and disinfecting agents) (Alcock et al., 2023; Reygaert, 2018).

1.2 Regulations and initiatives to combat AMR

In addition to the One Health framework, several other initiatives have been developed in order to combat the increasing concern of AMR. To address the global misuse and overuse of antimicrobial chemicals, in 2015, a Global Action Plan (GAP) was proposed by WHO, leading to the implementation at national levels, with national action plans. In 2023, 92 countries had functional AMR action plans, while 85 others were in the process of establishing them (TrACSS, 2023). To support proper prescription of antimicrobial chemicals, stewardships programs were developed, including the AWaRe (Access, Watch and Reserve) classification of antibiotics based on their appropriate use in treatment (World Health Organization, 2021). This is important as it highlights antibiotics that are used as first-line treatment (e.g., β-lactams) and therefore more commonly prescribed than last resort ones (e.g., glycopeptides) (Jovetic et al., 2010). Furthermore, surveillance programs have been launched to monitor the use of antimicrobial chemicals and the development and spread of AMR. One of such programs is the Global Antimicrobial resistance and Use Surveillance System (GLASS), which aims to collect standardized data across countries (WHO, 2015).

To monitor AMR in the environment, certain antimicrobial chemicals are included in monitoring programs for CECs. For example, at the European Union (EU) level under the Water Framework Directive, the Watch List for surface water monitoring is updated every two years and considers

substances that *"may pose a significant risk to or via the aquatic environment"*, including the risk for antimicrobial resistance development (Joint Research Centre, 2025). Proper monitoring of these substances requires accurate analytical methods and reliable PNECs. Table 1 shows the monitored antimicrobial chemicals since the first Watch List was established in 2015. Compared to antimicrobial chemicals, tracking of antimicrobial resistance genes remains relatively more challenging due to the absence of standardized methodologies for monitoring and assessment of their risk. As antimicrobial resistance genes are naturally present in the environment, defining their baseline levels is important for understanding whether their presence in a specific environment is influenced by pollution from anthropogenic sources. Baseline threshold levels indicating such impacts have been recently proposed for some genes by Abramova et al., (2023). Furthermore, high-priority resistance genes have been proposed, considering their risks in clinical settings (e.g., *aadA*, *blaCTX-M*, *ermB*, *sul1*, *qnrS*) (Zhang et al., 2021; Zhang et al., 2022), and selected biomarkers to support AMR monitoring in wastewater were suggested (Manaia, 2022). Also, joint effort of research scientists on collecting data on global levels of resistance genes in wastewater and environmental matrices led to the creation of resistant gene databases (e.g., Alygizakis et al., 2024; Cacace et al., 2019).

Table 1. Antimicrobial chemicals selected on the EU Watch List in past and recent years (EU 2015/495, 2018/840, 2020/1161, 2022/1307, 2025/439).

Antimicrobial chemicals	*2015*	*2018*	*2020*	*2022*	*2025*
Amoxicillin					
Clindamycin					
Ciprofloxacin					
Macrolides[a]					
Ofloxacine					
Oxytetracycline					
Sulfamethoxazole					
Trimethoprim					
Azoles[b]					

(a) Erythromycin, clarithromycin, azithromycin; (b) Clotrimazole, fluconazole (not included in 2025), imazalil, ipconazole, metconazole, miconazole, penconazole, prochloraz, tebuconazole and tetraconazole.

Wastewater is a major route for AMR dissemination, making the integration of monitoring programs and treatment strategies within existing wastewater

directives crucial. Since January 2025, the recast EU Urban Wastewater Treatment Directive (UWWTD) (EU 2024/3019) has come into force and introduced stricter requirements. For example, it has lowered the threshold to 1000 population equivalents (PE) in areas where wastewater must be collected and treated to at least a secondary level (2000 PE in the former UWWTD EU 91/271/EEC). Similarly, AMR monitoring in wastewater will newly apply to areas and/or WWTPs serving >100000 PE. More importantly, the recast directive has emphasised the need for enhanced efforts and investigation into future monitoring of the environmental impacts of small household agglomerations, such as decentralized, on-site sewage facilities (OSSF).

1.3 On-site sewage facilities and AMR dissemination

Globally, OSSF account for 24% of treated household wastewater, yet only 12% of this is considered safely treated (UN, 2024). This highlights that these decentralized systems do not necessarily guarantee a safe discharge into the environment. OSSF are commonly employed in rural and suburban areas where connection to the main sewage network is impractical. In Sweden, OSSF treat ~13% of household wastewater (Olshammar et al., 2015). Despite the increasing attention towards small household agglomerations, decentralized wastewater treatment systems often serve areas with small PE, for which secondary treatment and onwards are not mandatory by regulation.

Over the past 18 years, research on OSSF has expanded (Figure 1). Early on, it has focused on inorganic substances like metals. Then, it gradually expanded to include organic compounds and nutrients, and more recently, to microbial contaminants associated with AMR (**Paper II**). Since AMR was not a key focus in the early on studies, only limited selections of antimicrobial chemicals were examined, primarily sulfamethoxazole and trimethoprim, among a broader spectrum of organic pollutants. However, there has since been a growing focus on AMR, particularly concerning the presence of microbial contaminants like ARGs, resistant bacteria, and pathogens in OSSF systems (Figure 1). This shift reflects the increasing awareness of the One Health framework, as well as advances in (bio)analytical technologies that have improved the detection and

quantification of AMR contaminants in water. Despite this progress, available studies often examine chemical or microbial contaminants separately. Addressing both would add research significance as these contaminants interact together in AMR development and dissemination. The following sections summarize the existing scientific literature on AMR contaminants in OSSF.

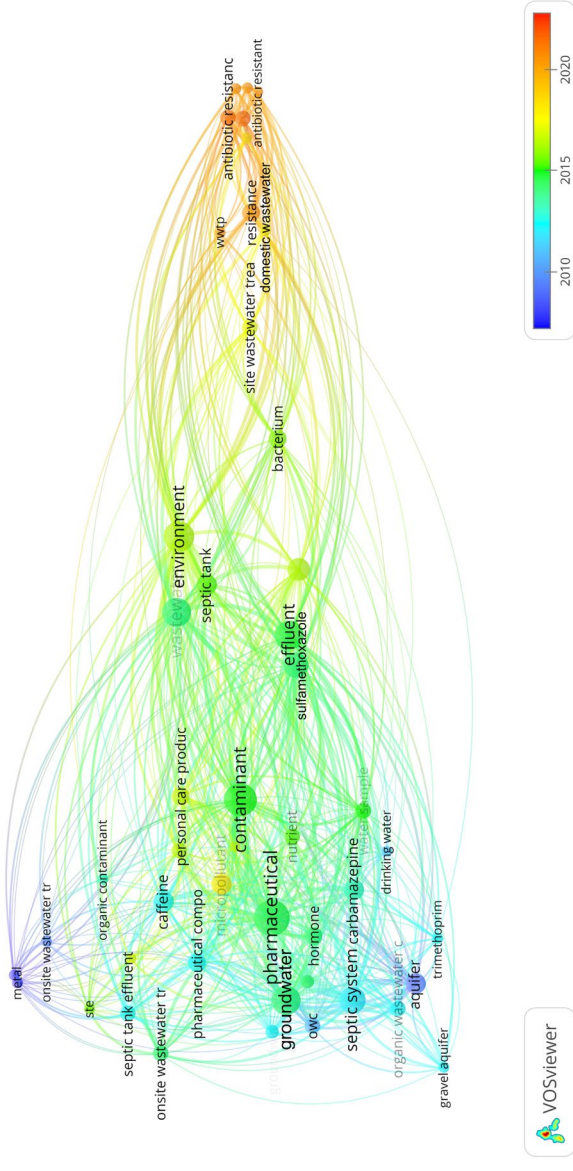

Figure 1. Distribution and connectivity of terms in abstract and titles of the articles obtained through literature search (section 3.3). The size of the circles represents how many times a term has been used. (made with VOSviewer).

1.3.1 Existing mitigation techniques at OSSF

With a common design of septic tanks and infiltration fields, OSSF are considered as diffuse sources of contamination to receiving aquatic environments (surface water or groundwater) (Blum et al., 2018). In the septic tank, which can be an open or close system, wastewater undergoes mainly primary treatment, where sedimentation separates the solid fraction from the liquid fraction. At this stage, biodegradation processes can also occur to some extent (Yates, 2011). The septic tank effluent is further treated via soil infiltration, with materials such as natural soil, sand and gravels. For natural soils to be suitable for wastewater infiltration, they must be permeable enough to prevent wastewater stagnation in the upper layers, as seen with sandy soils (Yates, 2011). As groundwater can be a recipient of effluent water following infiltration, an adequate infiltration zone between the ground surface and the groundwater table is essential for effective purification. The infiltration step serves as the last barrier to prevent contaminants entering the aquatic environment. Different physical and chemical mechanisms can prevent these AMR contaminants from leaching including adsorption, mechanical filtration, biodegradation (Gao et al., 2019; Schaider et al., 2017). The efficiency of these mechanisms can be influenced by both soil characteristics (e.g., texture, pH, cation exchange capacity) and contaminants properties (e.g., speciation, hydrophobicity) (Gao et al., 2019).

There are alternative OSSF designs, which incorporate secondary treatment, enhancing wastewater treatment beyond conventional septic tanks and infiltration fields. This includes aerobic treatment systems, trickling filter package plants, activated sludge processes with phosphorus removal, constructed wetlands, textile filters, denitrification tanks, aerated lagoons, nitrogen-removing biofilters, and sand filters (Du et al., 2014; Elliott et al., 2018; Hayward et al., 2019; Li et al., 2013; Subedi et al., 2015; Vidal et al., 2023).

1.3.2 AMR contaminants in OSSF wastewater

No matter the serving capacity or design of OSSF, the most frequently detected antimicrobial compounds in both raw and treated OSSF wastewater are the antibacterials sulfamethoxazole and trimethoprim, and the antimicrobial personal care products (PCPs) triclosan and triclocarban. Triclosan and triclocarban typically occurs in the µg/L (ppb) range with high

detection frequencies (Carrara et al., 2008; Conn et al., 2010; Hayward et al., 2019; Li et al., 2013; Subedi et al., 2015; Teerlink et al., 2012). These compounds are usually sufficiently removed (>90% removal efficiency) by the conventional design of OSSF with septic tanks followed by infiltration fields, mainly due to their hydrophobicity and high affinity with the solid fraction (high organic carbon-water partition co-efficient (K_{oc}); triclosan=4.56, triclocarban=3.61). In contrast, sulfamethoxazole and trimethoprim present in the ng/L (ppt) range, are not well removed in septic tanks (<20%) (Du et al., 2014). Alternative OSSF designs such as constructed wetlands efficiently removes trimethoprim, while good removal of sulfamethoxazole can be obtained using nitrogen removing biofilters (Clyde et al., 2021; Du et al., 2014). Other antimicrobial chemicals are found in the ng/L range, including the antifungal fluconazole, the fluoroquinolone ciprofloxacin, the macrolides azithromycin, clarithromycin, erythromycin and roxithromycin (Clyde et al., 2021; Du et al., 2014; Gao et al., 2019; Hayward et al., 2019). More rarely monitored but still found in the ng/L range in OSSF wastewater are climbazole, metronidazole, tetracycline and clindamycin (Gao et al., 2019; Hayward et al., 2019). Septic tanks do not effectively remove fluconazole, macrolides, clindamycin, climbazole and ciprofloxacin, but provide better removal for tetracycline and metronidazole (Gao et al., 2019; Hayward et al., 2019). Alternative OSSF systems (e.g., constructed wetlands) improve removal of macrolides (Du et al., 2014). Occasionally, antimicrobial chemical concentrations in treated wastewaters are found to exceed the PNEC for AMR selection ($PNEC_{AMR}$) (Bengtsson-Palme and Larsson, 2016), such as sulfamethoxazole ($PNEC_{AMR}$ = 16000 ng/L) measured at 37700 ng/L in effluent wastewater (Subedi et al., 2015).

Studies on the occurrence of antimicrobial resistance genes in OSSF shows that septic tanks do not reduce the gene abundance, and in some cases, can even contribute to the enrichment of ARG (i.e., β-lactams resistance genes) (Tan et al., 2021). This suggests that the conditions in septic tanks could favour AMR proliferation. The high-risk genes for clinical settings, *ermB*, *tetQ*, *tetO*, *sul1*, *blaTEM-1* and *qnrS*, were commonly found in OSSF effluent wastewater (Hayward et al., 2019; Ma et al., 2023). Additional treatment steps have shown improvements in ARG removal, including peat bio-filtration, biological aerated treatment, constructed wetlands and sand filtration (Hayward et al., 2021, 2019; Ma et al., 2023; Park et al., 2016).

1.3.3 AMR contaminants in the receiving environment

In OSSF-impacted aquatic environments, sulfamethoxazole and fluconazole were frequently found at concentrations occasionally high enough to pose risks for AMR selection, such as fluconazole in groundwater beneath an infiltration field in a silty-sand area (Phillips et al., 2015). Other antimicrobial compounds, including macrolides (0.1-89 ng/L) and tetracycline (3.9 ng/L), were also found in surface water and groundwater (Ferrell and Grimes, 2014; Gao et al., 2019). However, their concentrations remained well below their $PNEC_{AMR}$ thresholds (250-1000 ng/L), indicating low risk for AMR selection. Despite good removal of these chemicals through conventional OSSF treatment, the antimicrobial PCPs, triclosan (4.76-54.8 ng/L) and triclocarban (1.0-124 ng/L), can be found in the receiving water (Hayward et al., 2019; Li et al., 2013; Subedi et al., 2015; Yang et al., 2017, 2016). Although rarely detected, antivirals, i.e., acyclovir, nevirapine and oseltamivir, were reported in downstream groundwater (Fisher et al., 2016). As for antimicrobial resistance genes, Ma et al., (2023) reported genes conferring resistance to multidrugs, macrolide-lincosamide-streptograminB (MLSB) and bacitracin in receiving waters, with especially multidrug resistance genes being at comparable abundance to OSSF effluent wastewater.

1.4 Use of biochar for wastewater treatment

Biochar is a carbon-rich material derived from pyrolysis of biomass (feedstock), which is often discarded by other productive processes such as seed waste, garden waste or woodchips, but also from sewage sludge (Zhao et al., 2019). Key properties that can affect the suitability of biochar in removing micropollutants are the surface area (i.e., specific, external, microporous), pore size distribution and presence of functional groups on the surface, all of which are highly dependent on the feedstock type and pyrolysis conditions (Tomczyk et al., 2020). The main mechanism in the removal of micropollutants is adsorption, physically (*physisorption*) or chemically (*chemisorption*) (Alsawy et al., 2022; Tong et al., 2019). With increasing strength, the most common adsorption mechanisms include hydrophobic interactions, π-π interactions of aromatic rings, H-bonding and electrostatic interactions (Tong et al., 2019). Biochar exhibits comparable properties to granular activated carbon (GAC), which is well-known for its

efficiency in removal of micropollutants (Betsholtz et al., 2024). GAC has also been tested as quaternary treatment in municipal WWTPs (Svahn and Borg, 2024; Takman et al., 2023). However, GAC treatment is particularly sensitive to high levels of particulate and dissolved organic matter, which can clog GAC beds and compete for adsorption sites (Corwin and Summers, 2012). This leads to reduced removal efficiency for micropollutants and shorten life span (Beijer et al., 2017; Corwin and Summers, 2012). This could be worsen in OSSF settings as it receives mainly primary treatment. Instead, biochar can be a cost-effective alternative due to larger pore size distribution (Huggins et al., 2016). Yet, as the organic matter could also affect micropollutants removal with biochar, more investigation is needed (Kearns et al., 2021). Previous laboratory-scale studies reported biochar to effectively remove antimicrobial chemicals such as ciprofloxacin (Chemtai et al., 2024), lincomycin (Liu et al., 2016) and clarithromycin (Imreová et al., 2024). Additionally, biochar has shown to remove genetic material with up to 85% (Calderón-Franco et al., 2021) and >80% (Bimová et al., 2021) efficiency. For genetic materials, the main adsorption mechanisms were π-π interaction, electrostatic interaction and calcium ion bridge interaction (Fang et al., 2021).

2. Research needs and thesis objectives

The growing health concerns over AMR demand urgent action to counteract the development and spread of AMR across human, animal, and environmental compartments. This leads to the need for comprehensive understanding of the pollution sources and dissemination pathways by monitoring AMR contaminants from source to recipient, assessing the impacts and implementing related mitigation strategies. Three main research needs have been identified (Figure 2) including:

- Develop analytical methodologies relevant to AMR monitoring in water matrices of interest.
- Identify AMR dissemination pathways and dynamics.
- Improve AMR mitigation strategies.

To help addressing these needs and key knowledge gaps, this thesis specifically aims to investigate the role of OSSF as contributor to AMR in the environment (Figure 2). The specific objectives were:

I. To develop a new analytical methodology for quantification of antimicrobial chemicals in different water matrices and to assess the stability of antimicrobial chemicals under different scenarios (**Paper I**).

II. To critically examine the global state-of-the-art on AMR dissemination from OSSF and prioritize relevant antimicrobial chemicals based on their risks and environmental hazards (**Paper II**).

III. To examine the temporal co-existence dynamics between AMR determinants and chemical contaminants (antimicrobial and high-use chemicals) and dissemination pathways from OSSF to the associated groundwater environment (**Paper III**).

IV. To evaluate the suitability of biochar in mitigating AMR determinants and chemical contaminants, for potential, future applications at an OSSF site (**Paper IV**).

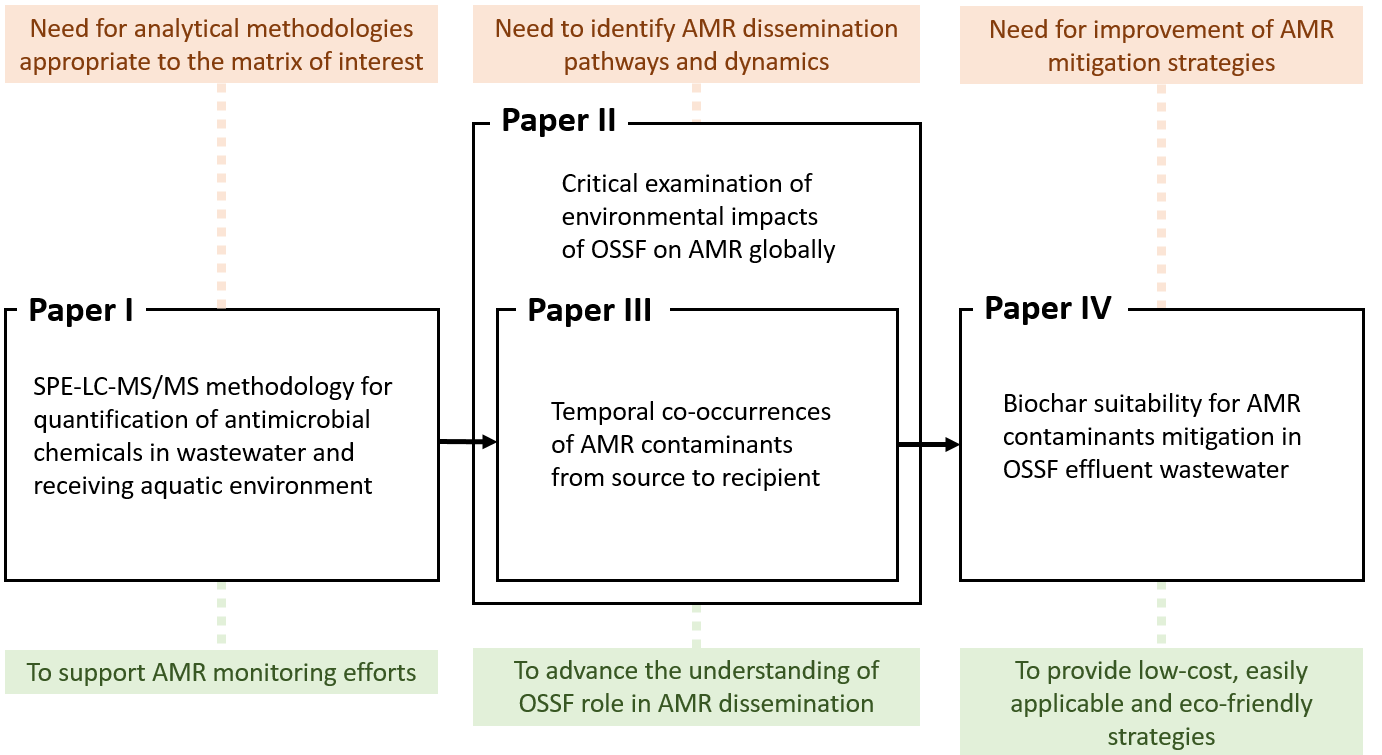

Figure 2. Schematic representation of research needs and how the thesis objectives address them.

3. Methodology

3.1 Chemicals of interest

This thesis assessed a range of antimicrobial chemicals of interest, including antibacterials, antifungals, antivirals, as well as some of their (bio)transformation products. In **Paper I**, method development and validation were performed, targeting 77 relevant antimicrobial chemicals for systemic use. This included 52 antibacterials (spanning across 17 classes), 14 antivirals, four antifungal and seven human metabolites (for details see **Paper I**). Their selection was based on (i) usage in Swedish clinical settings, (ii) occurrence in effluent wastewater of WWTP and in global surface water environment, (iii) requirements at EU level from the 3rd edited Watch List, (iv) metabolic excretion (transformation products), and (v) importance in the WHO AWaRe classification. The validated method using solid-phase extraction (SPE) as sample preparation for 35 chemicals in **Paper I** was further applied in **Paper III**, along with 21 high-use chemicals selected based on their high detection frequency in Swedish wastewater (Haalck, 2022; Haalck et al., 2024; Khan et al., 2024). **Paper IV** targeted chemicals that can be analysed via direct injection method, including antimicrobial chemicals validated in **Paper I**, and additional transformation products, selected based on their occurrence in global surface water environments (Löffler et al., 2023), and also high-use chemicals. As a literature synthesis work, **Paper II**, retrieved and prioritized 30 OSSF-related antimicrobial chemicals from a global perspective.

3.2 Targeted AMR genetic determinants

Relevant AMR genetic determinants were selected following a pre-screening of 384 genes in wastewater and groundwater samples from the studied OSSF site (**Paper III**). With these results, the selection was performed based on (i) their higher abundance in wastewater after OSSF treatment and in downstream groundwater compared to upstream levels; and (ii) their high risk prioritization in clinical settings (Zhang et al., 2021, 2022). A total of 48 genes were analysed in **Paper III**. The same set of genes was used in the biochar pre-selection of **Paper IV**, while 96 genes were analyzed for the column experiment in **Paper IV**. The gene selection included the 16S rRNA

gene, several types of mobile genetic elements (MGE) (i.e., plasmids, insertion sequences, transposons), integrons, antibiotic resistance genes (ARG) related to nine antimicrobial classes (i.e., aminoglycosides, beta-lactams, integrons, MLSB, phenicols, quinolones, sulfonamides, tetracycline, trimethoprim, vancomycin), as well as other resistances (e.g., multidrug resistance (MDR), mercury resistance). Taxonomic marker genes (*Bacteroidetes*, *Firmicutes*) were also included together with pathogen marker genes for *Shigella* spp (**Papers III** and **IV**), *Candida albicans*, *Candida auris*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Escherichia coli* and *Enterococci* spp (**Paper IV**).

3.3 Retrieval of literature data and information

Literature was compiled using the workflow of Khan et al. (2024). Literature search was conducted in Scopus and Web of Science (accesses on August 16, 2023) using terms related to OSSF and AMR contaminants (genetic determinants and antimicrobial chemicals) (see **Paper II** for details). A validation search confirmed the adequacy of the search strings. From 497 initial articles, 33 peer-reviewed studies were selected for further analysis after duplicate removal, abstract screening using Rayyan (Ouzzani et al., 2016) and manual review. Data and information were extracted and compiled including: (i) contaminant type; (ii) measured concentrations in wastewaters and receiving water; (iii) OSSF serving capacity; (iv) sampling methods; and (v) country.

3.4 Study site and sampling design

The investigated OSSF in **Paper III** is located in the Kalmar County, Southeast of Sweden and serves ~300 permanent inhabitants and treats 50-120 m^3 of wastewater per day (Figure 3A). The incoming wastewater undergoes primary treatment in an open septic tank. Afterwards, the septic tank effluent is treated in aerated ponds. This results in final effluent collected in a well at a pump station and intermittently pumped to an infiltration site. Pumping cycles are controlled based on wastewater volume. The infiltration site (Figure 3B) consists primarily of unsaturated material (sand and gravel) in the upper layer (5 m), overlying natural soil. The groundwater table is at approximately 6 m below ground.

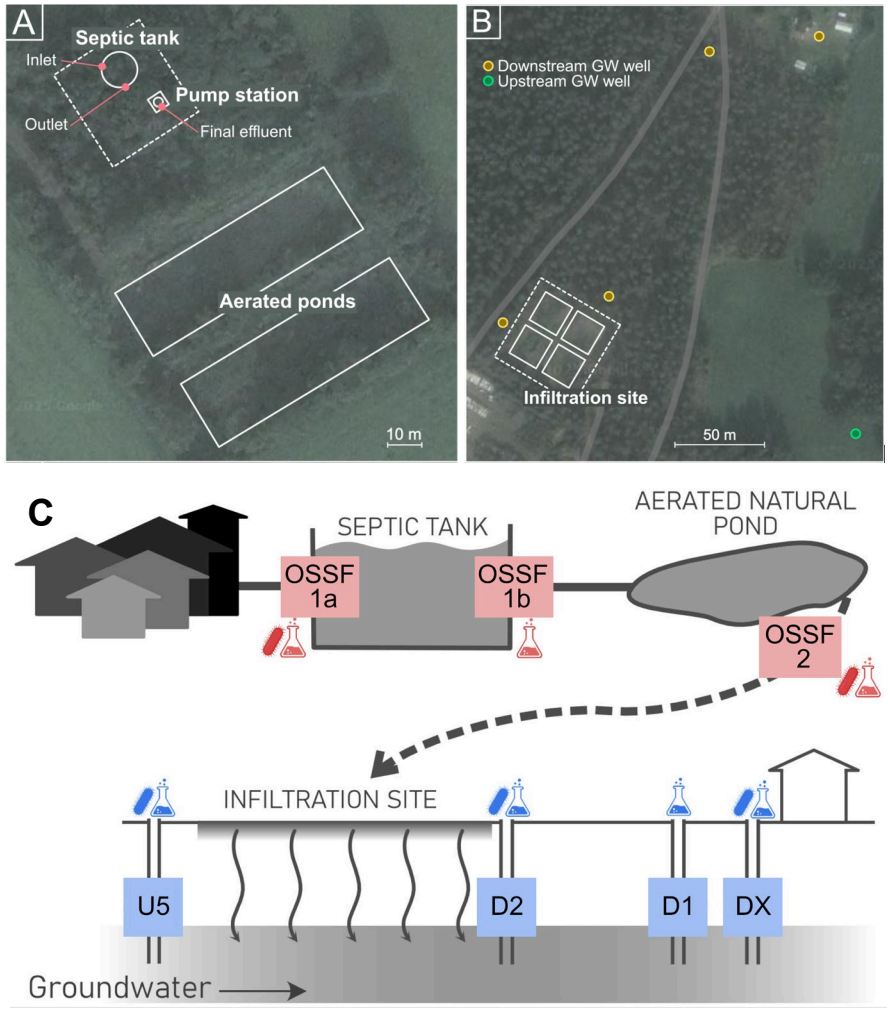

Figure 3. Satellite image of the sampling locations: (A) OSSF including septic tank and aeration ponds, (B) infiltration site with upstream and downstream groundwater wells, and C) schematic flow of the sampling points.

The sampling was designed to collect wastewater samples at three locations within the OSSF including septic tank inlet (OSSF 1a), septic tank outlet (OSSF 1b), and at the pump station (OSSF 2) (Figure 3C). To investigate the potential impact of the OSSF on groundwater resources, groundwater samples were collected at three wells downstream of the infiltration site, including two beneath the infiltration site, which were later combined (D2) due to insufficient water volumes, and two further downstream (D1, DX;

~200 m) (Figure 3C). Additionally, upstream groundwater (U5) was sampled as background level for chemicals and AMR determinants. Four sampling campaigns, each lasting up to five consecutive days, were performed over a year:

- Campaign 1 - March 2022
- Campaign 2 - August 2022
- Campaign 3 - October 2022
- Campaign 4 - February 2023

For wastewater collection, daily composite samples were obtained from 24 h time-integrated sampling (45 mL every 10 min). Groundwater samples were collected using a bailer as grab sampling. For chemical analysis, samples were stored in polypropylene (PP) bottles pre-rinsed with methanol and MilliQ water and frozen on-site at -20°C. For analysis of AMR determinants, wastewater (200-450 mL) and groundwater (1500-4500 mL) was filtered in triplicates on-site using sterile PES membrane filters (0.2 µm), which were immediately frozen at -20°C.

3.5 (SPE-)LC-MS/MS method development

A method for the quantification of antimicrobial chemicals using ultra-high liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS, Exion® LC, Sciex® Triple-Quad 3500) (Figure 4) and SPE for sample preparation was optimized and validated. For LC-MS/MS analysis, the choice of analytical column (Kinetex® EVO, biphenyl and C18), organic mobile phase (methanol *vs.* acetonitrile), additives (formic acid, acetic acid, ammonium acetate) and LC gradient was optimized considering good sensitivity and chromatographical separation. Additionally, mass spectrometry settings were optimized including declustering potential (DP), collision energy (CE), cell exit potential (CXP) and ion source parameters (temperature, voltage, curtain gasses) to ensure suitability and sensitivity of the ions for target analysis. Sample preparation via SPE was optimized by testing three different extraction sorbents: Oasis® HLB based on hydrophilic-lipophilic interactions, and the two mixed-mode ion-exchange sorbents Oasis® WCX and MCX. For LC-MS/MS validation, within-run and between-run accuracy and precision were evaluated using spiked standards.

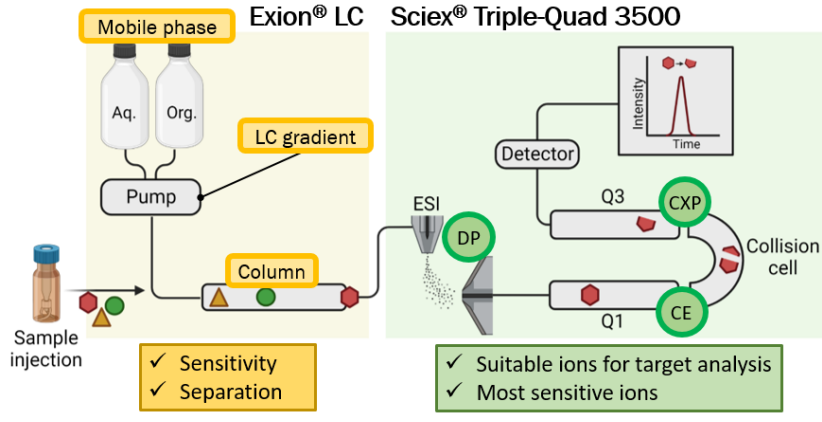

Figure 4. Schematic representation of the considered parameter in LC-MS/MS optimization (made with BioRender®).

For SPE-LC-MS/MS validation, within-run and between-run precision and extraction efficiency were evaluated on spiked tap water, groundwater, surface water, influent and effluent wastewater. Evaluation at different concentration levels (20, 50 and 150 ng/L) was performed only on tap water (as the only water matrix without background chemical concentrations), while the other matrices were validated at 50 ng/L for groundwater and surface water and 250 ng/L for influent and effluent wastewater (for details of equation and acceptance criteria see Table 2).

Instrumental detection limits (IDLs) and instrumental quantification limits (IQLs) were determined in neat standards as signal/noise ratio of 3 and 10, respectively. Method detection limits (MDLs) and method quantification limits (MQLs) were determined in influent and effluent wastewater, surface water and groundwater.

Table 2. Parameters used in method validation with their equation and criteria.

Parameter	*Equation*	*Acceptance criteria*
Recovery (%)	$\frac{C_{pre-spike}}{C_{neat\ standard}} \times 100$	50-150%
Accuracy (bias%)	$\frac{C_{measured} - C_{nominal}}{C_{nominal}} \times 100$	±25%
Precision (RSD%)	$\frac{s}{\bar{x}} \times 100$	<25%

3.6 Chemical analysis

Based on the developed method in **Paper I**, SPE was used to extract the chemicals in wastewater (40 mL) and groundwater (200 mL) in **Paper I** (demonstrating the method's applicability) and **Paper III**, prior to LC-MS/MS analysis (Exion® LC, Sciex® Triple-Quad 3500). Briefly, wastewater and groundwater samples were filtered (glass microfiber filters Whatman® GF/D), and after adjustment with 2 M hydrochloric acid to pH 6, addition of disodium ethylenediaminetetraacetate dihydrate (Na_2EDTA) and spiking of the internal standard mixtures (IS; 250 ng/L for wastewater, 50 ng/L for groundwater), they were loaded onto SPE cartridges Oasis® WCX (150 mg, 6cc, 30 µm). After washing and drying under vacuum, the analytes were eluted (5 mL MeOH + 5 mL 4% FA MeOH). Finally, the eluted samples were pre-concentrated under nitrogen at 35°C to 20 µL, and reconstituted to 200 µL with MeOH and MilliQ water (concentration factor of 250 for wastewater and 1000 for groundwater). Direct injection method with LC-MS/MS (Exion® LC, Sciex® Triple-Quad 6500+) was used for analysis of high-use chemicals (Haalck, 2022; Haalck et al., 2024) in **Paper III** and **Paper IV**, and for analysis of antimicrobial chemicals and transformation products (Löffler et al., 2025) in **Paper I** and **Paper IV**. Prior to analysis, samples were centrifuged (8000 rpm, 10 min, 4 °C) and spiked with IS (see **Papers I**, **III** and **IV** for more details). For all the chemical analyses, the analytes were separated on a Phenomenex® Kinetex® Biphenyl column (100×2.1mm, 1.7 µm) at a flow rate of 0.5 mL/min, with mobile phases of 0.1% formic acid in both water (A) and methanol (B) (ESI+) and 0.1% acetic acid in both water (A) and methanol (B) (ESI-). Total runtime was 15.5 min starting at 10% B (0-0.5 min), increasing to 20% B (curve -3, 0.5-2 min), to 75% B (2-7 min), and to 100% B (curve -4, 7-9 min), washing phase 100% B (9-12 min) and re-equilibration phase 10% B (12.1-15.5).

3.7 AMR determinants analysis

In **Papers III** and **IV**, environmental DNA was extracted from the filters obtained from on-site (waste)water filtration and analyzed using high-throughput qPCR (HT-qPCR) with a SmartChip™ system (TakaraBio, CA, USA). DNA extraction using DNeasy PowerWater Kit (QIAGEN) and analysis were performed by Resistomap Oy (Helsinki, Finland). More details

on the analysis were previously reported (Muziasari et al., 2016; Schmittgen and Livak, 2008; Stedtfeld et al., 2018; Zhu et al., 2013).

3.8 Biochar treatment experimental set-up

Five different types of biochars were tested for their ability in removing AMR contaminants from OSSF effluent wastewater. These biochar materials had a total surface area ranging from 14-335 m^2/g and were derived from seed waste, sewage sludge, wood/forest waste, garden waste or forest biomass. In the pre-selection experiment, OSSF effluent wastewater was spiked with target antimicrobial chemicals (50 µg/L). Parent and TPs were studied separately, as well as AMR determinants in unspiked effluent wastewater. Effluent wastewater was exposed to biochar for 21 days for AMR determinants (1:10 ratio; 50 g dry weight (dw) biochar in 500 mL effluent; sampled time points: 1, 7, 14, 21 days) and 14 days for chemical contaminants (4 g dw biochar in 40 mL effluent; sampled timepoints: 1, 3, 6, 24 hours and 14 days). Control samples (spiked or unspiked and without biochar) were analyzed at time 0, and after 10 and 21 days for AMR determinants, and 0, 1, 3, 6, 24 hours, and 14 days for chemicals. Granular activated carbon (GAC, Chemviron® Carbon) was included as a benchmark material. This experiment was conducted in triplicate under parallel conditions at room temperature.

Afterwards, column experiments (Figure 5) were conducted in triplicate, separately for AMR determinants and chemical contaminants (parents + TPs), accounting for the hydraulic conditions at the studied OSSF pump station (OSSF 2, section 3.4). From here, effluent wastewater is intermittently pumped to the infiltration site with reported median hydraulic retention times of 20-700 minutes. To simulate short retention times, columns were refilled with effluent every 20 minutes. Columns were built from 50 mL syringes, fitted with a polyester net and perforated plastic disc to retain 25 g dry weight (dw) of biochar. Effluent aliquots (25 mL) were added at 20-minute intervals, repeated 20 times for a total of 500 mL. For chemical contaminants, spiked effluent (50 µg/L antimicrobial chemicals) was sampled (1 mL) before and after each column pass. For AMR determinants, 500 mL of influent was collected, and a composite sample (~400 mL) was gathered from the 20 treated aliquots.

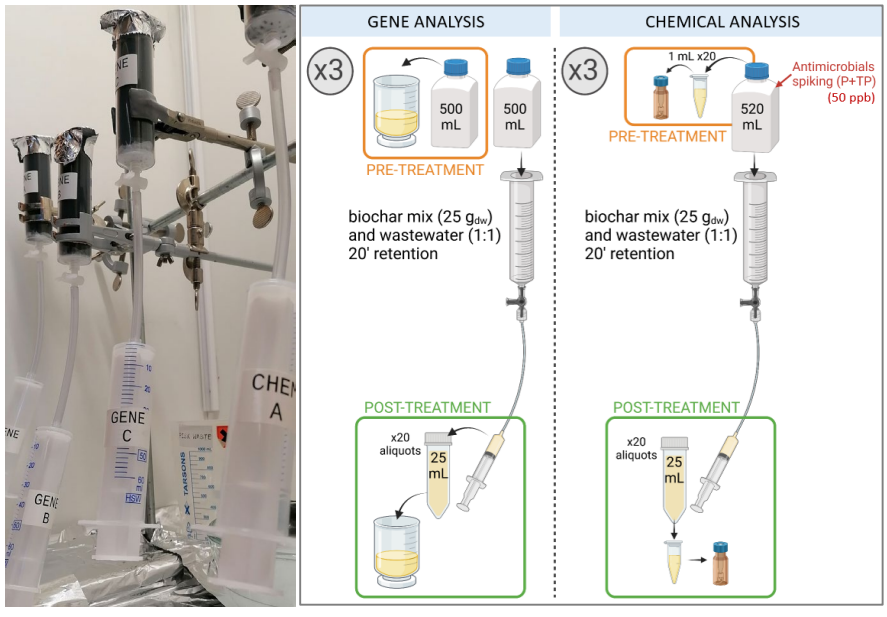

Figure 5. Column experiment set-up and its schematic diagram (BioRender®).

3.9 Data handling

All data analyses were performed using R (version 4.3.1), with Affinity Designer (version 1.9.1.979) used for figure editing. Mainly used R packages included *data.table*, *tidyverse*, *dplyr* for data processing, and *ggplot2* for visualization. Correlations and network analyses were visualized using *corrplot* and *visNetwork*. For statistical comparisons across sampling sites and campaigns in **Paper III** and before and after biochar treatment in **Paper IV**, the Kruskal-Wallis test was applied, followed by post-hoc Wilcoxon tests with Bonferroni adjustment of *p*-values. In **Paper III**, Spearman correlation analysis, linear regression, and network analysis were conducted to explore co-occurrence patterns between AMR determinants and chemical contaminants. In all statistical analyses, non-detected AMR determinants and chemicals with concentrations below the MDL were assigned a value of zero. Chemical concentrations between the MDL and the MQL were set to half the MQL. For chemical removal analysis in **Paper IV**, first-order kinetic models were fitted to determine the time required to remove 50% of the initial concentration. Removal efficiencies were also calculated based on the initial and final concentrations.

4. Results and discussion

4.1 Quantification of antimicrobial chemicals in the context of AMR

With growing attention to AMR in wastewater and the environment (Larsson and Flach, 2022), there is a critical need for robust and microbiologically sensitive analytical methods that enable meaningful monitoring of antimicrobial chemicals. In **Paper I**, such a methodology was developed and validated, including: (i) a direct injection method for quantifying 53 antimicrobials; (ii) and an SPE-based method for 35 antimicrobials in influent and effluent wastewater, surface water, and groundwater. Prior to validation, optimization was performed for LC-MS/MS analysis and sample preparation.

For LC-MS/MS analysis, among various tested conditions, the Phenomenex® Kinetex® Biphenyl column with 0.1% formic acid in both methanol and water (ESI+) and 0.1% acetic acid in both methanol and water (ESI−) provided the optimal chromatographic separation and sensitivity among the other tested columns and mobile phase compositions (see section 3.5). The use of the biphenyl column is unique, in contrast to most previous studies using C18 columns (Kumar Mehata et al., 2022). LC gradient was optimized to enhance good peak shapes for early eluted compounds and to have all analytes eluted before the column washing step. Instrumental detection and quantification limits (IDLs and IQLs) ranged from 0.01-12 ng/mL and 0.02-39 ng/L, respectively. Compared to other studies (Holton and Kasprzyk-Hordern, 2021; Li et al., 2009), this method showed equal sensitivity for certain tetracyclines and macrolides, trimethoprim, sulfamethoxazole and sulfonamides TPs, while higher sensitivity was observed for nitrofurantoin, fluoroquinolones (i.e., enrofloxacin, lomefloxacin), chloramphenicol, clindamycin, ampicillin, cefalexin, sulfadiazine, sulfamethazine, chlortetracycline and vancomycin.

For sample preparation, a SPE protocol was optimized. In the literature, hydrophilic-lipophilic sorbents (e.g., Oasis® HLB) are commonly used (Gros et al., 2013; Holton and Kasprzyk-Hordern, 2021; Kumar Mehata et al., 2022), but the potential of ion-exchange sorbents is overlooked. In this study,

among the tested sorbents (see section 3.5), the ion-exchange Oasis® WCX cartridge was selected for its consistent performance across diverse matrices, unlike Oasis® HLB cartridge, which showed variable recoveries, and Oasis® MCX, which underperformed likely due to acidification-related instability. A two-step elution sequence using pure methanol followed by 4% formic acid in methanol was found to be the most effective, balancing compound-specific elution performances observed between 2% and 8% acid solutions. Sample acidification and EDTA addition to pH 6 further improved extraction efficiency, particularly for macrolides.

The SPE-LC-MS/MS method validation demonstrated good precision (within- and between-day) and recoveries based on established criteria (section 3.5). MQLs were similar between groundwater (0.33-54 ng/L) and surface water (0.53-75 ng/L) and also between influent (11-650 ng/L) and effluent wastewater (2.5-460 ng/L). Comparing method sensitivity can be challenging as MQLs in previous studies are often determined using neat standards, whereas in this method, MQLs were assessed in the water matrices of interest. Compared to a previous study using HLB sorbent (Gros et al., 2013; Holton and Kasprzyk-Hordern, 2021), this method showed higher sensitivity for 18 antimicrobial chemicals with MQLs up to 10-fold lower.

Considering that antimicrobial chemicals can act as selective agents even at sub-MIC levels (Gullberg et al., 2011; Stanton et al., 2020), it is crucial that analytical methods are capable of detecting these low concentrations relevant to AMR selection monitoring. In this light, the *microbiological sensitivity* of the method was assessed by comparing MQLs with MICs (Bengtsson-Palme and Larsson, 2016) (Figure 6). MQLs below MICs (ratio <1) enable detection of antimicrobials at levels where selection pressure occurs without growth inhibition (sub-MIC window). MQLs equal to or above MICs (ratio ≥1) allow assessment of both inhibition and selection effects (traditional selective window). All MQLs in our method (ng/L range) were lower than MICs (µg/L range), indicating good microbiological sensitivity and suitability for AMR monitoring in environmental (waste)waters.

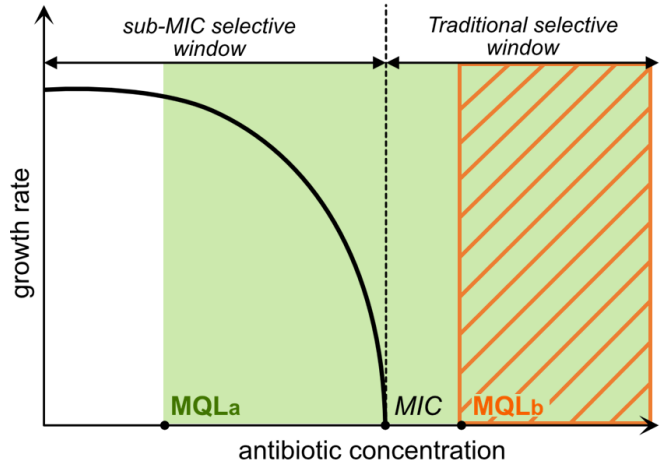

Figure 6. Exemplification of microbiological sensitivity assessment. MQL_a capture both sub-MIC and traditional selective window, while MQL_b only capture the traditional selective window, therefore with a decreased microbiological sensitivity (Figure adapted from Gullberg et al. (2011) with further additions of MQLs for this thesis).

4.2 Stability of the chemicals under different conditions and in different environmental (waste)waters

The stability of antimicrobial compounds during sampling, storage, and analysis is a key factor that can influence their detection. To support future sampling efforts, **Paper I** investigated the stability of 53 antimicrobial compounds under various conditions (e.g., storage in freezer or refrigerator, typical sewage conditions, use of preservatives) and assessed their sorption tendencies to glass and polypropylene (PP) containers (Figure 7).

The findings showed that antivirals, sulfonamides, macrolides, and fluoroquinolones were generally stable, while certain β-lactams (i.e., ampicillin, piperacillin, mecillinam), vancomycin, and meropenem were unstable under most tested conditions. Norfloxacin also showed instability in working solutions after six months. These results align with existing knowledge that β-lactams are particularly unstable, posing challenges for their detection (Prieto Riquelme et al., 2022). For storage of the working solutions -80°C was preferable. The use of preservatives (sodium azide and sodium metabisulfite), which improves the stability of drug residues in wastewater (Chen et al., 2013; González-Mariño et al., 2010; O'Brien et al., 2019), did not offer the same advantage for antimicrobial chemicals.

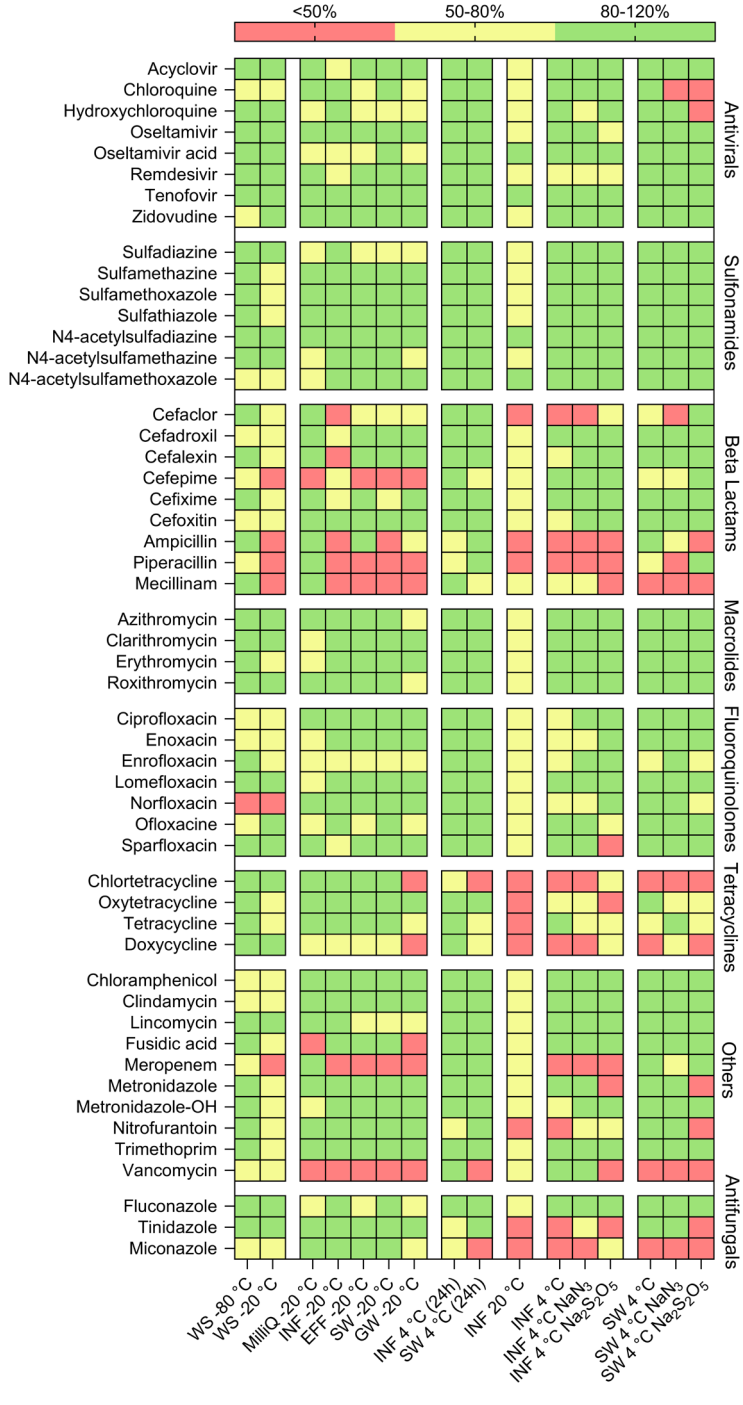
<50%
50-80%
80-120%
Acyclovir
Chloroquine
Hydroxychloroquine
Oseltamivir
Oseltamivir acid
Remdesivir
Tenofovir
Zidovudine
Antivirals
Sulfadiazine
Sulfamethazine
Sulfamethoxazole
Sulfathiazole
N4-acetylsulfadiazine
N4-acetylsulfamethazine
N4-acetylsulfamethoxazole
Sulfonamides
Cefaclor
Cefadroxil
Cefalexin
Cefepime
Cefixime
Cefoxitin
Ampicillin
Piperacillin
Mecillinam
Beta Lactams
Azithromycin
Clarithromycin
Erythromycin
Roxithromycin
Macrolides
Ciprofloxacin
Enoxacin
Enrofloxacin
Lomefloxacin
Norfloxacin
Ofloxacine
Sparfloxacin
Fluoroquinolones
Chlortetracycline
Oxytetracycline
Tetracycline
Doxycycline
Tetracyclines
Chloramphenicol
Clindamycin
Lincomycin
Fusidic acid
Meropenem
Metronidazole
Metronidazole-OH
Nitrofurantoin
Trimethoprim
Vancomycin
Others
Fluconazole
Tinidazole
Miconazole
Antifungals
WS -80 °C
WS -20 °C
MilliQ -20 °C
INF -20 °C
EFF -20 °C
SW -20 °C
GW -20 °C
INF 4 °C (24h)
SW 4 °C (24h)
INF 20 °C
INF 4 °C
INF 4 °C NaN3
INF 4 °C Na2S2O5
SW 4 °C
SW 4 °C NaN3
SW 4 °C Na2S2O5

Figure 7. (previous page) Stability of antimicrobial chemicals under different scenarios. From left to right: working solutions (WS) at -80°C and -20°C for 6 months; MilliQ, influent (INF) and effluent (EFF) wastewater, surface water (SW) and groundwater (GW) at -20°C for 8 weeks; INF and SW at 4°C for 24h; INF at 20°C for 24h; INF and SW with preservatives sodium azide (NaN_3) and sodium metabisulfite ($Na_2S_2O_5$) at 4°C for 9 days. Each cell represents the remaining % of chemical at the endpoint of the stability test (green = 80–120%, stable; yellow = 50–80%, partly degraded; red = <50%, highly degraded). 4-epianhydrotetracycline was not studied due to high IQL. Reproduced from **Paper I** (Ugolini and Lai, 2024).

4.3 Identification of OSSF as overlooked contributors to AMR and research needs

In **Paper II**, the literature synthesis work demonstrated that OSSF contributes to AMR development and spread, with a wide range of antimicrobial chemicals received by OSSF and their presence in receiving waters. It showed on a global scale that typical OSSF designs, i.e., septic tanks followed by infiltration fields, are often ineffective at removing many antimicrobials. OSSF can also act as reservoirs of ARGs and MGEs. Septic tanks generally failed to reduce ARG loads, and in some cases even increased resistance to beta-lactams.

Available studies on AMR contaminants in OSSF was limited, both in the numbers and geographic coverage, especially lacking in the Global South countries, suggesting a potential underestimation of their global impact. Only 6 out of 33 compiled studies provided data on AMR determinants, with four quantifying up to 10 genes only (Hayward et al., 2021, 2019; Ma et al., 2023; Osińska et al., 2020; Park et al., 2016; Tan et al., 2021). This highlights a critical research gap on quantitative data for AMR determinants, which hinders proper understanding of AMR development and dissemination in OSSF. Furthermore, integrated assessment combining antimicrobial chemical levels with AMR determinants is largely lacking, despite the One Health framework emphasizing such interdisciplinary perspectives.

The results of **Paper II** also emphasized the importance of sampling across treatment steps (e.g., influent *vs.* effluent) to better assess system efficiency. Additionally, a lack of standardized terminology for decentralized domestic wastewater treatment systems can complicate the process of literature retrieval, interfering with the identification of potentially effective OSSF

designs. To support future harmonization, standardized terms are suggested using "*on-site sewage facilities (OSSF)*" or "*on-site wastewater treatment (OSWT)*".

4.4 Prioritization of antimicrobial chemicals relevant to OSSF settings based on the literature

Paper II showed that a total of 74 antimicrobial chemicals covering different classes have been targeted in OSSF settings, of which 30 were quantified at least once in raw wastewater, effluent wastewater or receiving waters. These included three antivirals (i.e., acyclovir, nevirapine, oseltamivir), two antifungals (i.e., climbazole and fluconazole), 23 antibacterials for systemic use, and two antimicrobials used in PCPs (i.e., triclocarban, triclosan). To prioritize them, a scoring system based on Löffler et al. (2023) and Khan et al. (2024) was used (Table 3).

Table 3. Scoring system for prioritization of relevant antimicrobial chemicals in OSSF settings (MEC = measured environmental concentration; BCF = bioaccumulation factor). Persistence, mobility and bioaccumulation were predicted with the VEGA software (Benfenati et al., 2019).

Type	*Criteria*	*Score*
$Score_{AMR}$	MEC>$PNEC_{AMR}$	1
$Score_{eco}$	MEC>$PNEC_{eco}$	1
$Score_{EH}$	$\frac{Score_{persistence} + Score_{mobility} + Score_{BCF}}{3}$	1
$Score_{persistence}$	Persistence (half-life > 40 days)	1
$Score_{mobility}$	Mobility (water solubility > 0.15 mg/L and Koc ≤ 4.5)	1
$Score_{BCF}$	Bioaccumulation (log BCF > 3.3)	1

Antimicrobial chemicals in OSSF settings were assessed by their ecological ($Score_{eco}$) and AMR selection risks ($Score_{AMR}$) in relation to their measured environmental concentrations (MEC), as well as environmental hazard ($Score_{EH}$) in relation to their predicted fate in the environment (persistence, mobility and bioaccumulation) (Table 3, Figure 8). In wastewater-impacted waters, occasional AMR selection risk was found for ciprofloxacin, fluconazole and trimethoprim, and ecological risk for erythromycin-H_2O, clarithromycin, sulfamethoxazole, triclocarban and triclosan. Out of the 30

antimicrobial chemicals compiled, nine were predicted to be persistent, 25 were predicted mobile and none was predicted to be bioaccumulative. This assessment led to a prioritization of these compounds from high to low concern in OSSF settings, with erythromycin-H_2O, ciprofloxacin and triclocarban being the top three antimicrobial chemicals of concern. This finding further supports the growing emphasis on investigating antimicrobial transformation products, as highlighted in a recent review from Löffler et al. (2023) regarding their role in global surface water environments. Several priority antimicrobial compounds in our study (e.g., ciprofloxacin, triclosan, sulfamethoxazole, trimethoprim, clarithromycin, and amoxicillin) were also previously recognized in aquatic ecosystems worldwide (Yang et al., 2017), further reinforcing their relevance in environmental monitoring.

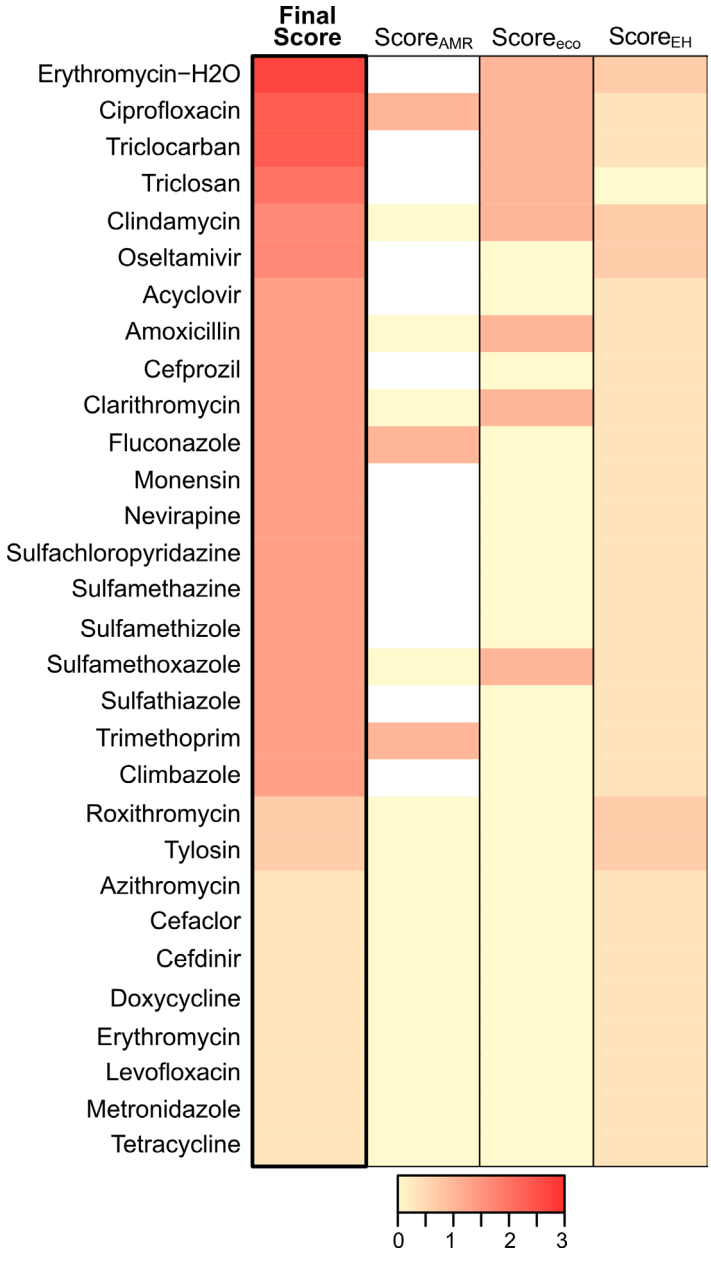

Figure 8. Prioritization of antimicrobial chemicals in OSSF settings based on their AMR selection and ecological risks and predicted environmental hazards.

4.5 Factors influencing dissemination from OSSF

From the data and information gathered in **Paper II**, the population served by OSSF varied greatly, from a few to thousands individuals. No distinct correlation was observed between antimicrobial chemical concentrations and OSSF serving capacity. However, the diversity of antimicrobial chemicals increased with the serving capacity. Additionally, demographics may also influence the diversity of detected chemicals (Conn et al., 2006; Fisher et al., 2016). OSSF are typically designed with septic tank and infiltration field, and with the limited removal efficiency with primary treatment (e.g. septic tank), contaminant removal heavily relies on soil properties (e.g., texture, pH, cation exchange capacity) in infiltration fields and contaminant characteristics (e.g., hydrophobicity, speciation). Aquifer vulnerability (e.g., shallow, unconfined aquifers) also affects contaminant dissemination. Antimicrobial chemicals are more impacted by seasonal variation compared to AMR determinants. Environmental parameters (e.g., pH, temperature, total organic carbon) can also affect AMR contaminant occurrences (Harrower et al., 2021; Ma et al., 2023), suggesting the importance of water chemistry when studying AMR dissemination. Although sampling methods does not impact AMR contaminants occurrences directly, it affects detection reliability. Especially for antimicrobial chemicals, grab samples, applied by most literature, may lead to under-detection, while time-integrated or flow-based sampling can better capture variation in the use of antimicrobial chemicals. For future studies, temporal variation, hydraulic retention times and sampling strategies should therefore be optimized.

4.6 Temporal patterns of AMR contaminants in a Swedish OSSF and receiving groundwater

In **Paper III**, 15 out of 35 targeted antimicrobial chemicals were measured in wastewater, with concentrations remaining similar after septic tank treatment (OSSF 1a: 0.46–860 ng/L; OSSF 1b: 0.44–816 ng/L), but decreasing after aerated ponds (OSSF 2: 0.18–145 ng/L) (Figure 9). Significant reductions ($p<0.05$) were observed during sampling campaigns 3 and 4. Overall, campaign 1 and 2 showed the lowest concentration levels while campaign 4 the highest. In groundwater, fewer antimicrobials were detected (up to 7), with fluconazole consistently present across all campaigns. Fluconazole was previously reported as ubiquitous in the

environment (Assress et al., 2020; Chen and Ying, 2015; Kahle et al., 2008). Below the infiltration site, the overall concentrations matched effluent levels in campaign 1 but were lower in campaign 4. Further downstream, groundwater wells showed similar concentrations.

In total, all 21 high-use chemicals (e.g., pharmaceuticals, sweeteners) were detected in wastewater with 100% frequency. Like antimicrobials, their concentrations remained unchanged after septic tank treatment (OSSF 1a: 134–380000 ng/L; OSSF 1b: 135–360000 ng/L), but declined after aerated ponds (OSSF 2: 15.0–194000 ng/L), especially in campaigns 2 and 3 (Figure 9). Unlike antimicrobials, no significant temporal variation was observed at the septic tank stages (p>0.05). In groundwater, up to 12 out of 21 high-use chemicals were detected at lower concentrations, with acesulfame consistently present. Acesulfame is considered as an ideal chemical marker for household wastewater (Buerge et al., 2009). High-use chemical concentrations decreased further downstream (5.6–218 ng/L) compared to below the infiltration site (8.2–51000 ng/L), indicating a dilution effect. The presence, although lower, of both antimicrobial and high-use chemicals in upstream groundwater suggests additional pollution sources around the area of the infiltration zone.

AMR determinants abundances (copies/mL) were significantly reduced (p<0.05) after septic tank and aerated pond treatment, but not their relative abundances to 16S rRNA (Figure 10). Copies/mL of certain genes, including resistance to aminoglycosides (*aadA2_3*, *aadA_1*), MLSB (*ermX_2*), sulfonamides (*sul1_2*, *sul3_1*), *MDR* (*ttgA*), phenicols (*catA3*), and MGE (*intI1_2, intI3, IncP_oriT*, *trfA*) and other resistance (*ttgB*), remained unchanged in at least one campaign (Figure 11A). For up to 19 genes relative abundances increased after treatment (Figure 11B), with integrons, sulfonamide, and trimethoprim groups significantly higher in at least one campaign (p<0.05). This implies that while OSSF treatment may reduce the total load of AMR determinants, it could selectively enrich for resistant populations and genetic elements (e.g., MGE) that are involved in HGT transfer. Although absolute and relative gene distribution were similar across sampling campaigns, there were occasional significant (p<0.05) temporal variation, influencing the groups of β-lactams, MGE, trimethoprim, aminoglycoside, and tetracycline. Campaign 2 (August 2022) had the lowest overall levels (Figure 10). These relative stable profiles were also reported

in WWTP (Brinch et al., 2020; Majeed et al., 2021). After soil infiltration, gene absolute abundances in groundwater (D2) were significantly lower compared to effluent wastewater (p<0.05), but were still higher than upstream levels. A total of 41 genes exceeded upstream abundances, with *ISPps* up to 90-fold higher. The tetracycline ARG *tet44* and *tetQ* were only measured below the infiltration site. Further reductions in gene abundance were observed at the downstream (DX) groundwater well, likely due to dilution effects. Of note, while the gene absolute abundances beneath the infiltration site were lower compared to effluent wastewater, the relative abundances for all genes except 12 (i.e., *Bacteroidetes*, *Firmicutes*, *aadA2_3*, *aadA_1*, *ermB_2*, *intI1_2*, *strB*, *tet44*, *tetQ*, *tetW*, *tnpA_4*, *trfA*) were found increased (Figure 11B). This suggests that the infiltration field act as potential hotspot for AMR determinants dissemination. This is further supported by the exceedance of the baseline abundance levels (Abramova et al., 2023) for certain genes, for example *blaCTX-M* (β-lactams), *ermX_2* (macrolides), *emrD_1* (MDR) and *ttgB* (other resistance). These genes are also highly relevant in clinical settings (Zhang et al., 2021, Zhang et al., 2022). Significant (p<0.05) temporal variation was observed for the relative gene abundances in downstream groundwater.

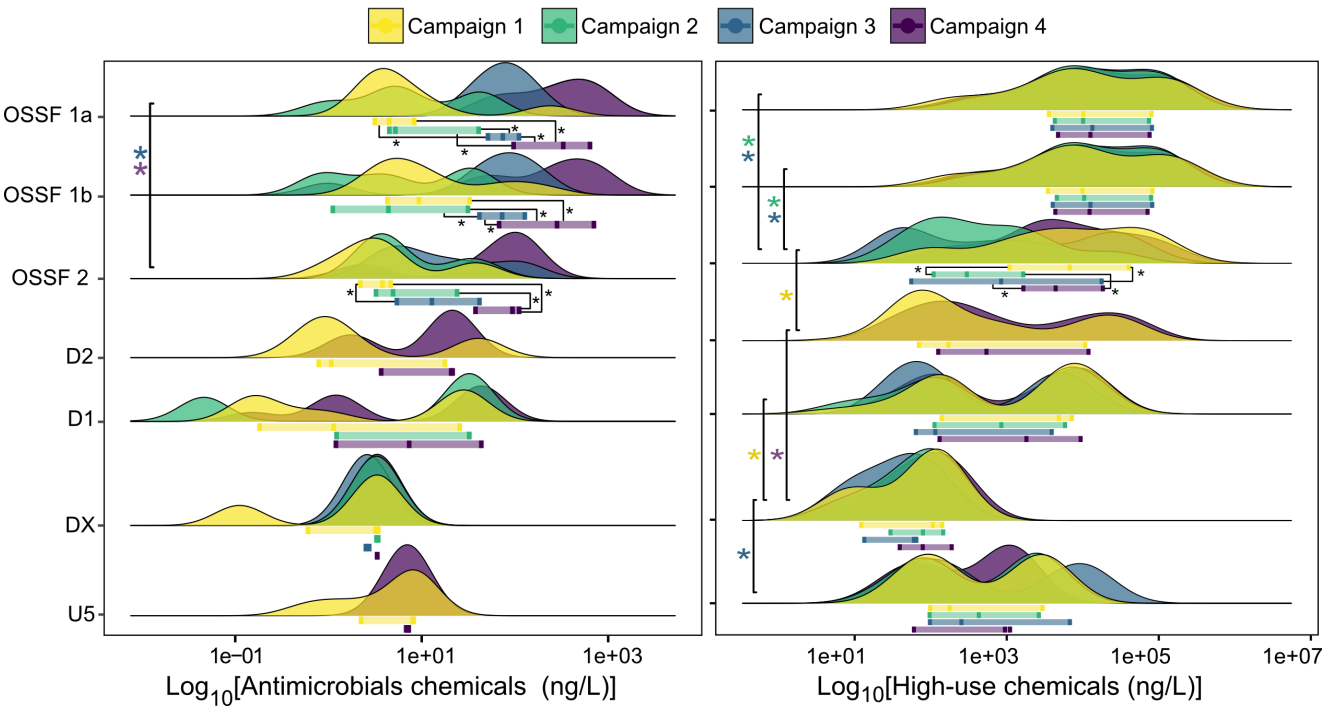

Figure 9. Distribution of antimicrobial chemicals and high-use chemicals across different sampling points (septic tank inlet (OSSF 1a), effluent wastewater after aeration ponds (OSSF 2), groundwater below the infiltration site (D2), further downstream (DX) and upstream (U5)) and campaigns. *Significant differences ($p<0.05$).

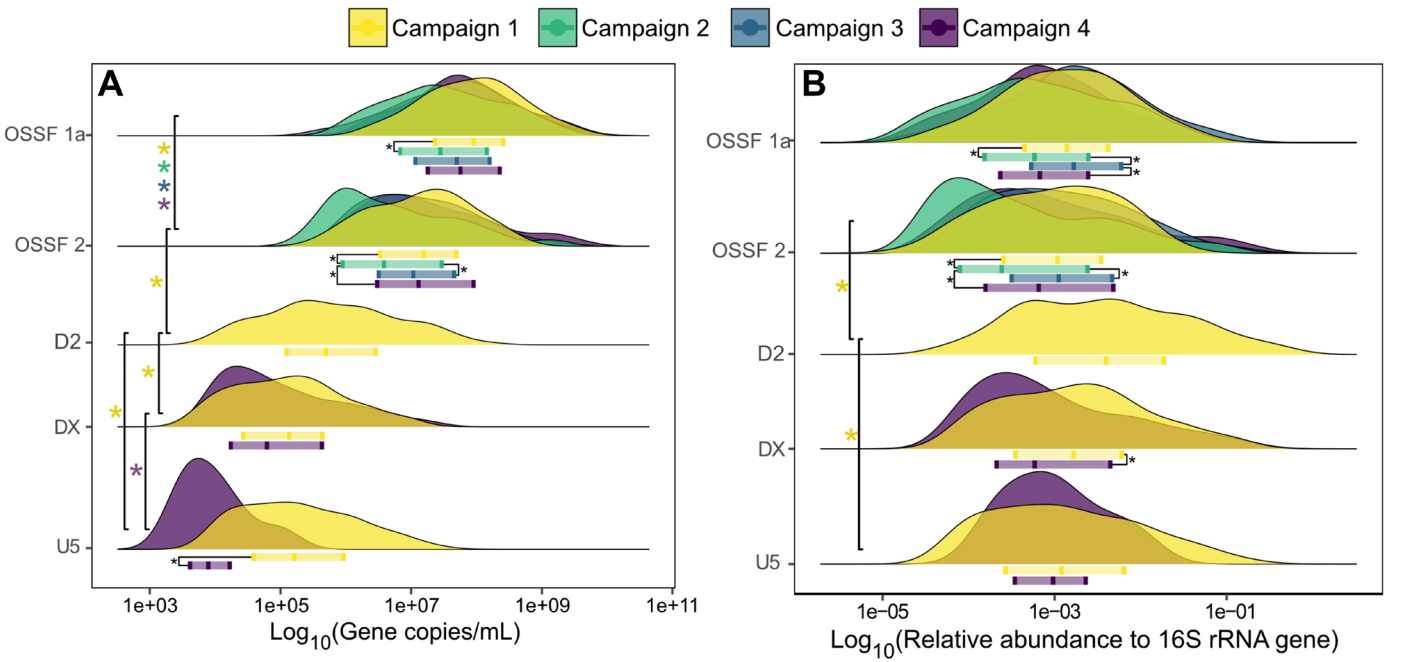

Figure 10. Distribution of AMR determinants in absolute abundances (copies/mL) and relative abundance to 16S rRNA across different sampling points (septic tank inlet (OSSF 1a), effluent wastewater after aeration ponds (OSSF 2), groundwater below the infiltration site (D2), further downstream (DX) and upstream (U5)) and campaigns. *Significant differences ($p<0.05$).

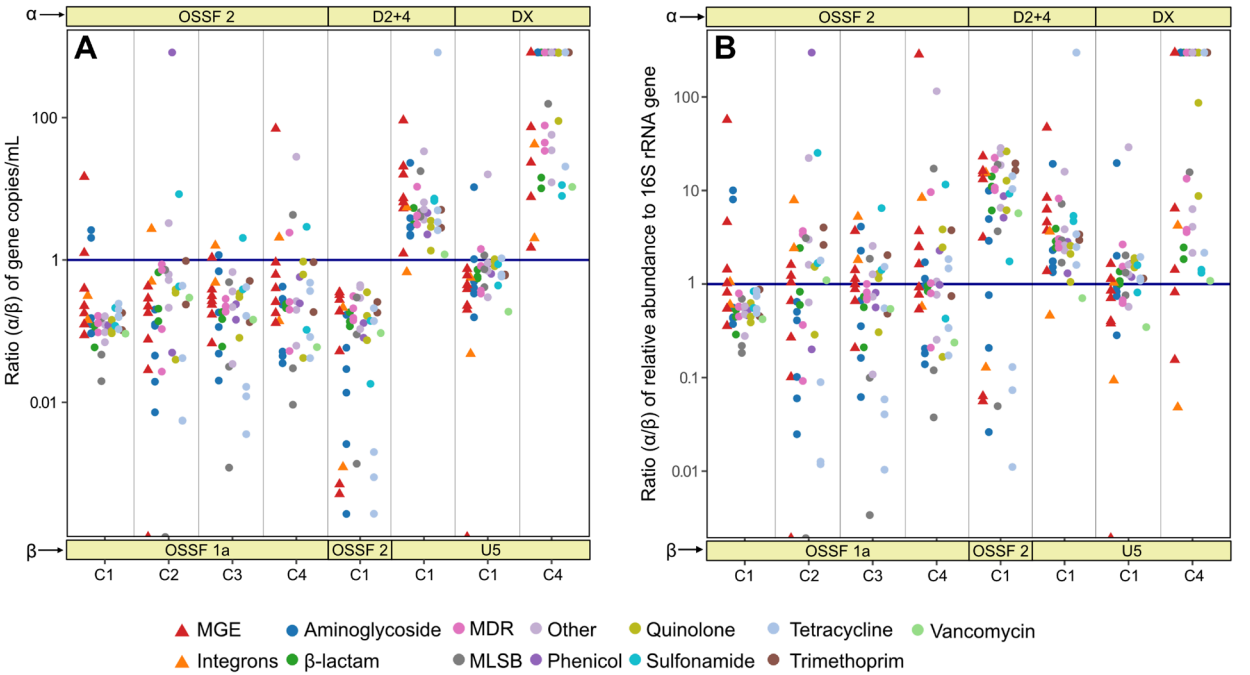

Figure 11. Comparison of individual AMR determinants colored by resistance group in absolute abundance (copies/mL) and relative abundance to 16S rRNA between sampling points. Ratio>1 indicate higher abundance in matrix α and ratio<1 higher abundance in matrix β.

4.7 Interactions of antimicrobial and high-use chemicals with AMR determinants in the dissemination

After OSSF treatment and infiltration into groundwater, a strong significant positive correlation was found between total AMR determinants and total chemical contaminants (ρ=0.79, p=2.1×10^{-6}), consistently for both antimicrobial (ρ=0.89) and high-use chemicals (ρ=0.79). Individual chemicals correlated differently with ARG groups, and especially with fluconazole and clarithromycin (antimicrobials) and several high-use chemicals (e.g., acesulfame, atenolol, citalopram) (Figure 12). In contrast, nicotine, paraxanthine, chloroquine, oseltamivir acid, and sulfamethoxazole showed no significant correlation (p>0.05).

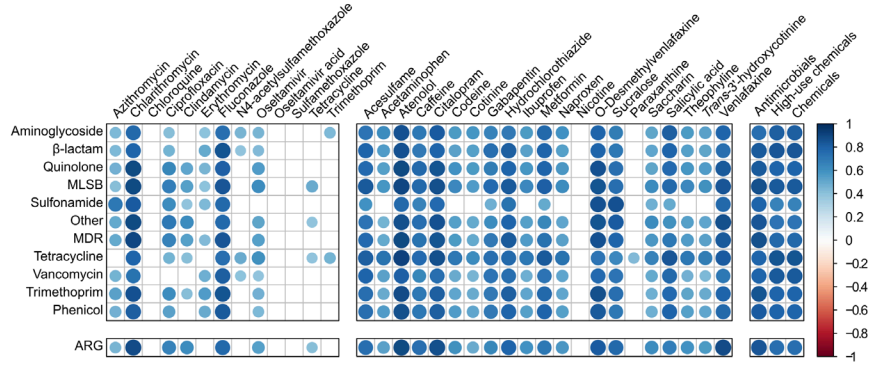

Figure 12. Spearman correlation analysis between ARG groups, antimicrobial chemicals and high-use chemicals. blank cells show no significant correlations (p>0.05).

Network analysis (Figure 13) revealed strong co-occurrences (ρ>0.8) between 39 genes and 18 chemicals, especially with atenolol, citalopram, and fluconazole. Six MGEs, two tetracycline resistance genes, and several other AMR genes were strongly associated with these chemicals. Linear regression also supported the interactions, showing a strong relation of AMR determinants with antimicrobial chemicals (R^2 = 0.9, p=2.37e-13) and a moderate relation with high-use chemicals (R^2 = 0.41, p=0.0004). Correlations between antimicrobial chemicals and AMR determinants have been reported in WWTP effluent water (e.g., Liu et al., 2023; Sun et al., 2023), reinforcing their role as selective pressure (Bengtsson-Palme and Larsson, 2016; Hendriksen et al., 2019; Tello et al., 2012). However, the relation with high-use chemicals (e.g., artificial sweeteners) are rarely

reported, although recent microbial studies reported their potential in triggering SOS responses, related to AMR development (Jia et al., 2021; Li et al., 2022; Wang et al., 2021, 2020; Yu et al., 2022, 2021). These results from **Paper III** suggest the potential need for better understanding the role of high-use chemicals in AMR development and dissemination, potentially including them in future monitoring strategies.

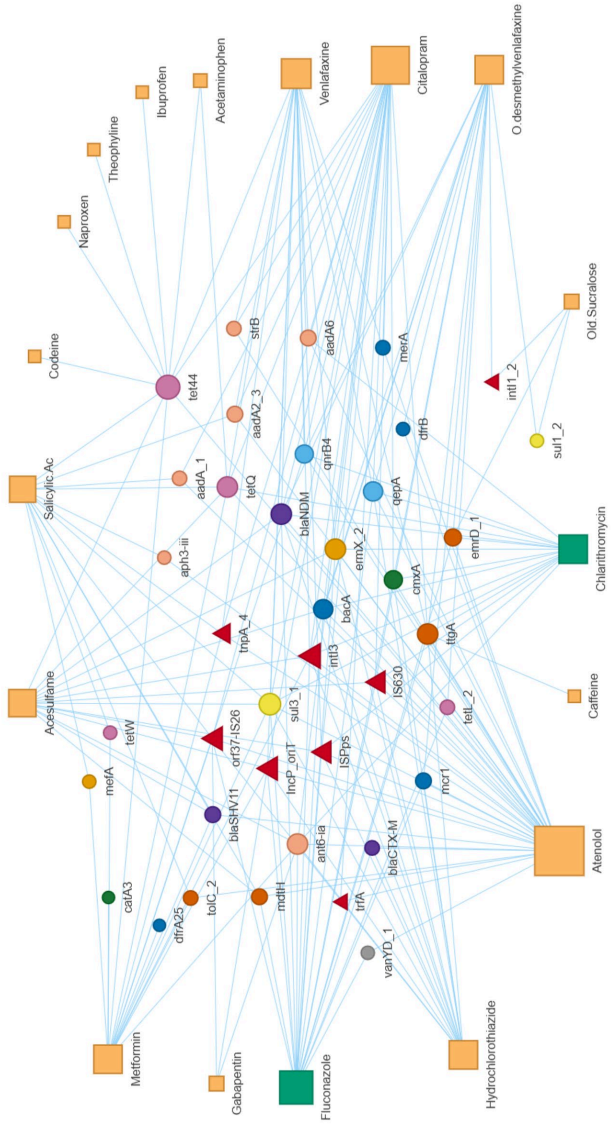

Figure 13. Network analysis of co-occurrences between AMR determinants and chemical contaminants ($\rho>0.8$, $p<0.01$).

4.8 The potential of using biochar as mitigation strategy in OSSF

Paper IV showed that the most suitable biochar for removal of antimicrobial chemicals and AMR determinants may differ.

In the pre-selection experiment, for removal of chemicals, biochar #8 with high specific surface area outperformed all other tested materials, including granular activated carbon (GAC) (tested as reference material) with median removal efficiency of 97% after 1-h contact time. Biochar #15 had the worst performance (median recovery 41%). Unlike all other biochar materials, biochar #15 has no carbonyl groups. At the experimental pH, this functional group, including e.g., carboxylic acid, tends to be negatively charged that favour the adsorption of positively charged antimicrobial chemicals via electrostatic interactions, which are stronger than hydrophobic and π-π interactions (Tong et al., 2019). For the removal of AMR determinants, physical filtration into the pores could be limited due to size exclusion. Given the smaller average pore size of all tested biochar (2-8 nm) compared to the size of bacteria (~500-1000 nm; Riley, 1999) and extracellular genetic material (~70 nm; Tsoi et al., 2010), such as plasmids, adsorption mainly occurs onto the external surface. Therefore, biochar with a higher proportion of external surface area over the specific surface area such as biochar #1 (60% compared to 25% of the other tested biochars), performed better and facilitated water diffusion between biochar particles.

The varying biochar properties required for removal of specific contaminant groups must be considered when implementing the mitigation strategy in the OSSF under investigation. Therefore, the following column experiment, considering the hydraulic conditions at the pumping station of the OSSF, was performed with a mixture of the two optimal biochars #1 and #8. This led to optimal removal for both chemicals contaminants (median >98% at each refilling step) and for AMR determinants (up to 85%). Overtime, decreasing removal efficiencies were observed for seven chemicals including 4-epi-anydrotetracycline, miconazole, oseltamivir acid, acesulfame, gabapentin, salicylic acid and saccharin, suggesting that these chemicals may have a higher tendency to leach out after repetitive wastewater loads. The taxonomic markers *Bacteroidetes* and *Firmicutes* were found reduced of 50% and the only two measured pathogens *Shigella spp* and *Acinetobacter*

baumannii were reduced of at least 25%. Significant (p<0.05) reductions were found for the resistance groups of β-lactams, MLSB, MDR, other resistance (e.g., mercury resistance), tetracyclines and MGE. After biochar treatment, seven AMR determinants increased in their absolute abundances, including *pbrT*, *tet(44), vanTC* and the genes ranked as high risk for clinical settings (Zhang et al., 2021; Zhang et al., 2022) coding for resistance to aminoglycosides (i.e., *APH(6)-Id, ANT(6)-Ia),* β-lactams *(*i.e., *blaNDM)* and MDR (i.e., *qacH).* All genes involved in mobilization of ARG were reduced up to 80%, highlighting the ability of biochar to counteract the occasional enrichment of these genes observed in **Paper III**. Overall, the column experiment showed promising results for removal of the AMR contaminants, but further investigation is needed to ensure durable efficiency of the system, before *in-situ* application at the OSSF.

5. Conclusions and future perspectives

Overall, this thesis addressed the need for assessing the contribution of OSSF to environmental AMR by providing a comprehensive understanding of their pollution with AMR contaminants. The findings emphasize that OSSF can act as important vectors of AMR dissemination, which thus should be considered in future monitoring, and deserve more mitigating efforts on combating AMR. Below, the main conclusions of this thesis related to the identified research needs (section 2) are drawn.

- **Develop analytical methodology appropriate to the matrix of interest**

In **Paper I**, I developed and validated a robust, microbiologically sensitive SPE-LC-MS/MS methodology for the quantification of antimicrobial chemicals in influent and effluent wastewater and aquatic environments (i.e., surface water, groundwater), which supports the (inter)national AMR monitoring effort. The use of ion-exchange sorbent Oasis® WCX was newly proposed for solid-phase extraction of 35 antimicrobial chemicals. This will support future AMR monitoring efforts. Furthermore, I assessed the stability of 53 antimicrobial chemicals under different scenarios, including working solutions, storage under frozen, refrigerated or typical sewage conditions, and the use of preservatives and glass or polypropylene materials. This will help minimize uncertainties in future planning of sampling and storage.

- **Identify AMR dissemination pathways and dynamics**

In **Paper II**, I examined the literature state-of-the-art on AMR dissemination from OSSF at the global level. The findings revealed that existing OSSF designs, which mainly rely on primary treatment, do not sufficiently remove AMR contaminants. As a result, these contaminants are often detected in receiving waters, posing occasional ecological risks and potential for AMR selection. In contrast, alternative design with secondary treatment better mitigated AMR dissemination.
Specifically, measured concentrations of antimicrobial chemicals in OSSF-impacted waters were prioritized based on a meta-analysis of their AMR selection, ecological risks and environmental hazard. Despite evidence

supporting the contribution of OSSF to AMR dissemination, the number of studies addressing this issue remains low, with limited geographical coverage. This emphasizes that OSSF are a largely neglected source of AMR contaminants. Furthermore, I identified a clear and critical knowledge gap on quantitative data for AMR determinants and on their co-occurrence with antimicrobial chemicals.

In **Paper III**, the focus was on the national, Swedish context, where I investigated an OSSF designed with septic tank and aerated pond treatments followed by sandy soil infiltration into the groundwater environment. I found that AMR determinants and chemical contaminants were insufficiently removed with this system, and occasional enrichment of mobile genetic elements was observed. In contrast to AMR determinants and high-use chemicals (e.g., artificial sweeteners), antimicrobial chemicals exhibited higher temporal variability, reflecting their relatively fluctuating consumption. Enrichment (higher relative abundance) of the resistome was observed in groundwater below the infiltration field compared to upstream. AMR determinants (copies/mL) strongly correlated with antimicrobial chemicals and high-use chemicals. This highlights the importance of considering the potential of high-use chemicals, beyond antimicrobial chemicals as future indicators of AMR dissemination. These results are valuable for decision-making in future environmental monitoring, regulations and implementation of mitigation strategies.

- **Improve AMR mitigation strategies**

Given the insufficient AMR contaminants mitigation of existing OSSF designs, **Paper IV** focused on evaluating the suitability of biochar as a low-cost, and ecological friendly strategy. Here, I showed that biochar is a promising material for mitigation of AMR contaminants. Optimal biochar properties varied: adsorption of chemicals was favoured by high specific surface area, while AMR determinants required a greater external surface area relative to the microporous one. This highlights that a mixture of different biochar is more suitable for efficiently mitigating both AMR contaminant types.

Considerations for future research:

Future research should further investigate microbial communities shifts during treatment and in impacted groundwater, to help identifying the ARG-carrier strains in the environmental compartment.

Additionally, the developed analytical method in **Paper I** and the sampling strategy used in **Paper III** could be applied to other OSSF with similar and alternatives designs at (inter)national level. This would broaden the current knowledge on these systems, additionally filling the gap identified in **Paper II**, as well as enabling the evaluation of system-specific impacts and the effectiveness of different mitigation approaches, particularly in light of the global variability in antimicrobial chemical usage.

The prioritization of antimicrobial chemicals in **Paper II** can be used for future decision-making for target analysis.

Future work on the method developed in **Paper I** could involve broadening of the chemical space.

Finally, up-scaling biochar treatment experiments from **Paper IV** could help understand the long-term performance as well as biochar durability, and reusability, for an ultimate application in real OSSF settings.

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Popular science summary

The environment is the main collector of all human-related activities. Within soil, water and air a great variety of contaminants circulates. Among these, there are contaminants which contributes to the insurgence of antimicrobial resistance (AMR) which is a growing global health challenge, since it reduces the efficiency of antimicrobial chemicals to treat infections. The main AMR contaminants are antimicrobial chemicals which are highly and often improperly used, and AMR determinants, which are genes present within microorganisms that give them the ability to fight antimicrobial chemicals. In areas far from the main cities, the wastewater that we produce is collected and treated in on-site sewage facilities (OSSF) and afterwards discharged in the environment. However, these treatment systems are not originally designed for removal of AMR contaminants and we do not know how well they work. Therefore, this thesis explores the extent to which OSSF contribute to the environmental dissemination of AMR. To start, I developed a sensitive laboratory method to accurately measure antimicrobial chemicals in influent and effluent wastewater and in the aquatic environment (e.g., rivers, lakes, groundwater), to be able to monitor the presence of chemicals from source to recipient. Afterwards, I explored the existing scientific literature to understand what we know so far about OSSF from around the world and about their role in AMR dissemination. From these data I found that OSSF are often overlooked sources of AMR contamination, and that there is still very little quantitative (how much) data on AMR determinants. Quantitative data are important when deciding risk threshold for impact assessment law-making. Also, there is not enough information on relationships between chemicals and AMR determinants, which could help us understand what influences AMR dissemination, ultimately preventing it. With the data from around the world on how much and which antimicrobial chemicals occur in OSSF, I prioritized them based on their ability to favour AMR, their impact on aquatic organisms, and their overall environmental hazard. This can support future decision-making. To further help filling the knowledge gap, I conducted a large field study at a Swedish OSSF site. By sampling both the wastewater treatment system and the surrounding groundwater, I found that these facilities do not sufficiently remove AMR contaminants, and the quantity of antimicrobial chemicals varies greatly over the year, which is in accordance with their infrequent use, while the quantity

of AMR determinants was more stable. In the receiving groundwater I found higher quantities of chemicals and AMR determinants compared to a reference groundwater upstream of the OSSF. This suggests that OSSF is an AMR dissemination pathway and deserves more attention in monitoring and regulatory efforts. I also observed strong links between AMR genes and chemical pollutants, suggesting that their presence is connected. The dissemination of these AMR contaminants from OSSF raise the question: how can we easily improve OSSF treatment to reduce the discharge of AMR contaminants? To help answer this, I tested biochar — a carbon-rich material which production follows the principle of circular economy, by using discarded material from other production processes (e.g., garden or forest waste) — as a potential solution. I found that biochars with a high specific (or total) surface area were better at removing chemical pollutants, while those with a larger external surface area were more effective at removing AMR-related genes. Combining different types of biochar provided the best results for reducing both types of contaminants. Overall, this research improves our understanding of how OSSF contribute to the environmental spread of AMR. It also offers valuable insights for better monitoring, regulation, and management strategies to help fight AMR and protect environmental and public health.

Populärvetenskaplig sammanfattning

Miljön är den viktigaste källan till all mänsklig verksamhet. I mark, vatten och luft cirkulerar ett stort antal olika föroreningar. Bland dessa finns föroreningar som bidrar till uppkomsten av antimikrobiell resistens (AMR), vilket är en växande global hälsoutmaning, eftersom det minskar effektiviteten hos antimikrobiella kemikalier vid behandling av infektioner. De viktigaste AMR-föroreningarna är antimikrobiella kemikalier som används i stor utsträckning och ofta på ett felaktigt sätt, och resistensfaktorer, som är gener i mikroorganismer som ger dem förmågan att bekämpa antimikrobiella kemikalier. I områden långt från de större städerna samlas det avloppsvatten som vi producerar upp och behandlas i decentraliserade avloppsanläggningar (OSSF) och släpps därefter ut i miljön. Dessa reningssystem är dock inte ursprungligen utformade för att avlägsna AMR-föroreningar och vi vet inte hur väl de fungerar. I den här avhandlingen undersöks därför i vilken utsträckning OSSF bidrar till spridningen av AMR i miljön. Till att börja med utvecklade jag en känslig laboratoriemetod för att noggrant mäta antimikrobiella kemikalier i inkommande och utgående avloppsvatten och i vattenmiljön (t.ex. floder, sjöar, grundvatten), för att kunna övervaka förekomsten av kemikalier från källa till recipient. Därefter utforskade jag den befintliga vetenskapliga litteraturen för att förstå vad vi hittills vet om olika OSSF från hela världen och deras roll i spridningen av AMR. Utifrån dessa data fann jag att OSSF ofta är förbisedda källor till AMR-kontaminering och att det fortfarande finns mycket lite kvantitativa data (hur mycket) när det gäller resistensfaktorer. Kvantitativa data är viktiga när man beslutar om gränsvärden för konsekvensbedömning i samband med lagstiftning. Det finns inte heller tillräckligt med information om sambanden mellan kemikalier och resistensfaktorer, vilket skulle kunna hjälpa oss att förstå vad som påverkar spridningen av AMR och i slutändan förhindra den. Med data från hela världen om hur mycket och vilka antimikrobiella kemikalier som förekommer i OSSF har jag prioriterat dem utifrån deras förmåga att bidra till AMR, deras påverkan på vattenlevande organismer och deras övergripande risk för miljön. Detta kan vara ett stöd för framtida beslutsfattande. För att ytterligare bidra till att fylla kunskapsluckan genomförde jag en stor fältstudie på en svensk OSSF. Genom provtagning av både avloppsreningssystemet och det omgivande grundvattnet fann jag att dessa anläggningar inte avlägsnar AMR-föroreningar i tillräcklig

utsträckning, och mängden antimikrobiella kemikalier varierar kraftigt under året, vilket är i enlighet med deras sparsamma användning, medan mängden resistensfaktorer var mer stabil. I grundvattnet fann jag högre mängder kemikalier och AMR-bestämmande ämnen jämfört med ett referensgrundvatten uppströms OSSF:en. Detta tyder på att OSSF är en spridningsväg för AMR och förtjänar mer uppmärksamhet i övervaknings- och regleringsarbetet. Jag observerade också starka kopplingar mellan resistens-gener och kemiska föroreningar, vilket tyder på att deras förekomst hänger ihop. Spridningen av dessa AMR-föroreningar från OSSF väcker frågan: hur kan vi på ett enkelt sätt förbättra OSSF-behandlingen för att minska utsläppet av AMR-föroreningar? För att hjälpa till att besvara denna fråga testade jag biokol - ett kolrikt material som produceras enligt principerna för cirkulär ekonomi genom att använda kasserat material från andra produktionsprocesser (t.ex. trädgårds- eller skogsavfall) - som en potentiell lösning. Jag fann att biokol med en hög specifik yta var bättre på att avlägsna kemiska föroreningar, medan de med en större yttre yta var effektivare på att avlägsna resistensrelaterade gener. Att kombinera olika typer av biokol gav de bästa resultaten för att minska båda typerna av föroreningar. Sammantaget ökar denna forskning vår förståelse för hur OSSF:er bidrar till den miljömässiga spridningen av AMR. Den ger också värdefulla insikter för bättre övervakning, reglering och strategier för att bekämpa AMR och skydda miljön och folkhälsan.

Riassunto scientifico semplificato

L'ambiente è il principale collettore di tutte le attività umane. Nel suolo, nell'acqua e nell'aria circola una grande varietà di contaminanti. Tra questi, vi sono contaminanti che contribuiscono all'insorgere della resistenza antimicrobica (AMR), che rappresenta una sfida crescente per la salute globale, poiché riduce l'efficacia delle sostanze chimiche antimicrobiche nel trattamento delle infezioni. I principali contaminanti responsabili per l'AMR sono le sostanze chimiche antimicrobiche, utilizzate in misura elevata e spesso in modo improprio, e i determinanti genetici dell'AMR, ovvero i geni presenti all'interno dei microrganismi che conferiscono loro la capacità di combattere le sostanze chimiche antimicrobiche. Nelle aree lontane dalle città principali, le acque reflue che produciamo vengono raccolte e trattate in impianti di depurazione in loco (OSSF) e successivamente scaricate nell'ambiente. Tuttavia, questi sistemi di trattamento non sono stati originariamente progettati per la rimozione dei contaminanti AMR e non sappiamo quanto siano efficienti. Pertanto, questa tesi esplora in che misura gli OSSF contribuiscono alla diffusione ambientale di AMR. Per iniziare, ho sviluppato un metodo di laboratorio sensibile per misurare accuratamente le sostanze chimiche antimicrobiche nelle acque reflue in entrata e in uscita e nell'ambiente acquatico (ad esempio, fiumi, laghi, acque sotterranee), per poter monitorare la presenza di sostanze chimiche dalla fonte al destinatario. In seguito, ho esplorato la letteratura scientifica esistente per capire cosa sappiamo finora sugli OSSF di tutto il mondo e sul loro ruolo nella diffusione dell'AMR. Da questi dati ho scoperto che gli OSSF sono spesso fonti trascurate di contaminazione da AMR e che ci sono ancora pochi dati quantitativi sui determinanti genetici dell'AMR. I dati quantitativi sono importanti quando si decide la soglia di rischio per la valutazione dell'impatto legislativo. Inoltre, non ci sono abbastanza informazioni sulle relazioni tra sostanze chimiche e determinanti della resistenza antimicrobica che potrebbero aiutarci a capire cosa influenza la diffusione della resistenza antimicrobica e, in ultima analisi, a prevenirla. Con i dati provenienti da tutto il mondo su quante e quali sostanze chimiche antimicrobiche sono presenti negli OSSF ho stabilito una priorità in base alla loro capacità di favorire la resistenza antimicrobica, al loro impatto sugli organismi acquatici e alla loro pericolosità ambientale complessiva. Questo può supportare il processo decisionale futuro. Per contribuire a colmare la lacuna di conoscenze ho

condotto un ampio studio sul campo in un sito OSSF svedese. Campionando sia il sistema di trattamento delle acque reflue sia le acque sotterranee circostanti, ho scoperto che queste strutture non rimuovono sufficientemente i contaminanti AMR e che la quantità di sostanze chimiche antimicrobiche varia notevolmente nel corso dell'anno, in accordo con il loro uso poco frequente, mentre la quantità dei determinanti genetici dell'AMR è più stabile. Nelle acque sotterranee riceventi ho trovato quantità più elevate di sostanze chimiche e di determinanti genetici dell'AMR rispetto all'acqua sotterranea di riferimento a monte dell'OSSF. Ciò suggerisce che l'OSSF è una via di diffusione della resistenza antimicrobica e merita maggiore attenzione negli sforzi di monitoraggio e normativi. Ho anche osservato forti legami tra i geni AMR e gli inquinanti chimici, suggerendo che la loro presenza è collegata. La diffusione di questi contaminanti AMR da OSSF solleva la questione: come possiamo migliorare facilmente il trattamento OSSF per ridurre lo scarico di contaminanti AMR? Per rispondere a questa domanda, ho testato il biochar, un materiale ricco di carbonio la cui produzione segue il principio dell'economia circolare, utilizzando materiali di scarto provenienti da altri processi produttivi (ad esempio, rifiuti di giardino o forestali), come potenziale soluzione. Ho scoperto che i biochar con un'elevata superficie specifica erano più efficaci nel rimuovere gli inquinanti chimici, mentre quelli con una superficie esterna più ampia erano più efficaci nel rimuovere i determinanti genetici dell'AMR. La combinazione di diversi tipi di biochar ha fornito i migliori risultati per la riduzione di entrambi i tipi di contaminanti. Nel complesso, questa ricerca migliora la nostra comprensione di come gli OSSF contribuiscano alla diffusione ambientale dell'AMR. Inoltre, offre spunti preziosi per migliorare il monitoraggio, la regolamentazione e le strategie di gestione per aiutare a combattere l'AMR e proteggere l'ambiente e la salute pubblica.

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Novel, alternative analytical methodology for determination of antimicrobial chemicals in aquatic environments and public use assessment: Extraction sorbent, microbiological sensitivity, stability, and applicability

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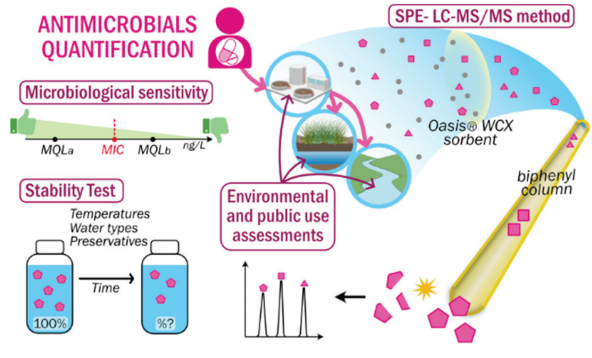
HIGHLIGHTS

- Novel use of Oasis® WCX sorbent for extraction of multiple antimicrobial classes.
- Unique example of evaluating the microbiological sensitivity of analytical methods.
- Extensive stability tests on 53 antimicrobials in sampling and storage conditions.
- Sodium azide is a better preservative than sodium metabisulfite.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Assessing antimicrobial chemicals from wastewater source to recipient water systems is crucial in planning effective, policy-related interventions for antimicrobial resistance (AMR) risk mitigation. However, the capability of related analytical methods for AMR assessment has not been explored previously. There is also a lack of knowledge on the effectiveness of alternative extraction sorbents with ion-exchange functions, and little information on chemical stability from sampling to analysis as well as preservative options. Hence, our study aims to address the clear need for advanced, broad-range and microbiologically-sensitive methodologies, paired with thorough stability assessments.

Results: Oasis® WCX ion-exchange was for the first time employed in solid-phase extraction (SPE) for antibacterials, antifungals, antivirals and human metabolites in various water matrices. Analysis was performed using

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liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) on a biphenyl analytical column. The optimized and validated method provided satisfactory accuracy, precision, and recovery for 53 compounds via LC-MS/MS direct injection and for up to 35 compounds via SPE-LC-MS/MS. Method quantification limits (MQLs) were determined in groundwater (0.33–54 ng L^{-1}), surface water (0.53–75 ng L^{-1}), effluent wastewater (2.5–470 ng L^{-1}), and influent wastewater (11–650 ng L^{-1}). As a novel approach, MQLs were compared with minimum inhibitory concentrations, to confirm our method's microbiological sensitivity for studying AMR. Stability assessment revealed that most compounds remained stable in standard solution at −80 °C for six months, in various waters at −20 °C for eight weeks, and during 24-h sampling at 4 °C. Sodium azide was a better preservative than sodium metabisulfite.

Significance: Our study is an added value to the analytical methodology for water measurements of antimicrobial chemicals, in which it provides a novel, alternative method that is robust and overall more sensitive than others using generic Oasis® HLB sorbents and C18 analytical columns in SPE-LC-MS/MS. Also, the comprehensive data on antimicrobial stability helps reduce methodological uncertainty for future studies. Our method shows sufficient microbiologically-sensitivity and thus is suitable for future (inter)national regulatory water monitoring of AMR.

1. Introduction

Antimicrobial resistance (AMR) occurs naturally within microbial communities during competition for resources and ecological niches, but use of antimicrobial chemicals (e.g., antibacterials, antifungals, antivirals) accelerates AMR development and spread. Despite this, production of antimicrobials continues to escalate, with many hundred thousand tons produced worldwide annually for human and veterinary usage [1,2]. Among high-income countries with lower consumption [3], Sweden alone recorded sales of ~70 tons of antibacterials in 2019 [4]. Macrolides (azithromycin) and cephalosporins have emerged as commonly prescribed antimicrobials for treatment of Covid-19, alongside antivirals [5–8]. Unintentional release of antimicrobials and related (bio)transformation products from wastewater treatment plants (WWTPs) to the environment has created a need for official monitoring data on these chemicals in the environment. Some antimicrobials (e.g., sulfamethoxazole, trimethoprim, fluconazole, ofloxacine) are on the EU Watch List, which requires Member States to provide aquatic occurrence data on them [9]. To support the growing discussion on regulating the release of antimicrobials and to help identify various types of these chemicals for up-to-date evaluation of AMR within the One Health perspective [10–12], new, advanced (more sensitive) analytical methodologies for detection of antimicrobial chemicals in (waste)waters are continuously on demand.

Solid-phase extraction (SPE) is a common sample preparation procedure for water extraction of antimicrobial chemicals [13]. Oasis® HLB is the typical choice of sorbent for SPE in many studies [13–15], whereas mixed-mode ion-exchange sorbents (e.g., Oasis® WCX and MCX) are rarely selected. Oasis® HLB has been widely used under different conditions to enhance extraction efficiency, e.g., for macrolides with addition of chelating agents (ethylenediaminetetraacetic acid disodium (Na_2EDTA)) in water samples[15–18], and for fluoroquinolones and tetracyclines with sample acidification (to pH 3) [15–19]. The latter implies that besides intermolecular attractions, ionic interactions could occur between these antimicrobial groups and the sorbent [16]. But, only a few studies have evaluated use of mixed-mode ion-exchange sorbents in this context. In one such study, Oasis® MCX as cation-exchange sorbent was used for extracting sulfonamides in wastewater, and sulfamethoxazole, erythromycin, and chloramphenicol in surface water, groundwater, and drinking water [20,21]. In another study, Oasis® MCX as cation-exchange sorbent was used in tandem with Oasis® HLB for extraction of antimicrobial chemicals in wastewaters and groundwater [17]. In contrast, use of Oasis® WCX as cation-exchange sorbent has been much less well explored, in only one study investigating extraction of three fluoroquinolones in wastewater using Oasis® WCX sorbents in SPE [22]. With Oasis® MCX sorbents (pKa<1), water acidification to low pH conditions (pH 2–3) is a common practice for enhancing protonation on the analytes in previous studies [17,20]. The Oasis® WCX sorbent (pKa≈5) is also suitable for extracting cationic analytes, but not at such low pH conditions as MCX could, because its negatively-charged function would not be displayed at water pH < 5. As most antimicrobials are basic molecules that can become positively charged in water as soon as below pH 7, WCX sorbents can be a valuable, alternative option when dealing with antimicrobial chemicals that are sensitive to degradation in very acidic conditions during water extraction. Examining the capability for capturing different classes of antimicrobial chemicals using SPE sorbents with ion-exchange function is essential, but remains overlooked. Moreover, while most studies report analytical detection limits for measuring antimicrobial chemicals in water, there is often limited understanding of the microbiological sensitivity of the methods when studying AMR. Bridging this knowledge gap can increase the future applicability of analytical methods across the disciplines of analytical chemistry and microbiology.

To date, antimicrobial stability tests have been performed at different temperatures (from −80 °C to 20 °C) and durations (1–30 days), including: (a) in solvent standards for six β-lactams [23]; (b) in deionized pure water for 56 antibiotics, with and without use of EDTA [24]; (c) in surface water for amoxicillin [25]; (d) in acidified wastewater for 12 sulfonamides, macrolides, and their metabolites [26]; (e) in wastewater for 17 antivirals [27]; and (f) in wastewater for 29 antibacterials, antivirals, and their metabolites [28]. These studies have mainly focused on limited classes of antimicrobial chemicals and in only one water matrix at a time. Information on the stability of antimicrobial chemical classes across different water matrices and conditions is particularly relevant for accurate measurements. Moreover, while sodium azide and sodium metabisulfite have been used as preservatives for drugs or pharmaceuticals in long-term storage of wastewater samples [29,30], their efficacy in preserving antimicrobial chemicals remains untested.

The main aim of this study was to develop better methodology for determination of antimicrobial chemicals in different water matrices. Specific objectives were to: (i) optimize and validate a new analytical method for extracting and analyzing various antimicrobial classes (antibacterials, antifungals, antivirals, human metabolites) in different water matrices (tap water, surface water, groundwater, effluent wastewater, and influent wastewater) using Oasis® WCX in SPE and a biphenyl column in LC-MS/MS; (ii) compare method quantification limits (MQLs) with minimum inhibitory concentrations (MICs) of antimicrobials, as a new approach to evaluate the microbiological sensitivity of the analytical method for its application in AMR assessment; (iii) examine the stability of antimicrobials in five scenarios, including standard solutions and different water matrices, and with(out) preservatives at different temperatures and durations; and (iv) assess method applicability in analysis of (waste)waters from hospitals, municipal WWTPs, on-site sewage facilities (OSSFs), and groundwater downstream of OSSFs. Our study also addressed a knowledge gap through comprehensive stability studies encompassing different antimicrobial classes in various scenarios, to help reduce uncertainties

regarding standard solution storage, sample storage, and water sampling.

2. Methods

2.1. Selection of target compounds

Target antimicrobial chemicals (Table S1) were selected considering (a) their usage in Sweden and concern over drug resistance in our clinical settings [4], (b) their occurrence in effluent from municipal WWTPs and in global surface water environments [31–33], (c) the need for monitoring data at EU level for the 3rd-edited Watch List [34,35], (d) their metabolic excretion and (e) their importance in the World Health Organization AWaRe Classification [36]. A few antivirals associated with treatment for Covid-19 were also included [7]. Most of the target antimicrobial agents have a high excretion rate (>40%) in unchanged form. For those with lower excretion rate, main metabolites that are still biologically active after excretion were considered. Altogether, 77 chemicals comprising antibacterials (n = 52 from 17 classes), antivirals (n = 14), antifungals (n = 4), and human metabolites (n = 7) were chosen (Table S1), and prioritized according to clinical and environmental relevance (Table S2). For chemicals and materials used, see SI.

2.2. Method validation

We validated the optimized analytical methodology, including instrumental analysis and sample preparation, for different method performance features suggested in the European Medicines Agency (EMA) bioanalytical method validation guidelines [37]. Instrumental analysis comprised high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS; Exion® LC, Sciex® Triple-Quad 3500). Calibration curves were constructed with 10-point concentrations over a range of 0.5–200 ng mL^{-1} (internal standard (IS) at 50 ng mL^{-1} at each calibration point). Accuracy (percentage bias, i.e., % deviation from the nominal value) and precision (percentage relative standard deviation, RSD) were evaluated at the lowest calibration level, in which within-run performance was validated using two replicates per day and between-run performance was evaluated over three different days. Linearity was evaluated using 10-point calibration curves (weighted 1/x) and the acceptable regression coefficient (R^2) was >0.99. Carry-over was determined by injecting a blank sample following the highest calibration standard, where an analyte signal <20% of instrumental detection limit (IDL) was accepted. IDL and instrumental quantification limit (IQLs) were determined from the lowest calibration point with signal-to-noise (S/N) ratio of 3 and 10, respectively.

Sample preparation was conducted using SPE, followed by LC-MS/MS measurement of analytes. This was validated with tap water, groundwater, surface water, and wastewater (influent and effluent). Since there was no possibility of obtaining wastewater, groundwater, and surface water samples free of the target analytes, within- and between-run precision and extraction efficiency of the method at different concentration levels were determined using spiked tap water, as in similar previous studies [38–41]. The validation is based on analyte concentrations that take into account the correction of responses between native analytes and IS mass-labeled compounds. Extraction efficiency in percentage was determined by comparing analyte concentrations measured in pre-spiked samples with those in a standard, as an evaluation of the overall procedural accuracy accounting for the SPE performance and existence of matrix effects during instrumental analysis. Recovery of 50–150% was considered satisfactory, as in previous studies [14,15,42]. Tap water samples were spiked at low, medium, and high levels (20, 50, and 150 ng L^{-1}; n = 5, n = 1, and n = 1, respectively on day 1 of validation; n = 2 for all levels on day 2 and 3 of validation). For tetracyclines, validation was performed as described above, but using quenched tap water, since formation of chlorinated tetracyclines impedes extraction and analytical detection (Fig. S1) [43]. This can be addressed by using ascorbic acid or potassium sulfite as quenching agents [44–46]. Potassium sulfite (27 mg L^{-1}) was selected, as it does not affect sample pH, and quenching overnight at room temperature was performed. All samples for SPE were spiked with IS (50 ng L^{-1}), except the post-spike samples. In every extraction batch, blank MilliQ water samples spiked with IS (50 ng L^{-1}) were included to check for potential contamination. Within-run precision (RSD, %) and extraction efficiency (recovery, %) were determined at the low level (20 ng L^{-1}, n = 5) from the day 1 validation batch. Between-run precision and extraction efficiency were evaluated at the low, medium, and high levels across the day 1–3 of validation. Furthermore, to assess within-run and between-run precision and extraction efficiency across day 1–3, four different water matrices were extracted and analyzed: (a) surface water (200 mL), (b) groundwater (200 mL), (c) effluent wastewater (40 mL), and (d) influent wastewater (40 mL), spiked at the mid-level (50 ng L^{-1} for surface water and groundwater, IS at 50 ng L^{-1}, n = 2; 250 ng L^{-1} for influent and effluent wastewater, IS at 250 ng L^{-1}, n = 2). Non-spiked samples of these four water matrices were included to evaluate background analyte concentrations (n = 2; IS at 50 ng L^{-1}). Method detection limits (MDLs) and MQLs, corresponding to S/N ratio of 3 and 10, respectively, were determined for all four water matrices.

The acceptance criteria for precision and accuracy were based on the EMA guidelines with slight adjustment (25% for precision, ±25% for accuracy), as justified previously [39], since the guidelines are primarily for validation of bioanalytical methods that encounter high analyte concentrations, whereas aquatic levels of the analytes in this study were rather low. Similar criteria have been applied in other studies [14,15,25].

2.3. Method application

2.3.1. Sample collection

We collected a total of six (waste)water samples from four different sites, including a municipal WWTP, OSSF, hospital and groundwater environment, as a proof-of-concept application for our developed method. Daily (24-h) composite influent and effluent wastewater samples were collected at ~4 °C using flow-proportional sampling at the municipal WWTP and using time-proportional sampling (every 10 min) at the OSSF. The OSSF in this study has wastewater treatments of a septic system and aeration pond. The effluent is subsequently discharged to the groundwater environment via soil infiltration. Such OSSFs in Sweden is commonly used in rural and sub-urban areas where connection to centralized wastewater plants is limited. OSSFs are widely overlooked when it comes to studying their potential of spreading antimicrobial chemicals. The hospital wastewater (daily composite) was collected from an onsite sewage tank using time proportional sampling (every 15 min). The groundwater downstream of the OSSF was grab-sampled. Aliquots of the samples were stored at −20 °C in polypropylene bottles pre-rinsed with MilliQ water and methanol (MeOH) until analysis.

2.3.2. Sample extraction

Wastewater (40 mL) and groundwater (200 mL) samples were filtered, followed by acidification to pH 6 with 2 M HCl and addition of Na_2EDTA (0.1 M) to the samples (3 mM). The samples were spiked with IS (50 ng L^{-1} for groundwater; 250 ng L^{-1} for wastewater) and loaded onto Oasis® WCX cartridges (150 mg, 6 cc, 30 μm), pre-conditioned with MeOH (5 mL) and pH 6 MilliQ water (5 mL). The cartridges were then washed with MilliQ water pH 6 (3 mL), followed by drying under vacuum for 40 min. Analytes on the cartridges were eluted with MeOH (5 mL), and then 4% formic acid in MeOH (5 mL). The eluent was concentrated to 20 μL under a gentle stream of pure nitrogen at 35 °C, and then reconstituted with MeOH (40 μL) and MilliQ water (140 μL) to a final extract (200 μL, 30% organic solvent content).

2.3.3. LC-MS/MS analysis

Sample extracts and 10-point calibration standards (0.5–200 ng

mL^{-1}, IS 50 ng mL^{-1}) were analyzed using LC-MS/MS in both positive and negative electron spray ionization (ESI) mode. Chromatographic separation (Fig. S2) was performed on a Phenomenex® Kinetex® Biphenyl column (100 × 2.1 mm, 2.6 μm) at a flow-rate of 0.5 mL min^{-1}. In positive ESI mode, the mobile phases were (A) 0.1% formic acid in MilliQ water and (B) 0.1% formic acid in MeOH. In negative ESI mode, the mobile phases were (A) 0.1% acetic acid in MilliQ water and (B) 0.1% acetic acid in MeOH. Injection volume was 10 μL. Total run time was over 15.5 min with the LC-gradient (Fig. S3A): 0–0.5 min, 10% B; 2 min, 20% B (curve −3); 7 min, 75% B (curve −4); 9–12 min, 100% B; 12.1–15.5 min, 10% B. The MS was operated in multiple reaction monitoring (MRM). For each analyte, two MRM transitions (Table S3) with the highest intensity were selected and used for quantification and qualification. Identification and confirmation of analytes were based on: (a) consistent retention time (±0.1 min) between samples and calibration standards, between the two MRM transitions, and with its corresponding mass-labeled compounds; (b) comparable concentrations (RSD ≤20%) quantified in the two MRM transitions; and (c) ion ratios (a tolerance from ±20% to ±50%) between samples and calibration standards [38,47,48].

2.4. Stability, preservative, and sorption studies

The experiments were performed in darkness under different conditions: *Working solutions* (storage at −80 °C and −20 °C): the working solution (10 μg mL^{-1}) in MeOH was analyzed at time zero (t0, once prepared) and in 1, 3, and 6 months. Dilutions to 50 ng mL^{-1} were prepared for analysis. *Sample storage in freezer* (at −20 °C): spiked water matrices (25 μg/L; MilliQ water, surface water, groundwater, influent wastewater, and effluent wastewater) were analyzed at t0 and in 2, 4, 6 and 8 weeks. *Sample storage in refrigerator* (at 4 °C): spiked surface water and influent wastewater (25 μg/L) were measured at t0, in 2 and 6 h, and in the following 1, 3, 5, and 9 days. *Typical sewage conditions* (storage at 20 °C): spiked influent wastewater (25 μg/L) was measured at t0 and in 1, 2, 6, and 24 h. *Preservatives:* sodium azide (NaN_3) and sodium metabisulfite ($Na_2S_2O_5$) were tested as preservatives (0.5 g/L) in surface water and influent wastewater (25 μg/L) at 4 °C; samples were analyzed at t0 and in 1, 3, 5, and 9 days. *Sorption to materials:* spiked MilliQ water (25 μg/L) was prepared in amber HPLC glass vials and polypropylene Eppendorf® tubes, kept at 4 °C for 3 days.

To avoid frost-and-thaw cycles, samples for each time point were already prepared ($n = 3$ for storage at −20 °C and the sorption experiment, $n = 1$ for working solutions, $n = 2$ for the other experiments). The samples were analyzed through direct injection onto LC-MS/MS, after the preparation steps including centrifugation (10 min, 4 °C, 8000 rpm), transfer of supernatant (180 μL) into vials, and addition of IS (50 ng mL-1). Stability of the analytes was evaluated by comparing the selected time points relative to t0. The experiments were performed separately for parent compounds and metabolites.

3. Results and discussion

3.1. Method optimization

3.1.1. LC-MS/MS analysis

Instrumental analysis was optimized (see SI for details) regarding ionization mode, MRM transition, analytical column (Kinetex® biphenyl, C18, EVO columns), mobile phase, and LC gradient. Briefly, two product ions with the highest intensity were chosen as quantification and confirmation MRMs for the analytes. With mobile phases of (A) MilliQ water and (B) MeOH with 0.1% formic acid each and a generic LC gradient (Fig. S3B), the biphenyl column allowed optimal elution, retention, and separation of the target analytes compared with a C18 column (early elution of some analytes) (Fig. S4) or EVO column (two compounds without elution). Replacing MeOH with acetonitrile in the mobile phase (B) for this column worsened analyte peak separation (Fig. S5). Hence, MeOH was deemed superior. Use of a biphenyl column is unique, with most previous studies mainly using a C18 column [13]. LC gradients were further optimized (Figs. S3C and S3A) to avoid analyte elution in the column wash step (Fig. S6). Optimal MeOH content per sample was 30% (Fig. S7). The same LC setting was tested for negative ESI with optimized mobile phases using 0.1% acetic acid. After optimization, 10 analytes were eliminated (Fig. S8) due to poor signals and/or retention in any column, or to not being eluted with any mobile phase.

3.1.2. Sample extraction

Extraction sorbents. Oasis® SPE cartridges (HLB, MCX, WCX) were evaluated to determine the most suitable extraction for the analytes, with consistent optimal recovery in two extreme water types, i.e., MilliQ water and influent wastewater. For each sorbent, recommended sample pH, washing solution, and elution solvents were used (Table S4). The optimal SPE sorbent was determined based on absolute extraction recoveries (%), numbers of analytes with >15% absolute recovery, and matrix effects (%) in the two water matrices. Absolute recoveries (%) were obtained as the analyte response (peak area) ratio of pre-spike samples (spiking before SPE) to post-spike samples (spiking after SPE). Matrix effects were assessed by comparing the response of analytes in post-spike samples with that in standard solutions at the same concentration.

The three sorbents showed varying extraction efficiency of the analytes in the two water matrices (Fig. 1, Table S5). For HLB, there was a substantial difference in absolute recovery between MilliQ water (25–75th percentile 28–87%, median 41%, mean 54%) and influent wastewater (77–105%, 97%, 87%). HLB also gave lower recovery in MilliQ water than the other two sorbents. Unlike HLB, WCX and MCX showed similar absolute recovery for MilliQ water (WCX: 50–95%, 78%, 72%; MCX: 61–92%, 79%, 77%) and influent wastewater (WCX: 45–92%, 69%, 68%; MCX: 69–98%, 87%, 78%). In fact, similar distribution pattern of the recovery data for the two water matrices was observed using WCX, suggesting consistent capability for extracting target analytes from water matrices lying between the two extreme water types. WCX also gave higher numbers of analytes with >15% absolute recovery in influent wastewater and showed better extraction compared with MCX, of macrolides, fluoroquinolones, and tetracyclines

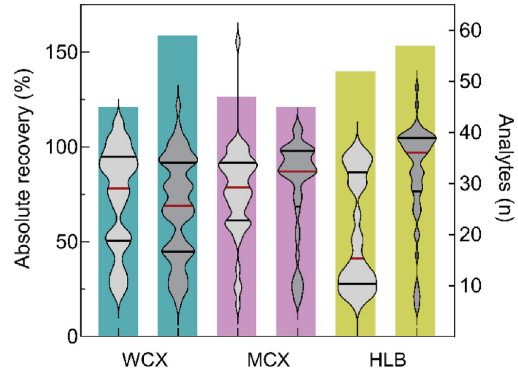

Fig. 1. Absolute recovery (%) in violin plots (left y-axis) and numbers of analytes with >15% absolute recovery in bar charts (right y-axis) using Oasis® HLB (yellow), MCX (pink) and WCX (turquoise) sorbents in MilliQ water (left of a sorbent) and influent wastewater (right of a sorbent) extraction. The violin plot (red line: median; black line: 25%tile and 75%tile) in light grey represents the absolute recovery for MilliQ water and in dark grey for influent wastewater of each sorbent. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in particular (Fig. S9). All three sorbent types generally showed a similar pattern of matrix effect for the analytes in influent wastewater (Fig. S10). For instance, lincomycin was subjected to ion suppression, and enrofloxacin to ion enhancement, irrespective of sorbent type. Analyte matrix effects varied only occasionally with sorbent type, e.g., for azithromycin with ion suppression using MCX and HLB, but with ion enhancement using WCX. The majority of analytes showed ion suppression and some were subjected to ion enhancement, regardless of sorbent type, particularly chloroquine, hydroxychloroquine, and the fluoroquinolone class. Ion enhancement of a fluoroquinolone (norfloxacin) with HLB sorbent has been reported previously [44].

Based on the above results, it was decided to proceed with WCX in further optimization and validation steps. The potential of WCX to extract a wide range of antimicrobial chemicals in different water matrices had not been explored previously, so the method optimization and validation performed in this study adds to current knowledge on water extraction-based analysis of antimicrobials using WCX as sorbent, instead of generic HLB.

Elution solution. We evaluated three serial 10-mL elution solutions, i.e., MeOH (5 mL) combined with 2%, 4%, or 8% formic acid (Fig. S11,

Table 1
Optimized and validated LC-MS/MS analytical method for analysis of the target antimicrobials.

Compound	linearity (R^2)	precision (within-run)[b,d]	precision (between-run)[c,d]	accuracy (between-run)[c,d]	IDL (ng mL^{-1})	IQL (ng mL^{-1})
ESI+						
Acyclovir[a]	0.9861	3	15	1	2.05	6.84
Ampicillin	0.9992	4	20	−16	0.01	0.03
Azithromycin[a]	0.9976	16	18	−21	0.02	0.05
Cefadroxil	0.9990	8	13	−2	0.06	0.19
Cefalexin	0.9936	5	25	−9	0.03	0.12
Cefepime	0.9788	6	18	−14	0.68	2.27
Chloroquine[a,e]	0.9953	0.1	7	6	5.42	18.1
Chlortetracycline[a]	0.9967	9	7	−13	0.17	0.55
Ciprofloxacin[a,e]	0.9950	0.1	34	−11	0.18	0.60
Clarithromycin[a]	0.9979	4	9	8	0.01	0.04
Clindamycin[a]	0.9925	9	7	−1	0.01	0.02
Enoxacin[a,e]	0.9978	13	12	−14	0.41	1.37
Enrofloxacin[a,e]	0.9964	10	31	14	0.01	0.04
Erythromycin[a]	0.9974	6	19	−10	0.01	0.04
Fluconazole[a]	0.9987	1	6	24	0.02	0.08
Hydroxychloroquine[a,e]	0.9931	4	37	11	1.99	6.63
Lincomycin	0.9954	1	9	18	0.01	0.03
Lomefloxacin[a,e]	0.9935	3	5	−14	0.01	0.03
Mecillinam	0.9977	8	5	17	0.02	0.05
Meropenem	0.9968	1	9	−6	0.19	0.65
Metronidazole[a]	0.9989	2	12	−2	0.03	0.09
Metronidazole-OH	0.9779	0.2	6	−17	0.05	0.15
Miconazole[a]	0.9944	11	19	−7	0.01	0.03
N4-acetylsulfadiazine[a]	0.9958	15	20	−3	0.02	0.06
N4-acetylsulfamethazine[a]	0.9933	8	25	16	0.05	0.17
Norfloxacin[a,e]	0.9939	16	12	23	0.52	1.73
Ofloxacine[a,e]	0.9983	1	10	−7	0.10	0.34
Oseltamivir[a]	0.9978	10	16	19	0.01	0.03
Oseltamivir acid[a]	0.9985	7	8	2	0.01	0.05
Oxytetracycline	0.9934	11	12	5	0.25	0.83
Remdesivir[a]	0.9985	12	14	−14	0.01	0.04
Roxithromycin[a]	0.9936	2	16	5	0.01	0.04
Sparfloxacin[a,e]	0.9985	4	9	6	0.04	0.15
Sulfadiazine	0.9946	1	16	21	0.01	0.03
Sulfamethazine[a]	0.9982	5	14	12	0.01	0.04
Sulfamethoxazole[a]	0.9982	1	16	−7	0.03	0.10
Sulfathiazole[a]	0.9943	8	17	−9	0.01	0.04
Tetracycline[a]	0.9984	12	8	−11	0.10	0.32
Tinidazole[a]	0.9960	9	17	5	0.02	0.08
Trimethoprim[a]	0.9969	1	17	25	0.03	0.11
Vancomycin	0.9934	2	8	−14	0.69	2.29
ESI-						
4-epianhydrotetracycline[e]	0.9971	7	13	−2	11.7	39.1
Cefaclor	0.9928	1	4	−11	0.59	1.98
Cefixime	0.9938	3	14	−5	0.54	1.81
Cefoxitin	0.9980	5	19	−7	0.11	0.37
Chloramphenicol[a]	0.9832	6	5	24	0.01	0.02
Doxycycline[a]	0.9768	4	48	4	1.89	6.28
Fusidic acid	0.9916	3	8	−2	1.51	5.03
N4-acetylsulfamethoxazole[a]	0.9988	9	2	7	0.01	0.04
Nitrofurantoin[a]	0.9990	4	21	8	0.02	0.06
Piperacillin	0.9985	7	19	11	0.04	0.14
Tenofovir	0.9976	2	22	−6	0.34	1.14
Zidovudine[a]	0.9970	22	18	−6	0.04	0.13

[a] In the extraction method;
[b] n = 2;
[c] Across three different days;
[d] At the lowest calibration point;
[e] Quadratic calibration curve.

Table 2
Method performance of SPE-LC-MS/MS analysis for the target antimicrobials in tap water, groundwater, surface water, influent and effluent wastewater.

Compound	Tap water								Groundwater				
	within-run precision	between-run precision			within-run recovery (%)	between-run recovery (%)			within-run precision	between-run precision	recovery (%)	MDL (ng L-[1])	MQL (ng L^{-1})
		low	med	high		low	med	high					
Acyclovir	12	16	10	10	84	71	72	95	11	23	107	7.48	25.0
Azithromycin	3	14	17	13	92	98	98	90	14	10	107	0.28	0.92
Chloramphenicol	5	6	10	7	85	85	88	99	3	11	83	0.24	0.80
Chloroquine[b]	–	–	–	7	–	–	–	130	13	10	144	2.37	7.89
Chlortetracycline[a]	3	3	–	–	81	71	–	–	3	7	83	1.2	3.99
Ciprofloxacin[b]	7	13	13	8	125	113	100	87	0.2	11	148	0.61	2.05
Chlarithromycin	9	5	29	21	55	57	61	71	14	15	65	0.26	0.85
Clindamycin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	–	–
Doxycycline[a]	4	–	–	26	132	–	–	101	–	–	–	14.8	49.3
Enoxacin[b]	–	–	5	8	–	–	145	118	8	21	150	1.70	5.65
Enrofloxacin	12	14	10	5	110	85	79	68	4	19	85	0.49	1.63
Erythromycin	11	14	3	12	107	103	87	93	1	11	122	16.3	54.3
Fluconazole	4	3	18	14	77	80	98	109	14	14	100	0.21	0.70
Hydroxychloroquine	–	–	–	9	–	–	–	144	–	–	–	0.92	3.07
Lomefloxacin[b]	7	14	9	–	84	94	119	–	7	31	112	0.27	0.91
Metronidazole	3	4	5	6	105	100	100	101	3	14	106	0.45	1.51
Miconazole	19	12	11	20	92	132	95	105	12	12	150	1.38	4.62
N4-acetylsulfadiazine	14	10	11	10	123	103	95	100	9	15	86	0.44	1.47
N4-acetylsulfamethazine	9	8	11	11	140	129	121	133	13	12	124	0.50	1.68
N4-acetylsulfamethoxazole	3	4	7	6	112	111	112	122	0.1	19	146	0.25	0.82
Nitrofurantoin	6	16	19	11	134	106	99	111	16	15	104	0.23	0.76
Norfloxacin	–	–	6	10	–	–	149	119	–	–	–	2.30	7.66
Ofloxacine[b]	10	5	12	10	93	91	95	92	7	23	85	0.34	1.14
Oseltamivir	6	9	14	13	84	78	81	92	1	21	114	0.2	0.66
Oseltamivir acid	–	–	–	–	–	–	–	–	–	–	–	0.10	0.33
Remdesivir	9	8	16	10	105	110	112	111	3	15	125	0.13	0.43
Roxithromycin	8	9	8	9	78	70	75	82	27	19	92	0.17	0.56
Sparfloxacin[b]	–	–	–	–	–	–	–	–	15	26	60	0.61	2.05
Sulfamethazine	7	11	12	14	81	83	79	85	2	3	93	1.01	3.35
Sulfamethoxazole	4	10	10	13	102	111	104	112	7	9	115	0.79	2.64
Sulfathiazole	8	9	20	11	56	55	49	55	10	11	125	0.52	1.75
Tetracycline[a]	5	22	17	13	92	84	85	101	1	11	103	1.15	3.83
Tinidazole	–	–	–	–	–	–	–	–	–	–	–	0.67	2.24
Trimethoprim	5	7	12	5	108	102	86	99	2	21	115	0.79	2.64
Zidovudine	10	19	11	7	103	94	89	87	10	16	120	0.51	1.71

aQuenched tap water; (-) The compound did not pass the validation in term of precision and/or recovery; (nd) Not detected; (na) Not available;
bQuadratic calibration curve.

Table S6). Two elution fractions (Table S4) were combined to maintain high throughput in sample analysis and sample pH was adjusted to 6. The sulfonamides group was eluted in high recovery (75–96%) with the MeOH fraction alone (Fig. S12), but showed reduced recovery (42–66%) in the serial elution with 2% formic acid. The decrease was even greater with 8% formic acid (18–55%) (Fig. S11, Table S6), indicating that greater acidity was not favorable for these chemicals in the eluted solution. Using 8% formic acid instead of 2% improved recovery for some chemicals (Fig. S11, Table S6), as protonation on WCX sorbent was facilitated. For instance, in influent wastewater, enhanced absolute recoveries were observed for fluoroquinolones, cephalosporins, chloroquine, hydroxychloroquine, clotrimazole, entacapone, lamivudine, linezolid, miconazole, oseltamivir, and oseltamivir acid. However, high acidity greatly reduced recovery for some other chemicals, e.g., penicillins, macrolides, darunavir, fusidic acid, and rifampicin. With 4% formic acid, recovery of all analytes was either improved or similar to that with 2% formic acid (Fig. S11, Table S6). Hence, 4% formic acid was chosen as a suitable compromise for improved recovery of different antimicrobial classes.

Na_2EDTA. Na_2EDTA, a metal chelating agent, is commonly used in antimicrobial analyses, e.g. [15–18,49,50]. In our study, overall recovery remained similar for most analytes with or without use of Na_2EDTA in sample preparation (Fig. S13, Table S6; see SI for details). A stronger influence was observed for two antimicrobial classes, cephalosporins of β-lactams and macrolides. Thus, Na_2EDTA was included in method validation, primarily for better extraction of macrolides (e.g., clarithromycin, erythromycin) due to their high relevance in European surface water environments [51].

3.2. Method validation

3.2.1. LC-MS/MS method

Of 67 analytes tested with the optimal LC-MS/MS method, 53 showed satisfactory between-run accuracy (from −21 to 25% bias) at the lowest calibration standards (Table 1), while 14 were excluded (Fig. S1). Within-run precision (RSD 0.1–22%) of these 53 analytes was also satisfactory. Almost all analytes showed satisfactory between-run precision (RSD 2–48%), with less satisfactory results observed for enrofloxacin (31%), ciprofloxacin (34%), hydroxychloroquine (37%), and doxycycline (48%). These analytes were still included in validation, due to satisfactory between-run accuracy. Linearity was generally good ($R^2 > 0.99$) for almost all analytes (Table 1), but slightly less satisfactory ($R^2 = 0.98$) for cefepime, metronidazole-OH, chloramphenicol, and doxycycline. Carryover was not observed for any analyte. IDL range was 0.01–12 ng mL^{-1} and IQL range was 0.02–39 ng mL^{-1}. Doxycycline, hydroxychloroquine, chloroquine, and 4-epianhydrotetracycline showed the lowest instrumental sensitivity (IDLs 2–12 ng mL^{-1}, IQLs 6–39 ng mL^{-1}). Compared with other studies [14,44], our method showed higher sensitivity for nitrofurantoin, enrofloxacin, lomefloxacin, chloramphenicol, clindamycin, ampicillin, cefalexin, sulfadiazine,

Surface water					Effluent wastewater					Influent wastewater				
within-run precision	between-run precision	recovery (%)	MDL (ng L^{-1})	MQL (ng L^{-1})	within-run precision	between-run precision	recovery (%)	MDL (ng L^{-1})	MQL (ng L^{-1})	within-run precision	between-run precision	recovery (%)	MDL (ng L^{-1})	MQL (ng L^{-1})
17	15	101	9.34	31.2	20	14	114	40.7	136	na	na	na	102	339
2	16	103	0.25	0.83	9	11	100	1.33	4.42	12	17	98	16.5	55.1
1	12	91	0.36	1.21	5	6	70	1.23	4.09	7	5	67	34.4	115
10	10	137	2.43	8.10	1	7	137	8.53	28.4	4	12	90	28.3	94.4
4	10	58	1.47	4.89	2	7	78	5.95	19.8	5	9	53	25.9	86.2
2	24	111	0.61	2.05	1	6	117	2.38	7.93	0.04	6	70	11.0	36.6
–	7	51	0.34	1.15	–	–	–	1.60	5.35	11	9	74	3.99	13.3
nd	nd	nd	–	–	nd	nd	nd	–	–	8	24	140	10.8	36.1
2	18	144	10.2	33.8	na	na	na	47.1	157	na	na	na	131	435
3	22	120	1.89	6.29	3	17	126	6.67	22.2	3	18	126	17.5	58.2
8	37	62	0.71	2.38	8	16	47	4.25	14.2	15	22	61	14.1	47.0
na	na	na	22.5	75.1	na	na	na	139	465	na	na	na	194	648
0.3	4	90	0.19	0.64	6	12	109	1.80	6.00	17	10	125	12.5	41.8
–	–	–	0.75	2.51	2	23	132	2.92	9.72	3	17	99	23.8	79.5
7	23	88	0.31	1.04	1	15	80	1.23	4.09	4	13	83	7.05	23.5
4	19	97	0.56	1.86	0.1	12	99	1.83	6.09	1	11	99	3.85	12.8
–	–	–	3.33	11.1	–	–	–	7.82	26.1	–	–	–	19.7	65.5
0.4	16	78	0.49	1.63	3	16	80	2.23	7.44	3	14	108	19.8	65.8
7	16	125	0.54	1.78	4	9	124	2.69	8.98	–	–	–	4.22	14.1
4	13	129	0.21	0.69	9	13	92	1.15	3.83	20	16	73	27.6	92.1
4	19	98	0.29	0.96	6	9	74	1.23	4.10	7	16	49	26.1	87.0
2	22	135	3.51	11.7	1	16	144	18.9	63.0	na	na	na	99.4	331
3	18	81	0.37	1.24	4	12	100	1.48	4.92	6	10	92	5.65	18.8
3	18	99	0.23	0.76	3	17	120	1.36	4.55	4	18	104	3.49	11.6
–	–	–	0.16	0.53	9	25	133	0.74	2.48	16	–	105	3.33	11.1
7	17	118	0.21	0.71	2	14	114	1.12	3.73	7	21	134	7.93	26.5
27	15	81	0.17	0.55	8	13	83	0.84	2.82	4	13	103	21.6	71.9
18	19	50	0.87	2.90	10	15	53	3.87	12.9	5	14	75	13.4	44.7
14	15	97	3.93	13.1	5	11	95	9.87	32.9	4	13	113	15.5	51.7
0.2	15	122	2.61	8.71	1	8	123	16.9	56.5	4	14	147	23.5	78.2
14	17	140	1.50	5.01	5	10	133	5.76	19.2	–	–	–	21.3	71.0
4	19	88	0.97	3.23	3	8	96	3.47	11.6	8	11	82	21.8	72.7
–	–	–	0.80	2.67	0.4	13	105	1.84	6.13	3	11	76	8.36	27.9
1	11	106	0.86	2.88	9	12	105	4.83	16.1	8	15	99	5.65	18.8
7	9	94	0.60	1.99	21	18	117	3.31	11.1	3	21	84	42.7	142

sulfamethazine, chlortetracycline, and vancomycin, and similar sensitivity for metronidazole, N4-acetylsulfadiazine, N4-acetylsulfamethoxazole, tetracycline, oxytetracycline, ciprofloxacin, ofloxacine, azithromycin, roxythromycin, erythromycin, trimethoprim, and sulfamethoxazole.

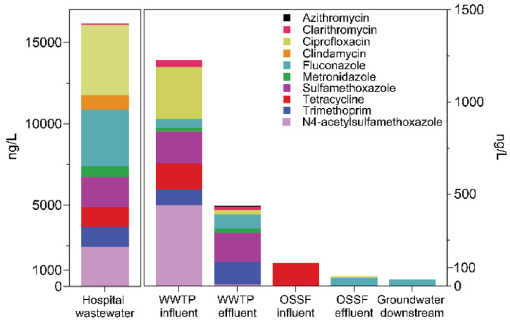

Fig. 2. Cumulative antimicrobial concentrations (ng/L) quantified in the six (waste)water samples. Left y-axis for hospital wastewater. Right y-axis for the other waters. The municipal WWTP services ~24 000 inhabitants with active sludge treatment followed by chemical precipitation. The OSSF services ~300 inhabitants with septic tank treatment and aeration pond before discharging through soil infiltration. The sampling at the WWTP and OSSF was conducted in March 2022. Hospital wastewater was collected in December 2022.

3.2.2. SPE-LC-MS/MS method

Of the 53 instrumentally validated analytes, 18 did not show satisfactory between-run recovery and/or precision at least in tap water and/or influent wastewater (Fig. S8). Thus, 35 remained for validation in different water matrices using the optimized SPE-LC-MS/MS method (Table 2). The number of analytes that passed validation varied with water matrix, with 21 validated in all water matrices (Fig. S14, Table 2) and six additional analytes validated for tap water (total 27), eight for groundwater (29) and surface water (29), nine for effluent wastewater (30), and seven for influent wastewater (28). In tap water, within-run precision at the low level (RSD 3–19%, $n = 5$) was satisfactory for 27 analytes, as was between-run precision at all three levels (low RSD 3–22%, medium RSD 3–29%, high RSD 5–26%). The analytes also showed acceptable within-run recovery (low 55–140%) and between-run recovery (low 55–132%, medium 49–149%, high 55–144%) in tap water. In groundwater, within-run (0.1–27%) and between-run (3–31%) precision was generally satisfactory, with acceptable recovery (60–150%), for 29 analytes. Precision was marginal for sparfloxacin (26%), roxythromycin (27%), and lomefloxacin (31%). Similar results were obtained for surface water, with overall satisfactory precision (within-run 0.2–27%, between-run 4–37%) and recovery (50–144%) for 29 analytes, and only roxythromycin (27%) and enrofloxacin (37%) showing less satisfactory precision. The wastewater matrices also showed satisfactory precision and recovery, for 30 analytes in effluent wastewater (within-run 0.1–21%, between-run 6–25%, recovery

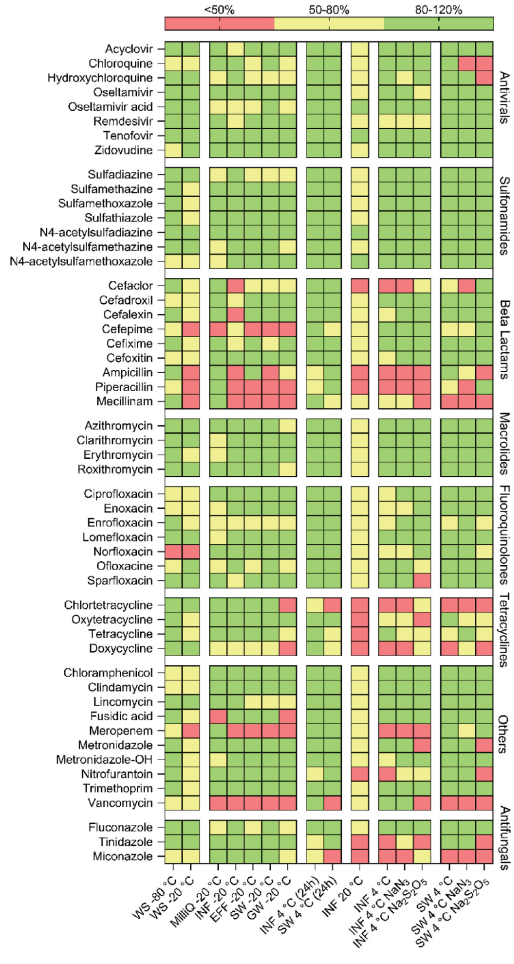

Fig. 3. Antimicrobial stability evaluations in different scenarios: working solutions (WS) at −80 °C and −20 °C for 6 months; MilliQ, influent (INF) and effluent (EFF) wastewater, surface water (SW) and groundwater (GW) in freezers at −20 °C for 8 weeks; INF and SW in refrigerators at 4 °C for 24h; INF at 20 °C for 24h; INF and SW with preservatives sodium azide (NaN_3) and sodium metabisulfite ($Na_2S_2O_5$) at 4 °C for 9 days. Each cell represents the remaining % of chemical at the endpoint of the stability test (green = 80–120%, stable; yellow = 50–80%, partly degraded; red = <50%, highly degraded). 4-epianhydrotetracycline was not studied due to high IQL. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

47–144%) and 28 analytes in influent wastewater (within-run 0.04–20%, between-run 5–24%, recovery 49–147%). Four analytes (clindamycin, hydroxychloroquine, oseltamivir acid, and tinidazole) were validated in only wastewater matrices (Fig. S14). Better extraction performance for antimicrobials in wastewater than in natural or pure waters has been reported previously [14]. Our recovery results for fluoroquinolones (norfloxacin, ofloxacine, ciprofloxacin) in effluent wastewater were similar to those in a previous study using WCX cartridges [22].

Similar sensitivity of the analytes was observed for groundwater (MDLs 0.10–16 ng L-1; MQLs 0.33–54 ng L-1) and surface water (MDLs 0.16–23 ng L^{-1}; MQLs 0.53–75 ng L^{-1}) (Table 2). Relatively high sensitivity (MQL <1 ng L^{-1}) in groundwater was seen for remdesivir, roxythromycin, oseltamivir, fluconazole, nitrofurantoin, chloramphenicol, clarithromycin, N4-acetylsulfamethoxazole, lomefloxacin, and azithromycin, while erythromycin (54 ng L^{-1}) and acyclovir (24 ng L^{-1}) showed the lowest sensitivity. These analytes were also among those showing the highest and lowest sensitivity in surface water. Sensitivity of the analytes was similar between effluent (MDLs 0.74–140 ng L^{-1}; MQLs 2.5–460 ng L^{-1}) and influent (MDLs 3.3–190 ng L^{-1}; MQLs 11–650 ng L^{-1}) wastewater. In effluent wastewater, relatively sensitive compounds (MQL <5 ng L^{-1}) were N4-acetylsulfamethoxazole, azithromycin, chloramphenicol, lomefloxacin, nitrofurantoin, ofloxacine, oseltamivir, oseltamivir acid, remdesivir, and roxythromycin, while the least sensitive (MQLs 140–460 ng L^{-1}) were erythromycin, doxycycline, and acyclovir. In influent wastewater, relatively sensitive compounds (MQL <15 ng L^{-1}) were oseltamivir acid, oseltamivir, metronidazole, and clarithromycin, while norfloxacin, erythromycin, doxycycline, and acyclovir were the least sensitive (MQLs 330–650 ng L^{-1}). Generally, oseltamivir had the lowest MQL in all water matrices (0.7–12 ng L^{-1}), while erythromycin and acyclovir showed the lowest sensitivity (25–650 ng L^{-1}). With the exception of erythromycin, our method achieved low MQLs for the macrolides, as in another study [14].

Our method sensitivity was determined using the water matrices themselves. Compared with a previous study [15] based on the same approach but using HLB as sorbent, our method showed lower MQLs (up to 7-fold lower) for ciprofloxacin, azithromycin, roxythromycin, tetracycline, sulfathiazole, and chlortetracycline in surface water and effluent wastewater, and for clarithromycin in influent wastewater. Ofloxacine, N4-acetylsulfadiazine, and N4-acetylsulfamethazine showed much lower MQLs (up to 10-fold lower) in surface water, effluent, and influent wastewater. Compared with another study using HLB and estimating MQLs based on recovery in a water matrix and concentration factor [14], even more compounds showed higher sensitivity with our method (azithromycin, chloramphenicol, ciprofloxacin, doxycycline, enrofloxacin, lomefloxacin, metronidazole, N4-acetylsulfadiazine, N4-acetylsulfamethoxazole, nitrofurantoin, ofloxacine, tetracycline, and trimethoprim). However, comparison of method sensitivity is challenging, since MQLs are often derived from neat standards in other previous studies.

We assessed the usefulness and sensitivity of our analytical methodology with relevance to knowledge of microbiology (Fig. S15). Minimum inhibitory concentration (MIC) divides AMR development into two selective windows: traditional (above MIC, with growth inhibition) and sub-MIC (without growth inhibition). In this light, MQLs of the analytes were compared with MICs reported previously [52] as ratio of microbiological sensitivity. At MQL ≥ MIC (ratio ≥1) (Fig. S15), the analytical method allows study of AMR due to both selective pressure and growth inhibition within the microbial community (i.e., traditional selective window). At MQL < MIC (ratio <1) (Fig. S15), the analytical method allows study of AMR considering selective pressure in the absence of growth inhibition, as AMR can still develop below MIC (i.e., sub-MIC selective window) [53–55]. All antibacterials in this study showed ratio <1, with MQLs in ng L^{-1} range and MICs in μg L^{-1} ranges, indicating that our analytical method is microbiologically applicable and can meaningfully contribute to monitoring AMR development.

3.3. Method application

Using the validated SPE-LC-MS/MS method, we detected 10 analytes in various types of wastewaters and groundwater (Fig. 2, Table S7). Antimicrobials are widely used in hospitals, and higher cumulative concentrations were observed in hospital wastewater compared with other municipal wastewaters. In particular, the levels of ciprofloxacin, fluconazole, metronidazole, tetracycline, and trimethoprim found in

hospital wastewater could risk promoting AMR (Table S7). In municipal effluent wastewater, fluconazole, metronidazole, sulfamethoxazole, and trimethoprim were found at unchanged concentrations compared with influent wastewater, while clarithromycin concentration only slightly decreased, suggesting very low removal efficiency for these compounds. Fluconazole and clarithromycin are reported to be recalcitrant substances in WWTPs using conventional activated sludge and aerobic granular sludge [56]. Removal of tetracycline, N4-acetylsulfamethoxazole, and ciprofloxacin at the municipal WWTP was efficient, with 10-fold reductions in their concentrations from influent to effluent wastewater. Similar removal was seen for tetracycline at the OSSF. Sulfamethoxazole appeared to be poorly removed, but removal may have been masked by re-formation of sulfamethoxazole following degradation (deacetylation reaction) of N4-acetylsulfamethoxazole during treatment at the WWTP [57]. Some compounds (metronidazole, sulfamethoxazole, trimethoprim, clarithromycin, N4-acetylsulfamethoxazole) were detected in hospital and municipal wastewater, but not in OSSF wastewater. Metronidazole is only used in the hospital sector [58], while sulfamethoxazole, trimethoprim, and clarithromycin are commonly used in primary care [58]. The absence of these antimicrobials in OSSF wastewater may be related to the small population that the OSSF serves. In groundwater, only fluconazole was found, at a similar level as in OSSF effluent wastewater, indicating a moderate AMR development risk (Table S7). Fluconazole has been widely reported in other aquatic environments [34,59–61].

3.4. Stability, preservative, and sorption studies

The stability, preservatives and sorption studies were performed for the 53 instrumentally validated analytes (Fig. S8).

Working solutions: At −80 °C, the analytes generally showed high stability, except for norfloxacin with <50% remaining (Fig. 3, Fig. S16). In fact, 36 analytes were highly stable (80–120%) after 6 months. However, at −20 °C only 21 analytes were stable (Fig. 3). The other analytes were reduced by at least 20%, with >50% degradation for most β-lactams, norfloxacin, and meropenem (Fig. S16). Storage at −80 °C helped maintain analyte stability in working solutions.

Sample storage in freezer: Overall, antivirals, sulfonamides, macrolides, fluoroquinolones, antifungals, and most other antimicrobials showed relatively high stability in the various water matrices tested (Fig. 3). Cefoxitin was stable in most water matrices, but most other β-lactams were highly degraded except in MilliQ water (Fig. 3, Fig. S17). Similar findings were made for meropenem. Vancomycin was completely degraded in influent wastewater (Fig. S17), but in the other water matrices its stability was undetermined, as it was non-detectable at t0. Except for doxycycline (greater degradation in all water matrices), tetracyclines were generally quite stable in most water types but showed low stability in groundwater. Similar results were obtained for fusidic acid.

Sample storage in refrigerator: Most analytes were highly stable in influent wastewater, with ampicillin, piperacillin, chlortetracycline, nitrofurantoin, tinidazole, and miconazole being relatively less stable (Fig. 3). Instability of β-lactams during sampling and transport has been reported previously [19,62]. However, except for chlortetracycline and miconazole, the β-lactams were stable in surface water. Degradation of at least 20% was seen for cefepime, mecillinam, tetracycline, and doxycycline in surface water, but not in wastewater. Chlortetracycline, vancomycin, and miconazole showed >50% degradation in surface water (Fig. S18). Caution on the degradation of these relatively less stable compounds during daily sampling of wastewater and surface water at 4 °C is worth in future studies.

Typical sewage conditions: Only oseltamivir acid, tenofovir, N4-acetylsulfadiazine, and N4-acetylsulfamethoxazole remained highly stable in wastewater at 20 °C, while 38 showed relatively lower stability (degradation of at least 20%). Tetracyclines, β-lactams (cefaclor and penicillins), nitrofurantoin, and antifungals (tinidazole and miconazole) were highly degraded (Fig. 3, Fig. S19). Similar results have been reported previously for tetracyclines and nitrofurantoin [28]. Instability of β-lactams was also identified as a challenge in recent wastewater-based surveillance for AMR [62]. While our results provide an initial understanding of the chemical stability at typical within-sewer temperature, future investigation using sewer reactors is needed to identify effects of other within-sewer characteristics, e.g., presence of biofilm, on degradation [63–65].

Preservatives: Stability of the compounds in influent wastewater and surface water was not substantially improved by use of a preservative agent (Fig. 3). Slight improvement was observed mainly in wastewater and for only a few compounds (cefalexin, cefoxitin, ciprofloxacin, enrofloxacin, metronidazole-OH, and nitrofurantoin). Generally, most compounds showed either similar or improved stability with NaN_3 than $Na_2S_2O_5$ in the two water matrices. In the presence of $Na_2S_2O_5$, hydroxychloroquine, ampicillin, mecillinam, sparfloxacin, oxytetracycline, doxycycline, metronidazole, vancomycin, and tinidazole, were more degraded (Figs. S20 and S21). While NaN_3 and $Na_2S_2O_5$ are reported to be useful for stabilizing drug residues in wastewater [29,30,66], our results suggest that they may not necessarily offer the same positive effect in preserving antimicrobial chemicals. Therefore, potential degradation should be considered in retrospective analysis for antimicrobial chemicals of water samples preserved with e.g., $Na_2S_2O_5$.

Sorption to materials: A ratio (plastic/glass materials) of <1 was obtained for vancomycin, remdesivir, miconazole, roxythromycin, clarithromycin, and tenofovir (Fig. S22), indicating their strong tendency to sorb to plastic in a pure water environment. This may partly explain the absence of vancomycin at t0 in most water matrices in the stability experiments. However, some analytes showed a ratio of >1 (Fig. S22), indicating higher preference for sorption to glass, including azithromycin, lomefloxacin, fusidic acid, sparfloxacin, oxytetracycline, tetracycline, ofloxacine, hydroxychloroquine, chloroquine, enrofloxacin, chlortetracycline, ciprofloxacin, enoxacin, doxycycline, and norfloxacin. Sorption of azithromycin to glass was seen in another recent study [67]. These results fill an existing knowledge gap on sorption behavior to plastics and glass for a range of antimicrobial chemicals, and can help in selecting suitable materials for sample storage or sampling in future studies.

4. Conclusions

Advanced analytical methods for detecting antimicrobial chemicals in water is constantly needed, considering the growing interest in investigating their aquatic occurrence at (inter)national level for monitoring and regulation purposes. We investigated the effectiveness of WCX sorbents for extracting various antimicrobial classes from water. The new method we developed was successfully validated for 53 compounds using LC-MS/MS with direct injection applicability, and for 35 compounds across different water matrices using SPE-LC-MS/MS. Most compounds excluded during method development and validation were not a high priority in the study context (Table S2). We refined the methodology with comprehensive knowledge of antimicrobial stability in different scenarios, to help minimize uncertainties related to storage of standard solutions and samples, and use of preservatives and materials. In a novel approach comparing MQLs with MICs, we assessed the microbiological sensitivity of the method and its suitability for studying the influence of antibacterials on AMR development. The method successfully detected 10 commonly used antimicrobials in hospital and municipal wastewater and in groundwater.

CRediT authorship contribution statement

Valentina Ugolini: Investigation, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Visualization. **Foon Yin Lai:** Conceptualization, Resources, Methodology, Validation, Data curation, Writing – review & editing, Supervision, Project

administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.342029.

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Supplementary Information (SI)

Novel, alternative analytical methodology for determination of antimicrobial chemicals in aquatic environments and public use assessment: Extraction sorbent, microbiological sensitivity, stability, and applicability

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SUPPLEMENTARY SECTION

Chemicals and materials

Reference standards of the targeted antimicrobial chemicals and mass-labelled chemicals (internal standard, IS) were purchased from Sigma-Aldrich, Santa Cruz Biotechnology and LGC Limited, as neat powders or organic solutions with purity ≥ 98%. For chemicals as neat powders, individual stock solution was prepared by dissolving the powders in methanol (MeOH) or acetonitrile (ACN). Chemicals as neat powders that were not dissolvable in these solvents were dissolved in MilliQ water and then diluted into MeOH or ACN as the stock solution with less than 5% of the aqueous content. Individual stock solution was obtained in a range of 50 to 1000 ng μL^{-1} and stored in amber bottles at -80°C. A mixture working solution of the native antimicrobial chemicals was prepared in MeOH at 10 ng μL^{-1} and kept in amber vials at -80°C. The organic solvents, MeOH and ACN, were LC-MS grade quality. Formic acid (>99%), acetic acid (>99%), ammonium acetate ($C_2H_7NO_2$, 25%), hydrochloric acid (HCl, 30%) were at analytical-grade or LC-grade. The solvents, acids and bases were purchased from Merck and Sigma-Aldrich. MilliQ water (LC-PAK) was generated at the laboratory from a Milli-Q® IQ-7000 purification system with filters of a 0.22 µm Millipak Express membrane and an LC-PAK polishing unit by Merk Millipore (Billercia, MA, USA). Solution of Na2EDTA (0.1M) was purchased from Fisher Scientific. Potassium sulfite (K_2SO_3), sodium metabisulfite ($Na_2S_2O_5$) and sodium azide (NaN_3) were purchased from Sigma-Aldrich. Waters® Extraction Manifold with 20 positions and a self-gauging vacuum system, as well as SPE cartridges, including Oasis® HLB (200 mg, 6 cc), MCX (150 mg, 6 cc) and WCX (150 mg, 6 cc) were purchased from Waters®. Glass microfiber filters (Whatman® GF/D) were purchased from Sigma-Aldrich. HPLC analytical columns (100x2.1 mm, 2.6 µm), including Kinetex® EVO, C18 and biphenyl columns, were purchased from Phenomenex®.

LC-MS/MS method optimization

Chemical standards (0.5 to 1 ng μL^{-1} in MeOH) were infused onto the MS to obtain the optimal ionization mode and MRM parameters of the analytes based on their peak intensity. The majority of our target analytes was ionized with positive ESI, as amino groups are easily protonated to result in $[M+H]^+$. Three analytes were noticed to be ionized as $[M+2H]^+$ (teicoplanin) and $[M+3H]^+$ (vancomycin and ceftazidime), due to multiple protonation in their highly complex molecular structures. Previous studies reported similar ionization of vancomycin and teicoplanin.[1,2] Some analytes showed higher peak intensity in negative ESI for $[M-H]^-$, as deprotonation was facilitated by hydroxyl groups. Two product ions among the highest intensity were chosen as the quantification and confirmation MRMs of the analytes (Table S3).

Different columns and mobile phases were evaluated to optimize the chromatographic separation under the same LC gradient program (Figure S3B) and flow rate (0.5 mL min^{-1}). Phenomenex® HPLC columns (100x2.1 mm, 2.6 µm), including Kinetex® EVO, C18 and biphenyl columns, were tested. For the positive ESI run, we focused on using (A) 0.1% formic acid Milli-Q water and (B) 0.1% formic acid MeOH, as the mobile phases, since protic solvents help enhance protonation in positive ESI. EVO column was excluded as meropenem and hydroxychloroquine did not elute within the run time. Comparing C18 and biphenyl columns, early elution (within the first minute of the run) of some analytes with C18 columns was observed, especially for lamivudine and hydroxy-metronidazole (Figure S4), whereas better separation and

retention of the target analytes were obtained using a biphenyl column. Thus, the biphenyl column was selected for further usage in our study. With this column, another mobile phase of (A) MilliQ water and (B) ACN with 0.1% formic acid each was tested. This, however, did not result in improved peak intensity, and even worsened the analytes' peak separation (Figure S5). Hence, MeOH is reassured as better organic solvent in our study.

A few analytes were excluded from this study for further validation because of poor signal intensity (i.e., teicoplanin) and poor retention (i.e., amikacin, ceftazidime, colistin, fosfomycin, imipenem, spectinomycin and tobramycin) in any of the three columns. This can be explained by the fact that these analytes are highly polar with very low logD values (-18 to -2 at pH 6.5) (Table S1). Amoxicillin was also excluded as it showed substantial degradation at the working solution, as previously reported.[3,4]

With the optimal column and mobile phases, LC gradients were further adjusted (Figures S3C and S3A) so that the more hydrophobic analytes, including darunavir, remdesivir, clotrimazole, miconazole, ritonavir, lopinavir, piperacillin and fusidic acid, did not elute during the column washing step with full organic solvent, reducing potential interferences (Figure S6).

To maintain high sample throughput in the future analysis, the biphenyl column and LC-gradient (Figure S3A) established were kept as our choice for optimizing the chromatography of the negative ESI analytes. Two mobile phase options were compared, including (i) MilliQ water and methanol with 10 mM ammonium acetate each, and (ii) MilliQ water and MeOH with 0.1% acetic acid each. Tenofovir, cefaclor, doxycycline, 4-epinhydrotetracycline, cefixime and cefotaxime were not eluted using the option (i). The chromatogram, considering analytes' peak intensities and separation, was generally better with the option (ii) than (i). Imipenem was not eluted with any of the mobile phase options and was therefore excluded from the study.

SPE-LC-MS/MS method optimization

Sample extraction

Na_2EDTA. The recovery overall remained similar for most of the analytes with and without the use of Na_2EDTA in sample preparation (Figure S13). The main influence observed was between two antimicrobial classes, cephalosporins of β-lactams and macrolides. With Na_2EDTA in influent wastewater, cephalosporins (except cefepime) were no longer recovered. This group of antimicrobial was not recoverable in MilliQ water with and without Na_2EDTA. Similar result in both water matrices was also noticed for ampicillin. For macrolides, the recovery remained similar with Na_2EDTA in influent wastewater, but largely improved in MilliQ water (e.g., clarithromycin, tylosin, vancomycin). Doxycycline was recovered in influent wastewater only with the presence of Na_2EDTA. As a chelating agent of metals, Na_2EDTA was commonly used in previous antimicrobial analyses.[e.g.5–10] Our results were consistent to other studies, especially the positive influence of Na_2EDTA on macrolides' recovery.[6,11] Unlike previous studies,[12] fluoroquinolones were not negatively influenced with Na_2EDTA in our study. Na_2EDTA was included for further method validation, primarily in favor of macrolides, supporting its importance as the newly-added priority substances (e.g., clarithromycin, erythromycin) in the European surface environment under the EU Water Framework Directive.[13]

SI FIGURES

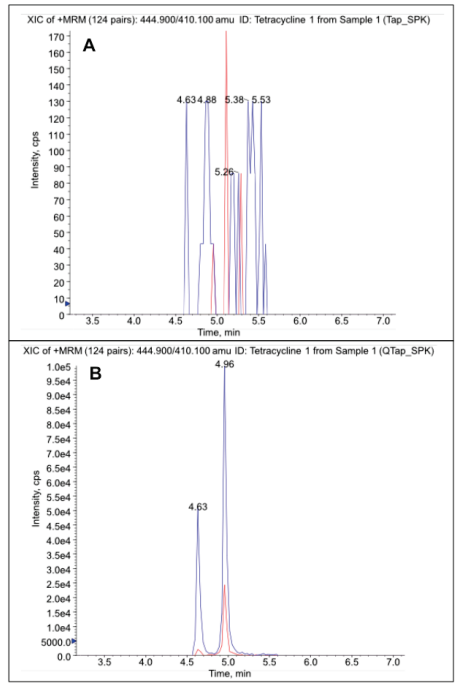

Figure S1. Signal intensity of tetracycline in tap water (50 ng mL^{-1}) without (A) and with (B) the quenching agent potassium sulfite.

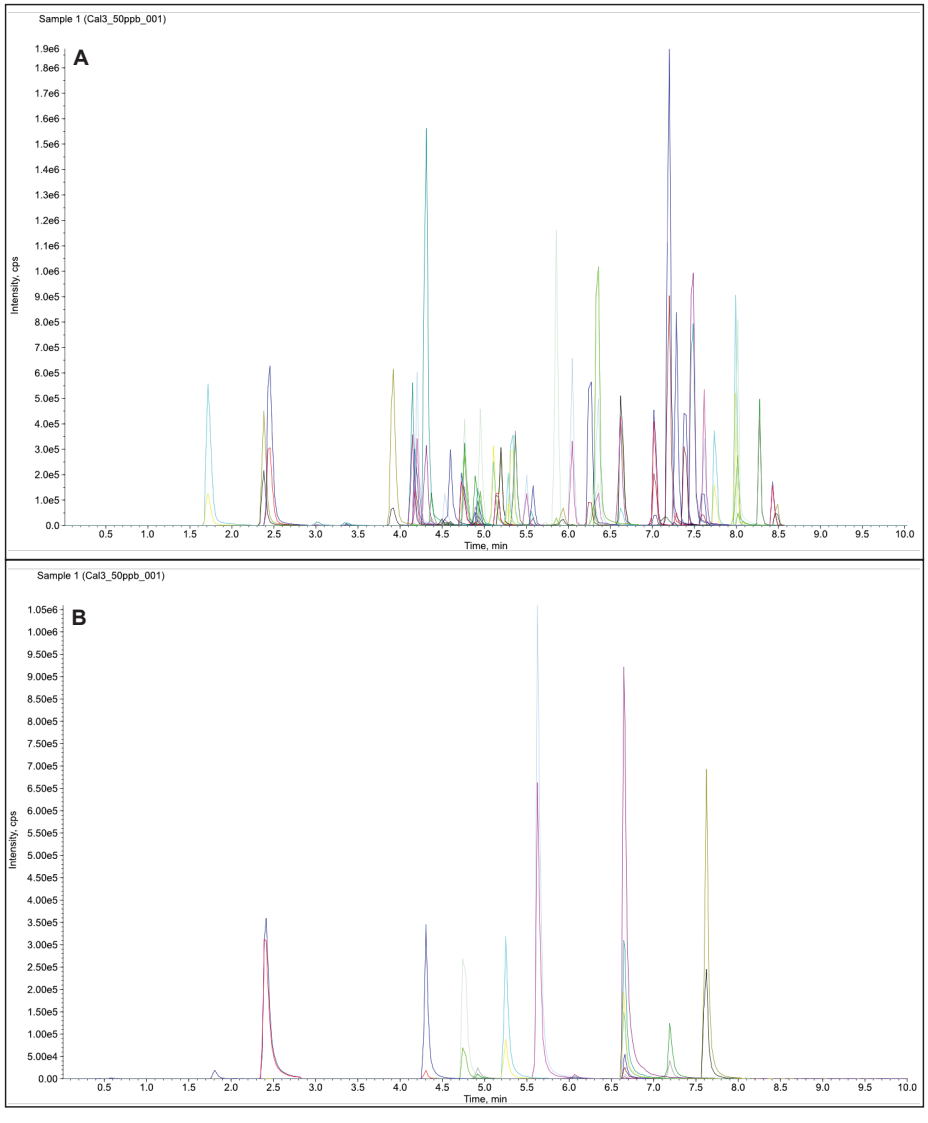

Figure S2. Chromatograms of a neat standard (50 ng mL^{-1}) using the developed LC-MS/MS method in A) ESI+ and B) ESI-.

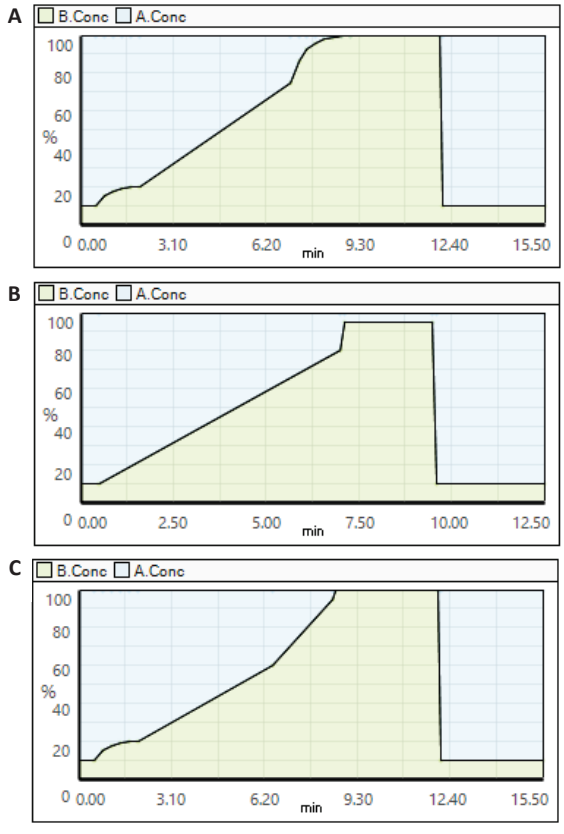

Figure S3. A) LC gradient: 0.5 min, 10% B (convex gradient, -3); 2 min, 20% B; 7 min, 75% B (convex gradient, -4); 9 min, 100% B; 12 min, 100% B; 12.1 min, 10% B; 15.5 min 10% B. Total run time 15.5 min. **B)** LC gradient: 0.5 min, 10% B; 7 min, 80% B; 7.1 min, 95% B; 9.5 min, 95% B; 9.6 min 10% B, 12.5 min, 10% B. The total run time was 12.5 min. **C)** LC-gradient A consisted of 0.5 min, 10% B (convex gradient, -3); 2 min, 20% B; 6.5 min, 60%; 8.5 min, 95%; 8.6 min, 100% B; 12 min, 100% B; 12.1 min, 10% B; 15.5 min, 10% B. Total run time 15.5 min.

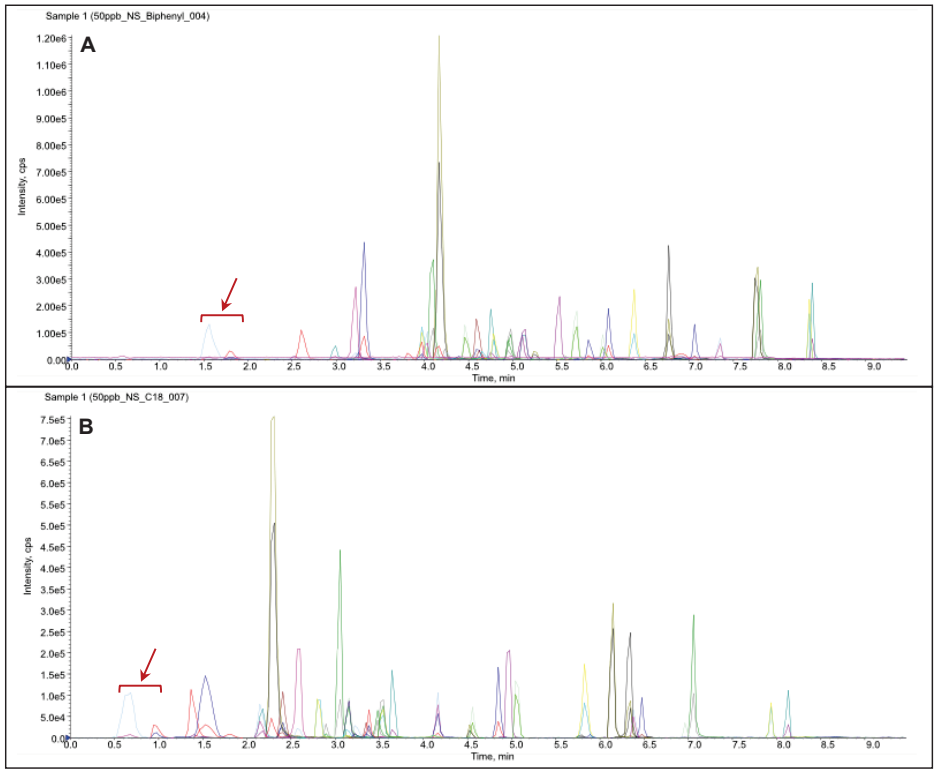

Figure S4. Comparison of chromatograms using different Phenomenex® HPLC-columns: (A) Kinetex® Biphenyl (100x2.1 mm, 2.6 µm) and (B) Kinetex® C18 (100x2.1 mm, 2.6 µm). Lamivudine and hydroxy-metronidazole (red arrow) were early eluted (RT <1 min) using Kinetex C18.

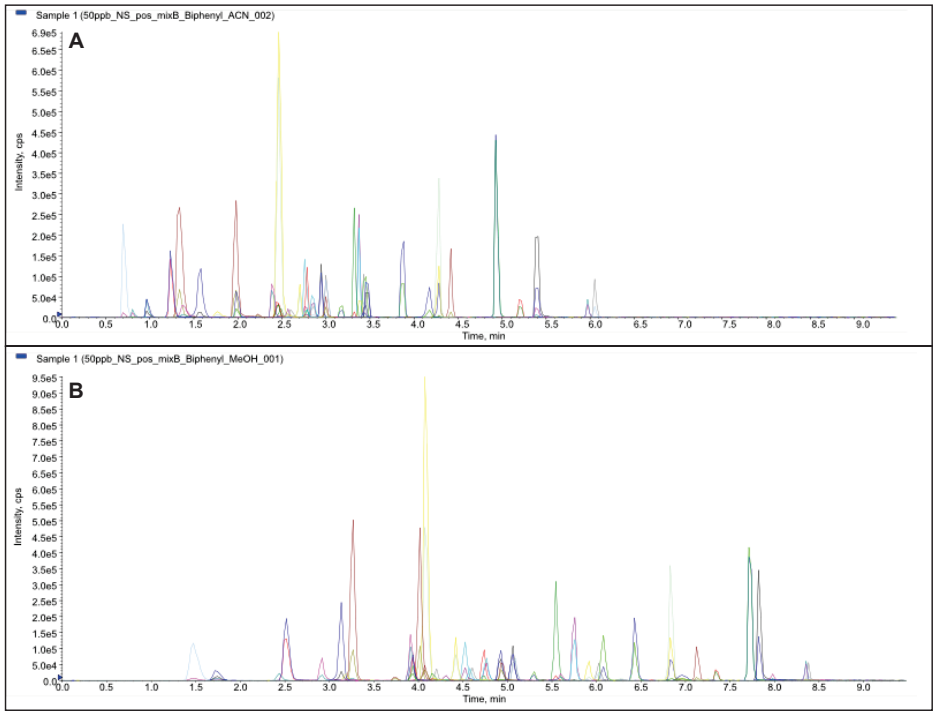

Figure S5. Comparison of chromatograms on a biphenyl column using different solvents as organic mobile phase: (A) acetonitrile with 0.1% FA and (B) methanol with 0.1% FA.

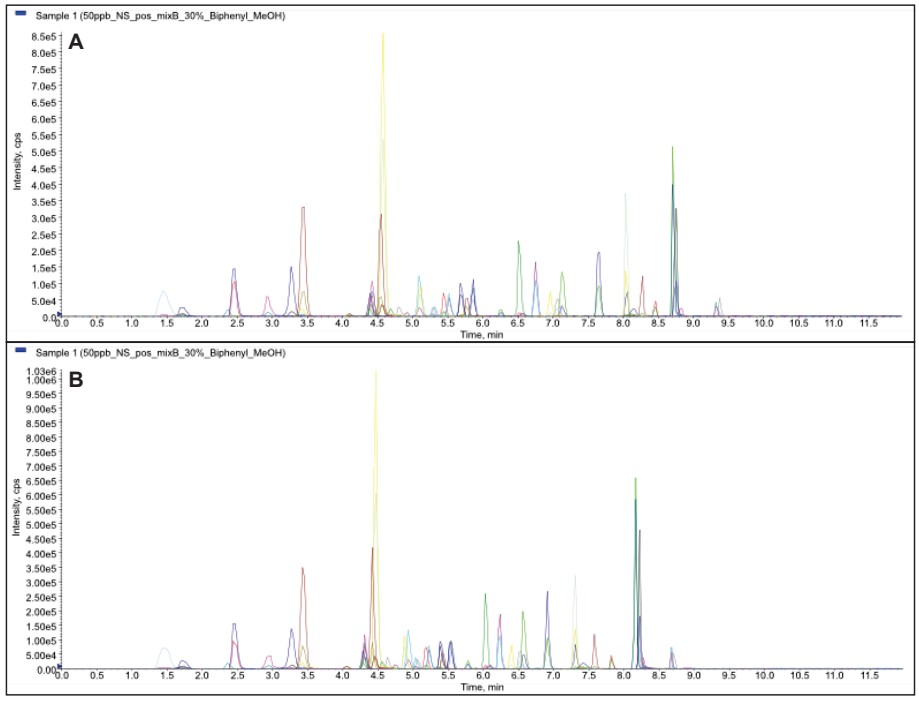

Figure S6. Comparison of chromatograms based on a biphenyl column with methanol with 0.1% FA as organic solvent but using different LC-gradient: (A) LC-gradient in Figure S3C and (B) LC-gradient in Figure S3A. All analytes were eluted before 9 min (column wash step) in (B).

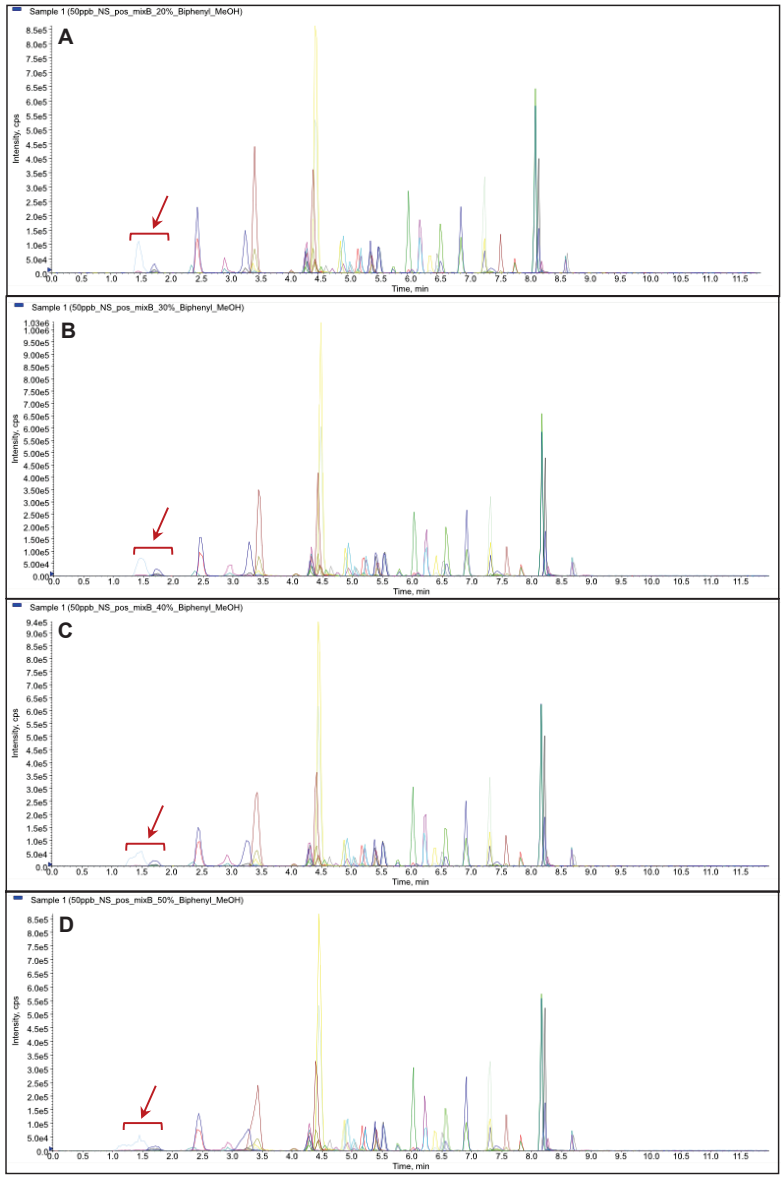

Figure S7. Comparison of chromatograms with different organic solvent content in samples: (A) 20%, (B) 30%, (C) 40% and (D) 50%. Note the difference in the early eluted peak (red arrow), where the peak quality decrease with increasing organic content.

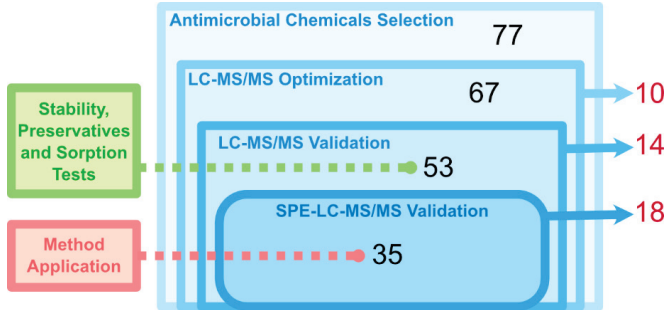

Figure S8. Numbers of the analytes that are included and eliminated during the method optimization and validation processes. Analytes (n=10) excluded after LC-MS/MS optimization are amikacin, amoxicillin, ceftazidime, colistin, fosfomycin, gentamicin, imipenem, spectinomycin, teicoplanin and tobramycin. Analytes (n=14) excluded after LC-MS/MS validation are abacavir, cefotaxime, clotrimazole, darunavir, emtricitabine, entacapone, lamivudine, linezolid, lopinavir, nevirapine, rifampicin, ritonavir, sulfapyridine and tylosin. Analytes (n=18) excluded after SPE-LC-MS/MS validation are 4-epianhydrotetracycline, ampicillin, cefaclor, cefadroxil, cefalexin, cefepime, cefixime, cefotaxime, cefoxitin, fusidic acid, lincomycin, mecillinam, metronidazole-OH, oxytetracycline, piperacillin, sulfadiazine, tenofovir and vancomycin. Eliminated analytes are not ranked in top priority (Table S2).

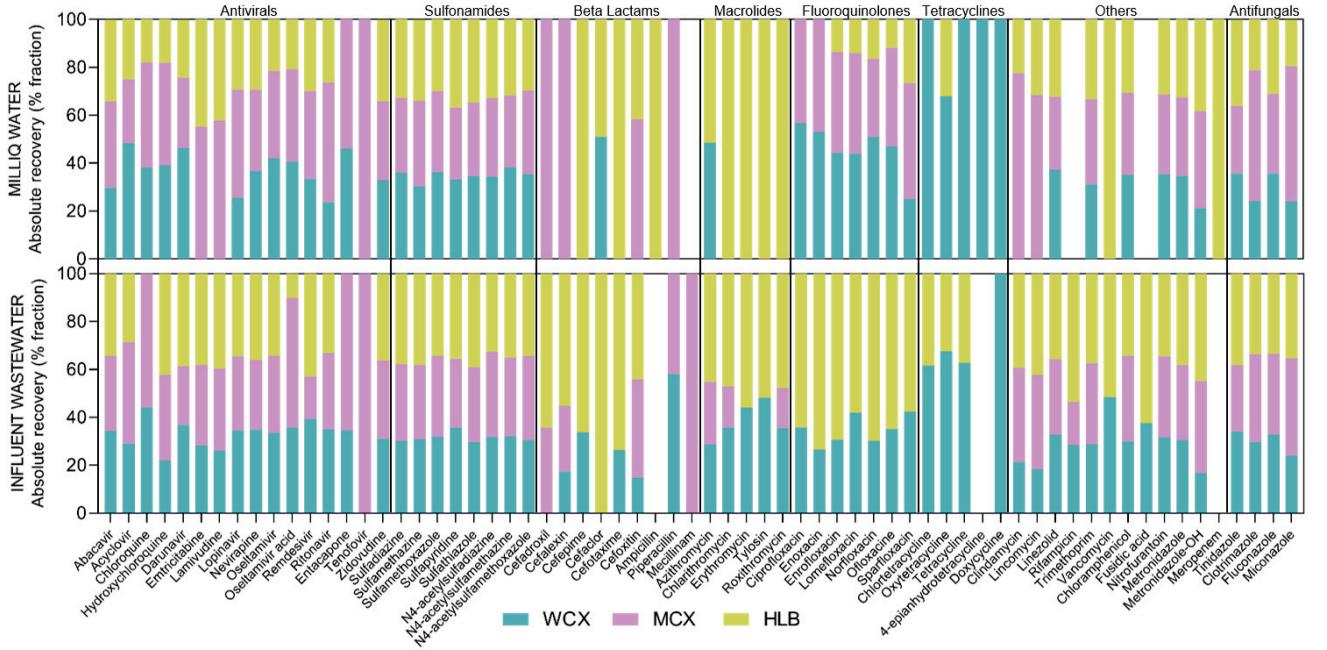

Figure S9. Proportion (%) of absolute recoveries using different sorbent types: Oasis® WCX (green), Oasis® MCX (pink) and Oasis® HLB (yellow) in MilliQ water (top) and influent wastewater (bottom).

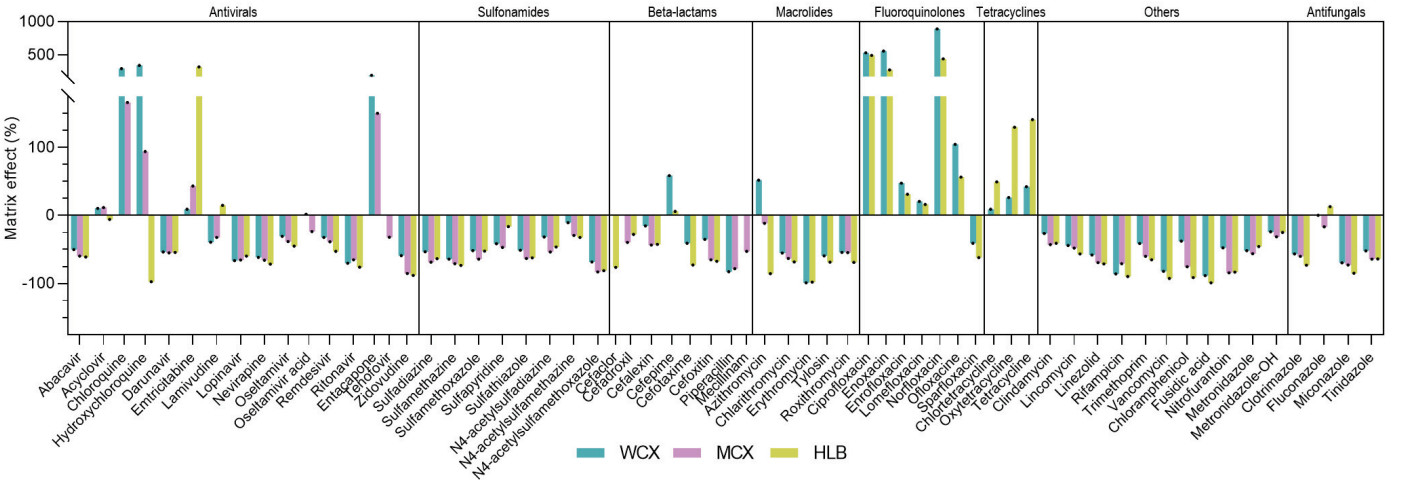

Figure S10. Comparison of matrix effects in influent wastewater using Oasis® WCX (green), Oasis® MCX (pink) and Oasis® HLB (yellow) sorbents.

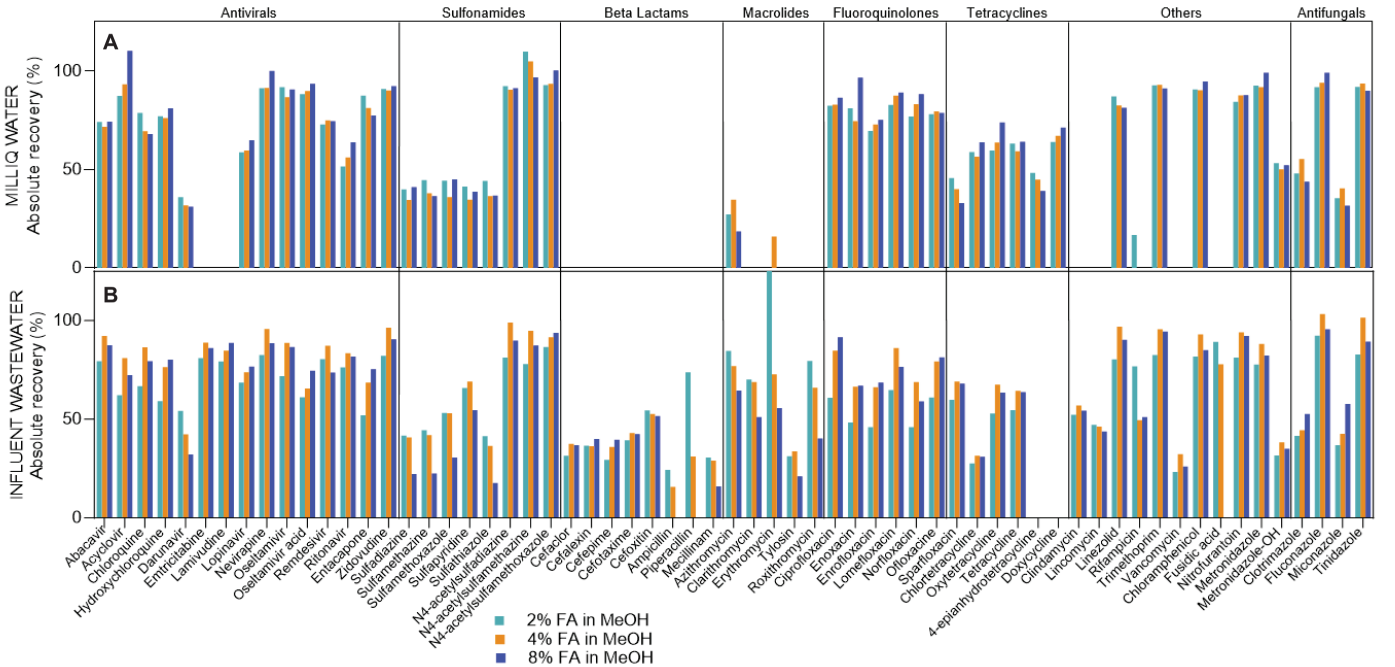

Figure S11. Absolute recoveries (>15%) of the analytes in (A) MilliQ water and (B) influent wastewater using WCX with three different serial elution solutions: (i) methanol and 2% formic acid methanol (green), (ii) methanol and 4% formic acid methanol (orange) and (iii) methanol and 8% formic acid methanol (blue).

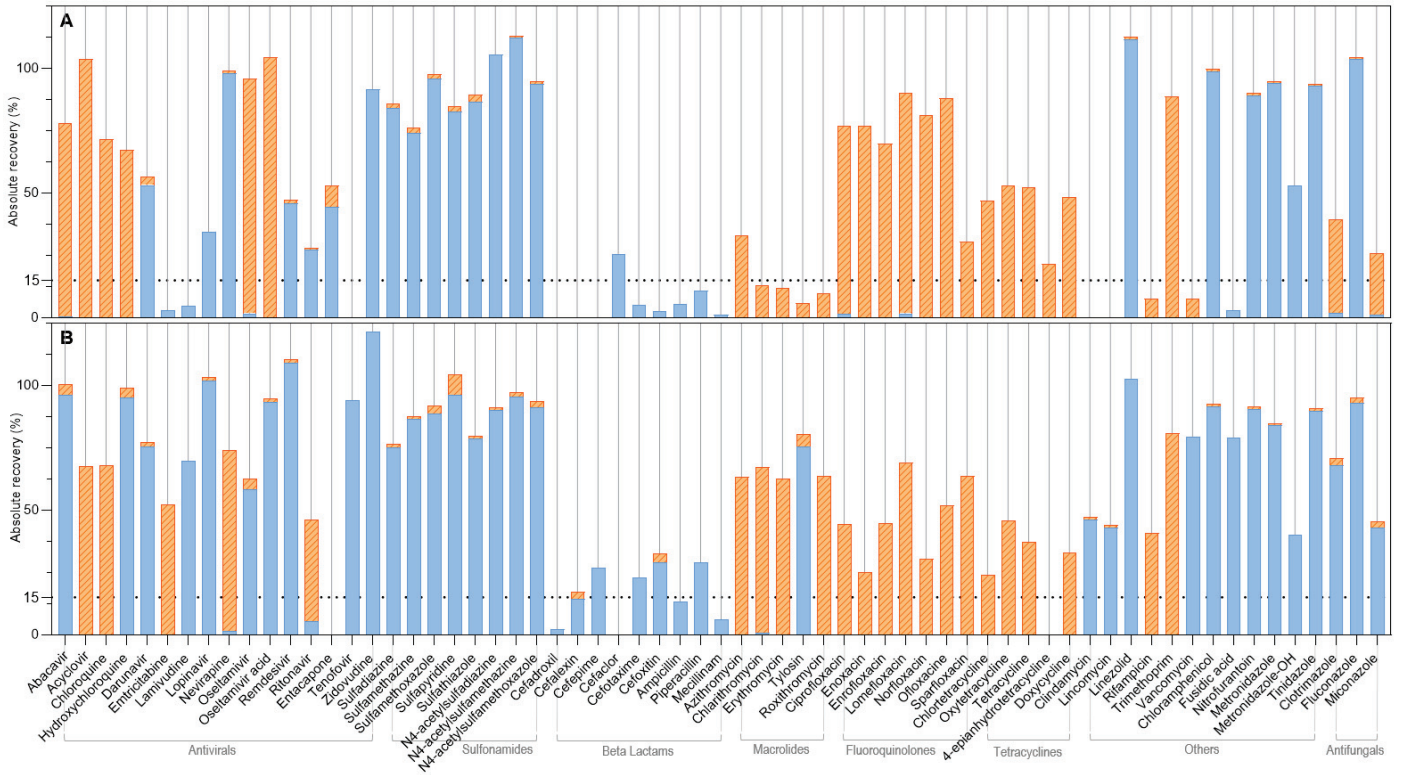

Figure S12. Absolute recoveries of the analytes in (A) MilliQ water and (B) influent wastewater using WCX and eluting with methanol (fraction 1, blue) and 2% formic acid in methanol (fraction 2, orange and line pattern).

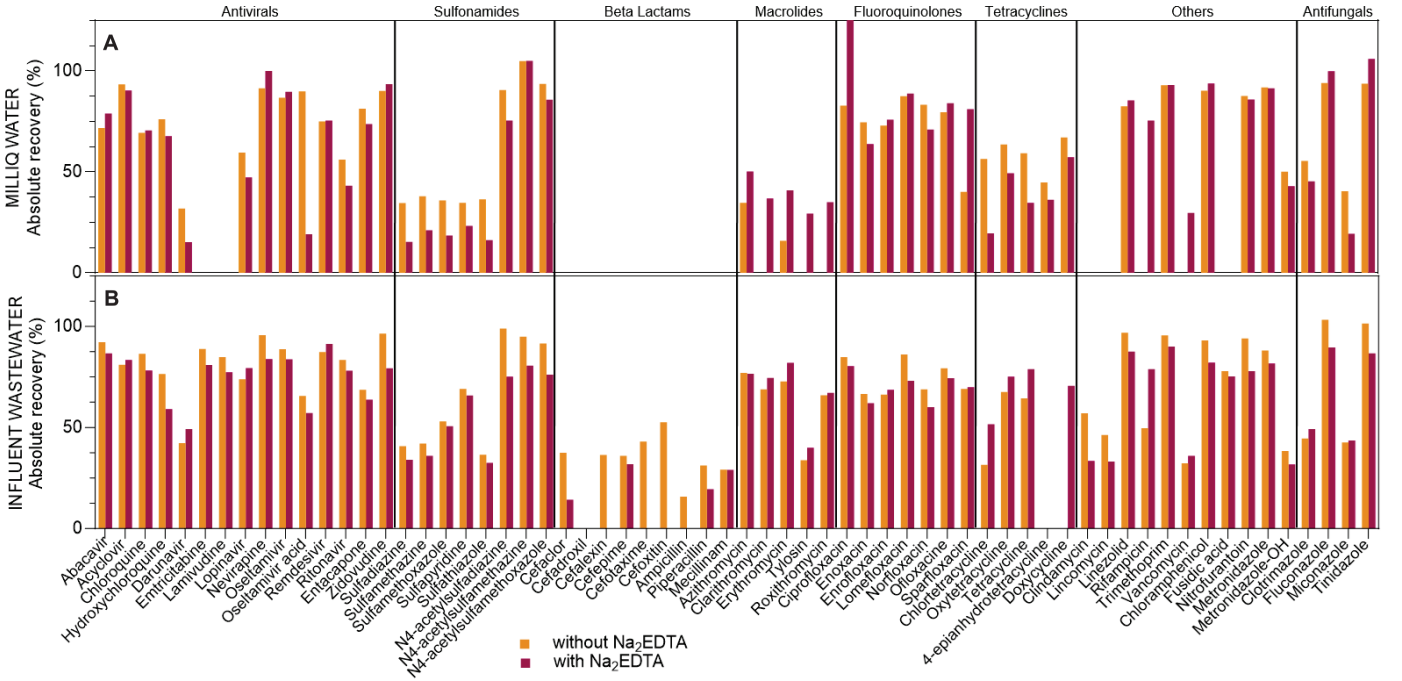

Figure S13. Absolute recoveries of the analytes in (A) MilliQ water and (B) influent wastewater with Na_2EDTA (red) and without Na_2EDTA (orange) addition in sample treatment prior to SPE using WCX and elution with a serial solution of methanol and 4% formic acid in methanol.

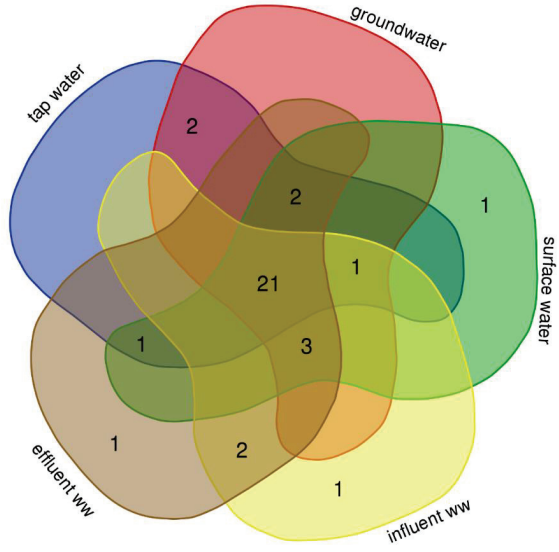

Figure S14. Venn diagram representing the number of compounds validated (fulfilled between-run recovery 50-150% and between-run precision RSD<25% at the mid-level) in different water matrices, including tap water (*n*=27), groundwater (*n*=29), surface water (*n*=29), influent wastewater (ww) (*n*=28) and effluent wastewater (ww) (*n*=30).

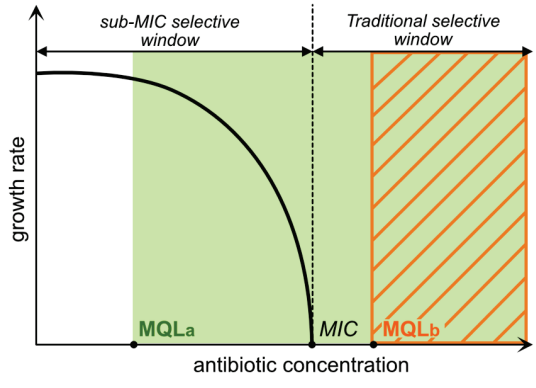

Figure S15. Schematic principle of microbiological sensitivity for our developed analytical method. The microbial community grows at a certain rate (y-axis) until the increasing antibiotic concentration (x-axis) cause growth inhibition (i.e., minimum inhibitory concentration (MIC)) (see black curve line). Sub-MIC (without growth inhibition) and traditional selective windows (with growth inhibition), at which AMR development occurs, are identified by Gullberg et al., 2011.[14] In scenario (a), the MQL_a is below MIC; the analytical method is therefore able to cover both windows, showing a high microbiological sensitivity. In scenario (b), the MQL_b is above MIC; the analytical method shows a lower microbiological sensitivity as it covers only the traditional selective window.

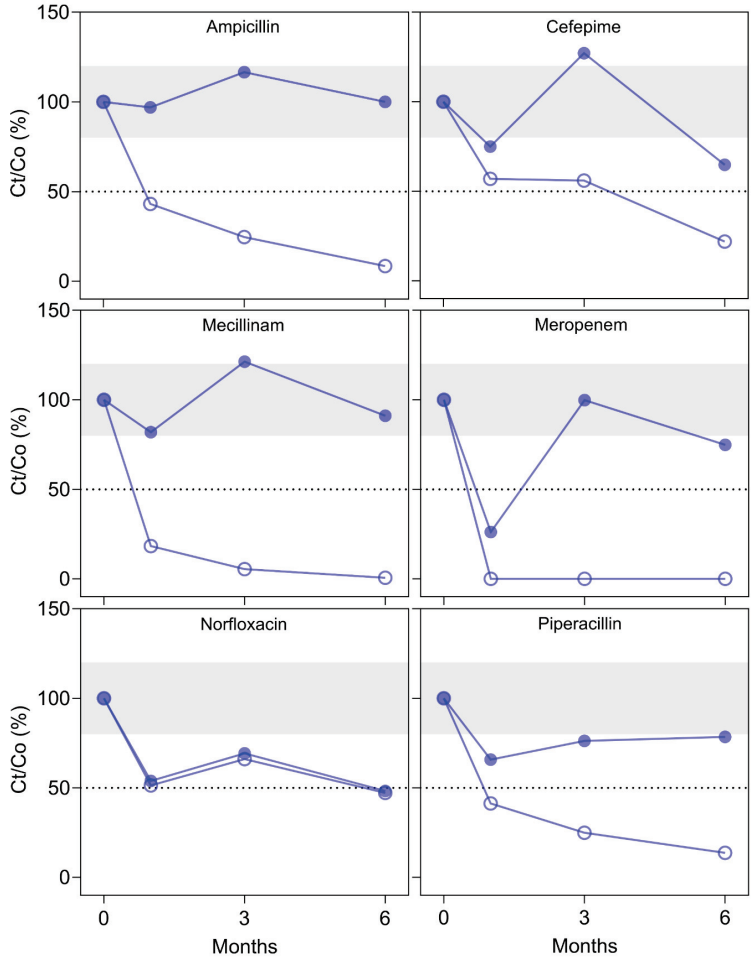

Figure S16. The most unstable antimicrobials (degradation >50%) in working solutions (10 µg mL^{-1}) stored for 6 months in methanol at -80 °C (filled circle) and at -20 °C (empty circle).

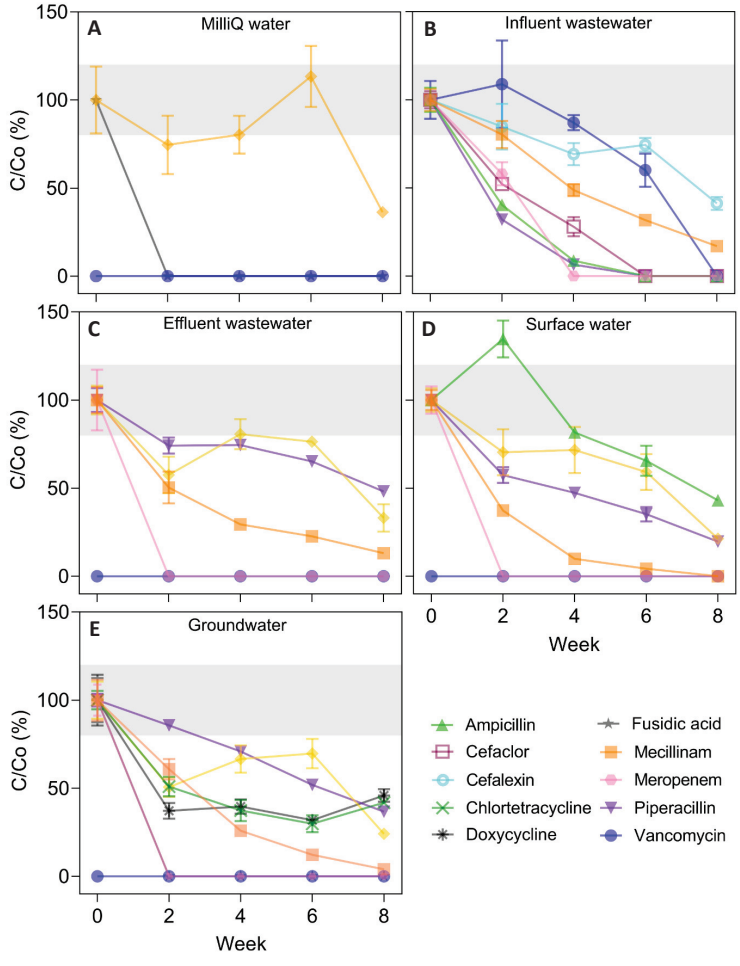

Figure S17. The most unstable antimicrobials (degradation>50%) in different water matrices, including A) MilliQ water, B) influent wastewater, C) effluent wastewater, D) surface water and E) groundwater, and stored up to 8 weeks at -20 °C. Vancomycin was only detected in influent wastewater at time 0 but not in other water matrices.

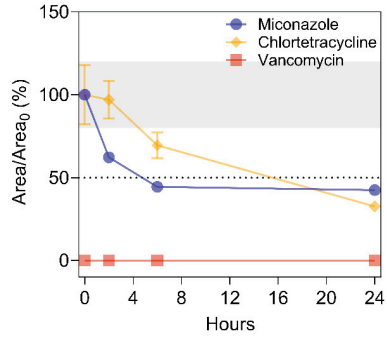

Figure S18. The most unstable antimicrobials (degradation >50%) in surface water stored at 4 °C for 24 hours.

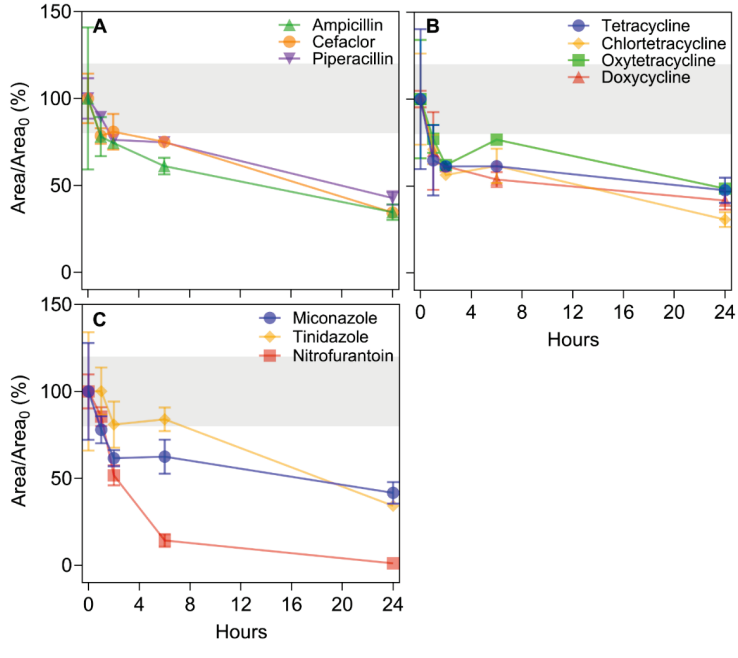

Figure S19. The most unstable antimicrobials (degradation >50%) in influent wastewater stored at 20 °C for 24 hours. A) β-lactams, B) tetracyclines and C) nitrofurantoin and antifungals.

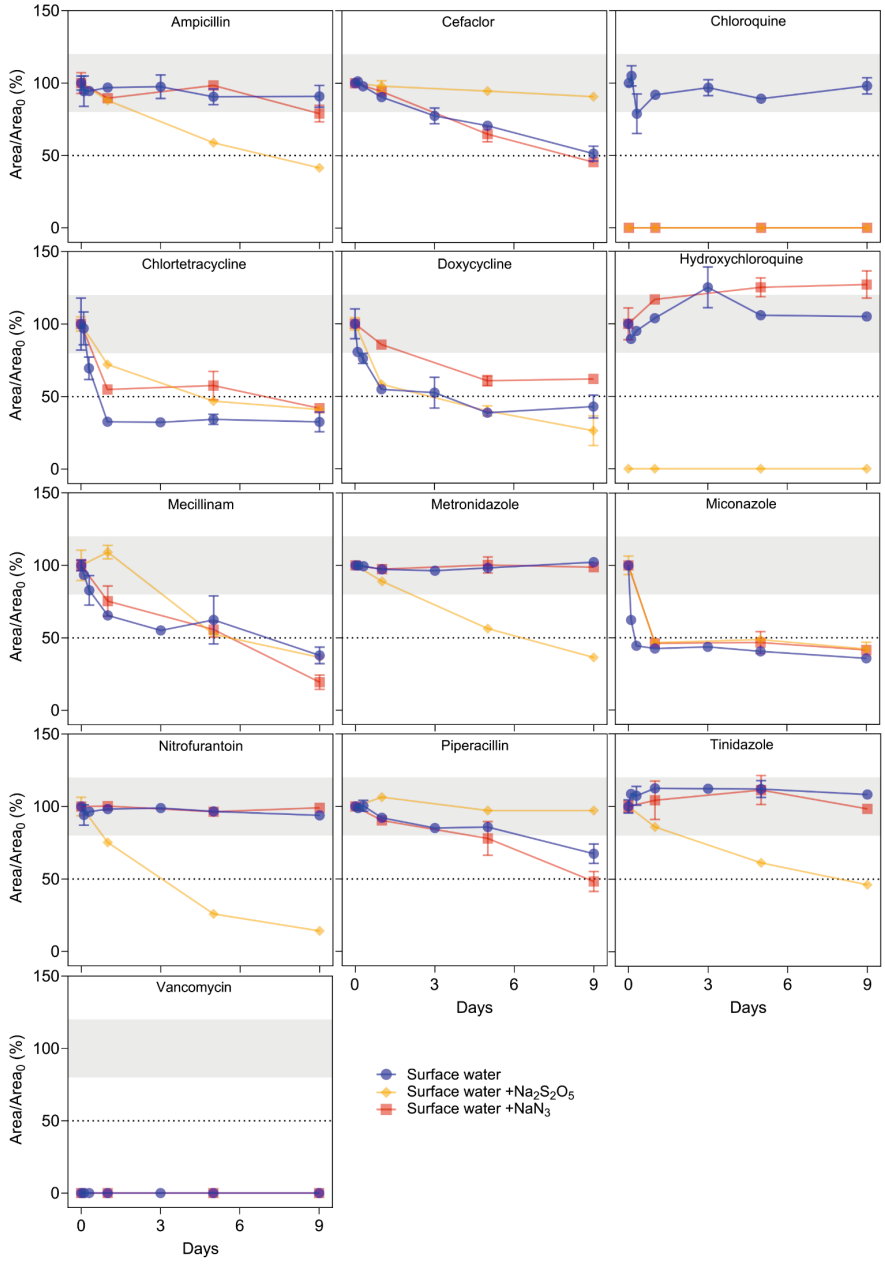

Figure S20. The most unstable antimicrobials in refrigerated conditions (4 °C) in surface water (●) and with the addition of the two biocides used as preservatives, sodium metabisulfite ($Na_2S_2O_5$, ♦) and sodium azide (NaN_3, ■).

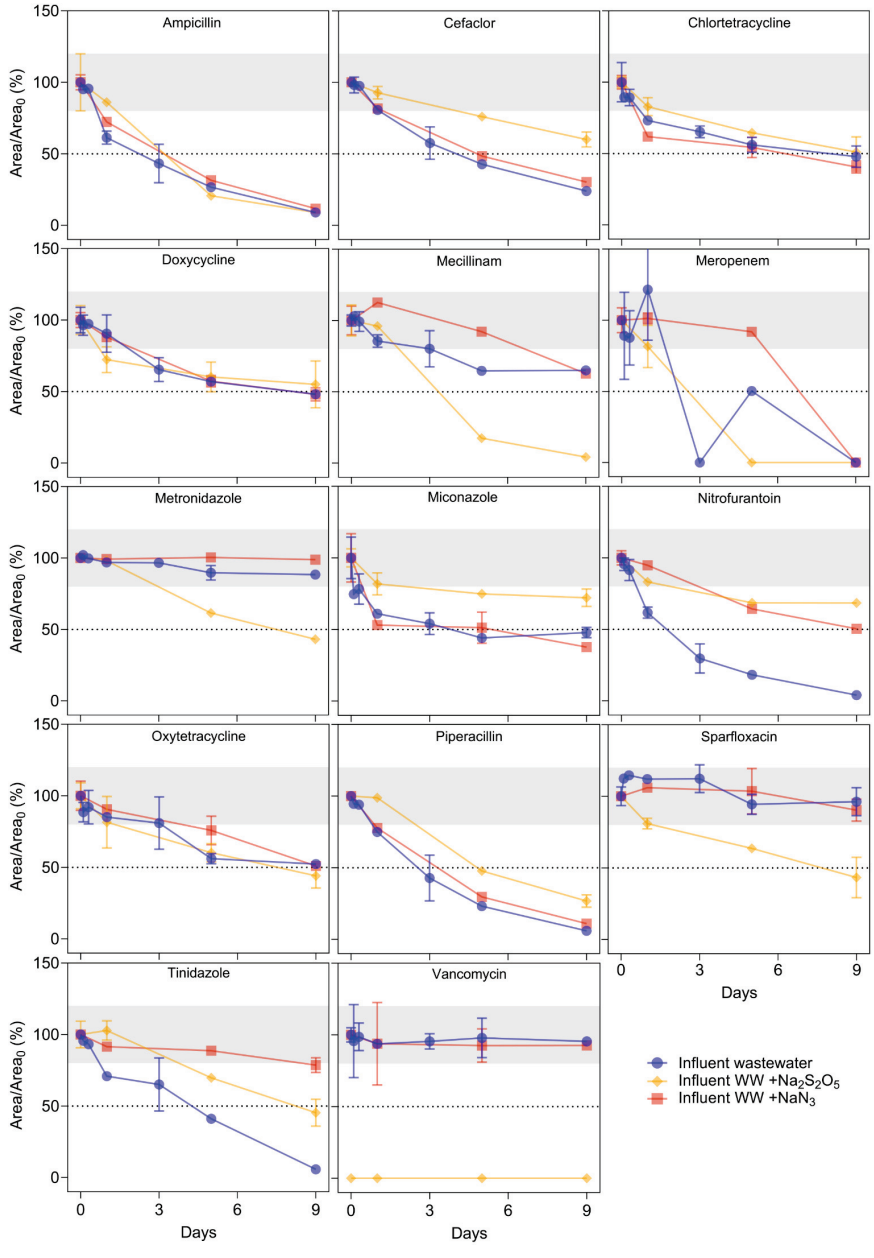

Figure S21. The most unstable antimicrobials in refrigerated conditions (4 °C) in influent wastewater (●) and with the addition of the two biocides used as preservatives, sodium metabisulfite ($Na_2S_2O_5$, ♦) and sodium azide (NaN_3, ■)

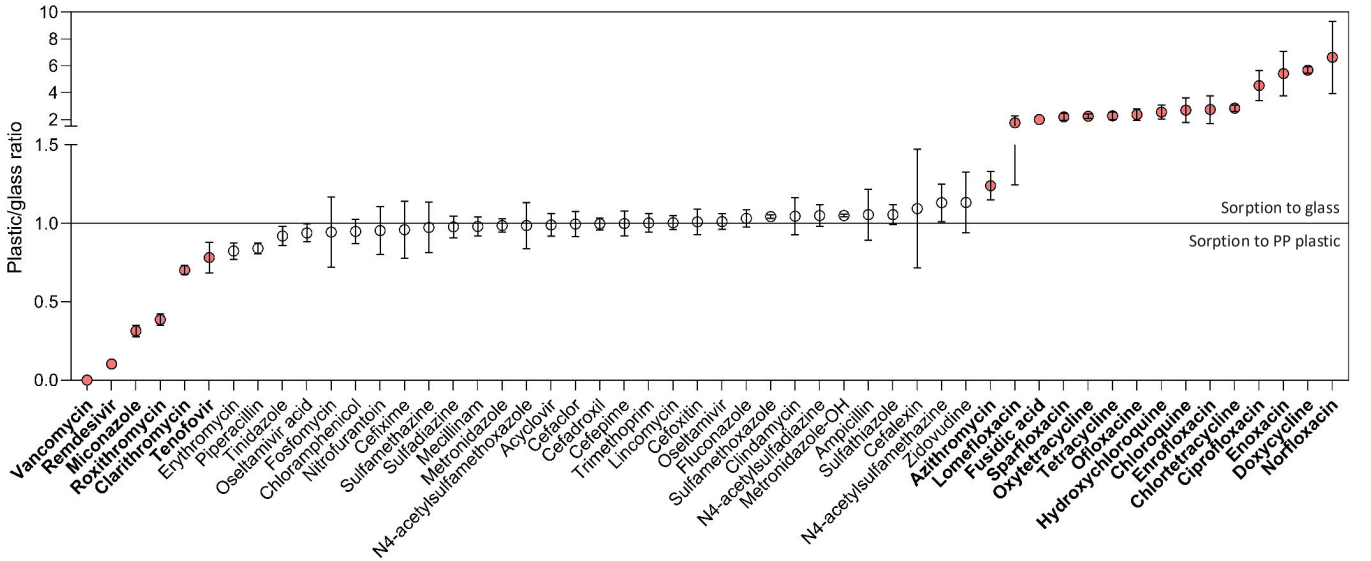

Figure S22. Sorption experiments of the analytes between materials of glass HPLC vials and PP plastic tubes.

SI TABLES

Table S1. List of antimicrobials included in the LC-MS/MS method development.

n	Analytes	Antimicrobial type		Class	MM (g/mol)	Structure	log k_{ow}[a]	logD[b,c]	State of charge[b,c]	pK_a[c,d]	pK_a[e]	Human excretion rate (%)
1	4-Epianhydrotetracycline	HM	antibacterial	tetracyclines	426.14	$C_{22}H_{23}ClN_2O_7$	1.77	-1.28	-0.0077	3.32, 8.13	4.5; 5.7	-
2	Abacavir	PC	antiviral	nucleoside reverse transcriptase inhibitors (NRTIs)	286.15	$C_{14}H_{18}N_6O$	1.22	-0.508	0.9867	0.76, 6.87, 15.43	0.4; 5.1	-
3	Acyclovir	PC	antiviral	synthetic nucleoside analogues	225.20	$C_8H_{11}N_5O_3$	-1.56	-1.55	-0.0001	0.6, 10.16	2.27; 9.3	-
4	Amikacin	PC	antibacterial	aminoglycosides	585.29	$C_{22}H_{43}N_5O_{13}$	-4.52	-19.15	3.9889	8.22, 8.42, 8.98	7.64; 8.05, 8.81	67-79[15]
5	Amoxicillin	PC	antibacterial	beta-lactams (penicillins)	365.10	$C_{16}H_{19}N_3O_5S$	0.87	-2.32	-0.0553	3.23, 7.22	2.9; 7.4	43-57[16]
6	Ampicillin	PC	antibacterial	beta-lactams (penicillins)	349.10	$C_{16}H_{18}N_3NaO_4S$	1.27	-2.02	-0.0541	3.24, 7.23	2.5; 7.3	50[17]
7	Azithromycin	PC	antibacterial	macrolides	748.51	$C_{38}H_{72}N_2O_{12}$	4.02	-3.990	1.9992	9.08, 11.16	8.74	6-12[18]
8	Cefaclor	PC	antibacterial	beta-lactams (cephalosporins)	367.04	$C_{15}H_{14}ClN_3O_4S$	0.34	-2.326	-0.055	2.63, 7.23	7.07	54[19]
9	Cefadroxil	PC	antibacterial	beta-lactams (cephalosporins)	363.39	$C_{16}H_{17}N_3O_5S$	0.12	-2.457	-0.0514	3.45, 7.22	2.48; 7.37; 9.64	>90
10	Cefalexin	PC	antibacterial	beta-lactams (cephalosporins)	347.09	$C_{16}H_{17}N_3O_4S$	0.53	-2.153	-0.0502	3.45, 7.23	2.53; 7.13	70-100[20]
11	Cefepime	PC	antibacterial	beta-lactams (cephalosporins)	480.12	$C_{19}H_{24}N_6O_5S_2$	-1.43	-3.45	0.0058	2.94, 3.69, 11,15	1.12; 3.07; 10.8	85
12	Cefixime	PC	antibacterial	beta-lactams (cephalosporins)	453.04	$C_{16}H_{15}N_5O_7S_2$	-1.3	-5.433	-1.9853	2.63, 4.10, 11.06	2.06; 2.7; 3.72	50
13	Cefotaxime	PC	antibacterial	beta-lactams (cephalosporins)	455.06	$C_{16}H_{17}N_5O_7S_2$	0.63	-3.427	-0.9956	2.86, 3.65, 11.04	2.21; 3.15; 10.87	54
14	Cefoxitin	PC	antibacterial	beta-lactams (cephalosporins)	427.05	$C_{16}H_{17}N_3O_7S_2$	0.29	-2.095	-0.9962	3.59, 10.97	2.75	85
15	Ceftazidime	PC	antibacterial	beta-lactams (cephalosporins)	546.10	$C_{22}H_{22}N_6O_7S_2$	-1.45	-5.938	-0.9892	2.54, 4.04, 10.88	2.91; 3.81	83-90
16	Chloramphenicol	PC	antibacterial	amphenicols	322.01	$C_{11}H_{12}Cl_2N_2O_5$	0.98	0.8787	0	10.89	11.03	9.5
17	Chloroquine	PC	antiviral	antimalarials	319.10	$C_{18}H_{26}ClN_3$	4.6	-0.695	1.9797	7.68, 10.33	4; 8.4; 10.2	-
18	Chlortetracycline	PC	antibacterial	tetracyclines	478.11	$C_{22}H_{23}ClN_2O_8$	-0.62	-2.92	-0.0568	-	3.33; 7.55; 9.33	>50 (fecal)
19	Ciprofloxacin	PC	antibacterial	fluoroquinolones	331.13	$C_{17}H_{18}FN_3O_3$	0.16	-0.936	0.2958	5.33, 8.77	3.01; 6.14; 8.7	60
20	Clarithromycin	PC	antibacterial	macrolides	747.48	$C_{38}H_{69}NO_{13}$	3.16	0.3554	0.999	9, 12.46	8.99	22
21	Clindamycin	PC	antibacterial	lincosamides	424.18	$C_{18}H_{33}ClN_2O_5S$	2.16	-0.521	0.9727	7.55, 12.41	6.9	13.6
22	Clotrimazole	PC	antifungal	imidazoles	344.10	$C_{22}H_{17}ClN_2$	5.44	5.5546	0.6473	6.26	5.49	0.5
23	Colistin	PC	antibacterial	polypeptides	1154.75	$C_{52}H_{98}N_{16}O_{13}$	-2.29	-22.68	4.9986	10.24	10	60
24	Darunavir	PC	antiviral	protease inhibitors (Pis)	547.24	$C_{27}H_{37}N_3O_7S$	2.9	2.8167	0.0002	2.39, 13.59	2.39; 13.59	-
25	Doxycycline	PC	antibacterial	tetracyclines	444.15	$C_{22}H_{24}N_2O_8$	0.81	-0.752	0.4422	6, 7.45	3.02; 7.97; 9.15	35-60
26	Emtricitabine	PC	antiviral	nucleoside reverse transcriptase inhibitors (NRTIs)	247.25	$C_8H_{10}FN_3O_3S$	-0.43	-1.31	0	14.29	10.1	-
27	Enoxacin	PC	antibacterial	fluoroquinolones	320.13	$C_{15}H_{17}FN_4O_3$	-0.12	-1.059	0.1992	5.1, 8.68	6; 8	>40
28	Enrofloxacin	PC	antibacterial	fluoroquinolones	359.16	$C_{19}H_{22}FN_3O_3$	0.77	-0.415	0.2946	5.32, 8.72	3.85; 6.19; 7.59	-
29	Entacapone	PC	antiviral	COMT inhibitors	305.29	$C_{14}H_{15}N_3O_5$	2.05	1.3784	-0.4504	6.09	4.5	-
30	Erythromycin	PC	antibacterial	macrolides	733.46	$C_{37}H_{67}NO_{13}$	3.06	-0.288	0.999	9, 12.45	8.89	25
31	Fluconazole	PC	antifungal	triazoles	306.10	$C_{13}H_{12}F_2N_6O$	0.49	0.5609	0.0002	1.7, 2.3, 12.68	1.76	80
32	Fosfomycin	PC	antibacterial	phosphonic antibiotics	138.06	$C_3H_7O_4P$	-2.1	-3.049	-1.0293	1.25, 7.82	2.5; 6.7	93-99
33	Fusidic acid	PC	antibacterial	fusidanes	516.35	$C_{31}H_{48}O_6$	5.54	3.0694	-0.9559	4.66	4.7	<0.5
34	Gentamicin	PC	antibacterial	aminoglycosides	477.32	$C_{21}H_{43}N_5O_7$	-1.31	-15.74	4.956	10.03, 12.55	10.12; 12.55	79
35	Hydroxychloroquine	HM	antiviral	Antimalarials	335.18	$C_{18}H_{26}ClN_3O$	3.72	-1.611	1.9795	7.68, 9.76, 15.59	4; 8.3; 9.7	-
36	Imipenem	PC	antibacterial	Beta-lactams (carbapenems)	299.35	$C_{12}H_{17}N_3O_4S$	-1.16	-3.798	0.0108	3.54, 11.8	3.44; 10.88	70
37	Lamivudine	PC	antiviral	nucleoside reverse transcriptase inhibitors (NRTIs)	229.26	$C_8H_{11}N_3O_3S$	-0.93	-1.237	0.0001	2, 14.29	4.3	-
38	Lincomycin	PC	antibacterial	Lincosamides	406.21	$C_{18}H_{34}N_2O_6S$	0.51	-2.279	0.9894	7.97, 12.37	7.79	40-60
39	Linezolid	PC	antibacterial	oxazolidinones	337.14	$C_{16}H_{20}FN_3O_4$	0.62	0.6367	0	-1.18, 4.85	5.2	65
40	Lomefloxacin	PC	antibacterial	fluoroquinolones	351.14	$C_{17}H_{19}F_2N_3O_3$	0.34	-0.506	0.4486	5.61, 8.79	5.49; 8.78	60

41	Lopinavir	PC	antiviral	protease inhibitors (PIs)	628.36	$C_{37}H_{48}N_4O_5$	5.42	4.6876	0	13.39	13.39	-
42	Mecillinam	PC	antibacterial	amidinopenicillins	325.15	$C_{15}H_{23}N_3O_3S$	-0.55	-0.548	-0.008	3.3, 7.92	3.3; 7.92	55
43	Meropenem	PC	antibacterial	Beta-lactams (carbapenems)	383.15	$C_{17}H_{25}N_3O_5S$	-1.29	-4.354	0.0018	3.47, 8.39	2.9; 7.4	70
44	Metronidazole	PC	antibacterial/ antiprotozoal	nitroimidazoles	171.16	$C_6H_9N_3O_3$	-0.02	-0.46	0.0011	3.03	2.55	60-80
45	Metronidazole-OH	HM	antibacterial/ antiprotozoal	nitroimidazoles	187.01	$C_6H_9N_3O_4$	-0.02	-1.277	0	1.45, 13.75	2; 13.3	-
46	Miconazole	PC	antifungal	imidazoles	413.99	$C_{18}H_{14}Cl_4N_2O$	5.66	5.6025	0.7499	6.48	6.65	40-50
47	N4-acetylsulfadiazine	HM	antibacterial	Sulfonamides	292.06	$C_{12}H_{12}N_4O_3S$	0.43	0.4068	-0.116	6.88	-0.2; 6.1	-
48	N4-acetylsulfamethazine	HM	antibacterial	Sulfonamides	320.10	$C_{14}H_{16}N_4O_3S$	0.98	0.6698	-0.1153	-0.97, 6.88	-1.1; 7.2	-
49	N4-acetylsulfamethoxazole	HM	antibacterial	sulfonamides	295.06	$C_{12}H_{13}N_3O_4S$	1.09	0.4105	-0.7251	0.38, 5.58	-3.4; 5.6	-
50	Nevirapine	PC	antiviral	non-nucleoside reverse transcriptase inhibitors (NNRTIs)	266.12	$C_{15}H_{14}N_4O$	1.81	2.4872	0.0018	3.28, 9.98	2.8;	-
51	Nitrofurantoin	PC	antibacterial	nitrofurans	238.03	$C_8H_6N_4O_5$	-0.27	-0.224	-0.0058	8.23	7.2;	20-30
52	Norfloxacin	PC	antibacterial	fluoroquinolones	319.13	$C_{16}H_{18}FN_3O_3$	-0.27	-1.046	0.3024	5.34, 8.77	3.11; 6.1; 8.6	42
53	Ofloxacine	PC	antibacterial	fluoroquinolones	361.14	$C_{18}H_{20}FN_3O_4$	0.11	-1.094	0.2233	5.17, 8.39	6.05; 8.22	72.5
54	Oseltamivir	PC	antiviral	neuraminidase inhibitors	312.40	$C_{16}H_{28}N_2O_4$	0.95	-1.668	0.9994	4.38, 9.29	7.6	-
55	Oseltamivir acid	HM	antiviral	neuraminidase inhibitors	284.17	$C_{14}H_{24}N_2O_4$	-1.78	-1.843	0.0449	9.26, 14.03	4.1; 9.3	-
56	Oxytetracycline	PC	antibacterial	tetracyclines	460.15	$C_{22}H_{24}N_2O_9$	-0.09	-4.579	-0.0371	-	3.27; 7.46; 8.94	50 (fecal)
57	Piperacillin	PC	antibacterial	beta-lactams (penicillins)	517.16	$C_{23}H_{27}N_5O_7S$	1.31	-2.735	-0.997	3.49	-4.3; 3.49	70
58	Remdesivir	PC	antiviral	nucleoside analogues	602.23	$C_{27}H_{35}N_6O_8P$		2.0072	-0.0001	0.65, 10.23	0.65; 10.23	-
59	Rifampicin	PC	antibacterial	rifamycins	822.41	$C_{43}H_{58}N_4O_{12}$	2.66	2.1852	0.8597	6.94, 7.53	1.7; 7.9	<30
60	Ritonavir	PC	antiviral	protease inhibitors (PIs)	720.31	$C_{37}H_{48}N_6O_5S_2$	5.03	5.2216	0.0006	2.84, 13.68	2.8; 13.68	-
61	Roxithromycin	PC	antibacterial	macrolides (semi-synthetic)	836.52	$C_{41}H_{76}N_2O_{15}$	3.24	0.0582	0.9992	-	9.17;	10
62	Sparfloxacin	PC	antibacterial	fluoroquinolones	392.17	$C_{19}H_{22}F_2N_4O_3$	0.41	-0.186	0.5122	5.72, 8.88	5.75; 8.79	10
63	Spectinomycin	PC	antibacterial	aminoglycosides	332.15	$C_{14}H_{24}N_2O_7$	-0.15	-4.69	1.7903	6.6, 8.98	6.8; 8.8	50
64	Sulfadiazine	PC	antibacterial	sulfonamides	250.05	$C_{10}H_{10}N_4O_2S$	-0.09	0.3499	-0.0927	2.01, 6.99	2; 6.48	44
65	Sulfamethazine	PC	antibacterial	sulfonamides	278.08	$C_{12}H_{14}N_4O_2S$	0.73	0.613	-0.092	2, 6.99	2.07; 7.49	10-20
66	Sulfamethoxazole	PC	antibacterial	sulfonamides	253.05	$C_{10}H_{11}N_3O_3S$	0.89	0.4777	-0.5794	1.97, 5.86	1.85; 5.6	15
67	Sulfapyridine	PC	antibacterial	sulfonamides	249.05	$C_{11}H_{11}N_3O_2S$	0.35	0.8383	-0.3663	2.14, 6.24	2.22; 8.58	40
68	Sulfathiazole	PC	antibacterial	sulfonamides	255.01	$C_9H_9N_3O_2S_2$	0.31	0.6013	-0.6513	2.04, 5.73	2.01; 7.11	-
69	Teicoplanin	PC	antibacterial/ antiviral	glycopeptides	1877.55	R- group A) $C_{10}H_{20}$		-2.285	-0.0775	3.23, 7.13	2.88	-
					1879.55	R- group B) $C_{10}H_{22}$						
					1894.55	R- group C) $C_{11}H_{24}$						
70	Tenofovir	PC	antiviral	nucleoside reverse transcriptase inhibitors (NRTIs)	287.21	$C_9H_9N_3O_2S_2$	-1.42	-3.460	-0.6297	1.35, 4.82	3.75	-
71	Tetracycline	PC	antibacterial	tetracyclines	444.15	$C_{22}H_{24}N_2O_8$	-1.3	-3.505	-0.0294	3.25, 8.96	3.32; 7.78; 9.58	60 (fecal+urine)
72	Tinidazole	PC	antibacterial/ antiprotozoal	nitroimidazoles	247.06	$C_8H_{13}N_3O_4S$	-0.25	-0.581	0.0019	3.28	4.7	60
73	Tobramycin	PC	antibacterial	aminoglycosides	467.26	$C_{18}H_{37}N_5O_9$	-3.8	-18.19	4.95	9.42, 12.43	7.55; 7.75; 9.1	85
74	Trimethoprim	PC	antibacterial	dihydropyrimidines	290.14	$C_{14}H_{18}N_4O_3$	0.91	0.2691	0.993	7.16	3.23; 6.76	50
75	Tylosin	PC	antibacterial	macrolides	915.52	$C_{46}H_{77}NO_{17}$	2.23	-0.080	0.9963	8.43, 12.45	3.31; 7.5	-
76	Vancomycin	PC	antibacterial	glycopeptides	1447.43	$C_{66}H_{75}Cl_2N_9O_{24}$	-0.75	-6.084	-	-	7.1	75
77	Zidovudine	PC	antiviral	nucleoside reverse transcriptase inhibitors (NRTIs)	267.24	$C_{10}H_{13}N_5O_4$	-0.35	-0.573	-0.0418	7.36	-3; 9.96	-

(a) data retrieved from CompTox®; (b) at pH 6; (c) data retrieved from Chemicalize®; (d) predicted; (e) experimental; (PC) parent compound; (HM) human metabolite; (-) data not available

Table S2. Prioritization of the selected antimicrobial chemicals according to environmental and clinical relevance. A maximum total score of 14.9 points is assigned, including 10 points for environmental relevance (5 points = presence in effluent wastewater,[21–23] 5 points = listed in EU watch list [24,25]) and 4.9 points for clinical relevance. Clinical relevance is further divided into whether a compound (a) is highly used in clinical settings (1 point) and (b) is susceptible to antimicrobial resistance (1 point) according to the SWARME report,[26] (c) belongs to the antiviral group (2 points)[27], and (d) is ranked in the WHO's AWaRe classification (0.3 points = Access; 0.6 points = Watch; 0.9 points = Reserve).[28] For example, among the selected compounds, ciprofloxacin (a score of 11.6) shows the highest environmental and clinical relevance, with the '.6' indicating its 'Watch' classification in WHO's AWaRe. A score for human metabolites (light blue colored) is not assigned (see section "Selection of target compounds").

Initial selection	LC-MS/MS validated	SPE-LC-MS/MS validated	Compound	Total score
x	x	x	Ciprofloxacin	11.6
x	x	x	Sulfamethoxazole	10.3
x	x	x	Trimethoprim	10.3
x	x	x	Erythromycin	6.6
x	x	x	Clindamycin	6.3
x	x	x	Azithromycin	5.6
x	x	x	Clarithromycin	5.6
x	x	x	Enoxacin	5.6
x	x	x	Norfloxacin	5.6
x	x	x	Ofloxacine	5.6
x	x	x	Roxithromycin	5.6
x			Amoxicillin	5.3
x	x	x	Doxycycline	5.3
x	x	x	Metronidazole	5.3
x	x		Sulfadiazine	5.3
x	x	x	Sulfamethazine	5.3
x			Sulfapyridine	5.3
x	x	x	Sulfathiazole	5.3
x	x	x	Tetracycline	5.3
x			Clotrimazole	5
x	x	x	Enrofloxacin	5
x	x	x	Fluconazole	5
x	x	x	Miconazole	5
x	x		Cefoxitin	2.6
x			Ceftazidime	2.6
x	x		Piperacillin	2.6
x			Teicoplanin	2.6
x	x		Ampicillin	2.3
x			Abacavir	2
x	x	x	Aciclovir	2
x	x	x	Chloroquine	2
x			Darunavir	2
x			Emtricitabine	2
x			Entacapone	2
x			Lamivudine	2
x			Lopinavir	2
x			Nevirapine	2
x	x	x	Oseltamivir	2
x	x	x	Remdesivir	2
x			Ritonavir	2
x	x		Tenofovir	2
x	x	x	Zidovudine	2
x			Colistin	1.9
x			Linezolid	1.9

x			Cefotaxime	1.6
x			Fosfomycin	1.6
x	x		Fusidic acid	1.6
x			Imipenem	1.6
x	x		Meropenem	1.6
x			Rifampicin	1.6
x			Tobramycin	1.6
x			Amikacin	1.3
x	x		Cefadroxil	1.3
x			Gentamicin	1.3
x	x		Mecillinam	1.3
x	x	x	Nitrofurantoin	1.3
x			Spectinomycin	1.3
x	x		Cefaclor	0.6
x	x		Cefepime	0.6
x	x		Cefixime	0.6
x	x	x	Chlortetracycline	0.6
x	x		Lincomycin	0.6
x	x	x	Lomefloxacin	0.6
x	x		Oxytetracycline	0.6
x	x	x	Sparfloxacin	0.6
x	x		Vancomycin	0.6
x	x		Cefalexin	0.3
x	x	x	Chloramphenicol	0.3
x	x	x	Tinidazole	0
x			Tylosin CRS	0
x	x		4-epianhydrotetracycline	
x	x	x	4-acetylsulfamethoxazole	
x	x	x	4-acetylsulfamethazine	
x	x	x	4-acetylsulfadiazine	
x	x	x	Oseltamivir acid	
x	x	x	Hydroxychloroquine	
x	x		Metronidazole-OH	

Table S3. MRM transition of the target antimicrobial chemicals and internal standards used in the validated LC-MS/MS method.

Compound	Q1	Q3 (quantifier; qualifier)	DP	CE (quantifier; qualifier)	CXP (quantifier; qualifier)	IS compound used [b]
ESI+						
Acyclovir [a]	226.1	167.1; 208.9	120	15; 11	12; 15	Oxazepam-d5
Ampicillin	349.9	106; 114	76	21; 43	8; 8	Ranitidine-d6
Azithromycin [a]	749.5	591.3; 157.9	120	41; 47	11; 11	[13C,2H3]-Azythromycin
Cefadroxil	363.8	113.9; 85.9	56	27; 67	8; 8	Oxazepam-d5
Cefalexin	347.6	158.1; 173.9	64	14; 21	12; 12	Ranitidine-d6
Cefepime	481.3	323.9; 167.1	35	24; 32	6; 12	Tetracycline-d6
Chloroquine *[a]	319.9	246.9; 142.1	106	29; 31	16; 10	[13C,2H3]-Azythromycin
Chlortetracycline [a]	478.9	444; 154	101	29; 37	24; 10	Tetracycline-d6
Ciprofloxacin *[a]	332	231; 288.1	91	49; 25	14; 12	Ofloxacine-d3
Clarithromycin [a]	748.2	158; 590.1	86	35; 28	11; 11	Diltiazem-d4
Clindamycin [a]	425	126.1; 376.7	34	33; 28	10; 13	Sulfamethoxazole-d4
Enoxacin *[a]	321	303.1; 233.9	90	29; 30	11; 15	Ofloxacine-d3
Enrofloxacin *[a]	360	316.1; 245.1	101	27; 37	12; 16	[13C,2H3]-Azythromycin
Erythromycin [a]	734.3	158.1; 576.3	105	38; 28	12; 10	[13C,2H3]-Erythromycin
Fluconazole [a]	306.9	238; 219.9	16	23; 25	14; 16	Lidocaine-d10
Hydroxychloroquine *[a]	336	247.1; 179	116	29; 49	18; 12	Ofloxacine-d3
Lincomycin	407.3	126.3; 359	100	33; 27	4; 6	Fluoxetine-d5
Lomefloxacin *[a]	352	308; 265.1	91	25; 33	20; 10	Ofloxacine-d3
Mecillinam	326	167.1; 139.1	116	31; 41	12; 10	cis-Sertraline-d3
Meropenem	383.9	141.1; 113.9	116	21; 35	10; 8	-
Metronidazole [a]	171.9	128; 82	50	19; 33	10; 8	Metronidazole-(ethylene)-d4
Metronidazole-OH	187.8	123.1; 144.1	65	18; 18	9; 10	Metronidazole-(ethylene)-d4
Miconazole [a]	416.7	160.9; 123	101	37; 93	12; 10	Sulfamethoxazole-d4
N4-acetylsulfadiazine [a]	293	197.8; 227	90	24; 26	14; 4	DEET-d10
N4-acetylsulfamethazine [a]	320.9	185.9; 124.1	106	29; 33	12; 10	DEET-d10
Norfloxacin *[a]	319.9	302.3; 276	90	30; 24	11; 10	Ofloxacine-d3
Ofloxacine *[a]	362	318.1; 261.1	80	27; 37	12; 18	Ofloxacine-d3
Oseltamivir [a]	313	166; 119.9	60	27; 41	12; 10	Oxazepam-d5
Oseltamivir acid [a]	285	138; 94	63	25; 39	10; 8	Oxazepam-d5
Oxytetracycline	460.9	426.1; 200.9	116	27; 49	16; 12	Tetracycline-d6
Remdesivir [a]	603	200; 229	101	53; 27	16; 6	Citalopram-d6
Roxithromycin [a]	837.5	679.3; 558.1	105	31; 34	13; 10	cis-Sertraline-d3
Sparfloxacin *[a]	393	349.1; 264	105	29; 49	12; 16	Caffeine-13C3
Sulfadiazine	251.1	156; 107.9	20	21; 32	12; 9	Diltiazem-d4
Sulfamethazine [a]	278.9	124.1; 92	86	31; 41	10; 8	Sulfamethoxazole-d4
Sulfamethoxazole [a]	253.9	92; 108	71	37; 33	8; 8	Sulfamethoxazole-d4
Sulfathiazole [a]	256	156.1; 108	72	21; 32	11; 8	Sulfamethoxazole-d4
Tetracycline [a]	444.9	410.1; 154	116	27; 35	16; 12	Tetracycline-d6
Tinidazole [a]	248.1	121; 128.1	65	22; 28	9; 9	Metronidazole-(ethylene)-d4
Trimethoprim [a]	291	230; 123	96	33; 33	14; 10	Oxazepam-d5
Vancomycin	483.9	364.1; 459.3	37	12; 7	12; 8	Sulfamethoxazole-d4
[13C,2H3]-Azythromycin [a]	753.7	595.3	120	42	11	
[13C,2H3]-Erythromycin [a]	738.4	580.1	109	28	10	
Caffeine-13C3 [a]	198.1	140.1	82	27	10	
cis-Sertraline-d3 [a]	308.9	275	56	17	10	
Citalopram-d6 [a]	331.1	116	111	35	8	
DEET-d10 [a]	202.1	119	131	25	10	
Diltiazem-d4 [a]	418.9	182	101	33	12	
Fluoxetine-d5	315	153.2	76	13	10	
Metronidazole-(ethylene)-d4 [a]	175.9	128	61	21	10	
Ofloxacine-d3 [a]	365	321.1	100	27	18	
Oxazepam-d5 [a]	292.2	273.9	100	23	14	

Ranitidine-d6	321	176	67	25	12	
Sulfamethoxazole-d4 [a]	257.9	112.1	94	33	10	
Tetracycline-d6 [a]	451.2	416.1	95	29	14	
ESI-						
4-epianhydrotetracycline *	425.3	254.7; 408.1	-99	-22; -21	-9; -7	-
Cefaclor	365.9	286; 175.9	-10	-14; -12	-9; -11	Hydrochlorothiazide-13C6
Cefixime	451.7	282.4; 124.1	-70	-14; -30	-19; -7	-
Cefoxitin	425.8	155.8; 111.9	-45	-12; -24	-11; -7	Hydrochlorothiazide-13C6
Chloramphenicol [a]	320.7	152.1; 120.9	-80	-22; -46	-11; -9	Hydrochlorothiazide-13C6
Doxycycline [a]	442.9	357.7; 239.7	-100	-30; -64	-13; -9	Oxybenzone-d5
Fusidic acid	515	220.9; 455.1	-125	-34; -28	-13; -7	Irbesartan-d7
N4-acetylsulfamethoxazole [a]	293.8	198; 133.9	-60	-22; -34	-7; -7	Bezafibrate-d4
Nitrofurantoin [a]	236.9	151.7; 123.8	-54	-16; -19	-5; -9	Hydrochlorothiazide-13C6
Piperacillin	515.8	233; 329.9	-60	-24; -18	-11; -15	Losartan-d4
Tenofovir	285.8	133.8; 107.1	-95	-30; -58	-11; -5	-
Zidovudine [a]	266.1	223; 193	-65	-15; -20	-7; -11	Oxybenzone-d5
Bezafibrate-d4 [a]	363.9	278	-80	-24	-11	
Hydrochlorothiazide-13C6 [a]	301.7	274.96	-100	-26	-5	
Irbesartan-d7	434	200.2	-115	-34	-9	
Losartan-d4	424.9	156.8	-105	-30	-9	
Oxybenzone-d5 [a]	231.8	186.1	-5	-14	-9	
Propylparaben-d7	185.9	135.9	-85	-22	-7	

* quadratic calibration curve; [a] in the extraction method; [b] isotopically labeled (IS) compounds were determined based on the absolute recovery of both IS and native compounds, expected concentrations in water extracts post-spiked with the compounds and expected concentrations in standard solutions. Similar assignment procedure is also employed elsewhere.[29]; '-': the analyte performs without IS compounds and is only considered up to the LC-MS/MS validation step in this study; nevertheless, inclusion of corresponding IS compounds are encouraged for future studies if possible. Similar approach was also applied in a recent study.[30]

Table S4. Recommended criteria including sample pH, washing solution and elution solution used during extraction sorbent optimization.

	Oasis® HLB	**Oasis® WCX**	**Oasis® MCX**
Sample pH	7-7.5	7-7.5	2
Washing solution	MilliQ water (3 mL)	MilliQ water (3 mL)	pH 2 MilliQ water (3 mL)
Elution solution	5 mL MeOH	Fraction 1 – MeOH (5 mL) Fraction 2 – 2% FA in MeOH (5 mL)	Fraction 1 – MeOH (5 mL) Fraction 2 – 5% NH_4OH in MeOH (5 mL)

Table S5. Absolute recoveries (>15%) of the analytes in MilliQ water and influent wastewater testing Oasis® HLB, MCX and WCX cartridges.

	MilliQ			**Influent wastewater**		
Cartridge type	**HLB**	**MCX***	**WCX***	**HLB**	**MCX***	**WCX***
ESI+						
Abacavir	90	95	78	100	91	100
Acyclovir	54	57	104	67	99	68
Ampicillin	15	-	-	-	-	-
Azithromycin	35	-	33	99	57	63
Cefadroxil	-	25	-	43	24	-
Cefalexin	-	27	-	54	27	17
Cefepime	26	-	-	53	-	27
Chlarithromycin	26	-	-	88	32	67
Chloroquine	34	83	72	-	86	68
Chlortetracycline	-	-	47	15	-	24
Ciprofloxacin	-	59	77	79	-	44
Clindamycin	21	72	-	87	87	47
Clotrimazole	34	88	39	80	87	71
Darunavir	30	36	57	104	66	99
Emtricitabine	64	79	-	103	91	77
Enoxacin	-	68	77	69	-	25
Enrofloxacin	22	68	71	102	-	45
Erythromycin	33	-	-	80	-	63
Fluconazole	91	98	105	97	98	95
Hydroxychloroquine	31	73	67	100	85	52
Lamivudine	64	88	-	106	92	70
Lincomycin	36	78	-	101	94	44
Linezolid	98	92	113	112	98	103
Lomefloxacin	29	86	90	95	-	69
Lopinavir	39	60	34	122	109	122
Mecillinam	-	-	-	-	19	-
Meropenem	22	-	-	-	-	-
Metronidazole	89	90	95	106	87	85
Metronidazole-OH	96	101	53	107	91	40
Miconazole	21	61	26	66	76	45
N4-acetylsulfadiazine	101	102	106	93	101	91
N4-acetylsulfamethazine	94	89	113	106	99	97
Nevirapine	79	92	99	106	86	103
Norfloxacin	27	54	84	72	-	31
Ofloxacine	22	77	88	96	-	52
Oseltamivir	49	83	96	76	71	74

Oseltamivir acid	53	99	104	18	95	63
Oxytetracycline	25	-	53	22	-	46
Remdesivir	42	51	47	104	43	95
Rifampicin	-	-	-	77	26	41
Ritonavir	31	59	28	104	100	110
Roxithromycin	20	-	-	86	30	64
Sparfloxacin	33	60	31	87	-	64
Sulfadiazine	78	74	86	96	81	77
Sulfamethazine	85	90	76	108	88	88
Sulfamethoxazole	81	91	98	99	98	92
Sulfapyridine	94	76	85	105	85	105
Sulfathiazole	89	79	89	105	84	80
Tetracycline	-	-	52	22	-	37
Tinidazole	95	74	94	102	74	91
Trimethoprim	95	102	89	105	95	81
Tylosin	25	-	-	86	-	80
Vancomycin	29	-	-	84	-	79
ESI-						
4-epianhydrotetracycline	-	-	22	-	-	-
Cefaclor	24	-	25	27	-	-
Cefixime	-	-	-	-	-	-
Cefotaxime	41	-	-	64	-	23
Cefoxitin	49	69	-	98	91	33
Chloramphenicol	87	98	100	106	111	93
Doxycycline	-	-	48	-	-	33
Entacapone	-	62	53	-	87	46
Fosfomycin	-	-	-	-	-	-
Fusidic acid	-	-	-	131	-	79
N4-acetylsulfamethoxazole	80	94	95	106	108	94
Nitrofurantoin	80	85	90	100	98	92
Piperacillin	-	17	-	-	21	29
Tenofovir	-	156	-	-	51	-
Zidovudine	96	92	92	110	99	94
*sum of absolute recoveries of fraction 1 and 2 (-) recovery below 15% or not recovered						

Table S6. Absolute recoveries (>15%) of the analytes in MilliQ water and influent wastewater using Oasis® WCX cartridges and testing different elution solutions.

	MilliQ				**Influent**			
Elution solution	**MeOH + 2%FA MeOH**	**MeOH + 4%FA MeOH**	**MeOH + 4%FA MeOH (Na_2EDTA)**	**MeOH + 8%FA MeOH**	**MeOH + 2%FA MeOH**	**MeOH + 4%FA MeOH**	**MeOH + 4%FA MeOH (Na_2EDTA)**	**MeOH + 8%FA MeOH**
ESI+								
Abacavir	74	72	79	74	79	92	87	88
Acyclovir	87	93	90	110	62	81	83	72
Ampicillin	-	-	-	-	24	16	-	-
Azithromycin	27	35	50	19	85	77	77	65
Cefadroxil	-	-	-	-	-	-	-	-
Cefalexin	-	-	-	-	37	36	-	40
Cefepime	-	-	-	-	30	36	32	40
Chlarithromycin	-	-	37	-	70	69	75	51
Chloroquine	79	69	71	68	67	86	78	80

Chlortetracycline	59	57	20	64	28	31	52	31
Ciprofloxacin	82	83	193	87	61	85	80	92
Clindamycin	-	-	-	-	52	57	34	54
Clotrimazole	48	55	45	44	42	45	49	53
Darunavir	36	32	15	31	54	42	49	32
Emtricitabine	-	-	-	-	81	89	81	86
Enoxacin	81	75	64	97	48	67	62	67
Enrofloxacin	70	73	76	75	46	66	69	69
Erythromycin	-	16	41	-	171	73	82	56
Fluconazole	92	94	100	99	92	103	90	96
Hydroxychloroquine	77	76	68	81	59	77	59	80
Lamivudine	-	-	-	-	79	85	77	89
Lincomycin	-	-	-	-	47	46	33	44
Linezolid	87	83	85	81	80	97	88	90
Lomefloxacin	83	88	89	89	65	86	73	77
Lopinavir	59	60	47	65	69	74	79	77
Mecillinam	-	-	-	-	31	29	29	16
Meropenem	-	-	-	-	-	-	-	-
Metronidazole	93	92	91	99	78	88	82	82
Metronidazole-OH	53	50	43	52	32	38	32	35
Miconazole	36	40	19	32	37	43	44	58
N4-acetylsulfadiazine	92	91	75	91	81	99	75	90
N4-acetylsulfamethazine	110	105	105	97	78	95	81	87
Nevirapine	91	92	100	100	83	96	84	89
Norfloxacin	77	83	71	88	46	69	60	59
Ofloxacine	78	80	84	79	61	79	74	81
Oseltamivir	92	87	90	91	72	89	84	87
Oseltamivir acid	88	90	19	94	61	66	57	75
Oxytetracycline	60	64	49	74	53	68	75	64
Remdesivir	73	75	75	75	80	87	91	74
Rifampicin	17	-	75	-	77	50	79	51
Ritonavir	52	56	43	64	76	84	78	82
Roxithromycin	-	-	35	-	80	66	67	40
Sparfloxacin	46	40	81	33	60	69	70	68
Sulfadiazine	40	35	15	41	42	41	34	22
Sulfamethazine	45	38	21	37	45	42	36	23
Sulfamethoxazole	44	36	19	45	53	53	51	31
Sulfapyridine	41	35	23	39	66	69	66	55
Sulfathiazole	44	36	16	37	42	36	33	18
Tetracycline	63	59	35	64	55	64	79	64
Tinidazole	92	94	106	90	83	102	87	89
Trimethoprim	93	93	93	91	83	96	90	95
Tylosin	-	-	29	-	31	34	40	21
Vancomycin	-	-	30	-	23	32	36	26
ESI-								
4-epianhydrotetracycline	48	45	36	39	-	-	-	-
Cefaclor	-	-	-	-	32	38	-	37
Cefixime	-	-	-	-	-	-	-	-
Cefotaxime	-	-	-	-	39	43	-	43
Cefoxitin	-	-	-	-	55	53	-	52
Chloramphenicol	91	90	94	95	82	93	82	85
Doxycycline	64	67	57	71	-	-	71	-
Entacapone	88	81	74	78	52	69	64	75
Fosfomycin	-	-	-	-	-	-	-	-
Fusidic acid	-	-	-	-	89	78	75	-
N4-acetylsulfamethoxazole	93	94	86	100	87	92	76	94
Nitrofurantoin	84	88	86	88	81	94	78	92

Piperacillin	-	-	-	-	74	31	20	-
Tenofovir	-	-	-	-	-	-	-	-
Zidovudine	91	90	94	92	82	96	79	91
(-) recovery below 15% or not recovered								

Table S7. Concentrations (ng L^{-1}) of the antimicrobials found in the water samples. Risk quotient (RQ) is estimated through dividing the measured concentration by the predicted no-effect concentration (PNEC). PNEC is derived from the minimum inhibitory concentrations.[31] RQ are reported for antibacterials in brackets for each water sample (low environmental risk RQ<0.1, moderate environmental risk 0.1<RQ<1, high environmental risk RQ>1). Bold values mean RQ>1.

Compound	**$PNEC_{MIC}$ (ng L^{-1})**	**Hospital wastewater**	**WWTP influent**	**WWTP effluent**	**OSSF influent**	**OSSF effluent**	**Groundwater downstream**
Azithromycin	250	<MQL	<MQL	4.6 (0.02)	<MQL	<MQL	<MQL
Clarithromycin	250	100 (0.4)	36 (0.1)	20 (0.1)	<MQL	<MQL	<MQL
Ciprofloxacin	64	4300 (**67**)	280 (**4.4**)	23 (0.4)	<MQL	8.9 (0.1)	<MQL
Clindamycin	1000	890 (0.9)	<MQL	<MQL	<MQL	<MQL	<MQL
Fluconazole	250	3500 (**14**)	51 (0.2)	77 (0.3)	<MQL	46 (0.2)	37 (0.1)
Metronidazole	125	680 (**5.4**)	17 (0.1)	20 (0.2)	<MQL	<MQL	<MQL
Sulfamethoxazole	16000	1900 (0.1)	170 (0.01)	160 (0.01)	<MQL	<MQL	<MQL
Tetracycline	1000	1200 (**1.2**)	140 (0.1)	<MQL	130 (0.1)	<MQL	<MQL
Trimethoprim	500	1200 (**2.5**)	86 (0.2)	120 (0.2)	<MQL	<MQL	<MQL
N4-acetylsulfamethoxazole	-	2400	440	11	<MQL	<MQL	<MQL

<MQL – below method quantification limit (see Table 2); (-) not available

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The environment is a reservoir of contaminants that contribute to antimicrobial resistance (AMR), a global health threat, primarily through wastewater discharges. This thesis investigates the role of on-site sewage facilities (OSSF) in the environmental spread of AMR. The results indicate that OSSF contribute to AMR dissemination and offer important insights for future monitoring, regulation, and mitigation efforts.

Valentina Ugolini received her doctoral education at the Department of Aquatic Sciences and Assessment at the Swedish University of Agricultural Sciences. She holds a M.Sc. in Environmental Sciences from the University of Natural Resources and Life Sciences (BOKU), Vienna and the University of Copenhagen (KU).

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