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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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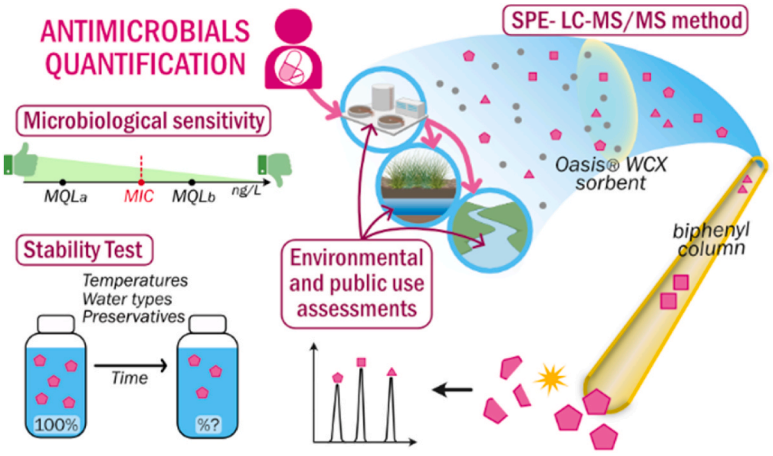
HIGHLIGHTS

- 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

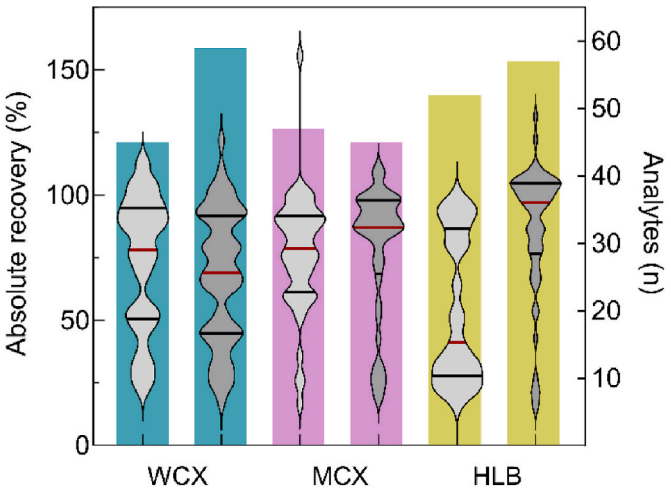

Fig. 1. Absolute recovery (%) in violin plots (left y-axis) and numbers of analytes with >15% absolute recovery in bar charts (right y-axis) using Oasis® HLB (yellow), MCX (pink) and WCX (turquoise) sorbents in MilliQ water (left of a sorbent) and influent wastewater (right of a sorbent) extraction. 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.

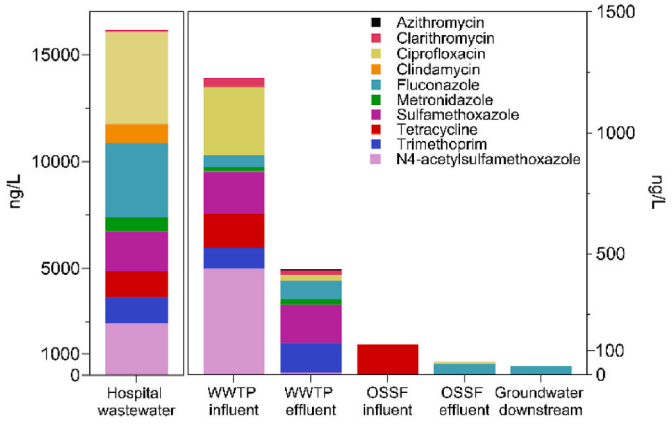

Fig. 2. Cumulative antimicrobial concentrations (ng/L) quantified in the six (waste)water samples. Left y-axis for hospital wastewater. Right y-axis for the other waters. The municipal WWTP services ~24 000 inhabitants with active sludge treatment followed by chemical precipitation. The OSSF services ~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

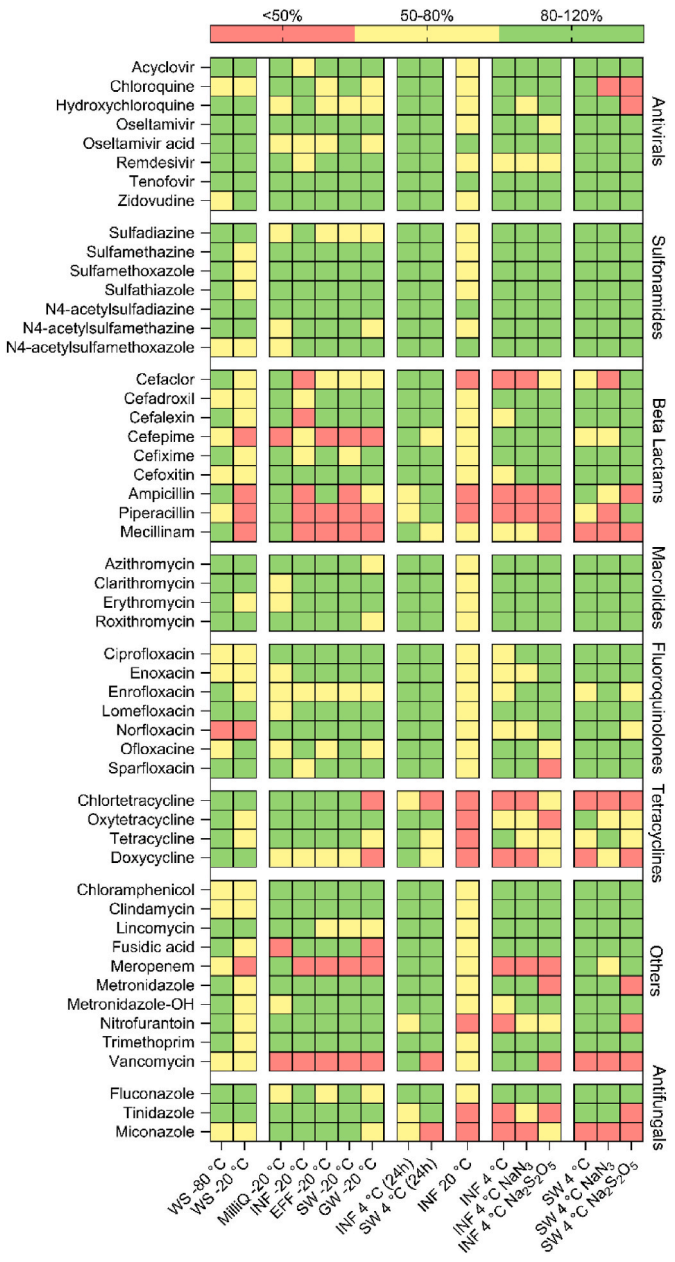

Fig. 3. Antimicrobial stability evaluations in different scenarios: working solutions (WS) at −80 °C and −20 °C for 6 months; MilliQ, influent (INF) and effluent (EFF) wastewater, surface water (SW) and groundwater (GW) in freezers at −20 °C for 8 weeks; INF and SW in refrigerators at 4 °C for 24h; INF 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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