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Towards a harmonized method for the global reconnaissance of multi-class antimicrobials and other pharmaceuticals in wastewater and receiving surface waters

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ABSTRACT

Antimicrobial resistance is a worldwide problem that is both pressing and challenging due to the rate at which it is spreading, and the lack of understanding of the mechanisms that link human, animal and environmental sources contributing to its proliferation. One knowledge gap that requires immediate attention is the significance of antimicrobial residues and other pharmaceuticals that are being discharged from wastewater treatment plants (WWTPs) on the dissemination of antimicrobial resistance in the environment. In this work we provide an approach to develop a harmonized analytical method for 8 classes of antimicrobials and other pharmaceuticals that can be used for global monitoring in wastewater and receiving waters. Analysis of these trace organic chemicals in the influent and effluent wastewater, and in the respective upstream and downstream receiving waters from different countries across the globe is not trivial. Here, we demonstrated that sample preparation using solid-phase extraction (SPE) not only provides a convenient and cost-effective shipping of samples, but also adds stability to the analytes during international shipping. It is important that SPE cartridges are maintained at cold temperature during shipment if the duration is longer than 7 days because a significant decrease in recoveries were observed after 7 days in the cartridges stored at room temperature, especially for sulfonamides and tetracyclines. To compensate for sample degradation during shipment, and matrix effects in liquid chromatography/mass spectrometry, the use of stable isotope labeled compounds should be employed when available and affordable. The importance of applying a defined tolerance for the ion ratios (Q/q) that have been optimized for wastewater and surface water is discussed. The tolerance range was set to be the mean Q/q of the analyte standard at various concentrations ± 40% for the influent, and ± 30% for the effluent, upstream, and downstream samples; for tetracyclines and quinolones, however, the tolerance range was ± 80% in order to minimize false negative and false positive detection. The optimized procedures were employed to reveal differences in antimicrobial and pharmaceutical concentrations in influent, effluent, and surface water samples from Hong Kong, India, Philippines, Sweden, Switzerland, and United States. The antimicrobials with the highest concentrations in influent and effluent samples were ciprofloxacin (48,103 ng/L, Hong Kong WWTP 1) and clarithromycin (5178 ng/L, India WWTP 2), respectively. On the other hand, diclofenac (108,000 ng/L, Sweden WWTP 2), caffeine (67,000 ng/L, India WWTP 1), and acetaminophen (28,000 ng/L, India WWTP 1) were the highest detected pharmaceuticals in the receiving surface water samples. Hong Kong showed the highest total antimicrobial concentrations that included macrolides, quinolones, and sulfonamides with concentrations reaching 60,000 ng/L levels in the influent. Antidepressants were predominant in Sweden, Switzerland, and the United States.

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1. Introduction

The World Health Organization's, 2014 report describes antimicrobial resistance (AMR) as a global threat to the "effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi" (World Health Organization, 2014) making death due to minor infections and cuts possible in a post-antimicrobial world. As the magnitude and frequency of human migration and movement increase due to globalization, the spread of resistant bacteria and antimicrobial resistance genes (ARGs) also increases (MacPherson et al., 2009) because bacteria and mobile genetic elements respect no geographic borders. One factor that contributes to the emergence of antimicrobial resistant bacteria is the presence of antimicrobials in the environment. Therefore, it is important to know how these chemicals enter the environment, to understand the pathways through which they disseminate, and to obtain knowledge on how their environmental occurrence may be controlled.

Antimicrobials and other pharmaceuticals are widely used for human and veterinary purposes (both therapeutic and prophylactic), as feed additives in fish aquaculture, and for growth promotion in livestock industries. Global antimicrobial use in animals alone for 2010 was estimated to be 63,000 tons, and is predicted to increase to 100,000 tons in 2030 (Van Boeckel et al., 2015). The large human consumption of these drugs and their intensive use in agriculture have resulted in the widespread occurrence of their residues in the environment. Antimicrobials are released into the environment in different ways, but primarily through the discharge of effluents from wastewater treatment plants (WWTPs) and water run-off from manure-treated agricultural lands.

WWTP effluents have been identified as one of the major sources of antimicrobials and other pharmaceuticals in the environment (Arnnok et al., 2017; Noguera-Oviedo and Aga, 2016; Vikesland et al., 2017). In one study, samples from a river impacted by WWTP effluent were found to contain antimicrobials such as sulfamethazine, amoxicillin, roxithromycin, and erythromycin; with sulfamethazine showing the highest concentration at 25.97 ng/L (Wu et al., 2016). In a separate study, the concentrations of different classes of antimicrobials detected in the influent were compared to those in the effluent to assess the efficiency of removal of these drugs. The most abundant antimicrobials were tetracyclines, fluoroquinolones, and macrolides. It was found that the amounts of these antimicrobials decreased in the effluent relative to the influent; the influent concentrations ranged from 111.1 ng/L to 30,049 ng/L, while the effluent ranged from 5 ng/L to 2014 ng/L (Tran et al., 2016).

Veterinary antimicrobials have also been shown to contaminate agricultural fields worldwide because animal manure is typically land-applied to fertilize croplands. Tetracycline, sulfamethazine, norfloxacin, erythromycin, and chloramphenicol have been found to leach from soil and enter the aquatic environment through surface run-off, especially with the occurrence of a heavy rainfall right after manure application. The introduction of veterinary antimicrobials from land-applied animal manure causes their accumulation in soil and in groundwater (Pan and Chu, 2016). As these chemicals come in contact with bacteria and other microorganisms in soil it is postulated that this exposure further augments the proliferation and spread of antimicrobial resistance in the environment (Pruden et al., 2006). Antimicrobials and ARGs that enter the environment both contribute to the development of antimicrobial resistance. It has been shown that low concentrations of antimicrobials detected in the environment can select for resistant bacteria, which implies that the consistent release of these antimicrobials from various sources, contribute significantly to the emergence and maintenance of resistance in the environment (Gullberg et al., 2011). On the other hand, ARGs have also been considered as emerging contaminants since their release into the environment contributes to the spread of resistance due to horizontal gene transfer (HGT), which allows for the sharing of these genes between pathogens and nonpathogens, and even gram – positive and gram – negative bacteria (Kruse and Sørum, 1994; Pruden et al., 2006).

Other non-antimicrobial pharmaceuticals and personal care products (PPCPs) may also contribute to the selection pressures that accelerate the evolution of antimicrobial resistant bacteria in the environment. For instance, the antidepressant fluoxetine has been recently shown to induce multiple AMR in *Escherichia coli* (Jin et al., 2018). This finding is notable because antidepressants are ubiquitous in surface waters, even in widely diluted large lakes, resulting in the bioaccumulation in fish (Arnnok et al., 2017). Furthermore, PPCPs can negatively impact biofilm respiration and growth (Rosi-Marshall et al., 2013). Improved understanding of the multitude of routes via which the above mentioned chemicals enter the environment can provide opportunities to prevent their spread, by enabling control strategies that target sources or identified critical control points (Larsson et al., 2018; Vikesland et al., 2017). The major sources of antimicrobial residues and other PPCP contamination are from the low- and middle-income countries (LMIC), where environmental regulations are poor and wastewater treatment facilities are lacking. Unfortunately, data on the occurrence of these contaminants from LMIC are very scarce, due to lack of resources and analytical capabilities (aus der Beek et al., 2016). Due to the limited knowledge on the environmental concentrations that might exert selection for resistant bacteria, it is important to conduct global-scale monitoring of antimicrobials to provide a basis for determining minimal selective concentrations in complex natural environments. Key to answering these questions are studies that include methodologies that allow for the accurate and sensitive measurements of these drug residues, irrespective of sample origin and the complexity of the matrix, which may vary significantly between countries of origin.

Recent studies on antimicrobials in wastewater involve analysis only of representative compounds for each class. In addition, most methods developed for these studies were applied to samples originating from a single location (Kimura et al., 2014; Novo et al., 2013; Östman et al., 2017; Senta et al., 2013). Analysis of several compounds from multiple classes of antimicrobials in samples originating from multiple locations around the world requires a different approach because of the numerous challenges that need to be addressed in sample collection, preservation, and analysis. First, simultaneous analysis of multiple compounds from different classes of antimicrobials is difficult because of the wide range of physical and chemical properties of the analytes that render efficient extraction and separation under one condition problematic. Second, wastewater and surface water samples from each country are likely to have unique matrix properties because of differences in the types of treatment processes employed and the varying government regulations on waste disposal. In addition, differences in antimicrobial consumption and prescription practices between countries also result in different patterns and types of antimicrobial residues in the environment (Centner, 2016; Wallinga et al., 2015). The analysis of a wide range of concentrations, different types of drug residues, and varying sample matrix complexity not only affect method detection limits, but also the chromatographic retention times of the compounds (Tran et al., 2016). In addition, for consistency of the global reconnaissance of antimicrobials, it is ideal to ship the samples to one location for analysis, hence the stability of the compounds during shipping must be well characterized. Other challenges regarding international shipment include high cost, potential breakage of sampling containers, and significant delays in delivery due to custom requirements for water samples that may be considered to be a potential biohazard.

This study aims to identify challenges and provide solutions to the problems encountered in the analysis of antimicrobials and other PPCPs in wastewater and surface water samples from different countries. The goal of the study is to develop a single sensitive and robust analytical method that can analyze several compounds from multiple classes of antimicrobials, including beta-lactams, ionophores, macrolides, quinolones, sulfonamides, antidepressant drugs and other PPCPs. A stability

study was performed to mimic the shipping conditions of samples when they are processed using solid-phase extraction (SPE) to preserve the analytes and minimize international shipping costs. This study also demonstrates the advantage of using isotopically labeled standards in overcoming the challenges of a global-scale reconnaissance study to collect data on antimicrobials and PPCPs in the environment. While global surveillance for antimicrobial resistance necessarily includes the monitoring of bacterial species, it is equally important to monitor residues of antimicrobials and PPCPs on a global scale. The resulting data from these studies can be used as guidance in environmental risk assessment for establishing regulations on emission limits of active ingredients of pharmaceuticals into the environment, and to serve as a comprehensive reference framework for future studies to understand the drivers of environmental antibiotic resistance. In this paper, important considerations in the analysis of antimicrobials and PPCPs are discussed and practical protocols that can be implemented in an identical manner in all countries, even those with limited resources, are presented.

2. Materials and methods

Acetaminophen (ACT), acetaminophen-d_4 (d_4-ACT), acetyl-sulfamethoxazole (ASMX), acetyl-SMX-d_4 (d_4-ASMX), amoxicillin (AXC), ampicillin (AMP), azithromycin (AZI), caffeine (CAF), carbamazepine (CBZ), cefapirin (CFP), ceftiofur (CFT), clarithromycin (CLA), demeclocycline (DMC, surrogate), dicloxacillin (DCXC), enrofloxacin (ENRO), erythromycin (ERY), ibuprofen (IBU), ibuprofen-d_3 (d_3-IBU), iopamidol (IOPA), lasalocid (LAS), maduramycin (MAD), meprobamate (MEP), metformin (MET), monensin (MON), naproxen (NPX), naproxen-d_3 (d_3-NPX), narasin (NAR), nigericin (NIG), nonactin (NON), norfloxacin (NOR), oxolinic acid (OXO), oxytetracycline (OTC), penicillin G (PENG), penicillin V (PENV), salinomycin (SAL), sarafloxacin (SARA), sulfachloropyridazine (SCP), sulfadiazine (SPD), sulfadimethoxine (SDM), sulfamerazine (SMR), sulfameter (SMT), sulfamethazine (SMZ), sulfamethizole (SMI), sulfamethoxazole (SMX), sulfamethoxazole-d_4 (d_4-SMX), roxithromycin (ROX), tetracycline (TC), tilmicosin (TIL), trimethoprim (TMP), trimethoprim-d_9 (d_9-TMP), and tylosin (TYL) were purchased from Sigma Aldrich. ^{13}C-erythromycin-H2O (^{13}C-ERY), ciprofloxacin (CIP), desvenlafaxine (DES), diclofenac (DIC), diclofenac-d_4 (d_4-DIC), and dilantin (DIL) were obtained from Cambridge Isotopes (Tewksbury, MA). Spiramycin (SPI, mixture of spiramycin I, II, and III) and sulfathiazole were purchased from ICN Biomedicals, Inc. (California, USA). Carbamazepine-d_{10} (d_{10}-CBZ), ciprofloxacin-d_8 (d_8-CIP) and caffeine-d_3 (d_3-CAF) were purchased from CDN Isotopes (Quebec, Canada). Anhydrotetracycline (ATC) chlortetracycline (CTC) was obtained from Acros Organics (New Jersey, USA). Diphenhydramine HCl (DPH), diphenhydramine-d_3 (d_3-DPH), bupropion HCl (BUP), bupropion-d_9 HCl (d_9-BUP), citalopram HBr (CIT), citalopram-d_6 HBr (d_6-CIT), paroxetine maleate (PRX), paroxetine-d_6 maleate (d_6-PRX), venlafaxine (VEN), venlafaxine-d_6 (d_6-VEN), desvenlafaxine (DES), desvenlafaxine-d_6 (d_6-DES), norfluoxetine oxalate (NFLX), norfluoxetine-d_6 oxalate (d_6-NFLX), norsertraline HCl (NSER), and norsertraline-$^{13}C_6$ HCl ($^{13}C_6$-NSER) were obtained from Cerilliant (Sigma-Aldrich, St Louis, MO). Barnstead NANOpure™ Diamond (Waltham, MA) purification system was used to obtain 18.2 MΩ water used throughout all experiments. LC-MS grade methanol and acetonitrile (Omnisolv™) was obtained from EMD Millipore Corporation (Billerica, MA). Formic acid (88%) was purchased from Fisher Chemical (Pittsburgh, PA).

2.1. Sample collection and solid phase extraction (SPE) protocol

2.1.1. Sample pre-treatment

Wastewater samples (0.5 L) were collected in amber glass bottles pre-rinsed with 10% nitric acid. After acidification to pH 2.5 ± 0.5 using 40% phosphoric acid, samples were filtered using 0.45 μm glass microfiber filters to remove particulate matter and microorganisms. Using a pipette, 2 mL of Na_2EDTA (5% *w/v* in water) was added to each sample. Subsequently, samples were spiked with surrogate standards (50 μL of a 1000 μg/L surrogate mixture solution).

2.1.2. SPE procedure

Oasis™ HLB cartridges (500 mg, 6 mL) were preconditioned with 6 mL acetonitrile, followed by 6 mL nanopure water. Samples were then loaded at a rate of 3–5 mL/min. Once the sample was completely loaded, cartridges were allowed to dry for a minimum of 30 min. Samples were wrapped in foil then secured in air-tight plastic bags then shipped on ice packs to the University at Buffalo. Upon receipt, samples were eluted with 8 mL of LC-MS grade acetonitrile. Using a turbo-evaporator, sample extracts were dried under N_2 gas at 35 °C. After drying, 100 μL of a 1000 μg/L d_{10}-CBZ (as internal standard) was added to the tubes followed by resuspension of the extracts in 900 μL of starting mobile phase. Samples were then transferred to 2 mL LC-MS amber vials that were previously desilanized for LC-MS/MS analysis.

2.2. LC-MS/MS analysis

Separation was carried out using an Agilent 1200 LC system (Palo Alto, CA) with degasser, chiller, quaternary pump, and autosampler. Two columns were tested for this study: a Waters Cortecs C18^{+}™ column (Milford, Massachusetts) with dimensions 2.1 × 150 mm and 2.7 μm particle size and a Waters Charged Surface Hybrid (CSH) column with dimensions 2.1 × 150 mm and 3.5 μm particle size. The signal-to-noise ratio of individual analyte signals was used to determine which column is more advantageous for the purposes of this study. The flow rate was set at 0.2 mL/min. The mobile phase consisted of a gradient program containing mobile phase A (aqueous 0.3% formic acid) and B (75 methanol/25 acetonitrile, v/v), starting with 90% A and 10% B that was kept constant for 3 min, followed by a linear ramp to 100% B from 3 to 22 min. This condition was kept for 5 min, then the mobile phase was returned back to 10% A within 4 min. The column was kept at this condition for another 14 min for column re-equilibration; total run time is 45 min. A Thermo TSQ Quantum Ultra (Waltham, Massachusetts) triple quadrupole mass spectrometer was used under positive heated electrospray ionization (+HESI) in timed selected reaction monitoring (tSRM or EZ method) mode. The details of the SRM transitions used are shown in Table S1 in the Supporting Information (SI). Ampicillin, amoxicillin, and cefapirin may degrade faster in the presence of acid. As an alternative to avoid degradation, the addition of acid before solid phase extraction can be skipped and the amount of acid additive in the mobile phase can be changed from 0.3% to 0.01%. However, this study includes a wide range of chemicals with different physico-chemical properties, and the existing method was found to work best for most of the compounds.

The mass spectrometer had the following spray settings: spray voltage (3000 V), ion sweep gas pressure (0 arbitrary units), vaporizer temperature (350 °C), sheath gas pressure (40 arbitrary units, N_2), auxiliary gas pressure (35 arbitrary units, N_2), capillary temperature (325 °C), collision gas pressure (1.5 mTorr, Ar), cycle time (0.300 s), and Q1 peak width (0.70 FWHM).

2.3. Test of stability in solid phase extraction cartridges

Influent wastewater samples collected from a local plant ($N = 21$) in Amherst, NY (USA) were spiked with 50 μL of a 1000 μg/L mixture containing all target sulfonamides, macrolides, PPCPs, quinolones, SSRIs, and tetracyclines mentioned above. Samples were prepared and extracted by SPE; the SPE cartridges were stored either at uncontrolled room temperature (23–27 °C) or inside a − 4 °C freezer to simulate "worst" and "best" case scenarios, respectively, during international shipping. In order to eliminate the possibility of degradation of analytes due to humidity in the SPE cartridges, the sorbent was dried thoroughly

under vacuum after loading of the water samples, prior to shipment. Three samples were eluted and analyzed on the same day of extraction to serve as the reference condition, during which sample degradation is unlikely to occur. Then, triplicate SPE cartridges were taken from each condition after seven, fifteen, and twenty-eight days of storage, for elution and analysis, to determine recoveries of each of the spiked analytes. The entire stability study was conducted for a period of four weeks.

2.4. Effect of matrix on the chromatographic retention times, and importance of quantification by isotope dilution

Aliquots (190 µL) were taken from each type of sample (extracts of wastewater and surface water) from 6 different countries (Hong Kong, India, Philippines, Sweden, Switzerland, and the United States); a total of 20 samples were spiked with 10 µL (5% of total volume) of a 1000 ng/L standard mix. These spiked extracts were analyzed using the optimized LC-MS/MS method to determine whether the differences in sample matrix will have a significant effect on the chromatographic retention times of the analytes.

Isotope dilution was used for quantification whenever a stable-isotope labeled analogue is available for each target analyte. Known amounts of labeled analogues were spiked into the water samples prior to SPE, serving both as surrogate standards to account for recoveries and as reference standards for quantification. For analytes that did not have a labeled surrogate, structurally similar isotope-labeled internal standards that have similar physico - chemical properties and retention times that are sufficiently close to the target analyte were used for quantification. For the latter method, 380 µL of each unknown sample was obtained and split into two. The first aliquot was spiked with 10 µL of the standard mix and the second aliquot was spiked with 10 µL of the starting mobile phase; both of these samples were analyzed by LC-MS/MS in consecutive runs. The recoveries of the surrogate standards were monitored in order to account for any degradation of the analytes that might occur in the SPE cartridges, and to compensate for the differences in ionization efficiencies resulting from the high variability in the sample matrices. The ratios of the quantitative ion (Q) over the qualitative ion (q) were also monitored, together with the retention time for quality assurance, as described in our previous publication (Angeles and Aga, 2018).

3. Results and discussions

3.1. Optimization of chromatographic separation and mass spectrometric fragmentation

In developing an analytical method for the quantification of a mixture of analytes with a wide range of polarities and acid-base properties, the first critical step is choosing the right chromatographic column. Between the two LC columns examined, it was found that the signal-to-noise (S/N) ratio of the analytes were higher using the Waters Cortecs C18$^+$™ column (Cortecs) with a solid core column and smaller particle size (2.7 µm) compared to Waters Charged Surface Hybrid (CSH) with a fully porous particle and a larger particle size (3.5 µm); these columns have the same dimensions. A summary of the S/N ratios and the signal improvement observed when using the Cortecs column is shown in Table 1. The signal improvement was calculated by dividing the S/N of the peaks obtained with the Cortecs column by the S/N obtained using the CSH column. Across the board, there is significant improvement in the S/N ratio for all of the compounds studied when using the Cortecs column. This improvement translates to lower detection limits, which is a critical factor in the trace analysis of antimicrobials in wastewater. Therefore, subsequent analysis used the Cortecs column. Different separation gradients were also tested, but only the gradient program that provided the optimum separation of analytes belonging to the same class of antimicrobials is presented in this paper.

Table 1
Comparison of the signal-to-noise (S/N) ratios for selected analytes using two different columns.

Signal-to-noise ratio (peak area)			
Analyte	Cortecs $C_{18}{}^+$	Charged surface hybrid (CSH)	Percent improvement
Macrolides			
Clarithromycin	1.7E + 07	2.7E + 06	630
Erythromycin	5.2E + 06	8.7E + 05	590
Roxithromycin	5.7E + 05	8.7E + 05	65
Spiramycin 2	6.7E + 06	1.4E + 06	500
Spiramycin 3	1.0E + 07	1.8E + 06	560
Tilmicosin	2.9E + 06	4.9E + 05	590
Tylosin	6.8E + 05	1.5E + 05	460
Quinolones			
Ciprofloxacin	1.9E + 04	5.7E + 03	340
Enrofloxacin	8.1E + 03	1.9E + 03	430
Norfloxacin	1.9E + 03	4.1E + 03	47
Oxolinic acid	6.6E + 03	1.2E + 03	540
Sarafloxacin	1.3E + 04	6.9E + 03	200
Antidepressants			
Bupropion	2.5E + 04	4.6E + 03	530
Citalopram	6.4E + 04	1.6E + 04	390
Norfluoxetine	6.9E + 04	2.0E + 04	350
Norsertraline	2.5E + 04	1.0E + 04	240
Paroxetine	2.9E + 04	2.6E + 04	110
Venlafaxine	1.3E + 04	3.3E + 03	400
Sulfonamides			
Sulfachloropyridazine	2.1E + 06	1.4E + 04	15,000
Sulfadiazine	3.7E + 04	4.9E + 04	76
Sulfadimethoxine	1.6E + 07	3.5E + 06	440
Sulfamerazine	6.3E + 06	1.4E + 04	45,000
Sulfamethazine	7.2E + 06	1.3E + 06	540
Sulfamethizole	9.0E + 05	2.1E + 05	430
Sulfamethoxazole	2.3E + 03	6.9E + 02	330
Sulfathiazole	2.5E + 04	1.2E + 04	210

One of the limiting factors in developing an LC-MS/MS method for multiple classes of compounds is the number of SRM transitions that can be incorporated into a single run without losing method sensitivity. The loss in sensitivity is a consequence of the MS scanning for a finite number of mass transitions in a finite amount of time; a longer dwell time is preferred for a more accurate measurement. When the MS is set to scan for a higher number of mass transitions per unit time, the effective dwell time per mass transition is decreased, which lowers the number of data points collected per chromatographic peak. This limitation can be circumvented by using the EZ™ method feature of the instrument software such that only specific SRM transitions are scanned for a specific period of time. By doing so, the number of points collected per analyte peak is increased, thereby providing a more accurate measurement, resulting in higher S/N ratios. The ability to create an EZ™ method provides a means by which a single method can analyze multiple classes of compounds that would normally be performed in separate analytical runs, thereby reducing analysis time.

Due to the differences in matrix complexity that can be expected for samples coming from different countries, it is possible that retention time shifts in chromatographic separation will occur in an unpredictable manner. In addition, because different countries use different antimicrobials, not all analytes will be present in all samples, thus making it challenging to determine if the absence of an analyte peak in the expected chromatographic retention time is truly a negative detection or due to a retention time shift. Since the positive detection of a compound in a timed-SRM MS method is highly dependent on the analyte's retention time, significant differences in matrix that lead to retention time shifts may cause a false-negative detection of a compound. Therefore, the robustness of the chromatographic method was tested by spiking aliquots of influent and effluent wastewater, and

surface waters collected from upstream and downstream of the WWTPs, with a known amount of the standard mix. The volume of the spiked standard was only 5% of the total volume to prevent significant changes in matrix characteristics due to dilution. The goal of this test was to assess the effect of the different matrices on the analyte retention times and determine if the matrix effects are significant enough to result in false negatives. The average retention times and standard deviations for the target analytes are summarized in Table S1. Peak shifts can be assessed by looking at the magnitude of the standard deviations; a low standard deviation indicates reproducible retention times. No significant retention time shifts were observed for all of the analytes; erythromycin exhibited the largest shift (0.7 min), but still within the time range before and after retention time. A significant retention time shift is when the analyte signal is not observed because of elution outside the time range set for the mass spectrometer to scan for a specific MS/MS transition. Since the method was set to scan for a mass transition within 1.5 min before and after the expected retention time, false-negative detection was prevented. It is also a reflection of the effectiveness of the SPE procedure in minimizing matrix effects that could affect chromatography.

3.2. Storage temperature affects analyte recoveries and stability in the SPE cartridge

The stability of antimicrobials in the SPE cartridges was tested under two different storage conditions, −4 °C and room temperature (23–27 °C), to simulate "best case" and "worst case" scenarios during international shipping, respectively. SPE cartridge comparison was not performed due to continued success with using the cartridge used in this study compared to other cartridges (Arnnok et al., 2017; Mullen et al., 2017; Su et al., 2014; Tso et al., 2011). Performing SPE in the country of sample origin after sample collection was advantageous based on two grounds: first, shipping and handling costs can be minimized since only SPE cartridges would be shipped internationally instead of glassware filled with liquid samples, and second, breakage and loss of valuable samples could be avoided. Shipping SPE cartridges rather than bottles of water samples also prevented issues regarding sample safety since no biologically active material is being transported. Courier service companies prohibit the shipment of samples containing flammable solvents such as methanol and acetonitrile, therefore, shipping of SPE-eluate in vials was not considered.

To study the analyte stability at a "worst-case" scenario during shipping, the SPE cartridges were stored in a room without temperature control to mimic conditions where the SPE cartridges may thaw out during shipment, which could happen due to potential delays in shipment release from government customs. Fig. 1 summarizes the results obtained for the stability of the different analytes, presented based on compound class. Sulfonamides and tetracyclines showed the highest degradation compared to the other compound classes irrespective of storage condition. For sulfonamides stored at room temperature, the recovery dropped from 54% to 43% then 28% between 8, 15, and 28 days of storage in the SPE cartridge, respectively. When stored at −4 °C, an improvement in sulfonamide recovery was observed for the first 8 days (74%) after which recovery decreased to 33% and 23% when eluted from the cartridge after 15 days and 28 days, respectively. For tetracyclines stored at room temperature, the trend in recovery was decreasing at 36%, 28%, and 21% recovery for elution at 8, 15, and 28 days, respectively. Cold storage showed improvement in tetracycline recovery at 58%, 53%, and 37% recovery after 8, 15, and 28 days, respectively. In general, a decreasing trend in analyte recovery can be observed over time for all compound classes. However, no generalizations regarding compound class stability can be established since different compounds have different stabilities irrespective of compound class.

To determine whether there is a statistical difference between the recoveries of each compound during the different extraction dates, Two-Way ANOVA was performed at a 99% confidence level. Whenever a statistical difference between the first week and second week is observed, a compound was deemed to have started degradation and is considered to be stable in the SPE cartridge for one week only. Similarly, when there is no statistical difference between the recoveries after 1 day and 8 days of storage, but there is statistical difference between the recoveries after 8 days and 15 days of storage, the analyte was considered to be stable for at least two weeks in the SPE cartridge. Compounds that do not have statistically different recoveries for all sampling points were considered stable for more than 28 days. Table 2 presents a classification of individual compound stabilities in the SPE cartridges for the two storage conditions. The compounds are listed in the first column when their recoveries in the 8-day samples were significantly different from the initial analyte recoveries in day 0 (reference value, set as 100%). A significant difference in recovery suggests that either (1) analyte degradation occurred during storage, (2) analytes irreversibly bound to the SPE packing material, or (3) interactions between the analytes and co-extracted matrix prevented efficient recovery or detection of the analytes in the final LC-MS/MS analysis. Taking the case of ciprofloxacin for instance, there is significant difference in the analyte recoveries between day 0 and day 8 in both room temperature and cold storage, where a 40% drop in the recovery was observed. Therefore, ciprofloxacin (CIP) was classified as a compound with a stability window less than 7 days, and was listed in the first column of Table 2 for both storage conditions. For sulfamethizole (STZ), the percent recovery after 7 days was 79 ± 15, which is not significantly different from the recovery in day 0, but is significantly different from the recovery in day 15 (33 ± 1) under the cold storage condition. In contrast, degradation was observed after 7 days, with a 60% drop in recovery between the day 0 and day 7, under uncontrolled room temperature conditions. Therefore, STZ is listed in the second column under cold storage (stable for up to 15 days), but is listed in the first column under uncontrolled room temperature storage (stable only for up to 7 days). Using the same logic, acetyl-sulfamethoxazole (ASMX) did not show significant decrease in its recovery until after day 15, under both storage conditions. Therefore, ASMX is listed in the third column and is considered stable for up to 15 days of storage in the SPE cartridge.

Table 2 provides guidance on how long SPE cartridges should be stored under refrigerated and under uncontrolled room temperature conditions, and indicates what analytes are most affected during long storage in the SPE cartridge. It is obvious and not surprising that cold storage increases the stability of the compounds, showing high recoveries of the analytes from the SPE cartridges even after one week of storage. This guideline is helpful in creating protocols for international collaborations. By keeping the cartridges cold during shipment, the integrity of the samples can be maintained. Taken from a different perspective, it also implies how there may be an underestimation of antimicrobial loading when the SPE cartridges do not get analyzed within a reasonable time scale (less than 7 days). Ideally, all samples should be eluted and analyzed by LC-MS/MS within two days after receipt. The number of analytes that degrade during the first 7 days of storage decrease from 33 compounds to 23 compounds when the cartridges are kept cold. The benefit of cold storage disappears when the cartridges are not eluted within 15 days as 38 compounds degrade within this time frame whether kept in cold or room temperature storage. Improved class stability is observed for macrolides and sulfonamides.

In actual samples, differences in the extent of degradation of the analytes in the SPE cartridges could be accounted for by correcting the analyte recoveries using the observed recoveries of the isotopically-labeled surrogate standards. Since the surrogate standards have the same physico-chemical properties as the corresponding native analytes, degradation and matrix suppression can be corrected for using the surrogate recovery data. Because the accuracy of quantification depends on the accurate spiking of the surrogates, the personnel performing the

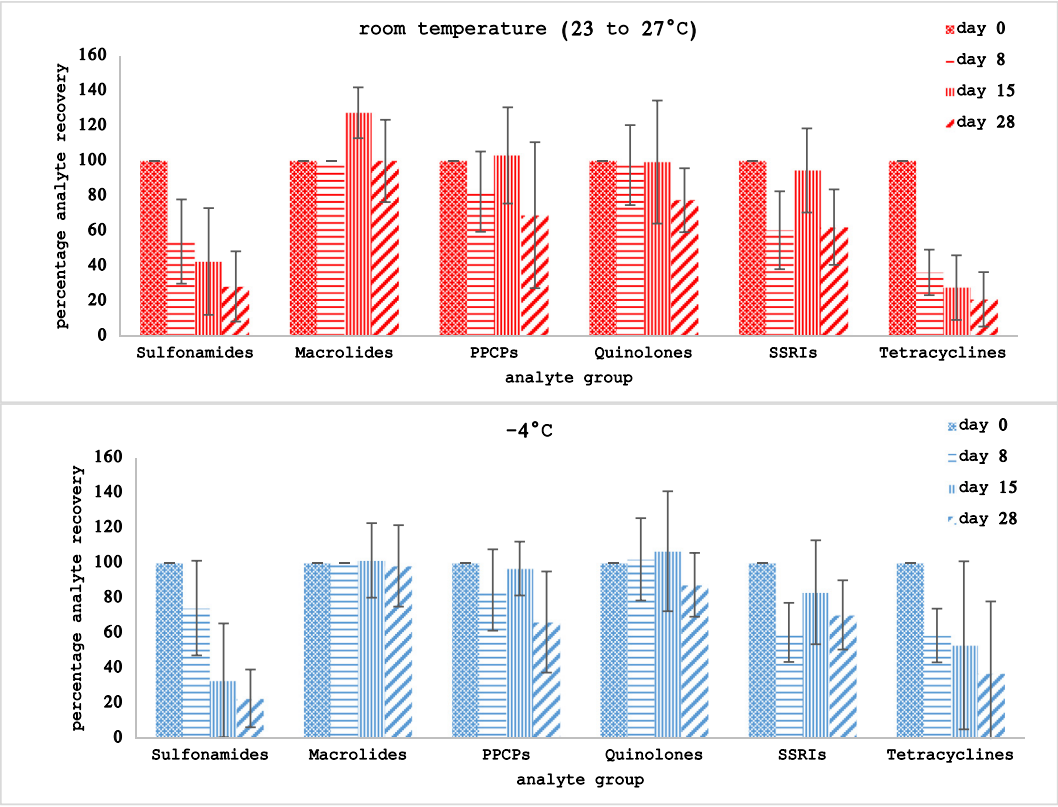

Fig. 1. The stability of analytes inside a solid phase extraction (SPE) cartridge differs by compound class. Sulfonamides, SSRIs (antidepressants), and tetracyclines show faster degradation while macrolides, quinolones, and other pharmaceuticals and personal care products (PPCPs) show longer stability inside the cartridge. SSRI: selective serotonin reuptake inhibitor.

Table 2
Using two-way ANOVA and a *P*-value of 0.01, differences in the stability of individual compounds from day 0 to day 28 were compared. It can be observed that the stability of the compounds in the cartridge get extended to longer days when the cartridges are kept in −4 °C compared to room temperature. The decrease in number of analytes that degrade within 7 days is evident with the decrease in the number of analytes that show significant difference in stability; 33 analytes degrade within the first 7 days compared to 23 analytes. Improved class stability is observed for macrolides and sulfonamides.

Stability in cartridge

		Between 0 and 7 days	Between 7 and 15 days	Between 15 and 21 days	Greater than 21 days
Cartridge stored in room temperature	Tetracyclines	ATC, CTC, DMC, OTC, CTC			
	Quinolones	ENRO, CIP	OXO	SARA	NOR
	PPCPs	IOPA, MET, CAF, DIC	ACT, NAP	IBP	MEP, CBZ, TMP
	Macrolides	TIL, SPI2, SPI3, CLA, ROX	AZI		ERY, TYL
	Sulfonamides	SMX, STZ, SMR, SMI, SMZ, SMT, SCP, SDM	SPD	ASMX	
	SSRIs	BUP, DIL, DES, NSER, VEN, NFLX, SER, CIT, PRX			
Cartridge stored at −4 °C	Tetracyclines	ATC, CTC, OTC, CTC	DMC		
	Quinolones	ENRO, CIP	OXO, SARA		NOR
	PPCPs	MET, CAF, DIC	ACT, IBP, MEP, NAP, CBZ, TMP		IOPA
	Macrolides		AZI		TIL, SPI2, SPI3, ERY, CLA, ROX, TYL
	Sulfonamides	SPD, SMR, SMZ, SMT, SDM	SMX, STZ, SMI, SCP	ASMX	
	SSRIs	BUP, DIL, DPH, DES, NSER, NFLX, SER, CIT, PRX	VEN		

extraction in the different sampling sites across the globe must be well trained prior to the sampling campaign. This training can be accomplished by sharing extraction protocols to the different laboratories together with the preparation of instructional videos and actual hands-on training, whenever possible.

The ion ratio (Q/q), which is the area of the quantifier ion (Q) divided by the area of the qualifier ion (q), has been used as an additional criterion for reporting analyte detection (Mol et al., 2015). For this

study, a tolerance range for Q/q of all target analytes in wastewater and surface water has been set be equal to the mean Q/q of the analyte standard ± 40% for influent samples, and mean Q/q ± 30% for the effluent, upstream, and downstream samples (Angeles and Aga, 2018). For tetracyclines and quinolones, however, a wider tolerance range was implemented, using mean Q/q ± 80% to avoid false negatives. However, for these compounds all positive peaks need to be verified carefully by inspecting the peaks individually before reporting any positive detections (Angeles and Aga, 2018).

Ciprofloxacin was the antimicrobial with the highest levels detected, with a concentration of 48,103 ng/L found in the influent wastewater sample from Hong Kong. This value was validated with the spiked surrogate and met all the criteria for positive detection. First, the peak for ciprofloxacin came out at the retention time that agrees with the retention time of the surrogate standard. Second, both quantifier and qualifier ions are present (Fig. S1). Third, the Q/q was within the acceptable range of 0.93 to 8.29 (Angeles and Aga, 2018); in the influent and effluent samples, the Q/q were 2.28 and 2.79, respectively, which are within the set range.

3.3. Different countries show differences in antimicrobial concentrations

WWTP samples were received from Hong Kong, India, the Philippines, Sweden, Switzerland, and the United States and included influent, effluent, upstream, and downstream samples. Analysis of samples revealed differences in the antimicrobial profiles, which are shown in Fig. 2. The contaminant loading is presented as a total of all the antimicrobials detected, sorted into four compound classes, sulfonamides, macrolides, tetracyclines, and quinolones. A difference in the concentration scale, due to the higher levels of antimicrobials in the wastewater compared to surface water, should be noted in the figures corresponding to the influent, effluent, upstream, and downstream samples. It is also notable that the antimicrobial levels are higher in samples originating from Hong Kong and India compared to samples originating from the Philippines, United States, Sweden and Switzerland. The influent from one of the WWTPs sampled in Hong Kong showed the highest antimicrobial concentrations, which were mainly quinolones (CIP, NOR) and macrolides (ROX, CLA, AZI), as listed in Table S2. The second highest concentrations of antimicrobials were observed in one of the WWTPs in India, which was mainly macrolides (CLA, AZI, A-ERY). As can be seen in Fig. 2 and Table S2, it can be deduced that quinolones (especially CIP) and macrolides (especially CLA and AZI) are the most heavily used antimicrobials in all countries. It is not surprising then that ciprofloxacin has been documented to be present at high concentrations in sediment (Kristiansson et al., 2011) and surface water (Mutiyar and Mittal, 2014) samples from India. Interestingly, other classes of antibiotics were detected at much lower concentrations; the highest sulfonamide levels were detected in United States WWTP 2 influent (1941 ng/L for sulfamethoxazole) and the highest tetracycline levels detected was in India WWTP 2 (241 ng/L for tetracycline).

When it comes to the effluent, clarithromycin was the highest detected antimicrobial (5178 ng/L in India WWTP 2), followed by sulfamethoxazole (1301 ng/L in Philippines WWTP 2), as shown in Table S2. Notably, in samples originating from India WWTP 2, clarithromycin and the macrolide-metabolite anhydro-erythromycin were found in the µg/L concentrations in the upstream sample (Table S3), indicating sources other than WWTP effluents. Effluents samples and surface water samples from Sweden, Switzerland, and the United States had generally very low in antimicrobial concentrations, and mostly below the detection limits. Some of the factors that may contribute in the high antimicrobial concentrations found in the environment include easy access to antimicrobials without prescription and the lack of access to toilets and sanitation facilities (Laxminarayan and Chaudhury, 2016). The macrolides remain in the µg/L level in the downstream sample. Ciprofloxacin and norfloxacin were also detected in the upstream

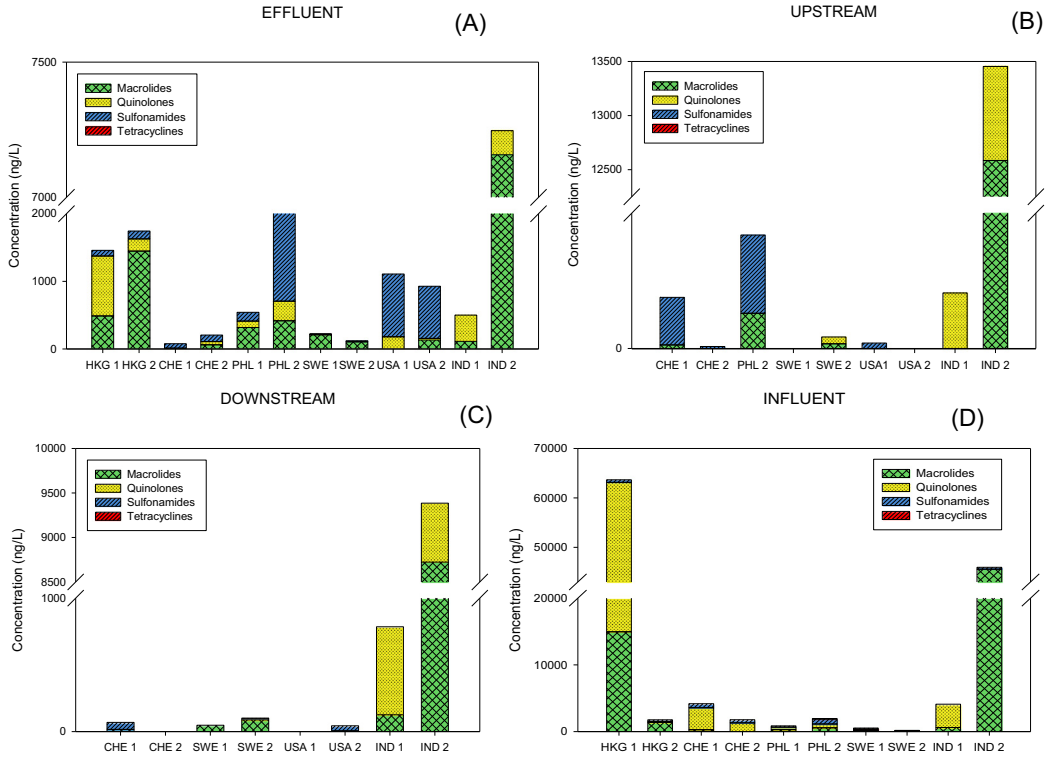

Fig. 2. The amounts of antimicrobials found in each sample in the influent (A), effluent (B), upstream (C), and downstream (D), are presented as total analyte loading in ng/L. Higher levels can be observed in the influent compared to the effluent samples. The influent samples from Hong Kong showed the highest antimicrobial loading among all countries studied (CHE – Switzerland, HKG - Hong Kong, IND - India, PHL – Philippines, SWE – Sweden, USA – United States of America). Two wastewater treatment plants were sampled per country and are labeled 1 and 2.

sample from India 2 at 635 ng/L and 233 ng/L, respectively (Table S3). A similar study, though with a more limited number of analytes, showed similar trends in Indian environmental samples (Fick et al., 2009).

Antidepressants, and other pharmaceuticals such as acetaminophen, caffeine, carbamazepine, diclofenac, iopamidol, and trimethoprim, were also analyzed in the samples because they may have the capability of exerting selection pressures on bacteria. A detailed summary of the concentrations at which these non-antimicrobial analytes were found is presented in Table S4 and S5. The most commonly detected PPCP was caffeine, which was present in 40 out of 41 samples, while diclofenac had the highest measured concentration at 108,000 ng/L (Table S5) in a sample downstream of a Swiss WWTP. For the antidepressants, the most frequently detected compound was venlafaxine, which was found in 24 out of 41 samples. The antidepressant norfluoxetine, on the other hand, is the compound detected with the highest concentration, of 984 ng/L in the United States wastewater influent sample (Table S4). As a result of the collective interplay among the physical, chemical, microbial and genetic components of the various environments, differences in the types and concentrations of antimicrobial and other compounds may be associated with corresponding ARG profiles characteristic of each environment. Antidepressants were detected at higher concentrations in Sweden, Switzerland, and the United States, which was expected since these countries are among the highest consumers of antidepressants. This reflects the fact that the United States is the top consumer of antidepressants in the world, where 11% of the people above 12 years old are prescribed to take these drugs (Pratt et al., 2017).

Certain challenges were encountered during surface water collection. Downstream sample collection was not possible in Hong Kong, as the WWTP effluent is discharged to the ocean where differentiating an impacted and unimpacted site was not feasible, and sample collection was dangerous. For samples coming from the Philippines, WWTP 1 was inside a building receiving wastewater from both residential and commercial sources, while the downstream sample from WWTP 2 was in an unsafe location thus preventing the collection of the sample. For the samples successfully collected, similar general trends in contaminant concentrations were observed such that they are higher in wastewater than surface water samples. Though it was difficult to discern conclusions regarding the differences in contaminant levels when comparing upstream and downstream samples, the relatively higher concentrations found in downstream samples demonstrated that WWTPs are important point sources of antimicrobials and PPCPs in many aquatic systems. One should not mistakenly attribute that the difference in contaminant loading between these two wastewater samples is due to removal, but it must be kept in mind that these samples may be from different batches of received wastewater. It is emphasized here that the goal of the present study was not to assess removal rates from the different WWTPs, but rather to develop a robust method that can be used for the global monitoring of antimicrobials and PPCPs in WWTP effluents and receiving waters from different countries. The results presented in this paper demonstrate the applicability of the developed sample preparation protocols and analytical method that can be adopted for international collaborative studies. For studies that aim to assess WWTP removal efficiencies of antimicrobials or other PPCPs, it is strongly recommended to collect composite samples in order to be able to draw out accurate conclusions regarding the treatment efficiencies. It is also important to collect water samples in various sampling points in the receiving waters and record the surface water flow rates and volumes to better estimate the predicted environmental concentrations of antimicrobials and PPCPs.

In conclusion, this paper provides a cost-effective and easy to implement sample preparation and analytical approaches for 8 classes of antimicrobials and other pharmaceuticals that can be used for global monitoring in wastewater and receiving waters. Because many researchers from upper middle and high income countries are partnering with LMIC to monitor occurrence of antibiotics and PPCPs globally, it was important to investigate critical steps in the sample preparation, shipping, and analysis to determine the effect of temperature and length of storage on the stability of target analytes during international shipment. Here, we demonstrated the robustness of the chromatographic method for different types of sample matrices ranging from influent and effluent wastewater, to surface waters collected from upstream and downstream of the wastewater treatment plants. In addition, the importance of applying a defined tolerance for the ion ratio (Q/q) optimized for wastewater and surface water to minimize false negative and false positive detection, was demonstrated. This tolerance range was set to be the mean ion ratio of the standard at various concentrations ± 40% for the influent, and ± 30% for the effluent, upstream, and downstream samples. For tetracyclines and quinolones, however, the tolerance range was 80%. This study successfully developed a general method for the cost-effective analysis of antibiotics and PPCPs from LMIC environments, while maintaining sample integrity and producing high quality data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.01.025.

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