ARTICLE

Occurrence and stability of veterinary antibiotics in Upper Pampanga River Basin located in Nueva Ecija, Philippines

Gerardo V. Ilagan*[1,2], Ian Ken D. Dimzon[1,3], and Armando M. Guidote Jr.[1,3]

[1]Department of Chemistry, School of Science and Engineering, Ateneo De Manila University, Katipunan Ave., Quezon City, 1108 Philippines
[2]Chemistry Department, College of Science, Central Luzon State University, Science City of Muñoz, Nueva Ecija, 3120 Philippines
[3]Philippine Institute for Pure and Applied Chemistry (PIPAC), Ateneo De Manila University Campus, Katipunan Ave., Quezon City, 1108 Philippines

ABSTRACT

The occurrence of antimicrobial resistance (AMR), resulting from improper use and handling of antibiotics , poses a significant threat to global health, food security, and sustainable development. One major cause of AMR is the misuse of antibiotic substances in the livestock and veterinary industries, where 93,000 tonnes of antibiotics were sold and consumed by the livestock industry in 2017, with an expected increase of 11.5% by 2030. Approximately 80% of livestock receive antibiotic treatment, and up to 75% of these compounds are excreted, eventually entering environmental waters and affecting aquatic biota. In the Philippines, data on antibiotic contamination, particularly in aquatic environments, remain scarce. This study investigates the presence and fate of seven veterinary antibiotics—amoxicillin (AMX), ampicillin (AMP), penicillin G (PenG), tetracycline (TET), oxytetracycline (OTC), sulfamethazine (SMZ), and sulfamethoxazole (SMX)—in the surface waters of the Upper Pampanga River and its tributaries in Nueva Ecija. Water samples from various localities were analyzed using a validated LC-MS/MS-ESI-Positive (MRM) method, following an optimized Solid Phase Extraction (SPE) procedure. The method demonstrated fitness for purpose in determining antibiotics in river water with R^2 values of >0.999 at a working range of 0.5-3.0 ng/mL, detection limits ranging from 0.046–0.065 ng/mL, and recovery values between 63.0–113% with precision values of 0.89-4.14% RSD. Five of the seven targeted antibiotics—PenG, TET, OTC, SMZ, SMX—were detected above the established limits, with concentrations ranging from 0.170 to 9.35 ng/mL. OTC, SMZ, and SMX were consistently found across all sampled sites, with OTC having the highest concentration (9.35 ng/mL) detected in Science City of Muñoz, where five antibiotics were present. Moreover, the stability test revealed that the antibiotics follow pseudo-first-order kinetics, with half-lives of 1.58-2.74 days, which is shorter than those previously reported in the literature. This finding may indicate that the substances present in the matrix catalyze the degradation of antibiotics thus affecting their stability. This study is the first to report the occurrence of antibiotics in surface waters of the Upper Pampanga River Basin. It is also the first to report the stability of these antibiotic substances in the said area. Further studies are recommended to investigate the degradation mechanisms of these substances and assess their ecological impact.

INTRODUCTION

Antibiotics are small-molecule compounds of variable origins administered at controlled doses to inhibit or kill pathogens in humans and animals. In the livestock industry, antibiotics are also utilized as growth promoters to enhance animal's resistance to stress-related illness (Bentley and Bennet, 2003). Despite their importance in the twentieth-century medicine, concerns persist regarding overuse, disposal, and environmental impacts. One major consequence of antibiotic overuse is the emergence of antibiotic-resistant bacteria. Misuse and overuse of antimicrobials across human, animal, and plant sectors accelerates the evolution of drug-resistant pathogens, contributing to AMR-a phenomenon in which bacteria no longer respond to commonly used antibiotics, making infections more difficult to treat (Khalid Ahmed et al., 2024).

A key driver of AMR is extensive antibiotic use in livestock production (Low et al., 2021). Global veterinary antibiotic sales reached 93,309 tonnes in 2017 and are projected to rise to 104,709 tonnes by 2030 (Tiseo et al., 2020). Up to 75% of administered antibiotics may be excreted (Xu et al., 2022), and these residues can enter aquatic environments. Conventional wastewater

*Corresponding author
Email Address: **gerardo.ilagan@student.ateneo.edu**
Date received: 04 September 2025
Dates revised: 25 January 2026
Date accepted: 26 January 2026
DOI: https://doi.org/10.54645/2026191HEM-38

KEYWORDS

antimicrobial resistance (AMR), antibiotics, liquid chromatography-tandem mass spectrometry, Upper Pampanga River Basin

treatment focuses primarily on particulate removal and may not effectively eliminate many antibiotics and metabolites. Antibiotics may also be transported in the aqueous phase or sorbed to suspended solids, which can later release these compounds back into water. In aquaculture, antibiotics may enter aquatic systems through feed leaching or direct treatment. Once present, antibiotics can undergo abiotic and biotic transformations that affect bioavailability and bioactivity and may contribute to AMR (Segura et al., 2009).

Determination of antibiotics in water samples is challenging due to the low analyte concentrations, matrix complexity, and the diverse physicochemical properties of antibiotics. Environmental waters also often contain mixtures of antibiotic classes, requiring highly selective methods. In lieu of these considerations, Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) is well-suited because many antibiotics are polar, non-volatile, and thermally labile. Other screening approaches often rely on LC-MS/MS for confirmation and quantification (Hernandez et al., 2007).. Antibiotic analysis typically requires sample clean-up and pre-concentration to mitigate matrix effects, commonly performed using solid-phase extraction (SPE) with hydrophilic-lipophilic balance (HLB) cartridges. Through this analytical method, successful identification of multiclass antibiotics in river water through untargeted analysis (Ibañez et al, 2009) was realized. It was also crucial for the simultaneous quantification of 6 antibiotics in groundwater through targeted analysis (Kivits et al., 2022).

In the Philippines, studies on antibiotic consumption and environmental fate-particularly in aquatic systems-remain limited. Surveys in swine and poultry farms have identified several commonly used antibiotics. It was reported that that enrofloxacin (36%), amoxicillin (27.0%), colistin (17.0%), and tetracyclines (39% for backyard farms) were the most used antibiotics (Barroga et al., 2020). While local work has often focused on residues in livestock products, recent studies have begun to examine antibiotics in surface waters, including sulfonamides detected in major river and bay systems. For instance, SMZ was detected in Agusan River system and Macajalar bay with concentration of 765 ng/mL in the bay area and 58.4 ng/mL upstream (Mariano et al., 2023). SMX was detected in Metro Manila's aquatic systems; Laguna Lake (34.3 ng/L), Pasig River (63.0 ng/L), and Manila Bay (34.3 ng/L) Despite these reports, comprehensive assessments of common veterinary antibiotics remain scarce, particularly in the Upper Pampanga River Basin, Nueva Ecija. Regulatory limits for antibiotics in Philippine aquatic environments have also not been established.

This study developed an optimized SPE-LC-MS/MS method to simultaneously assess seven commonly used veterinary antibiotics in the Philippine livestock industry: beta-lactams (AMX, AMP, and PenG), tetracyclines (TET and OTC), and sulfonamides (SMZ and SMX). The validated method was applied to surface waters of the Upper Pampanga River Basin in Nueva Ecija, where numerous livestock farms are located near Pampanga River tributaries.

MATERIALS AND METHODS

Chemicals and Standards

The standards for the antibiotics AMX, AMP, PenG, OTC, TET, SMZ, and SMX were provided by the Arphilake Project and the Philippine Institute for Pure and Applied Chemistry (PIPAC). Solvents such as the LC-MS grade acetonitrile, methanol, and water were purchased from Chemline Scientific Corporation, while the Strata-X HLB SPE 200 mg/3mL cartridges were purchased from Phenomenex USA. The Department of Chemistry at Ateneo De Manila University provided other reagents such as formic acid (LC-MS and AR Grade), Ammonium Hydroxide (AR Grade), and EDTA (AR Grade). Individual stock solutions with a concentration of 100 µg/mL were prepared by dissolving the appropriate amount of antibiotic with suitable solvents and stored in a freezer maintained at -20°C and amber vials to prevent degradation. A 500 ng/mL mixed antibiotic working standard was prepared from individual stock solutions and diluted with 0.1% formic acid (F.A.) in water. This standard, which was prepared on a weekly basis was used for method development, spiking in recovery experiments, and preparation of calibration standards.

Instrumentation

A triple quadrupole Shimadzu 8040 Series LC-MS/MS located at PIPAC, Ateneo de Manila University campus, was used for this study. Chromatographic separation was performed using an Intersustain C-18 column (2.1 x 150mm, 3 µm particle size), which was maintained at 40°C. The mobile phases used for this study were water (A) and acetonitrile (B), each modified with 0.1% formic acid, and delivered at a flow rate of 0.2ml/min. The mobile phase was delivered via binary gradient: 0min, 5% B; 3min, 20%B; 4 min, 30%B; 12 min, 99%B; 15 min, 5% B and maintained for 3 min for column re-equilibration. Injection volume was 10µL. Mass separation was performed on a triple quadruple mass spectrometer, with analytes ionized in positive electrospray ionization (ESI+) mode. Nitrogen was used as the nebulizing gas at 2L/min and as the drying gas at 15L/min, while the desolvation line (DL) temperature was set at 250°C and the heat block temperature was set at 350°C. The collision energies, using argon as the collision induced dissociation (CID) gas, were optimized for each antibiotic. Two (2) multiple reaction monitoring (MRM) transitions were selected per compound (Q for quantification transition and I for identification transition), and the ratio between the peak areas of the Q and I was measured. The data for LC-MS/MS analysis were acquired and processed using Shimadzu Lab solutions software, and imaging and graphics were processed using OriginLab Pro 2025.

Method Development and Validation

The validation of the analytical method to determine the extent of occurrence of antibiotics in environmental waters was carried out by evaluation of linearity, limit of detection, limit of quantitation, recovery, and precision, which was based on the Eurachem Guide (2014). Linearity is evaluated using the square of the regression coefficient (R^2), and linearity was assumed when the R^2 value for each antibiotic is ≥ 0.999. The limit of detection was estimated through the signal-to-noise ratio (S/N) of 3 using the formula $LOD = \frac{Lowest\ Spike\ Conc*3}{S/N}$, while the limit of quantitation was estimated as $LOQ = LOD * 3.3$. Recovery tests were conducted where the matrix sample was spiked with a known concentration of analyte and analyzed through the entire method. The % recovery was calculated using the formula $\%Recovery = \frac{C1-C2}{C3} x100\%$, where C1 is the concentration of the spiked sample, C2 is the concentration of the blank, and C3 is the theoretical spike concentration. Acceptable recovery values are between 60-115% since the expected concentration is at 1-10 ng/mL. Precision was measured using the same set of samples used in the recovery test. The relative standard deviation (RSD) values were calculated and considered satisfactory when the RSD values were ≤20% in repeatability conditions.

Optimization of SPE Conditions

To improve the extraction of antibiotics in water samples, the conditions for SPE were optimized based on solution pH, elution solvents, and the addition of ethylenediaminetetraacetic acid (EDTA) in samples. The solution pH is a crucial factor in the successful determination of antibiotics, as it influences the chemical properties and extraction efficiencies. At the same time, EDTA addition allows the chelation of minerals present in the sample. The impact of each optimization parameter on extraction efficiency was evaluated using triplicate analyses, and the mean recoveries guided the selection of final SPE conditions.

Sampling Sites

The sampling sites selected for this study were primarily located in the province of Nueva Ecija, except the municipality of Arayat in Pampanga, where the convergence point of the different tributaries was located. The said sampling sites were selected due to the presence of significant livestock activities in their location, and where different tributaries of the Pampanga River pass through, shown in Figure 1. Table 1 summarizes the general information of the different sampling sites including the sampling parameters.

Table 1: The Different Sampling Sites Selected for the Study

Code	City	Tributary Represented	Sampling Coordinate	Sampling Parameters			
				Sampling Time	pH	Temp, °C	TDS, µg/mL
SJC	San Jose City, Nueva Ecija	Talavera River	15.755435, 121.013914	0814H	8.36	28.9	196
SCM	Science City of Muñoz, Nueva Ecija	Ilog Baliwag	15.720936, 120.880771	1638H	8.35	31.0	186
PAL	Palayan City, Nueva Ecija	Cabu River	15.524355, 121.063284	1042H	7.96	31.1	260
PEN	Municipality of Peñaranda, Nueva Ecija	Peñaranda River	15.348885, 121.063085	1140H	8.99	31.9	225
CAB	Cabanatuan City, Nueva Ecija	Pampanga River	15.464899, 120.935706	1431H	8.19	31.5	223
ARA	Municipality of Arayat, Pampanga	Pampanga River (Convergence Point)	15.166238, 120.783160	1311H	8.46	32.6	226

Sampling

The sampling of river water was conducted on May 18, 2025. Samples were collected at 0.5m depth using a bucket via grab sampling. The collected samples were tested for sampling parameters such as pH, temperature, and total dissolved solids, and immediately transferred to a clean, pre-treated 1L amber glass bottle without headspace. The samples were preserved using 1% sodium azide and were stored in a cooler at 4°C and away from light during transport to the laboratory. Upon arrival at the laboratory, the samples were filtered using a 0.7 µm glass microfiber filter to remove the particulates. Solid-phase extraction and analysis were done within 48 hours of storage to avoid degradation.

Solid Phase Extraction and LC-MS/MS Analysis

The extraction and pre-concentration were realized using HLB SPE cartridges. Conditioning of the SPE column was done with 2 mL of methanol 3 times, followed by equilibration with 2 mL of 0.1%FA in water. A 100 mL sample was loaded onto the SPE column and allowed to pass through with the column with the aid of a vacuum pump at a rate of 1.5mL/min. After loading, the columns were washed with 3 mL of neutral water twice. The SPE columns were dried under vacuum for 5 minutes and eluted using methanol of different pH levels. The eluate was collected and dried via nitrogen gas purging and was reconstituted with 1 mL of water and transferred to an amber vial for LC-MS/MS analysis.

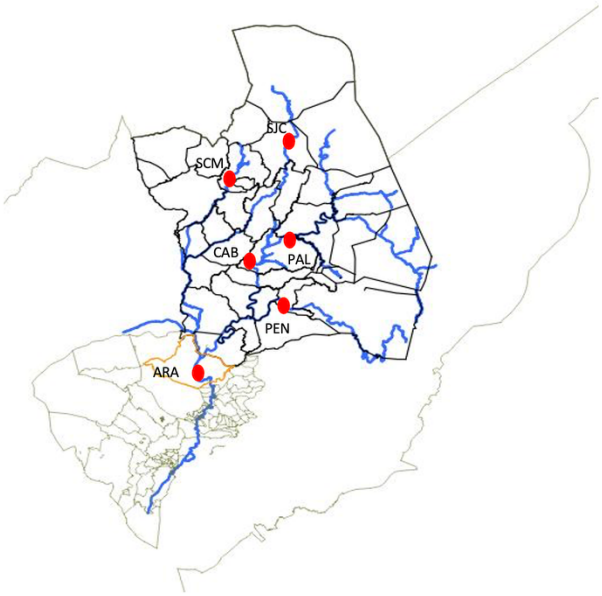

Figure 1: Map of Upper Pampanga River Basin depicting the sampling sites.

The samples were analyzed in the LC-MS/MS based on the optimized conditions that resulted from the method development and validation section of this study. Concentrations of antibiotics in the samples were calculated by the external standard method based on the peak area of the quantification. The Q/I ion ratio must be within the ±30% range relative to the average Q/I ion ratio of standards to confirm the presence of the analyte, which was adapted from the SANTE/11813 (2017).

Stability Test

The stability of antibiotics was evaluated using previously established methods with context-specific modifications (Gozlan et al., 2013; Fabregat-Safont et al., 2021). In this study, a river water sample was spiked with 30-50 ug/mL of each antibiotic and stored in a clear glass container under ambient temperature and lighting conditions in order to simulate realistic environmental exposure. In

contrast to the cited studies which used higher spiking concentrations, refrigerated storage or simulated matrices, this approach used in study aimed to reflect the more realistic river conditions. Analysis was performed and antibiotic concentrations were monitored at defined time intervals (0, 3, 6, and 10 days), to assess the behavior of these antibiotics under these conditions.

RESULTS AND DISCUSSION

Optimization of Detection Conditions

The first step for this study was to optimize the detection conditions of the liquid chromatography-tandem mass spectrometry in order to detect and quantify the seven antibiotics belonging to three major classes commonly used in the livestock industry, as summarized in Table 2. A Q_3 scan was performed to identify the precursor mass ($[M+H]^+$) of each antibiotic, which is followed by the determination of ion transitions through product ion scan and subsequent determination of optimized collision energies. Two product ion transitions were selected for multiple reaction monitoring (MRM) acquisition mode for better selectivity, shown in the chromatogram in Figure 2. The working range was determined using calibration points prepared from a mixed antibiotic standard solution (0-100 ng/mL), and all antibiotic substances had an R^2 value of ≥ 0.999. However, since the expected concentration from the river samples based on literature is between 0-5 ng/mL (Tlili et al., 2016), the most appropriate linear range for this study was determined using a 6-point calibration (0.5, 1.0, 1.5, 2.0, 2.5 & 3.0 ng/mL). The method was developed around these linear range and all antibiotic substances have an R^2 value of ≥ 0.999. The instrument detection and quantitation limits were estimated through signal-to-noise (S/N) ratio. They are summarized in Table 3 below. To ensure consistency, the mean quantifier to identifier (Q/I) ion ratio for each ion transition must have an RSD value of ≤20%.

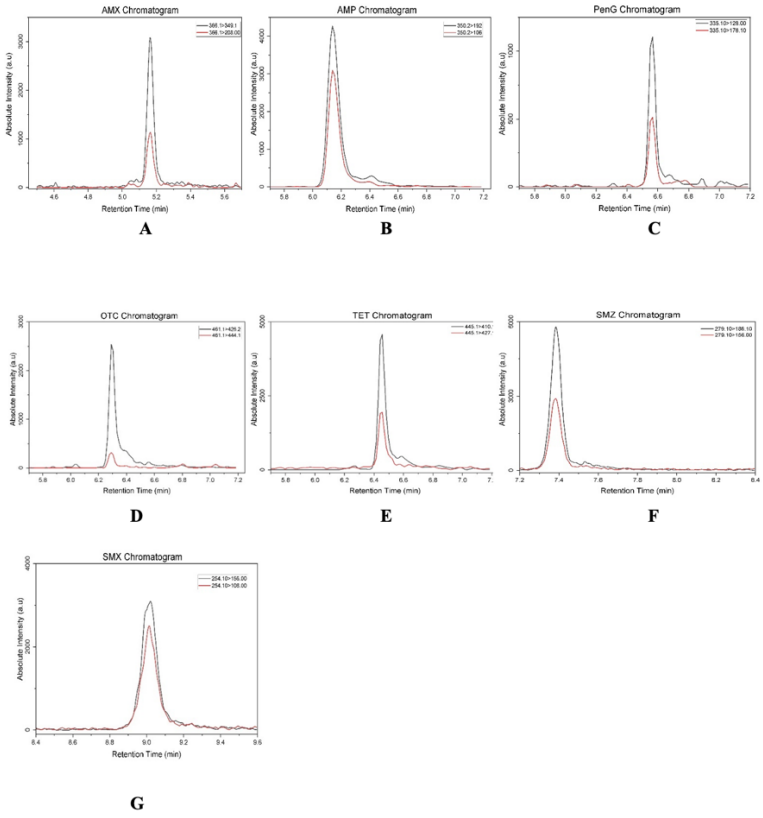

Figure 2: LC-MS/MS chromatogram of the antibiotic standards, including their ion transitions: (A) AMX, (B) AMP, (C) PenG, (D) OTC, (E) TET, (F) SMZ, and (G) SMX.

Table 2: Optimal conditions for the detection of antibiotics

Antibiotic	Exact Mass, amu	Retention Time, min	Precursor Ion $[M+H]^+$, m/z	Product Ions (Quantifier, Identifier), m/z	Collision Energies (CE), eV
AMX	365.1045	5.1	366.2	349.1, 208.0	9.0,13.0
AMP	349.1096	6.1	350.1	106.1, 192.1	30.0, 17.0
PenG	334.0987	6.6	335.1	128.0, 176.1	28.0, 26.0
OTC	460.1482	6.3	461.2	426.2, 444.1	21.0, 17.0
TET	444.1533	6.4	445.1	410.1, 427.1	20.0, 14.0
SMZ	278.0837	7.4	279.1	186.1, 156.0	18.0, 20.0
SMX	253.6521	9.0	254.1	156.0, 108.0	16.0, 30.0

Table 3: Performance characteristics of the LC-MS/MS method

Antibiotic	R^2	LOD, ng/mL	LOQ, ng/mL	Mean Q/I ion ratio (n=6)	% RSD, Q/I ion ratio (n=6)
AMX	0.9996	0.049	0.162	2.71	9.70
AMP	0.9991	0.065	0.213	2.06	5.21
PenG	0.9994	0.048	0.157	1.96	9.64

OTC	0.9995	0.054	0.180	8.59	3.34
TET	0.9993	0.046	0.152	2.30	4.89
SMZ	0.9995	0.047	0.156	2.23	1.56
SMX	0.9998	0.048	0.158	1.39	7.30

Optimization of Extraction Conditions

SPE has been consistently used as the primary sample preparation technique for the analysis of antibiotics in different sample matrices such as soil and water samples. However, the inherent nature of the river water matrix for this study necessitates the optimization of extraction conditions to maximize the recovery of antibiotics. Since the mechanism of retention and elution is heavily dependent on the polarity of the analytes, the optimization focuses on three parameters: elution solvent, pH of solution, and level of EDTA addition. While methanol is a widely accepted elution solvent, the different properties of antibiotics suggest the need to modify methanol to different pH levels. In this study, 5% formic acid (F.A.) in methanol, 7% ammonium hydroxide (NH_4OH) in methanol, and a combination of 5% F.A. in methanol followed by 7% NH_4OH in methanol were tested, which were spiked with 20 ng/mL of each antibiotic, shown in Figure 3A. The 5% F.A. in methanol eluted PenG and TC, while 7% NH_4OH in methanol eluted PenG and SMZ at acceptable recovery levels. When the elution solvents were combined, SMX and SMZ were eluted at acceptable recovery levels. Improvements in the recovery levels were noticed for the rest of the antibiotics.

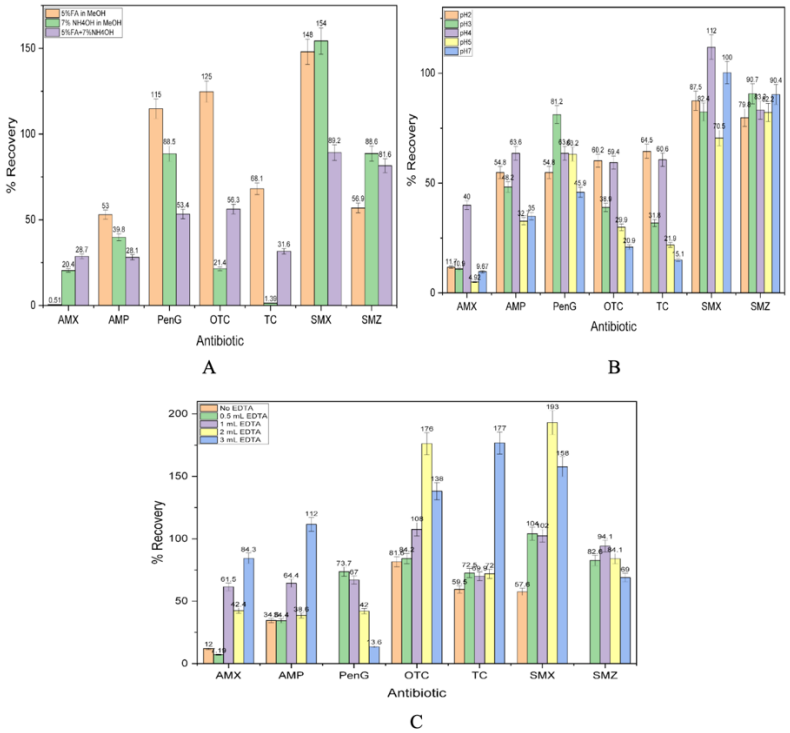

Figure 3: Recovery of antibiotics based on different optimization conditions: (A) elution solvent used. (B) solution pH, and (C) EDTA addition.

The extraction optimization proceeded with studying the effect of solution pH on the antibiotic recovery, since antibiotic substances behave differently at different pH levels due to the presence of multiple pK_a values. Solutions of different pH levels (pH 2, 3, 4, 5, and 7) were prepared and spiked with 2.0 ng/mL of each antibiotic and subjected to SPE extraction using the optimized elution solvent. The results show (Fig. 3B) that the recoveries were improved at pH 4, and almost all antibiotics are within the acceptable recovery levels. While AMX has a recovery value of < 60%, the recovery value at pH 4 was 40%. The varying recovery rates observed in the optimization of elution solvent at different conditions and the solution pH could be attributed to the amphoteric properties of the antibiotics being studied. β-lactams contain a carboxylic acid group with pK_a values of 2-3, and for AMX and AMP, an amino group with pK_a values of 7-8. Tetracyclines can form salts with both acids and bases in aqueous solution due to the presence of 3 pK_a values, while sulfonamides can be weakly alkaline due to anilinic nitrogen or can be weakly acidic due to N-H bonds in the sulfonamidic group (Tong et al., 2009).

River water is considerably laden with minerals such as Na^+, Ca^{2+}, Mg^{2+}, K^+. HCO_3^-, Cl^- and SO_4^{2-} and other minerals (Sugiyama et al., 2016). These may affect or interfere with antibiotics of interest, thus a metal complexing agent such as EDTA are added to the water sample to chelate some metal ions, such as Ca^{2+} and Mg^{2+} that bind with antibiotics. This improve the extraction of the antibiotics, and therefore, the effect of EDTA addition was also studied. A 2% solution of EDTA was prepared and added to the matrix at different levels (0, 0.5, 1.0, 2.0, and 3.0 mL), as shown in Figure 3C. It can be observed that all antibiotics are within the acceptable recovery levels when the solution is added with 1.0 mL of EDTA solution. However, excessive addition of EDTA may increase the recovery values of OTC, TET, and SMX beyond acceptable levels. Higher recovery rates beyond the acceptable recovery values relative to the higher amount of EDTA added to the solution were also observed in a previous study (Wang et al., 2023). This can be attributed to the enhanced matrix effects, in particular, in matrices that contain higher amounts of humic acid. Moreover, the higher recovery rates, even at lower pH extraction, can be attributed to the high formation constants of the ions present in the matrix with EDTA. Based on the extraction condition optimizations, the SPE procedure was revised. The samples must be added with 1.0 mL of 2% EDTA solution, adjusted to pH four before sample loading, and then eluted with 5% F.A. in methanol, followed by 7% NH_4OH in methanol.

Table 4: Recovery and RSD results for the proposed method

Antibiotic	Day 1		Day 2		Inter-Day Precision		Reference
	%Recovery*	%RSD*	%Recovery*	%RSD*	%Recovery	%RSD	
AMX	63.0	3.48	77.99	4.23	70.5	15.1	Fabregat-Safont et al, 2021
AMP	62.9	0.55	77.94	5.26	70.4	15.1	Fabregat-Safont et al, 2021
PenG	110	4.14	90.97	1.01	101	13.5	Fabregat-Safont et al, 2021
OTC	74.2	1.50	70.19	1.55	72.2	3.97	Zhang et al, 2024
TET	79.4	2.19	94.61	1.76	87.0	12.4	Zhang et al, 2024
SMZ	81.7	3.64	85.44	4.49	83.6	3.14	Zhou et al, 2012
SMX	103	0.89	96.24	5.56	99.8	5.03	Tlili et al, 2016

*n=3

Recovery and precision tests were conducted based on the developed SPE-LC-MS/MS method, as summarized in Table 4. A river water sample was spiked with 2 ng/mL of each antibiotic and was allowed to run through the entire method in triplicate. In repeatability conditions (intra-day), the % recoeedof 0.55% to 5.56%. In reproducibility conditions (inter-day), the % recovery ranged from 70.4% to 101%, with % RSD values of 3.97% to 15.1%. These results imply that the method provides acceptable recovery and precision in trace level antibiotics found in river water, thereby demonstrating that the method was successfully validated. Relevant studies with similar SPE-LC-MS/MS protocols that targets structurally related antibiotics in river water matrices are cited in Table 4. For instance, recoveries of β-lactam antibiotics were made possible through the use of Oasis HLB SPE cartridges, which highlights its applicability to this antibiotic class (Fabregat-Safont et al., 2021). Moreover, tetracyclines were extracted from the surface water through pH adjustment (Zhang et. al., 2024), which aligns to this study's approach of adjusting the pH to improve extraction. SPE extraction was also employed to extract multi-residue sulfonamides in environmental waters through similar ion transition monitoring (Zhou et al., 2012; Tlili et al., 2016). While these cited studies differ in sampling sites, brand of SPE kits and target antibiotics per reference, they employed similar SPE-LC-MS/MS workflows and targeted structurally related antibiotics, providing contextual relevance for the methodological approach and analytical scope of this study. The developed SPE-LC-MS/MS method for this study offers improvements by considering the cocktail nature of antibiotics found in nature, extracting these substances at suitable pH levels, and minimizing interference from other substances. In addition, this study takes into account the Q/I ion ratio as another criterion in determining the occurrence of antibiotic substances in the environment.

Testing the River Waters of the Upper Pampanga River Basin

The validated method was applied to the analysis of river waters of the different tributaries of Upper Pampanga River Basin, an economically important body of water that is vital for the agriculture and livestock industry of the Province of Nueva Ecija. The occurrence of the seven veterinary antibiotics was determined from six sampling sites: SJC, SCM, PAL, PEN, CAB, and ARA. A sample chromatogram of analyzed river water sample from SCM in comparison with each antibiotic standard is shown in Figure 4. Table 5 and Figure 5 present the individual concentration of each compound. A quality control (QC) sample of river water spiked with 2.0 ng/ml of antibiotics was also analyzed. AMX and AMP

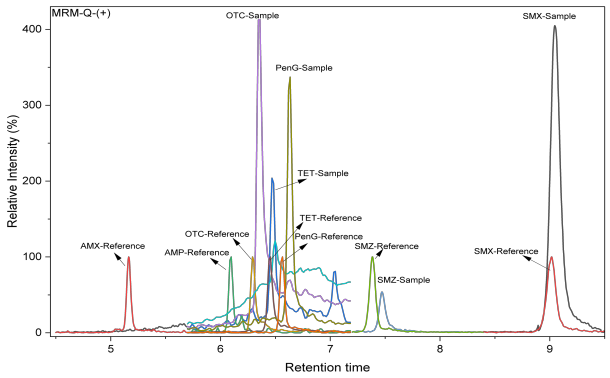

Figure 4: LC-MS/MS chromatogram showing the reference compounds in comparison with river water sample taken from SCM.

were not detected on all sampling sites, while OTC, SMX, and SMZ were present in all sampling sites. Five out of seven antibiotics were detected in SCM and ARA, with antibiotic concentration ranges for these areas being 0.69-9.35 ng/mL and 0.77-6.28 ng/mL, respectively. OTC has the highest concentrations at SCM (9.35 ng/mL) and ARA (6.28 ng/mL), followed by SMX at SCM (7.25 ng/mL) and SJC (6.89 ng/mL). PenG, a β-lactam antibiotic, was detected in all sampling sites except PEN, with the highest concentration at ARA (4.13 ng/mL). In contrast, TET was detected only in SCM (1.41 ng/mL) and ARA (0.77 ng/mL). It can also be noted that, generally, as the sampling point goes downstream, the antibiotic concentration decreases, but suddenly increases at sampling point ARA.

Table 5: Individual concentrations of antibiotics in different sampling sites

Antibiotic	Sampling Location and Concentration (ng/mL)						QC Sample	
	SJC	SCM	PAL	PEN	CAB	ARA	% Recovery*	% RSD*
AMX	n.d	n.d	n.d	n.d	n.d	n.d	90.2	3.64
AMP	n.d	n.d	n.d	n.d	n.d	n.d	77.9	8.12
PenG	0.44	3.17	2.11	n.d	1.56	4.13	71.3	1.29
OTC	1.02	9.35	3.98	1.58	2.82	6.28	96.2	5.56
TET	n.d	1.41	n.d	n.d	n.d	0.77	70.2	1.55
SMZ	0.17	0.69	5.7	0.35	0.57	0.91	85.4	4.49
SMX	6.89	7.25	3.24	2.06	1.36	1.38	113.7	1.46

n.d: not detected; *: n=3

The high concentration and persistence of OTC may be attributed to the presence of Ca^{2+} and Mg^{2+} in the matrix, as it establishes

strong complexes with OTC (Leal et al., 2018). Upon the addition of EDTA, Ca^{2+} and Mg^{2+} are chelated, thereby releasing OTC and enabling its detection. On the other hand, sulfonamides particularly SMX are frequently detected in wastewater and are not completely degraded through conventional wastewater treatment processes. As a result, more advanced methods, such as ozone treatment, are required for their removal (Liu et al., 2021). Consequently, these compounds are often still detected in river water. Studies involving PenG reported that wastewater treatment plants that employ anaerobic, hydrolysis, and two aerobic treatment processes in succession reduce the PenG concentration to as low as 1.68 ug/L. However, such a low concentration can also be attributed to the transformation of PenG to its degradation products such as penillolic and pencilloloc acids (Li et al., 2007). Thus, any detection of PenG in river water may be attributed to the poor wastewater treatment plants in farms nearby. While AMX is reported to be one of the most commonly used antibiotics in the Philippines (Barroga et al., 2020), it is not detected in all sampling sites, which can be attributed to the instability of AMX and thus, determination of AMX in the aquatic environments tends to be challenging (Fabregat-Safont et al., 2021).

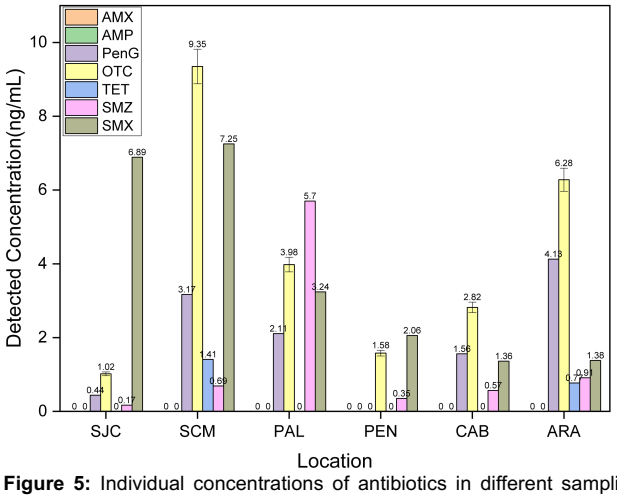

Figure 5: Individual concentrations of antibiotics in different sampling sites.

In the different Philippine rivers, the occurrence of SMX (63.0 ng/L) in Pasig River (Suzuki et al., 2013), SMZ (58.4 ng/mL) in Agusan River (Mariano et al, 2023), cefalexin (4.45 ng/L), doxycycline (3.99 ng/L), erythromycin (47.1 ng/L), levofloxacin (2.95 ng/L), sulfamethoxazole (102 ng/L) and trimethoprim (24.0 ng/L) in Angat, Lower Pampanga, Marikina and Pasig Rivers (Sta Ana et al., 2024) were reported. For this study, the sampling points were chosen near livestock farms; thus, it can be inferred that the majority of the detected antibiotics originated from the farms near the sampling points. However, this is not true for ARA, as this sampling point is situated in the downstream section of the river, where several communities along the banks of the Upper Pampanga River upstream of this location may contribute to the discharge of antibiotics. It can be inferred, based on the results of this study, that PenG, OTC, TC, SMZ, and SMX were detected in the Upper Pampanga River Basin and its tributaries and can be attributed to the livestock growing operations in the area. This is the first study to report the presence of these antibiotics in the aquatic environment of the Upper Pampanga River Basin. Finally, while the antibiotic concentrations reported in this study are relatively low, they can be considered either subinhibitory or non-lethal, which may induce diverse biological responses in bacteria, triggering different cellular responses that may include an altered antibiotic resistance/tolerance profile of the bacteria (Bernier & Surette, 2013). This study recommends that policymakers and other relevant agencies consider antibiotic testing and monitoring of livestock farms, and set a minimum regulatory limit for the most commonly used antibiotics in the Philippine livestock industry.

Stability Test

The method developed was also used to determine the concentration of antibiotics in a stability experiment, which aimed to determine the stability of the antibiotics in the river water matrix. The results shown in Figure 6 and Table 6 revealed that the antibiotics followed a pseudo-first-order reaction, which assumes that the concentration of the matrix is much greater than the concentration of the antibiotics tested. The results show that AMX has the largest rate constant and shortest half-life at 1.58 days, while the sulfonamide SMZ has the lowest rate constant and the longest half-life at 2.74 days. The stability test also revealed that the β-lactam antibiotics have half-lives of less than 2 days when compared to the rest of the antibiotics, which can be attributed to the poor stability of the β-lactam ring structure in environmental waters (Borrull et al., 2020). Thus, this antibiotic class are reported to have half-lives of 1.4-9.3 days at pH 7-9 (Mitchell et al., 2014). Sulfonamides are known to be persistent antibiotic pollutant that is usually resistant to hydrolysis degradation between pH 7-9 resulting to longer half-lives of up to > 1 year (Bialk-Bielinska et al., 2022). However, in the presence of light, the half-lives of sulfonamides were 10.5-12.9 days (Zhang et al., 2013).

Table 6: Kinetic data of different antibiotics in the river water matrix

Antibiotic	Matrix Conditions			Amount of Spike	Kinetic Parameters		
	pH	Temp, °C	TDS, ppm	ng/mL	K_{obs}, ng/mL/day	$t_{1/2}$, days	R^2
AMX	8.18	22.6	183	30	0.439	1.58	0.901
AMP					0.364	1.90	0.929
PenG					0.403	1.72	0.990
OTC				50	0.338	2.05	0.919
TET					0.278	2.50	0.967
SMZ					0.253	2.74	0.994
SMX					0.315	2.20	0.939

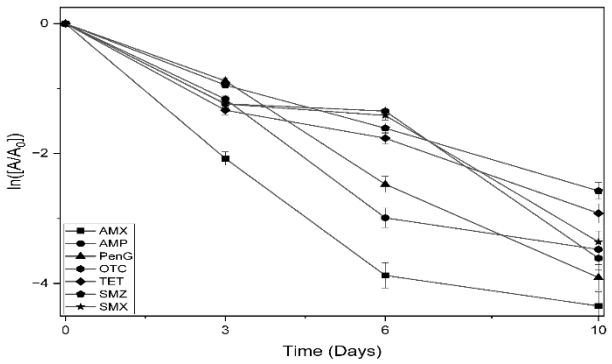

Figure 6: Antibiotic degradation kinetic plot.

Tetracyclines are also known to be stable in the environment and are known to degrade through hydrolysis. However, their degradation products are highly soluble, stable, and are usually more toxic than their parent tetracycline. At pH 7, tetracyclines have half-lives of 66-112 hours and 136-224 hours at pH 9 (Zhong et al., 2022). Based on the data from this study, the rapid degradation of antibiotics compared to the literature may be attributable to the presence of other substances in the matrix that affect the stability of antibiotic substances in the matrix, as evidenced by the lower R^2 values when calculated through pseudo-first-order kinetics. While EDTA addition chelated the divalent cations such as Ca^{2+} and Mg^{2+}, some minerals present in river water matrix such as HCO_3^-, Cl^-, and SO_4^{2-} may affect the degradation behavior of antibiotics. The presence of Cl^- ions showed lower degradation rates for OTC and SMZ ion but showed higher degradation rates for PenG at increasing Cl^- concentration in the presence of UV radiation since Cl^- ions could react with HO• and Cl• to form radicals with weak oxidizing capacity such as ClHO• and Cl_2^-•. These radicals of weak oxidizing capacity could be decomposed back to HO• and Cl• leading to the decreased degradation rates of OTC and SMZ and increased degradation rate of PenG as a function of increasing concentration (Bin Khalid et al., 2025). In the same study, the presence of low SO_4^{2-} concentration increases the degradation of penicillin G, oxytetracycline and sulfamethazine by 100%, 17% and 21%, respectively. This can be attributed to the lower amount of SO_4^{2-} ions that are converted to SO_4^-• radicals, which promotes degradation. Finally, HCO_3^- ions promotes degradation of antibiotics, particularly PenG, OTC and SMX as the concentration of HCO_3^- increases, since HCO_3^- ions react with HO• radical producing a weak oxidant CO_3^-•that can sustain degradation over longer timeframe and can perform targeted oxidation in complex or balanced pH systems (Bin Khalid et al., 2025; Li et al., 2025). Thus, future research may focus on elucidating the specific degradation mechanisms of these antibiotics in the aquatic environment of the Upper Pampanga River Basin and on determining the occurrence of their degradation products.

CONCLUSION

In this work, a method for the simultaneous determination of the occurrence of the seven veterinary antibiotics was validated and applied to the surface waters of Upper Pampanga River in Nueva Ecija. An SPE-LC-MS/MS method has been optimized based on the collision energies of the quantifier and identifier, the ratio of the quantifier and identifier, solution pH, elution solvents, and the addition of EDTA. The developed method has an instrument limit of detection of 0.046-0.065 ng/mL, quantitation limit of 0.152-0.213 ng/mL, with recovery values of 63.0-110% and RSD values of 0.55-4.14% in repeatability conditions, while recovery values of 70.4-101% and RSD values of 3.14-15.1% were obtained in reproducibility conditions.

The results show that 5 out of 7 antibiotics were detected in the river, with the highest occurrence at the Science City of Muñoz and the Municipality of Arayat. OTC, SMZ, and SMX were present in all sampling sites, with OTC exhibiting the highest concentration at 9.35 ng/mL, followed by SMX at 6.28 ng/mL in SCM and ARA, respectively. These measured values are enough to induce biological responses in bacteria, which may result in an altered resistance/tolerance profile of the bacteria. The antibiotics are degraded in the river matrix, as evidenced by a kinetic study conducted, where β-lactam antibiotics have the shortest half-lives compared to tetracyclines and sulfonamides. While hydrolysis is the primary degradation pathway for these antibiotics, their degradation is enhanced by the presence of various substances in the matrix. Due to the presence of antibiotics in the Upper Pampanga River Basin and their reduced stability in its waters, it is recommended that poultry and livestock farms invest in improved wastewater treatment facilities to reduce the continuous discharge of these substances into the surface waters in the area. At the convergence point, some antibiotics are re-emerging, indicating that the communities near the river may have been contributing to the continuous discharge of antibiotics.

While this is the first study to report the occurrence and fate of the most common veterinary antibiotics in the Upper Pampanga River Basin using the validated SPE-LC-MS/MS method, future work may be needed to determine the degradation pathways these antibiotics may take in the surface waters of the Upper Pampanga River Basin and determine the extent of occurrence of these antibiotic degradation products.

ACKNOWLEDGMENTS

The authors acknowledge the support provided by the Science Education Institute, Department of Science and Technology of the Republic of the Philippines, for the provision of a scholarship grant for GVI in the completion of his MS in Chemistry. The authors also thank Ms. Erin N. Bondoc, a DOST-CIP fellow for the technical assistance during the initial phase of the study. The authors acknowledge the support provided by the Arphilake Project, which provided the antibiotic standards used for this study, and the Philippine Institute for Pure and Applied Chemistry (PIPAC) for the technical support, particularly in the usage of the Shimadzu 8040 LC-MS system. AMG acknowledges the gratitude of support through the FEBTC David G. Choa Professorial Chair Trust Fund for the academic year 2024-2025.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

GVI wrote the first draft and revisions of this manuscript. GVI is also responsible for the conduct of sampling, SPE optimizations, analysis and data processing. IKD contributed to the conception and design of the study, particularly the method validation portion and supervision. AMG contributed to the conception and design of the study, guidance, review, and editing of the manuscript.

REFERENCES

Barroga, T. R. M., Morales, R. G., Benigno, C. C., Castro, S. J. M., Caniban, M. M., Cabullo, M. F. B., Agunos, A., de Balogh, K., & Dorado-Garcia, A. Antimicrobials Used in Backyard and Commercial Poultry and Swine Farms in the Philippines: A Qualitative Pilot Study. Frontiers in Veterinary Science 2020; 7: 1–8. https://doi.org/10.3389/fvets.2020.00329

Bernier, S.P. and Surette, M.G. Concentration-dependent activity of antibiotics in natural environments. Frontiers in Microbiology 2013; 4:20Feb 13;4(20): 1-14. https://doi.org/10.3389/fmicb.2013.00020
.

Białk-Bielińska A, Stolte S, Matzke M, Fabiańska A, Maszkowska J, Kołodziejska M, Liberek B, Stepnowski P, Kumirska J. Hydrolysis of sulphonamides in aqueous solutions. Journal of Hazardous Materials. 2012;221–222:264–274. https://doi.org/10.1016/j.jhazmat.2012.04.044

Bentley R. and Bennett J.W. What is an antibiotic? Revisited. In: Laskin A.K., Bennett J.W., and Gadd G.M. ed. Advances in Applied Microbiology. Volume 52. San Diego: Elsevier (USA), 2003: 303-327

Bin Khalid Z, Kelso C, van de Merwe and F I. Impact of ions co-occurring in water on antibiotics degradation by UV-based advanced oxidation process. Chemosphere. 2025;394:144798. https://doi.org/10.1016/j.chemosphere.2025.144798

Borrull J, Colom A, Fabregas J, Borrull F, and Pocurull E. Liquid chromatography tandem mass spectrometry determination of 34 priority and emerging pollutants in water from the influent and effluent of a drinking water treatment plant. Journal of Chromatography A 2020;1621:461090. https://doi.org/10.1016/j.chroma.2020.461090

European Union Reference Laboratory for Residues and Pesticides. SANTE/11813/2017-Guidance Document on Analytical Quality Control and Method Validation Procedures for Pesticide Residues and Analysis in Food and Feed. 2017. Retrieved from: https://eurl-pesticides.eu/userfiles/file/EurlALL/SANTE_11813_2017-fin.pdf

Fabregat-Safont, D., Pitarch, E., Bijlsma, L., Matei, I., & Hernández, F. Rapid and sensitive analytical method for the determination of amoxicillin and related compounds in water meeting the requirements of the European Union watch list. Journal of Chromatography A 2021; 1658(462605). https://doi.org/10.1016/j.chroma.2021.462605

Gozlan, I., Rotstein, A., & Avisar, D. Amoxicillin-degradation products formed under controlled environmental conditions: Identification and determination in the aquatic environment. Chemosphere 2013; 91(7): 985–992. https://doi.org/10.1016/j.chemosphere.2013.01.095

Hernández, F., Sancho, J. V., Ibañez, M., & Guerrero, C. Antibiotic residue determination in environmental waters by LC-MS. Trends in Analytical Chemistry 2007: 26(6): 466-485 https://doi:10.1016/j.trac.2007.01.012

Ibáñez, M., Guerrero, C., Sancho, J. V., & Hernández, F. Screening of antibiotics in surface and wastewater samples by ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry. Journal of Chromatography A 2009; 1216: 2529–2539. https://doi.org/10.1016/j.chroma.2009.01.073

Khalid Ahmed S., Hussein S., Qurbani K., Hussein Ibrahim R., Fareeq A., Ali Mahmood K., and Mohamed M.G. Antimicrobial resistance: Impacts, challenges and future prospects. Journal of Medicine, Surgery and Public Health. 2024;2:100081. https://doi.org/10.1016/j.glmedi.2024.100081

Kivits, T., Broers, H. P., Beeltje, H., van Vliet, M., & Griffioen, J. Presence and fate of veterinary antibiotics in age-dated groundwater in areas with intensive livestock farming. Environmental Pollution 2018;241: 988-998. https://doi.org/10.1016/j.envpol.2018.05.085

Leal JF, Santos EBH, and Esteves VI. Oxytetracycline in intensive aquaculture: water quality during and after its administration, environmental fate, toxicity, and bacterial resistance. Reviews in Aquaculture 2018;1–19. https://doi.org/10.1111/raq.12286

Li D, Yang M, Hu J, Zhang Y, Chang H, Jin F. Determination of penicillin G and its degradation products in a penicillin production wastewater treatment plant and the receiving river. Water Research. 2008 Jan;42(1–2):307–317. doi: 10.1016/j.watres.2007.07.016

Li Y, Teng J, Wu J, Zhang S, Zhao Z, and Li L. Mechanistic insights into carbonate radical-driven reactions: Selectivity and the hydrogen atom abstraction route. Journal of Hazardous Materials. 2025;485:136930. https://doi.org/10.1016/j.jhazmat.2024.136930

Liu X, Huang Y, Su X, Tian S, Li Y, and Yuan R. Oxidation of sulfadiazine and sulfamethoxazole through O_3, UV, and UV/O_3 processes. Desalination and Water Treatment 2021;222:346–353. https://doi.org/10.5004/dwt.2021.27088

Low, C. X., Tan, L. T.-H., Ab Mutalib, N.-S., Pusparajah, P., Goh, B.-H., Chan, K.-G., Letchumanan, V., & Lee, L.-H. Unveiling the Impact of Antibiotics and Alternative Methods for Animal Husbandry: A Review. Antibiotics 2015; 10(5): 578. https://doi.org/10.3390/antibiotics10050578

Magnusson B. and Örnemark U. (eds.) Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, (2nd ed. 2014). ISBN 978-91-87461-59-0. Available from http://www.eurachem.org

Mariano, S. M. F., Angeles, L. F., Aga, D. S., Villanoy, C. L., & Jaraula, C. M. B. Emerging pharmaceutical contaminants in key aquatic environments of the Philippines. Frontiers in Earth Science: Hydrosphere 2023; 7. https://doi.org/10.3389/feart.2023.1124313

Mitchell SM, Ullman JL, Teel AL, Watts RJ. pH and temperature effects on the hydrolysis of three β-lactam antibiotics: Ampicillin, cefalotin, and cefoxitin. Science of the Total Environment. 2014;466–467:547–555. https://doi.org/10.1016/j.scitotenv.2013.06.027

Sta. Ana KM, Montalaba MA, Galera KC, Espino MP. Detection of multiclass antibiotics in Angat, Pampanga, Marikina, and Pasig Rivers. 38th Philippine Chemistry Congress Book of Abstracts, July 22–24, 2024, Davao City. Poster Presentation A3-42.

Segura, P. A., François, M., Gagnon, C., & Sauvé, S. Review of the Occurrence of Anti-infectives in Contaminated Wastewaters and Natural and Drinking Waters. Environmental Health Perspectives 2009; 117(5):675-684. https://doi.org/10.1289/ehp.11776

Sugiyama M, Wu S, Hosoda K, Mochizuki A, and Hori T. Method for the preparation of artificial lake and river waters. Limnology and Oceanography: Methods 2016;14(5):343–357. https://doi.org/10.1002/lom3.10094

Suzuki, S., Ogo, M., Miller, T. W., Shimizu, A., Takada, H., & Siringan, M. A. A. (2013). Who possesses drug resistance genes in the aquatic environment? Sulfamethoxazole (SMX) resistance genes among the bacterial community in the water environment of Metro-Manila, Philippines. Frontiers in Microbiology 2013;4(102):1-12 https://doi.org/10.3389/fmicb.2013.00102

Tiseo, K., Huber, L., Gilbert, M., Robinson, T. P., & Van Boeckel, T. P. Global Trends in Antimicrobial Use in Food Animals from 2017 to 2030. Antibiotics 2020; 9(12); 918. https://doi.org/10.3390/antibiotics9120918

Tlili I, Caria G, Ouddane B, Ghorbel-Abid I, Ternane R, Trabelsi-Ayadi M, and Net S. Simultaneous detection of antibiotics and other drug residues in the dissolved and particulate phases of water by an off-line SPE combined with on-line SPE-LC-MS/MS: Method development and application. Science of the Total Environment 2016;563–564:424–433. http://dx.doi.org/10.1016/j.scitotenv.2016.04.101

Tong L, Li P, Wang Y, and Zhu K. Analysis of veterinary antibiotic residues in swine wastewater and environmental water samples using optimized SPE-LC/MS/MS. Chemosphere 2009;74(8):1090–1097. http://doi:10.1016/j.chemosphere.2008.10.051

Wang J, Ye KX, Tian Y, Liu K, Liang LL, Li QQ, Huang N, and Wang XT. [Simultaneous determination of 22 antibiotics in environmental water samples by solid phase extraction-high performance liquid chromatography-tandem mass spectrometry]. Se Pu 2023;41(3):241–249. https://doi.org/10.3724/SP.J.1123.2022.06004

Xu, C., Kong, L., Gao, H., Cheng, X., & Wang, X. A review of current bacterial resistance to antibiotics in food animals. Frontiers in Microbiology 2022;13: 822689. https://doi.org/10.3389/fmicb.2022.822689

Zhang Y, Xu J, Zhong Z, Guo C, Li L, He Y, Fan W, and Chen Y. Degradation of sulfonamide antibiotics in lake water and sediment. Environmental Science and Pollution Research 2013;20(4):2372–2380. https://doi.org/10.1007/s11356-012-1121-8

Zhong SF, Yang B, Xiong Q, Cai WW, Lan ZG, Ying GG. Hydrolytic transformation mechanism of tetracycline antibiotics: Reaction kinetics, product identification, and determination in WWTPs. Ecotoxicology and Environmental Safety. 2022;229:113063. https://doi.org/10.1016/j.ecoenv.2021.113063

Zhou JL, Maskaoui K, and Lufadeju A. Optimization of antibiotic analysis in water by solid-phase extraction and high-performance liquid chromatography–mass spectrometry/mass spectrometry. Analytica Chimica Acta 2012;731:32–39. https://doi.org/10.1016/j.aca.2012.04.021