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Occurrence of enrofloxacin in overflows from animal lot and residential sewage lagoons and a receiving-stream

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Abstract

Enrofloxacin (ENRO), a fluoroquinolone, was quantified in overflows from an animal lot and residential sewage lagoons and in a receiving-stream (Gans Creek). The concentrations of ENRO in samples was determined by high-performance liquid chromatography – tandem mass spectrometry. In total, ninety samples including duplicates were analyzed during several monthly sampling campaigns. The samples collected represented the residential sewage lagoon overflow (RLO), animal lot lagoon overflow (ALLO), the combined overflows (RLO and ALLO), and Gans Creek (upstream, midstream and downstream positions). The frequency of detection of ENRO was 90% for RLO and 100% for both ALLO and Gans Creek. The highest concentration of ENRO (0.44 µg/L) was found in ALLO sample collected during high precipitation. ENRO levels found in RLO samples ranged from < LOQ to 259 ng/L and the highest value observed also coincided with high flow. The levels of ENRO found in Gans Creek ranged from 17–216 ng/L. A preliminary ecotoxicological assessment was conducted through calculation

of the risk quotients (RQs) for organisms based on the ratio of the measured environmental concentrations in this study to the predicted-no-effect-concentrations (acute and chronic effect) data. From the RQs, high risks were observed for *Microcystis aeruginosa* (cyanobacteria; RQ = 4.4); *Anabaena flosaquae* (cyanobacteria; RQ = 1.3); and *Lemna minor* (aquatic vascular plant; RQ = 2.0). The long-term effects of mixtures of PHCs on Gans Creek watershed are probable.

Keywords: Environmental science, Pharmaceutical chemistry, Ecology

1. Introduction

Over the last fifty years, the agriculture industry has grown astronomically especially in the developed countries. The use of a wide range of pharmaceuticals for disease therapy in livestock has produced a positive impact on agricultural productivity. Other advances such as engineered nanoparticles is being considered (Servin and White, 2016; Giovanni et al., 2015) for massive applications in agriculture. Notwithstanding, many questions remain unanswered on the ecotoxicological effects and the potential risks of several ubiquitous chemicals to public health. For instance, antibiotics are widely used in human and veterinary health medicine. Belonging to several therapeutic classes, they are administered to control and treat various disease conditions in humans and animals (Thiele-Bruhn and Beck, 2005; Thompson et al., 2009). At sub-therapeutic levels, they also serve as growth promoters in animal agriculture (Ronquillo and Hernandez, 2017; Hoff et al., 2016).

There is global increased attention on the prevalence of pharmaceutical chemicals (PHCs) and their metabolites in the environment due to the potential ecosystem and human health risks. Antibiotic chemicals, in particular, are generally biologically active, ubiquitous, and highly soluble in water. Rang et al. (1999) reported that about 30% to 90% of an administered antibiotic dose to humans and animals are excreted in urine as the active compound. Moreover, it is well known that some antibiotic compounds and their metabolites are often associated with incomplete degradation during conventional wastewater treatments (Rodriguez-Mozaz and Weinberg, 2010; Li et al., 2012). Consequently, they are recognized as environmental contaminants from their physical and chemical behaviors (Puckowski et al., 2016).

The dangerous release of various PHCs into the environment is on the rise due to no regulations, inadequate regulations, lack of enforcement of policies, personnel shortages, and possibly the lack of knowledge on the potential public health risks. Nonetheless, many countries such as the United States, Canada, and the European Union member states have adopted significant measures to manage the health and environmental risks posed by PHCs in the environment. Antibiotics and their

metabolites reach aquatic systems through various sources, such as discharges from sewage lagoon systems, municipal wastewater treatment plants (WWTPs), household wastes, dumped surplus medicines and unused prescriptions, aquaculture wastes, and agricultural runoffs (Meyer et al., 2000; Gibs et al., 2013). In North America, lagoons are frequently used for human and animal wastewater storage (Hoque et al., 2014). However, their inefficiencies and failures have also been linked to surface water contaminations (USEPA, 2005).

Advances in sample preparations and chemical instrumentations have enabled the accurate identification and quantification of PHCs and their metabolites in aquatic systems in the ng/L to µg/L range. Previous studies found various chemicals in samples from production plant effluents (Larsson et al., 2007; Sanderson, 2011), confined animal feeding operations (Meyer et al., 2000; Khan et al., 2008), sewage and wastewater treatment plants (WWTPs) (Hoque et al., 2014; Du et al., 2014; Zuehlke et al., 2004), surface waters (Thomas and Hilton, 2004; Peng et al., 2008; Wang et al., 2011; Bernot et al., 2013; Jaimes-Correa et al., 2015), groundwater (Phillips et al., 2015; Paíga and Delerue-Matos, 2016), finished drinking water (Kleywegt et al., 2011; Valcárcel et al., 2011), sediment (Zhao et al., 2016) and biota (Alvarez-Muñoz et al., 2015; Xie et al., 2015). Some of the identified compounds were acetaminophen, salicylic acid, ibuprofen, ketoprofen, nimesulide, carbamazepine, fluoxetine, sertraline, lincomycin, sulfamethoxazole, tetracycline, oxytetracycline, azithromycin, ciprofloxacin, enrofloxacin (ENRO), erythromycin, ofloxacin, sulfamethazine, sulfamethoxazole, trimethoprim, atenolol, codeine, hydrocodone, etc.

The prevalence of antibiotic compounds in aquatic bodies affects ecological processes and invariably poses potential human and veterinary health risks. Among the many risks, is antibiotic resistance which is estimated at 700, 000 deaths per year (Bengtsson-Palme and Larsson, 2016). Bioactive chemicals and their metabolites pose potential adverse effects from consumption of meat, fish (Cepurnieks et al., 2015) and involuntary low-dose medication of large population groups (Bottoni and Caroli, 2015). Furthermore, there is the possibility of the spread of antibiotic resistance genes to animals and humans from wastewater and from animal manure/sewage sludge applications (Fatta-Kassinos et al., 2011; Hoff et al., 2016). The severity of antibiotic resistance in humans prompted the World Health Organization to implement surveillance systems (Prigitano et al., 2017) to protect public health. Therefore, it is necessary to conduct assessment studies of PHCs in the environment.

The persistence of exogenous substances in aquatic environments permits the need to report the influence of point source pollution especially from agricultural and residential lagoon systems on inland streams. Data reporting the impact of ENRO from residential lagoons is scarce in the literature. Nonetheless, previous studies

found ENRO in surface waters from Hangzhou, China (Tong et al., 2011) and Galicia, Spain (Iglesias et al., 2014). ENRO levels found in Seines River, France was below 10 ng/L (Tamtam et al., 2008) and below the detection limit in Lake Michigan, USA (Blair et al., 2013). Besides, ENRO was detected in aquaculture grown fish in Vietnam at a rate of 10.8% (Uchida et al., 2016). Numerous studies on ENRO focused on influent and effluent samples from WWTPs (Hoque et al., 2014; Li et al., 2012; Du et al., 2014), the receiving-stream, and its ecotoxicity on aquatic organisms (Chen et al., 2016; Sanderson, 2011; Jiang et al., 2014). The inadequacies of WWTP in antibiotics removal are evident in most past works. There are knowledge gaps on the occurrence, fate, and toxicity of antibiotic chemicals transported from animal lot and residential lagoon systems especially on rural watersheds.

The aims of this present work were to: (1) determine the concentrations of ENRO antibiotic in overflow samples from two sewage lagoon systems (human and animal) and in a rural Missouri receiving-stream (Gans Creek) during several sampling campaigns; and (2) investigate the ecotoxicity of ENRO on aquatic organisms in the receiving-stream through a preliminary risk assessment based on calculated risk quotient (RQ) derived from the ratio of measured environmental concentration (MEC) to predicted-no-effect-concentrations (PNEC). The ecological hazard of ENRO was examined on various test organisms belonging to several taxonomic groups – *Microcystis aeruginosa* (cyanobacteria), *Anabaena flosaquae* (Cyanobacteria), *Daphnia magna* (invertebrate), *Moina macrocopa* (invertebrate), and *Lepomis macrochirus* (fish). ENRO antibiotic is classified in the therapeutic drug group, the fluoroquinolones and it is a broad spectrum antibacterial agent that acts against both gram-positive and gram-negative bacteria. Most respiratory diseases in livestock are treated with ENRO and the mechanism of drug action follows the bacterial DNA replication process through the DNA gyrase enzyme. The present study allowed for first-time identification and comparison of ENRO concentrations from two contrasting sewage lagoon systems in the Lower Missouri-Moreau watershed.

2. Materials and methods

2.1. Gans Creek (Lower Missouri-Moreau watershed)

Fig. 1 presents the area of study in the current work and the sampling sites. Missouri state covers an area of 69,000 square miles and the population is approximately 5.9 million (U.S. Census Bureau, 2010). The lengths of classified and unclassified streams in Missouri are 22,370.3 and 82,126 miles, respectively (Missouri Department of Natural Resources, 2013). Gans Creek is located in the County of Boone (Columbia, Missouri, USA). Gans Creek is one of the several streams in the Lower Missouri-Moreau watershed (Basin/HUC: 10300102) and it

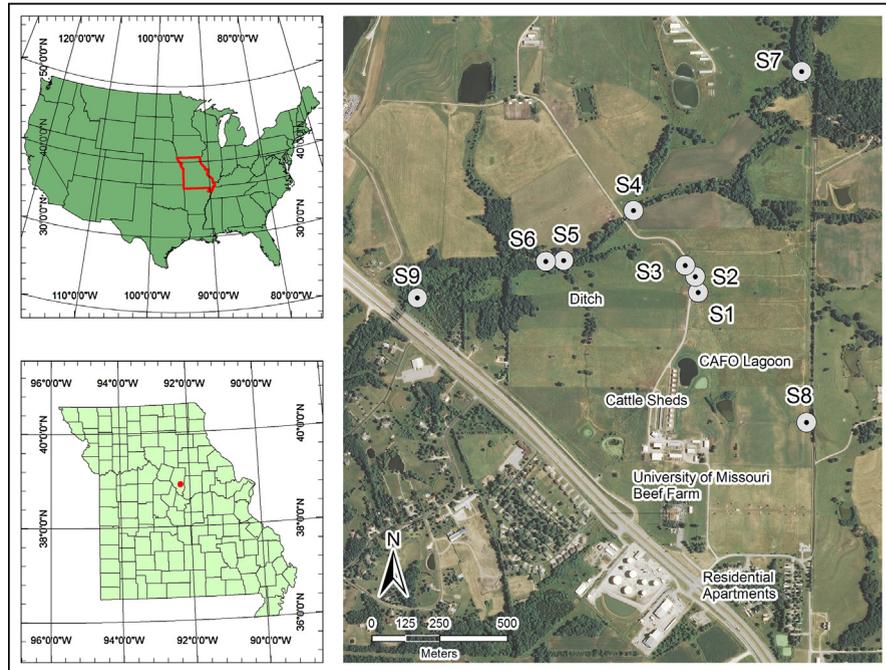


Fig. 1. Map showing Gans Creek and the sewage lagoon overflow sampling positions. Sample no. S1 (ALLO, animal lot sewage lagoon overflow); sample no. S2 (RLO – 2, residential sewage lagoon overflow sample 2); sample no. S3 (the combined overflows of sample nos. S1 and S2); sample no. S4 (Gans Creek at mid-stream point before entry of ALLO and RLO); sample no. S5 (the combined overflows of S1 and S2); sample no. S6 (Gans Creek at the mid-stream point after entry of S1 and S2); sample no. S7 (Gans Creek upstream sample); sample no. S8 (RLO – 1, residential sewage lagoon overflow sample 1); and sample no. S9 (Gans Creek at a downstream point).

is a tributary of the Little Bonne Femme Creek in the south of Columbia. The elevation of the area ranged from 183 to 274 m and land use can be described as 17.2% cropland, 35.7% grassland, 37.3% forest, 1.7% wetland, 6.9% development, and 1.3% water. The soil is characterized as silty loam with an average annual precipitation (Fig. 2) of 0.1 inches per year during the period of study. Corn, soybean, poultry, hog, and beef are the major agricultural products in Missouri.

2.2. Sewage lagoons

Lagoon systems are considered small ponds that are used to receive, hold and treat wastewaters. They serve small municipalities, farms, and industries because of their low cost of installation, simplicity, and ease of operation (Hoque et al., 2014; USEPA, 2002). During this study, the University of Missouri beef research farm had seven hundred animals (mainly cattle). Five hundred of them were fed concentrate meals and the remaining grazed the fields close to the research facility. The liquid wastes generated were channeled to a constructed sewage lagoon system (Fig. 1). During dry times, stored lagoon wastewater was intermittently sprayed on the grazing fields and runoffs traveled through a ditch into Gans Creek. The animal

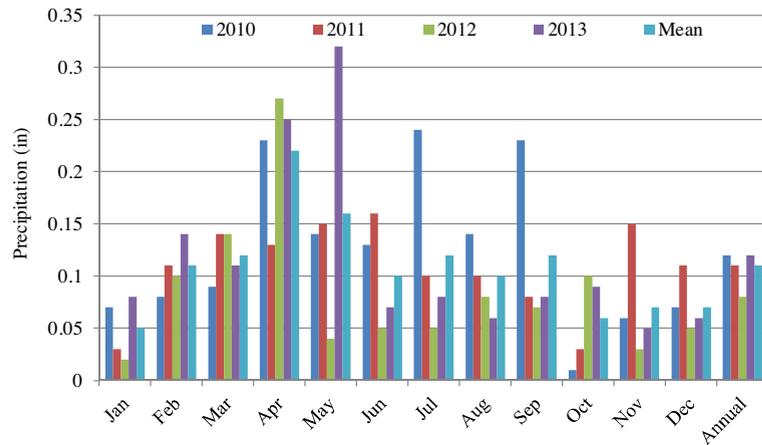


Fig. 2. Monthly and annual precipitation for the period 2010–2013 in Columbia, Missouri (Source of data: National Weather Service Forecast Office, 2014).

lot lagoon with berms was constructed about four decades ago. The lagoon surface area and depth are approximately 0.006 km² and 3.0 m, respectively. The size of a typical lagoon is determined by the number of animals housed and subsequently, the capacity of the wastewater lagoon. The bottom of the lagoon is a compacted claypan, and to our knowledge, aerators are not installed. In addition, the lagoon was under no form of treatment.

We also evaluated the residential sewage lagoon system (within a close proximity to the beef facility) and its overflow channel. An overflow pipe from the aerated single cell lagoon at irregular intervals transported liquid wastes through the same channel utilized by the beef farm. Information on the size and treatment of the residential lagoon was not available.

2.3. Sample collection

The potential sources of antibiotic compounds and their connectivity to Gans Creek were taken into consideration in the design of the sampling points. Nine sampling points (S1–S9; Fig. 1) were delineated as follows: sample no. S1 (ALLO, animal lot sewage lagoon overflow); sample no. S2 (RLO – 2, residential sewage lagoon overflow sample 2); sample no. S3 (the combined overflows of sample nos. S1 and S2); sample no. S4 (Gans Creek at mid-stream point before entry of ALLO and RLO); sample no. S5 (the combined overflows of S1 and S2); sample no. S6 (Gans Creek at the mid-stream point after entry of S1 and S2); sample no. S7 (Gans Creek upstream sample); sample no. S8 (RLO – 1, residential sewage lagoon overflow sample 1); and sample no. S9 (Gans Creek at a downstream point). Samples (in duplicates) were collected by the grab method in the morning hours each month in pre-cleaned amber bottles. The sampling campaigns lasted from November 2011 through April 2012 except in February 2012. Field blanks were prepared at the

sampling sites. Sampling containers were rinsed twice with the aliquots of the sample to be collected before filling the bottles. Thereafter, samples along with the field blanks were appropriately coded for easy identification. All samples were placed on ice in a container and transported to the laboratory for further processing and analysis.

2.4. Antibiotic assay

The target analyte, ENRO was selected for investigation based on the information provided by the beef research manager on the chemicals used at their facility. Nonetheless, information was lacking on the types of antibiotics prescribed or taken by individuals at the residential apartments. Specifically, Baytril, the trade name for ENRO, was used to control cattle infections. The antibacterial is known as the most commonly used fluoroquinolone to treat bovine and porcine infections (Grobbel et al., 2007) such as colibacillosis, salmonellosis, mycoplasmosis and pasteurellosis (Bona et al., 2016). ENRO was purchased as a standard from Sigma-Aldrich (St. Louis, Missouri, USA). The group name, CAS number, chemical formula, and molecular weight of ENRO are shown in Table 1 and the chemical structure is given as Fig. 3. Isotopic standard, enrofloxacin-d₅ (ENRO-d₅) used to check for matrix effect in our analytical process was purchased from Fisher Scientific (St. Louis, Missouri, USA).

2.5. Supplies, reagents and instrumentation

The purity of all chemicals used during the experiments was $\geq 97\%$. HPLC-grade ammonium acetate, formic acid, and acetonitrile were supplied by Fisher Scientific. Isotopically labeled ENRO-d₅, ENRO standard and Whatman Anotop PTFE syringe filters (0.2 μm) were also acquired from Fisher Scientific. The ultrapure water (18.2 M Ω /cm resistivity at 25 °C with total organic carbon < 3 $\mu\text{g/L}$) system was acquired from Millipore Corporation (Billerica, MA, USA). A Waters Alliance 2695 HPLC coupled to Waters Acquity TQD triple quadrupole

Table 1. Enrofloxacin (ENRO) described by physicochemical properties, therapeutic group name, CAS number, chemical formula, and molecular weight. CAS=Chemical Abstract Service.

Therapeutic Group Name	Function	Compound	CAS number	Chemical formula	Molecular wt. (g/mol)	Log K _{ow}	Water solubility	pK _a
Fluoroquinolone	Bactericidal agent	ENRO <i>1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid</i>	93106-60-6	C ₁₉ H ₂₂ FN ₃ O ₃	359.4	0.70 ^a	1.10 g/L ^b @ pH 5.0; 25 °C	6.38 ^c

^a USEPA, 2017. Sourced from: <https://www.epa.gov/tsc-screening-tools> (accessed May 8, 2017).

^b Gagliano and McNamara, 1996.

^c Nowara et al. (1997).

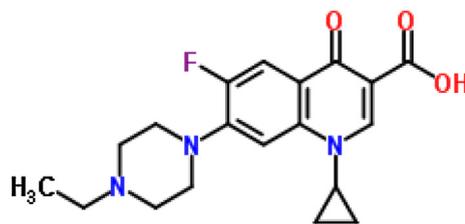


Fig. 3. Chemical structure of enrofloxacin (ENRO). Source: <https://http://www.chemspider.com/> (accessed June 16, 2017).

mass spectrometer (HPLC-MS/MS) equipment (Waters Corporation, Milford, Massachusetts, USA) was utilized to determine the concentrations of ENRO in samples. The Waters IntelliStartTM software was utilized to optimize the ionization energy, capillary and cone voltage, desolvation gas flow, the collision energy, and multiple reaction monitoring mode (MRM) transition ions (precursor and product ions).

2.6. Sample preparation and analysis

Aqueous samples were filtered and directly injected into the HPLC-MS/MS which allowed for measurement of the analyte in the ng/L range. Due to the high sensitivity of modern analytical equipment, direct injections of antibiotic compounds are common in the analysis of antibiotic chemicals (Vosough et al., 2015; Denadai and Cass, 2015). Briefly, a sample aliquot (10 mL) was filtered through a Whatman Anotop PTFE syringe filter (0.2 μ m) and the concentrations of the target compounds were quantified using HPLC-MS/MS. The target chemicals in injected samples were separated by a Phenomenex Kinetex C₁₈ (100 mm x 4.6 mm; 2.6 μ m particle size) reverse-phase column (Torrance, CA, USA). The mobile phase utilized for the separation was 10 mM ammonium acetate and 0.1% formic acid in water (A) and 100% acetonitrile (B). The gradient conditions were 0–0.5 min, 2% B; 0.5–7 min, 2–80% B; 7.0–9.0 min, 80–98% B; 9.0–10.0 min, 2% B; and 10.0–15.0 min, 2% B at a flow rate of 0.5 ml/min. The re-equilibration time between each analysis was 5 min.

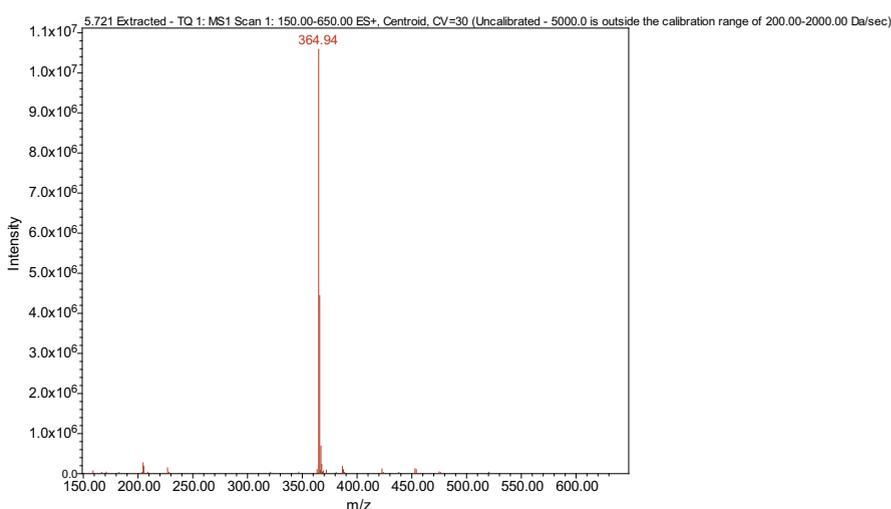
The MS/MS system was operated using electrospray ionization (EI) in the positive ion mode with a capillary voltage of 1.5 kV. The ionization source and the desolvation temperature were programmed at 150 °C and 450 °C, respectively. The MS/MS system operated in the MRM mode with a collision energy of 30 V. The molecular parent ions were screened and the product ions used for the quantifications were determined from the spectra obtained by injecting of 20 μ L of 1000 μ g/L pharmaceutical standard solutions. The predominant fragment ions were selected for quantification. The conditions for ENRO detection were as follows: mode (+); range of programmed MRM time mode: (5.5–7 s); molecular weight of the parent ion – P (m/z 360); and molecular weight of the product ion –

D (m/z 342). The acquired ions of ENRO in the analysis of beef kidney were 360, 342, and 316 (Hindle and Meng, 2004) and the MRM transitions of ENRO during the analysis of pharmaceuticals and personal care products in water were 360→342 and 360→316 (Ferrer et al., 2008).

2.7. Quality assurance

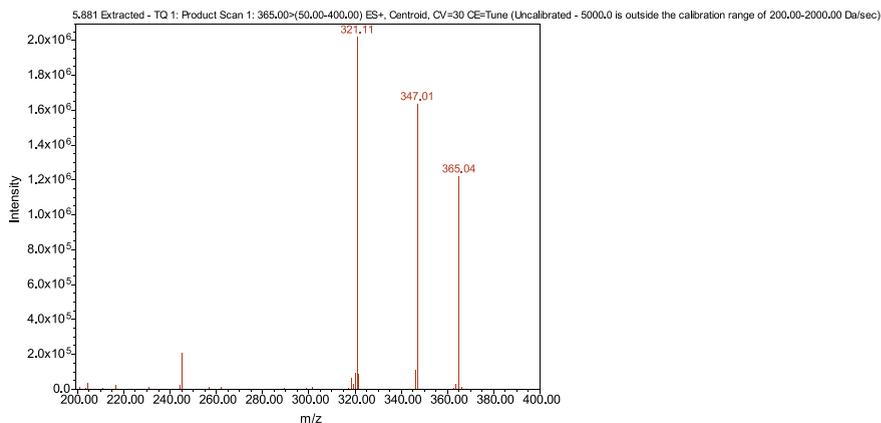
Figs. 4–7 shows the mass/charge (m/z) ratios of ENRO- d_5 and ENRO molecular/product ions. A high purity stock standard solution of each target compound was diluted appropriately with 100% acetonitrile. Each calibration curve was obtained from the results of injection of working calibration standard solutions (0.01, 0.05, 1, 50, 100, 250, and 500 $\mu\text{g/L}$). The ratio of the peak area to internal standard was plotted against concentration. The calibration setting was polynomial and the coefficient of determination (R^2) of an individual curve was greater than 0.98. Each standard injection was performed in duplicate for reproducibility. No contaminant was found in the field blanks analyzed along with other samples in each sequence of samples.

The limit of detections (LOD) of target compounds was performed with appropriate diluted working standard. The LOD and the limit of quantitation (LOQ) were determined as the concentration with signal-to-noise ratios of 3 and 10, respectively (Gaffney et al., 2015). In the current work, the LOD and LOQ of ENRO were 5 ng/L and 15 ng/L , respectively. The method was validated through the recovery of ENRO from a spiked solution at 0.5 $\mu\text{g/L}$ and the recovery was 91%. The relative standard deviations of analysis of standards were $\leq 8\%$. Matrix effect was not observed during the analysis of samples. ENRO- d_5 was analyzed



Molecular ion $[M + H]^+$ spectra of ENRO- d_5 (m/z 365).

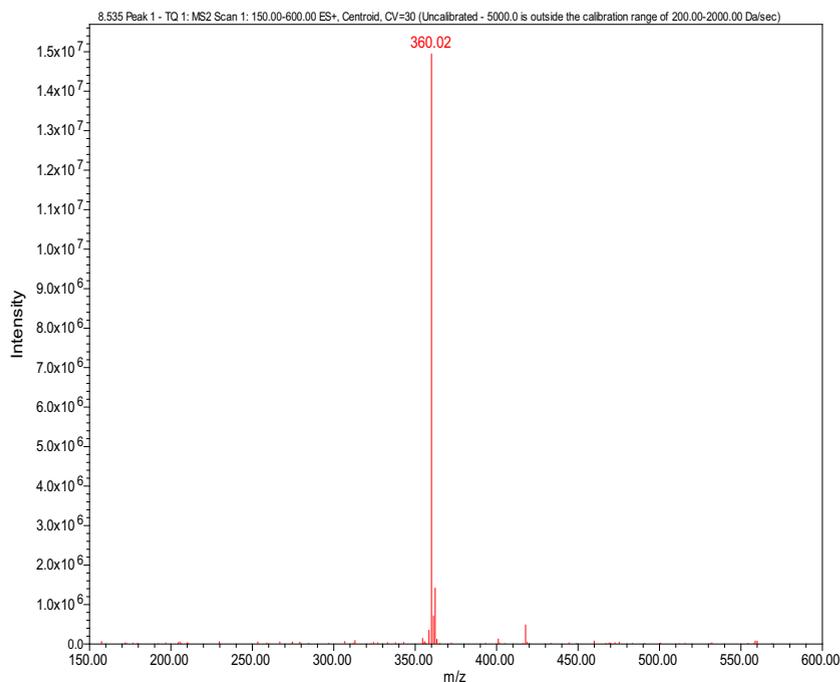
Fig. 4. Molecular ion $[M + H]^+$ spectra of ENRO- d_5 (m/z 365).



Production spectra (m/z 347) of ENRO-d₅.

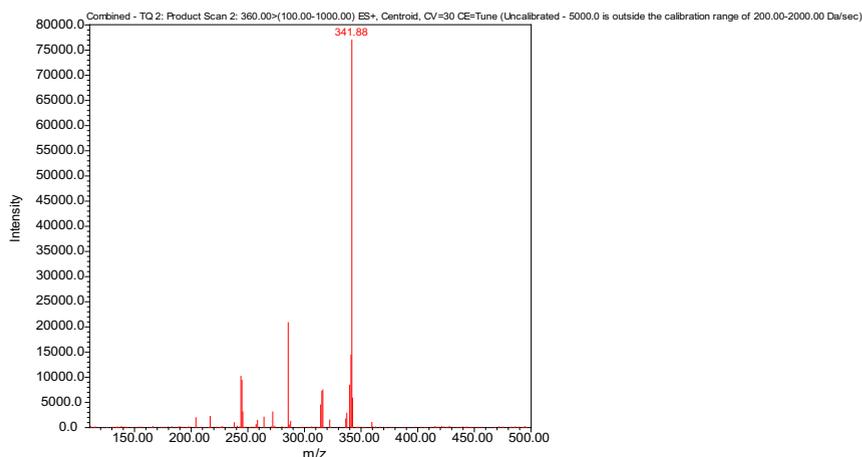
Fig. 5. Product ion spectra (m/z 347) of ENRO-d₅ (m/z 365).

under the same conditions with samples to check for matrix effect during our experiments. The recovery rates (%) for the diluted ENRO standards (0.1, 0.5 and 1 µg/L) were 110, 91, and 109, respectively. Similarly, the recoveries of 0.1 µg/L and 0.5 µg/L ENRO-d₅ standards were 109% and 100%, respectively.



ENRO (molecular ion: m/z 360).

Fig. 6. Molecular ion [M + H]⁺ spectra of ENRO (m/z 360).



ENRO (production: m/z 342).

Fig. 7. Product ion spectra (m/z 342) of ENRO (m/z 360).

2.8. Chromatographic data processing

The detection and integration of chromatographic peaks were performed using Waters Empower Chromatography software. The charts were prepared using Microsoft Excel 2010.

2.9. Preliminary ecotoxicological assessment

Antibiotics and their metabolites impact the biological functions of single species and the whole trophic chain. The degree of impacts is controlled by factors such as concentration, toxicity, target specificity, exposure duration, solubility, bioavailability, the sensitivity of the organisms, the toxicity of metabolites, and the combined effect of chemical mixtures in the aquatic system. Environmental indicators such as Daphnid (*Daphnia magna*), aquatic vascular plant (*Lemna minor*), green algae and fish belonging to different trophic levels have been used in previous studies for ecotoxicological assessments of chemicals (Robinson et al., 2005; Cunningham et al., 2006; Pro et al., 2003).

In the current work, the ecotoxicological risk of ENRO was estimated through acute and chronic effects data (European Chemicals Agency, 2008; European Medicines Agency, 2006). The PNEC values for acute and chronic toxicities have been derived using the equations (OECD, 1992) as follows:

$$\text{PNEC (acute toxicity)} = (\text{LC}_{50} \text{ or } \text{EC}_{50})/1000 \quad (1)$$

$$\text{PNEC (chronic toxicity)} = (\text{NOEL})/10 \quad (2)$$

Where EC_{50} = the lowest median effective concentration and NOEL = no-observed-effect-level. PNEC was calculated by dividing the EC_{50} Eq. (1) and NOEL Eq. (2) by the assessment factors of 1000 and 10, respectively (Tamura et al., 2013). The EC_{50} and NOEC values for aquatic organisms were obtained from published values (Ebert et al., 2011; Robinson et al., 2005; Table 2) for acute and chronic toxicities to organisms in various taxonomic groups. The risk quotient (RQ) expressed as the ratio of the MEC to PNEC Eq. (3) was used to assess the ecotoxicity of ENRO. RQ was calculated from the relationship below.

$$\text{Risk quotient (RQ)} = \text{MEC/PNEC} \quad (3)$$

The worst case scenario is to assume MEC as the highest concentration (Chen et al., 2016) found for all samples in the present work and therefore, the highest level of ENRO found in Gans Creek was used in RQ estimation. Previous studies (Hernando et al., 2007; Chen et al., 2016) used the following RQ classifications to assess ecotoxicity where low toxicity is from 0.01 to 0.1; medium toxicity from 0.1 to 1; and high toxicity from greater than 1. In the current work, literature EC_{50} or LC_{50} values (Table 2) were used to evaluate the potential acute toxicity of ENRO to various organisms. Irrespective of the period of sampling, the maximum concentration (ng/L) of ENRO found were 216 for Gans Creek; 440 for ALLO; and 259 for RLO.

3. Results and discussion

3.1. ENRO concentrations in overflows and the receiving-stream

Fig. 8 presents the temporal trend of ENRO concentrations in overflows and the receiving-stream samples. ENRO antibiotic was positively identified and quantified in overflows from the animal lot and residential sewage lagoon systems and in Gans Creek at trace levels. ENRO was frequently detected in most samples (>89% frequency) with a minimum below the LOQ (15 ng/L). The ALLO site (sample no. S1; overflow sample) showed a positive identification of ENRO and exhibited the highest value (0.44 $\mu\text{g/L}$) for samples analyzed. The ENRO level for sample no. S1 (March 2012) was approximately 2 and 3 folds higher than those for sample nos. S2 and S8 (RLO-2 and RLO-1), respectively. Furthermore, the levels for ALLO (sample no. S1) were at least 2 folds higher than those observed in Gans Creek during March 2012. ENRO was not positively identified in sample no. S8 (RLO-1) collected in December 2011. In Missouri, the month of March represented a rainy period and which coincided with times of high discharge from both lagoons. Overall, the frequency of detection of ENRO was 90% for sample nos. S2 and S8 (RLO sites).

The levels of ENRO in RLO samples ranged from < LOQ to 259 ng/L (the highest value was found also in a sample collected in March 2012). The maximum values

Table 2. Ecotoxicity data of enrofloxacin (ENRO, values in $\mu\text{g/L}$) antibiotic from the literature.

Test species	NOEL	End point	Test duration	Ecotoxicity value	PNEC ^a (Acute)	PNEC ^a (chronic)	Reference
<i>Lepomis macrochirus</i> *	18,600	LC ₅₀	96 h	216,000	216	21,600	Gagliano and McNamara, 1996
<i>Daphnia magna</i> **	23,000	EC ₅₀	48 h	79,900	79.9	7,990	Gagliano and McNamara, 1996
<i>Moina macrocopa</i> **		EC ₅₀	48 h	56,700	56.7	5,670	Park and Choi, 2008
<i>Microcystis aeruginosa</i> ***		EC ₅₀	5 d	49	0.049	4.9	Robinson et al., 2005
<i>Anabaena flosaquae</i> ***	19.1	EC ₅₀	3 d	173	0.173	17.3	Ebert et al., 2011
<i>Desmodesmus Subspicatus</i> ****	500	EC ₅₀	3 d	5,568	5.568	556.8	Ebert et al., 2011
<i>Pseudokirchneriella subcapitata</i> ****		EC ₅₀	3 d	3,100	3.1	310	Robinson et al., 2005
<i>Lemna minor</i> *****	30	EC ₅₀	48 h	107	0.107	10.7	Ebert et al., 2011

EC₅₀, median effect concentration; NOEL, no observed effect level.

^a AF, assessment factor value used in calculation = 1000.

* Fish.

** Invertebrates.

*** Cyanobacteria.

**** Algae.

***** Macrophyte.

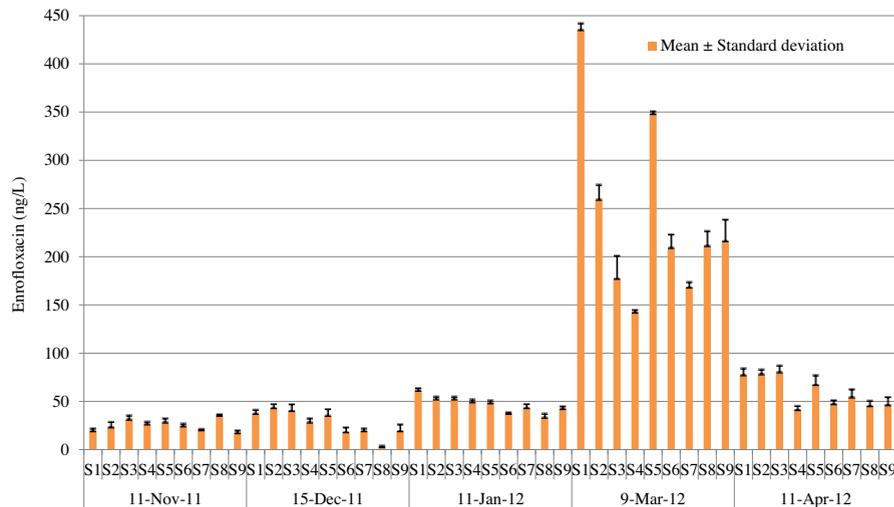


Fig. 8. Temporal trend of ENRO concentrations at the study sites.

of ENRO in ALLO and RLO samples (This study) were higher than the value established in a WWTP influent sample from Thailand, Bangkok (90.3 ng/L; Li et al., 2012), and an effluent wastewater in Coimbra, Portugal (spring: 211.5 ng/L and autumn: 53.7 ng/L; Seifrtová et al., 2008). It should be noted that previous published data on ENRO may be influenced by geographical locations and the sensitivities of the analytical methods employed in studies. Comparison of ENRO levels between the two lagoon systems (human and animal) indicated that the animal lot lagoon had a greater influence on the receiving-stream. This may be as a result of the volume of wastes generated, the number of animals treated, the frequency of treatment, the land application of animal lagoon effluent in the watershed, and the discharged amount of wastewater from the lagoon.

The occurrence of ENRO in this present study was 100% in Gans Creek. This was higher than the 11% detection frequency found in Ebro River, Spain (Gros et al., 2010). Transport of ENRO into Gans Creek was most prominent in March 2012. This antibiotic was equally identified upstream (S7; 0.17 $\mu\text{g/L}$) during the rainy period which suggested antibiotic contamination of the upper reaches of Gans Creek due to diffuse pollution. ENRO concentration for Gans Creek (This study) was below the 332 ng/L level described for Wangyang River, China (Jiang et al., 2014) but higher than the value found in surface water (55 ng/L; Thom Canal) in the Mekong Delta, Vietnam (Giang et al., 2015) and in surface water from Galicia, Spain (118.4 ng/L; Iglesias et al., 2014).

3.2. Risk quotient (RQ) for the receiving-stream

Fig. 9 shows the preliminary ecological risk assessment of ENRO in Gans Creek based on the calculated RQ values derived from acute and chronic toxicity data for various taxonomic groups. From the ecotoxicity assessment, ENRO posed a high

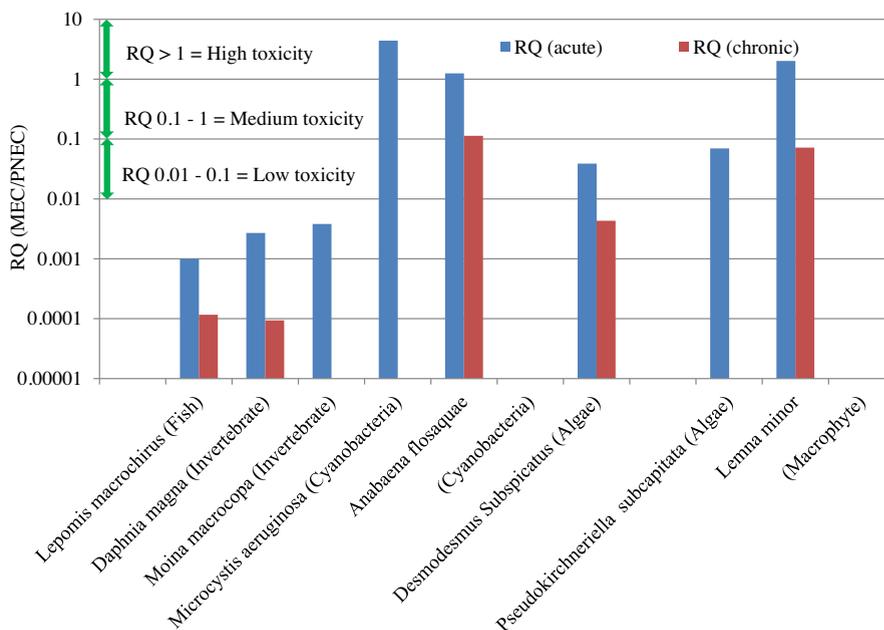


Fig. 9. Ecological risk assessment of Gans Creek (receiving-stream) based on acute and chronic toxicity values.

risk to both cyanobacteria and macrophytes. The RQ (acute toxicity) was in the order: *Microcystis aeruginosa* (Cyanobacteria; RQ = 4.4) > *Anabaena flosaquae* (Cyanobacteria; RQ = 1.3) > *Lemna minor* (aquatic vascular plant; RQ = 2.0). However, there was a low risk of acute toxicity associated with algae (*Desmodesmus subspicatus*; RQ = 0.04), and (*Pseudokirchneriella subcapitata*; RQ = 0.07). Medium risks due to chronic exposures of cyanobacteria (*Anabaena flosaquae*; RQ = 0.11), and a macrophyte (*Lemna minor*; RQ = 0.07) in the receiving-stream was probable. The RQs (< 0.01; acute) indicated minimal ecotoxicity hazards for the invertebrates (*Daphnia magna*) and (*Moina macrocopa*); and fish (*Lepomis macrochirus*) in Gans Creek watershed. Despite the less potential harm to fish from ENRO exposure, the long-term adverse effects from both single and combination of many PHCs can produce profound effects on biological communities.

Available data in the literature are mostly for acute ecotoxicity of various antibiotic compounds but chronic tests are also important to reflect on the adverse effects. Antibiotic chemicals have the potential to harm aquatic organisms especially algae and this can seriously impact the food chain. Under laboratory conditions, the environmental release of ENRO posed a risk to *Rhinella arenarum* tadpoles (Peltzer et al., 2017). Moreover, Bona et al. (2016) found that exposure to ENRO led to increased toxicity over *Daphnia magna* generations. But in a previous study, ENRO residual concentration after an application had no toxic effects on exposed aquatic organisms (Andrieu et al., 2015). ENRO is known to affect the shoots and

leaves of plants (Tasho and Cho, 2016). ENRO was detected in surface water from Hangzhou, China but the found levels posed no risk to the ecosystem (Tong et al., 2011). Besides, Jiang et al. (2014) found that ENRO concentrations in Wangyang River (China) posed a high risk to aquatic organisms. Data is still lacking on the ecotoxicity of single chemicals and there are knowledge gaps on the ecotoxicity of chemical mixtures and their chronic effects. Regarding drug compounds discharged into aquatic systems, the environmental concentrations are usually several magnitudes lower than the toxicological no-observed-effect-level (Seilar, 2002) probably due to dilution of the target compounds, the rate of removal/ degradation from the water column, and several other factors.

4. Conclusions

The occurrence of ENRO in overflow samples from two lagoon systems (human and animal lot) and in a receiving-stream (Gans Creek) in the Lower Missouri-Moreau watershed was investigated during this study. ENRO was frequently detected in samples analyzed and the levels found were in the ng/L to µg/L range. Our finding supports other studies on the concentration ranges for pharmaceutical chemicals transported into surface waters. Also, our observed values indicated the influence of pharmaceuticals from human and animal wastes transported into the Gans Creek watershed. The highest concentrations of ENRO in the overflows and stream samples were directly linked to periods of high discharge and precipitation. Overall, this current work indicated that wastewaters from animal agriculture and human septic systems are important point sources of PHCs into receiving-surface waters. Apart from the point source contributions, diffuse sources also accounted for the abundance of ENRO in upstream of the study area. Rural streams can be heavily impacted depending on the amount and frequency of wastewater they receive.

The maximum concentration of ENRO found in the receiving-stream in this study showed strong ecotoxicity (high risk, $RQ > 1$) for cyanobacteria (*Microcystis aeruginosa* and *Anabaena flosaquae*), and aquatic vascular plant (*Lemna minor*). Fish (*Lepomis macrochirus*) had a less toxicological effect from ENRO with RQs at 0.001 (acute) and 0.00001 (chronic). Notwithstanding, the long-term adverse effects of various antibiotics in Gans Creek watershed is probable.

The occurrence of antibiotic chemicals in aquatic bodies is a global problem and this study validates other works that reported the impact of antibiotics on rural streams. Globally, increasing the distance between the receiving-stream and the lagoon, efficient lagoon/wastewater treatment technologies, building resilient lagoons that are not prone to leakage or failure, proper dilution and discharge of lagoon wastewater, and allowance on lagoon size that accounts for high precipitation times may overall reduce the impact of pharmaceuticals and their

metabolites on receiving-surface waters. Too many chemicals are transported into surface waters and therefore, more effective measures should be instituted to address the problems of antibiotics. Current ecological assessments are very challenging, laborious, and expensive and therefore, new ways to assess the risks of whole ecosystems are imperative. Moreover, cost-effective approaches for chemical monitoring, toxicity testing and assessment of whole ecosystems will be valuable towards the understanding of the short and long-term effects of antibiotic chemicals and their metabolites in the environment.

Declarations

Author contribution statement

Abua Ikem, Chung-Ho Lin: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Bob Broz: Conceived and designed the experiments; Wrote the paper.

Monty Kerley: Conceived and designed the experiments; Wrote the paper.

Ho Le Thi: Performed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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