

# Determination of fluoroquinolone antibiotics in wastewater effluents by liquid chromatography–mass spectrometry and fluorescence detection

Haruhiko Nakata <sup>a,b,\*</sup>, Kurunthachalam Kannan <sup>b,c</sup>,  
Paul D. Jones <sup>b</sup>, John P. Giesy <sup>b</sup>

<sup>a</sup> Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan

<sup>b</sup> Department of Zoology, National Food Safety and Toxicology Center, Institute of Environmental Toxicology, Michigan State University, East Lansing, MI 48824, USA

<sup>c</sup> Wadsworth Center, New York State Department of Health, Empire State Plaza, P.O. Box 509, Albany, NY 12201-0509, USA

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## Abstract

The occurrence of quinolone antibiotics (QAs) was investigated in wastewater effluents and surface river/lake waters in the US and Canada by using solid-phase extraction with mixed phase cation exchange disk cartridge and liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography fluorescence detection (LC–FLD). Ofloxacin (OFL) was detected in secondary and final effluents of a wastewater treatment plant (WWTP) in East Lansing, Michigan, at concentrations of 204 and 100 ng/l, respectively. The mass flow calculation, estimated by multiplying the OFL concentration in the final effluent by the average influent volume of the WWTP, showed that the discharge of OFL to the river was 4.8 g/day. The OFL concentrations in wastewater effluents measured in this study are comparable to or less than those observed in several European countries. QAs were not detected in river and lake waters analyzed in this study, which may be due to dilution effects and to the higher detection limits, relative to those reported previously. OFL concentrations were  $\approx 1$ – $2$  orders of magnitude lower than the  $EC_{50}$  concentrations for environmental bacterium. However, greater concentrations of other QAs in sewage sludge from WWTPs may result in cumulative effects. Considering that the sewage sludge is applied to the land as fertilizers, soil-dwelling organisms could experience greater exposures to such antibiotics. Monitoring studies of QAs in sewage from WWTPs and in sediment/soil near aquaculture facilities and livestock farms will be necessary for the evaluation of the environmental distribution and risk of these compounds.

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## 1. Introduction

In recent years, public concern about the environmental occurrence of pharmaceuticals and personal care products (PPCPs) has been increasing. Numerous

\* Corresponding author. Address: Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan. Tel./fax: +81 96 342 3380.

E-mail address: nakata@sci.kumamoto-u.ac.jp (H. Nakata).

PPCPs have been detected in waste and natural water resources, sediments, soils, and aquatic biota. While the PPCPs are found at relatively low concentrations (ng/l range) in natural waters (Terns, 1998; Korpin et al., 2002), elevated concentrations of antibiotics (several mg/kg levels) have been reported in manure (Hamscher et al., 2002), sewage sludge, and sludge-treated soils (Golet et al., 2003). While most PPCPs are considered to be polar and are thus thought not to be persistent in the environment, these studies suggest that some PPCPs, such as clofibrac acid and lopromide, are less readily degradable and may cause subtle effects to the ecosystems (Daughton and Ternes, 1999). However, little is known about the environmental levels, behavior, and potential chronic effects of long-term and low-level exposures of PPCPs as well as their degradation products. Furthermore, there is a serious lack of information regarding the development of antibiotic-resistance in bacteria due to the frequent use and exposure to pharmaceuticals as they accumulate in the environment.

Among various pharmaceuticals, antibiotics belonging to the quinolone group, including fluoroquinolones (FQs) are of particular environmental concern. Their chemical structures are shown in Fig. 1. The quinolone antibiotics (QAs), such as pipemidic acid (PIP), ofloxacin (OFL), norfloxacin (NOR), ciprofloxacin (CIP), lomefloxacin (LOM), enrofloxacin (ENR), difloxacin (DIF), sarafloxacin (SAR), and tosfloxacin (TOS), comprise an important class of pharmaceuticals, which have been widely used for the last 20 years in Europe and the United States (Golet et al., 2002b). QAs are active against many Gram-negative and Gram-positive bacteria, and function by inhibiting DNA gyrase, a key enzyme in DNA replication (Bryan et al., 1989). CIP, NOR, and OFL are licensed for use in human medicine (Alder et al., 2001). As of November 1999, the global value of CIP sales exceeded 1.3 billion dollars; the second leading quinolone is OFL, with sales of approximately 900 million dollars.

In general, ENR and SAR have been used in veterinary medicine (Boxall et al., 2003). In the US, SAR and ENR have been approved for use in livestock, but the use of SAR for laying hens has always been prohibited by the US Food and Drug Administration (US FDA) (Chu et al., 2002). The US FDA has also proposed banning ENR for use in poultry farming, because of the evidence that it contributes to the development of FQ-resistant *Campylobacter* infections in humans (Schaefer, 2002). The concentrations of QAs have been monitored in poultry, pigs, cattle, and cultured fishes, as a result of increasing concerns regarding food safety (Hernandes-Arteseros et al., 2002). Also, maximum residue limits (MRLs) of several QAs in animals have been established by the European Union (EU) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The EU has set a MRL of 100–300 µg/kg (ppb) for the sum of ENR and its metabolites and CIP in muscle, kidney, and liver of livestock (Hernandes-Arteseros et al., 2002).

Investigations of the occurrence of FQs in wastewater effluents and natural waters have been conducted in several European countries, such as Switzerland (Golet et al., 2002a), France, Italy, Sweden, and Greece (Andreozzi et al., 2003). (Golet et al., 2002a, 2003) analyzed FQs in raw sludge, wastewater effluents, and river water samples in Switzerland. They found that a large proportion of FQs entering wastewater plants (88–92% of the FQs mass flow) is removed during the treatment processes, with the remaining FQs in the effluents further reduced as the water flows downstream. It was also suggested that sewage sludge is the main reservoir and source of FQs residues (Golet et al., 2003). However, since most of these studies have been conducted in European countries, little information is available on the occurrence of FQs in wastewater effluents and natural waters in the United States.

In this study, we determined QAs, such as PIP, OFL, NOR, CIP, LOM, ENR, DIF, SAR and TOS, in waste-

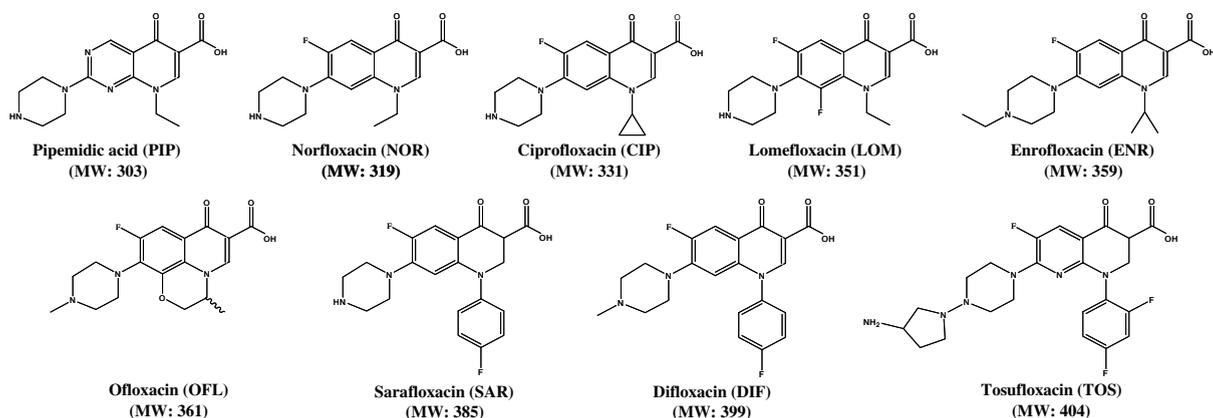


Fig. 1. Chemical structures and molecular weights of quinolone antibiotics analyzed in this study.

water effluents and river/lake waters in the US (Michigan) and Canada (the western Lake Ontario) using a liquid chromatograph interfaced with a mass spectrometer (LC–MS) and liquid chromatography fluorescence detector (LC–FLD).

## 2. Materials and methods

### 2.1. Chemicals

Standards of pipemedic acid (PIP), norfloxacin (NOR), ofloxacin (OFL) and lomefloxacin hydrochloride (LOM) were obtained from Sigma-Aldrich (St. Louis, MO). Ciprofloxacin hydrochloride (CIP) and Enrofloxacin (ENR) were purchased from ICN Biomedicals Inc. (Aurora, OH) and Fluka Chemical (Buchs SG, Switzerland), respectively. Difloxacin hydrochloride (DIF) and sarafloxacin hydrochloride (SAR) were obtained from Abbott Laboratory (North Chicago, IL). Tosufloxacin tosylate (TOS) was provided by Toyama Chemical Co. Ltd. (Tokyo, Japan). The purities of chemicals used in this study ranged 89% for DIF to 99.5% for SAR. All QA solutions were prepared in either methanol or dimethyl sulfoxide (DMSO). HPLC-grade acetonitrile (Burdick & Jackson, Muskegon, MI), methanol (Mallinckrodt Baker, Phillipsburg, NJ) and Milli-Q organic-free water (Millipore, Bedford, MA) were used in this study.

### 2.2. Samples

Secondary treatment and final effluent water samples ( $n = 3$ ) were obtained from a WWTP in East Lansing, Michigan (Fig. 2). Samples were collected in glass bottles and immediately stored in the dark at 4°C until analysis. The treatment processes at the WWTP include aerial grit removal, flow equalization, primary clarification, course bubble air activated sludge, secondary clarification, disinfection, rapid sand filtration, dechlorination, and post filtration aeration. Secondary effluent refers to effluent obtained from a clarifier (after particle settlement), while the final effluent refers to effluent obtained after secondary clarification. The average influent volume is 12.6 million gallons per day (MGD) at this WWTP. Nine samples of river and lake waters were collected from Detroit ( $n = 2$ ), Lansing ( $n = 3$ ), and Petosky ( $n = 3$ ) in Michigan, and from western Lake Ontario ( $n = 1$ ) in Canada (Fig. 2). All samples were obtained during August and October of 2002.

### 2.3. Sample preparation

The QAs were determined according to methods described previously (Golet et al., 2001) with some modifications. Briefly, 150–500 ml of each water sample was filtered through a 0.45 µm cellulose nitrate membrane filter, and its pH was adjusted to 3 by the addition of formic acid. The analytes were concentrated from water

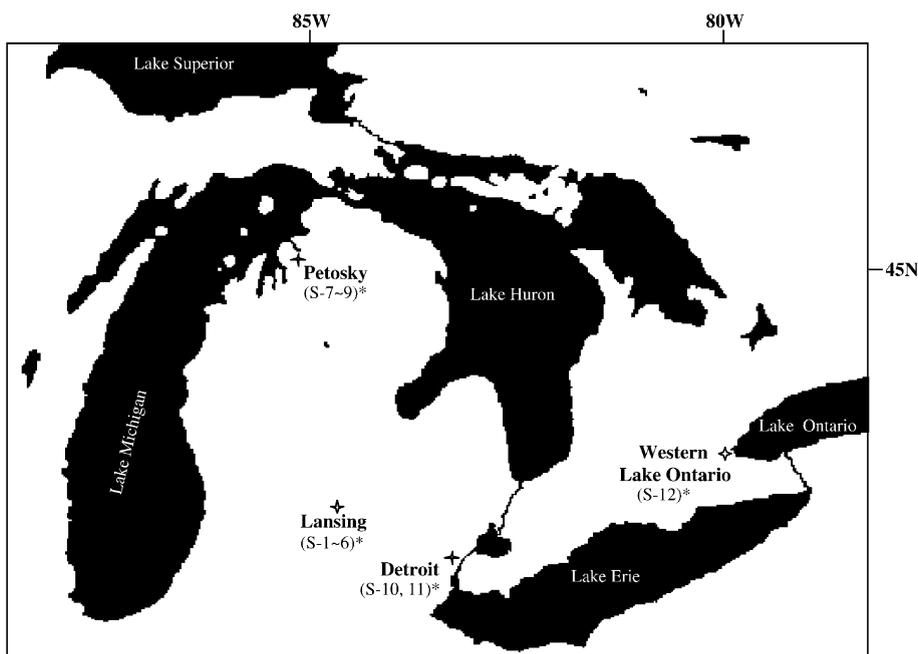


Fig. 2. Sampling sites of wastewater effluents and natural waters. \*:S = sample number, corresponding to the numbering in Table 2.

samples by solid-phase extraction using mixed-phase cation exchange (MPC) disk cartridges (3M Empore, St. Paul, MN). The MPC cartridge was preconditioned with methanol (8ml) and Milli-Q water at pH 3.0 (8ml). The flow speed of the extraction was set at 1–2 ml/min using a vacuum manifold. After extraction, the disk cartridges were eluted with 4 ml of 5% ammonia solution in methanol. The eluted solvents were evaporated to near-dryness under a gentle stream of nitrogen, and were reconstituted with 2 ml of 5% NH<sub>4</sub>OH/15% MeOH/water for injection into LC–MS and LC–FLD.

#### 2.4. LC–MS and LC–FLD analysis

Determination of QAs was performed on a high-pressure liquid chromatograph (HP-1100, Hewlett Packard) interfaced with a mass spectrometer (VG Platform, Fisons Instruments) with atmospheric pressure electrospray ionization in positive mode (ESP<sup>+</sup>). The mobile phases were a mixture of water and acetonitrile (98:2, pH: 3.0, phase A) and acetonitrile (phase B), respectively. The elution gradient was 5:95 (A:B) initially, and was programmed to 45:65 (A:B) in 25 min. The LC column, YMC ODS-AQ S-3 (4.0 × 50 mm, Waters) was used for the separation of FQs prior to detection by LC–MS. The analysis was performed at a flow rate of 0.2 ml/min, and column temperature was maintained at 23 °C. A sample solution of 20 µl was injected using a HP 1090 auto-sampler (Hewlett Packard). The desolvation gas flow rate was 200 ml/min and the desolvation temperature was and 200–300 °C. The cone voltage and capillary voltage were set at 50 V and 2–3 kV, respectively. Parent ion (MH<sup>+</sup>) of each FQs was monitored at *m/z* 304, 362, 320, 332, 352, 360, 386, 400, and 405 for PIP, OFL, NOR, CIP, LOM, ENR, SAR, DIF, and TOS, respectively. The chromatograms of the standard mixture and individual QAs are shown in Fig. 3.

Since electrospray ionization is a soft ionization technique, only one ion (parent MH<sup>+</sup>) could be monitored in the SIM mode. Therefore, for confirmation of the re-

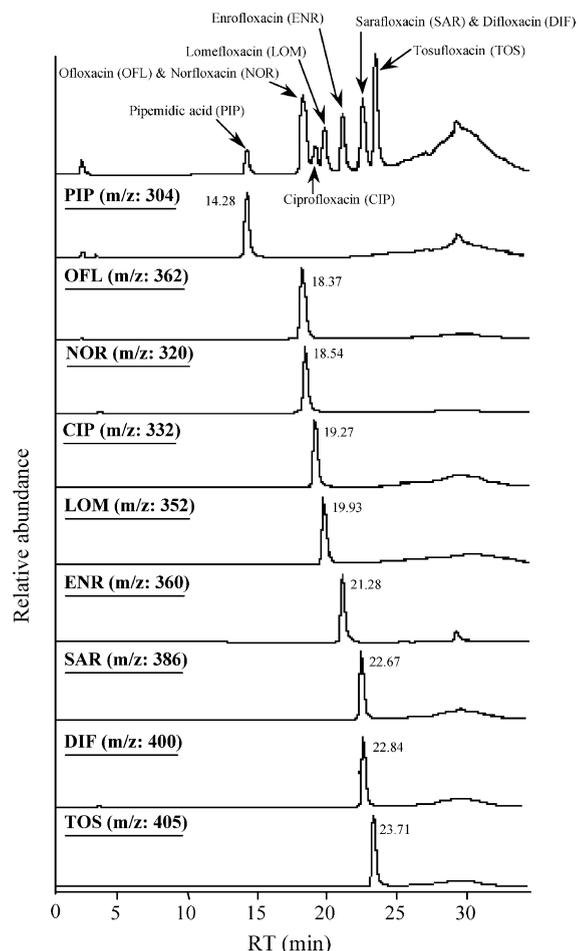


Fig. 3. LC–MS chromatograms of mixed and individual QA standards.

sults, we also used LC–FLD (LC: Perkin Elmer Series 200, FLD: Hewlett Packard 1046A) for determination of QAs. The FLD was set at an excitation wavelength of 278 nm and an emission wavelength of 445 nm, except

Table 1

Average recoveries (%), linear regression data, and squares of correlation coefficients ( $r^2$ ) for standard curve of quinolone antibiotics

Compound	MW	RT <sup>a</sup> (min)	Spiked amount of std (ng) <sup>b</sup>	Recovery (%)	Linear range (µg/l)	$r^2$
Pipemidic acid	303.3	14.28	200	84 ± 12	25–500	0.99941
Ofloxacin	361.4	18.37	200	87 ± 9.0	25–500	0.99967
Norfloxacin	319.3	18.54	200	90 ± 6.0	25–500	0.99979
Ciprofloxacin	331.4	19.27	200	85 ± 13	25–500	0.99980
Lomefloxacin	352.0	19.93	200	92 ± 6.2	25–500	0.99927
Enrofloxacin	359.4	21.28	200	107 ± 12	25–500	0.99972
Sarafloxacin	385.4	22.67	200	82 ± 4.3	25–500	0.99998
Difloxacin	399.4	22.84	200	93 ± 10	25–500	0.99988
Tosfloxacin	404.4	23.71	200	75 ± 7.2	25–500	0.99982

<sup>a</sup> Retention time of LC–MS.

<sup>b</sup> Two hundred nanograms of individual standards in 200 ml of river water that did not contain any QAs.

Table 2

Concentrations of quinolone antibiotics (ng/l) in wastewater effluents and river/lake waters collected from Michigan, USA and the Lake Ontario in Canada

Sample number	Sample name	Location	Sampling date	PIP	OFL	NOR	CIP	LOM	ENR	SAR	DIF	TOS
<i>WWTP effluents</i>												
1	Secondary effluent 1	East Lansing, MI	August, 2002	<31	<b>204</b>	<45	<19	<41	<34	<44	<26	<49
2	Secondary effluent 2	East Lansing, MI	August, 2002	<31	<8.6	<45	<19	<41	<34	<44	<26	<49
3	Final effluent	East Lansing, MI	August, 2002	<31	<b>100</b>	<45	<19	<41	<34	<44	<26	<49
<i>Natural waters</i>												
4	Red Ceder River	East Lansing, MI	September, 2002	<31	<8.6	<45	<19	<41	<34	<44	<26	<49
5	Grand River 1	Lansing, MI	September, 2002	<31	<8.6	<45	<19	<41	<34	<44	<26	<49
6	Grand River 2	Lansing, MI	September, 2002	<31	<8.6	<45	<19	<41	<34	<44	<26	<49
7	Petosky River 1	Petoskey, MI	July, 2002	<31	<8.6	<45	<19	<41	<34	<44	<26	<49
8	Petosky River 2	Petoskey, MI	July, 2002	<31	<8.6	<45	<19	<41	<34	<44	<26	<49
9	Petosky River 3	Petoskey, MI	July, 2002	<31	<8.6	<45	<19	<41	<34	<44	<26	<49
10	Detroit River 1	Detroit, MI	October, 2002	<31	<8.6	<45	<19	<41	<34	<44	<26	<49
11	Detroit River 2	Detroit, MI	October, 2002	<31	<8.6	<45	<19	<41	<34	<44	<26	<49
12	Lake Ontario	Canada, ON	October, 2002	<31	<8.6	<45	<19	<41	<34	<44	<26	<49

for OFL, which as at 500 nm. The mobile phase flow rate was maintained at 0.5 ml/min. The LC column, Discovery RP-Amide C16 (4.0 × 50 mm, Supelco) was used for the determination of FQs by LC-FLD. The mobile phase and gradient condition of the LC-FLD were similar to those for LC-MS.

### 2.5. Quality control

Quality control samples included spike recovery tests through the entire analytical procedure. Two hundred milliliters of river water that did not contain detectable quantities of QAs was spiked with 200 ng of a standard mixture containing nine QAs. Three replicate analyses were performed, and the average recoveries of FQs ranged from 75% to 107% (Table 1). Procedural and instrument blanks were analyzed for each set of six samples, to control for laboratory contamination and analytical interferences. The concentrations were reported as below the detection limit if the peak height was not greater than the specified threshold (signal to noise ratio of 2). The detection limits of LC-MS for PIP, OFL, NOR, CIP, LOM, ENR, SAR, DIF, and TOS in water samples were 31, 8.6, 45, 19, 41, 34, 44, 26, and 49 ng/l, respectively (Table 2).

## 3. Results and discussion

Among the QAs analyzed in this study, only OFL was detected in secondary and final effluents of the WWTP, at concentrations 204 and 100 ng/l, respectively (Table 2). The LC-MS chromatograms of QA standards and of the wastewater effluent are shown in Fig. 4. The retention time of the OFL standard was 18.37 min, a

value in agreement with that of water samples that contained OFL (Fig. 4). Similar results were obtained by LC-FLD when the retention time of OFL was compared between the standard and water samples (Fig. 5).

OFL has been used to treat various bacterial infections such as bronchitis, gonorrhoea, skin infections, and urinary tract infections etc. The US FDA has classified OFL as a pregnancy category C drug: there is a lack of information on the effects on the human fetus. We occasionally identified LOM peaks in LC-MS chromatograms. However, the peak heights were not greater than the specified threshold (2 times the noise level), and thus they could not be quantified. Similarly, LOM was not found by LC-FLD.

High concentrations of OFL have been found in the effluents from sewage treatment plants in European countries, such as France (330–510 ng/l), Italy (290–580 ng/l), and Greece (460 ng/l) (Table 3). The OFL concentrations measured in our study were comparable to or less than those observed in the previous studies from Europe. CIP and NOR were detected in the effluents of WWTPs in Switzerland (Golet et al., 2002a) and several European countries (Andreozzi et al., 2003), although they were not detected in our study. Such geographical differences might be due to varying patterns of FQ usage among countries and regions, as well as the relatively higher detection limit of FQs in this study. The highest concentrations of FQs in Europe were found in mixed wastewater streams, which also received wastewater collected from airport (Golet et al., 2002a).

The OFL concentration in the final effluent from the WWTP was approximately 50% less than the concentration found in the secondary treatment effluent (Table 2). While the number of samples analyzed is limited, such a decrease indicates a partial removal of OFL during the

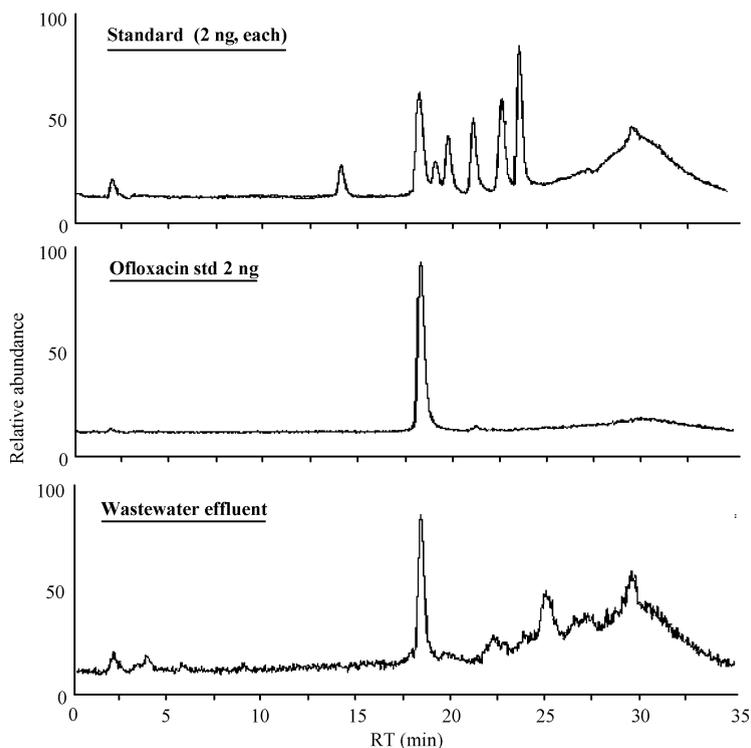


Fig. 4. LC-MS chromatogram of QA mixtures (total), ofloxacin standard, and wastewater effluent (SIM mode,  $m/z$ : 362).

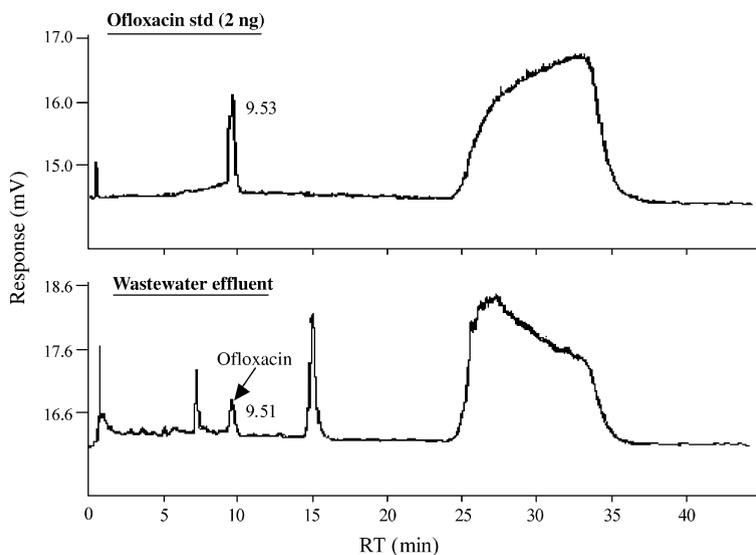


Fig. 5. LC-FLD chromatograms of ofloxacin standard and wastewater sample (excitation: 278 nm, emission: 500 nm).

wastewater treatment processes. A similar result has been reported in Switzerland (Golet et al., 2002a, 2003). It was reported that a large fraction of the CIP and NOR entering wastewater plants is removed during whole treatment processes, ranging from 88% to 92%

reduction (Golet et al., 2003). In contrast, elevated concentrations of CIP and NOR (several mg/kg) were determined in sewage sludge and sludge-treated soils (Golet et al., 2002b, 2003). While we have not analyzed sewage sludge, it is likely that large amounts of QAs are present

Table 3  
Comparison of fluoroquinolone concentrations (ng/l) in wastewater effluents from the US and European countries

Compound	USA (this study)	France <sup>a</sup>	Italy <sup>a</sup>	Sweden <sup>a</sup>	Greece <sup>a</sup>	Switzerland <sup>b</sup>	
						Raw sewage	Final effluent
Ofloxacin	100–204	330–510	290–580	120	460	<5.0	<5.0
Lomefloxacin	<41	180–290	180–320	130	290	<3.0	<3.0
Norfloxacin	<45	50–70	60–70	30	70	255–553	36–73
Ciprofloxacin	<19	60	40–70	30	70	313–568	62–106

<sup>a</sup> Andreozzi et al., 2003.

<sup>b</sup> Golet et al., 2002a.

in sludge samples, due to the strong sorption to organic particles of these compounds (Tolls, 2003).

The mass flow of OFL was estimated based on the OFL concentration in the final effluent (100 ng/l) and the average influent volume of the WWTP (12.6 MGD). The amount of discharge of OFL to the river is calculated at 4.8 g/day, a value approximately two orders of magnitude greater than the CIP and NOR discharges from a WWTP into a river ( $32 \pm 14 \mu\text{g/day}$  CIP and  $25 \pm 9 \mu\text{g/day}$  NOR) in Switzerland (Golet et al., 2002a). However, QAs were not detected in any natural water collected from Lansing, Detroit, Petosky, and the Lake Ontario at a detection limit of 8.6–49 ng/l (Table 2). This may be due to dilution effects of QAs in river/lake water and to the higher detection limits of these compounds.

Toxic assessment of PPCPs including QAs has been made by considering their effects on wastewater and environmental bacteria. Hartmann et al. (1998) identified that FQs, especially CIP, play a role as the main source of *umuC* genotoxicity of hospital wastewater. OFL was highly toxic to the marine bacterium, *Vibrio fischeri*, with an  $EC_{50}$  of  $14 \mu\text{g/l}$  (Backhaus et al., 2000). Kummerer et al. (2000) conducted the Growth Inhibition Test (GIT) using the Gram-negative environmental bacterium, *Pseudomonas putida*. They reported that the  $EC_0$ ,  $EC_{50}$ , and  $EC_{100}$  values of OFL were <10, 10, and  $40 \mu\text{g/l}$ , respectively, and that these were comparable to or lower than the values of CIP ( $EC_0$ : 10,  $EC_{50}$ : 80,  $EC_{100}$ :  $320 \mu\text{g/l}$ ). OFL concentrations in wastewater effluents were two orders of magnitude below  $EC_0$  and  $EC_{50}$  values obtained from bacterial test. Considering that QAs concentrations in natural waters are generally 1–2 orders of magnitude lower than those in wastewater effluents, QAs may pose only a limited risk of toxicity in aquatic environments.

In contrast, recently, the potential health effects of biosolids, which are treated sewage sludge used as fertilizers, have been investigated, due to the risk of exposure to various pathogens and mixture of chemicals, including PPCPs (Lewis and Gattie, 2002; Renner, 2002). Greater concentrations of CIP and NOR were detected in sewage sludge from a WWTP (1.4–2.4 mg/kg of dry wt.) in

Switzerland (Golet et al., 2003). This result may suggest that the soil-dwelling organisms and the bacteria could experience exposure to such drugs and their metabolites (Boxall et al., 2003). Considering these facts, monitoring studies of QAs in sewage from WWTPs and in sediment/soil near aquaculture facilities and livestock farms will be necessary for the evaluation of the environmental distribution and risk of these compounds.

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