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Enantiomeric profiling of quinolones and quinolones resistance gene *qnrS* in European wastewaters

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Abstract

Wastewater-based epidemiology (WBE) was applied for the first time in seven cities across Europe with the aim of estimating quinolones consumption via the analysis of human urinary metabolites in wastewater. This report is also the first pan-European study focussed on the enantiomeric profiling of chiral quinolones in wastewater. By considering loads of (fluoro)quinolones in wastewater within the context of human stereoselective metabolism, we identified cities in Southern Europe characterised by both high usage and direct disposal of unused ofloxacin. In Northern European cities, *S-(-)*-ofloxacin loads were predominant with respect to *R-(+)*-ofloxacin. Much more potent, enantiomerically pure *S-(-)*-ofloxacin was detected in wastewaters from Southern European cities, reflecting consumption of the enantiomerically pure antibiotic. Nalidixic acid, norfloxacin and lomefloxacin were detected in wastewater even though they were not prescribed according to official prescription data. *S,S-(-)*-moxifloxacin and *S,S-(-)*-moxifloxacin-*N*-sulphate were detected in wastewater due to metabolism of moxifloxacin. For the first time, average population-normalised ulifloxacin loads of 22.3 and 1.5 mg day^{-1} 1000 people^{-1} were reported for Milan and Castellón as a result of prulifloxacin metabolism. Enrichment of flumequine with first-eluting enantiomer in all the samples indicated animal metabolism rather than its direct disposal. Fluoroquinolone loads were compared with *qnrS* gene encoding quinolone resistance to correlate usage of fluoroquinolone and prevalence of resistance. The highest daily loads of the *qnrS* gene in Milan corresponded with the highest total quinolone load in Milan proving the hypothesis that higher usage of quinolones is linked with higher prevalence of quinolone resistance genes. Utrecht, with the lowest quinolones usage (low daily loads) had also one of the lowest daily loads of the *qnrS* gene. However, a similar

trend was not observed in Oslo nor Bristol where higher *qnrS* gene loads were observed despite low quinolone usage.

Keywords: chiral antibiotics; enantioselective analysis; wastewater-based epidemiology; antibiotic resistance; (fluoro)quinolones; biomarkers

1. Introduction

Although it is intuitively likely that regions with high rates of antibiotic prescription should also be those characterised by high levels of antibiotics and antimicrobial resistance (AMR) in the environment, evidence for such putative correlations has so far proved elusive. Data on antibiotic usage in both hospitals and communities are available from the European Surveillance of Antimicrobial Consumption Network (ESAC-Net), and data on AMR are provided by the European Antimicrobial Resistance Surveillance Network (EARS-Net). These surveillance networks, which provide critical data for informing effective AMR management strategies and policies, rely on national sales, reimbursement data and information taken from national drug registers. Unfortunately, a delay of one or two years is likely before epidemiological data are published, which limits the usefulness of the data to address challenges in real-time. Wastewater-based epidemiology (WBE) could provide a viable option enabling real time estimation of antibiotics consumption, as well as verification of spatial and temporal trends in antibiotics use (and also direct disposal).

WBE relies on the detection and quantification of indicators, so-called biomarkers, which profile real usage of a substance through characteristic human urinary excretion patterns. Examples of this approach are well documented in the spatial and temporal community-wide monitoring for illicit drugs use (Castrignanò, Yang et al. , Thomas, Bijlsma et al. 2012, Ort, van Nuijs et al. 2014, Castrignanò, Lubben et al. 2016, Castrignanò, Mardal et al. 2017), new psychoactive substances (Reid, Derry et al. 2013, Bade, Bijlsma et al. 2017) alcohol, tobacco and caffeine use (Castiglioni, Senta et al. 2014, Baz-Lomba, Salvatore et al. 2016, Gracia-Lor, Rousis et al. 2017), counterfeit medicine use (Causanilles, Cantillano et al. 2018), exposure to pesticides (Rousis, Gracia-Lor et al. 2017) and endocrine disruptors (Lopardo, Petrie et al. 2019) and general public health (Ryu, Gracia-Lor et al. 2016). The selection of potential biomarkers is based on a full understanding of human pharmacokinetics along with biomarker stability in the environmental matrix. This also includes stereoselective metabolism and/or stereoselective enrichment or depletion of the enantiomeric composition of the chiral drug and/or transformation products that can occur respectively in humans and in the environment. Furthermore, chirality is an important phenomenon utilised in WBE to: (i) distinguish between the use and the misuse of drugs, due to different enantiomeric signature of prescription medication and illicit usage , as well as (ii) verify the origin of a drug residue through the distinction between consumption and direct disposal of unused drugs due to stereoselective metabolism of most drugs in humans (Kasprzyk-Hordern and Baker 2012, Emke, Evans et al. 2014).

This study focusses on (fluoro)quinolones as they are classified, due to their importance in human medicine, to be the “highest priority critically important antimicrobials” by the World Health Organization (WHO) (http://www.who.int/foodsafety/cia/en/ 2017). A number of studies have addressed the presence of antibiotics or antibiotic resistance genes (ARGs) in the environment within the context of risk to the environmental and human health (Kümmerer 2009) (Pruden, Pei et al. 2006, Zhang, Zhang et al. 2009), but only a few reports correlated antibiotic loads to the presence of ARGs (Rodriguez-Mozaz, Chamorro et al. 2015). There is only a single study that addressed antibiotic profiling in the context of both metabolic profile and stereochemistry (Castrignano, Kannan et al. 2018). There are therefore two key knowledge gaps: (1) understanding the occurrence of antibiotics in the environment and their impacts on AMR and (2) possibility of using WBE approaches to estimate usage of antibiotics. Indeed, wastewater fingerprinting for

biomarkers of quinolones use could transform surveillance and management approaches leading to a reduction of quinolones usage in the monitored areas and ultimately protecting public health.

Therefore, the aim of this study is to:

(i) assess daily loads of selected antibiotics in wastewaters from several European locations,
(ii) undertake enantiomeric profiling of chiral antibiotics with an aim to compare potency of ABs (antibiotics) used across Europe and to verify the origin of ABs through the distinction between consumption and direct disposal of unused drugs,
(iii) measure the level of quinolones resistance gene *qnrS* in wastewater,
(iv) compare quinolones and gene resistance loads in the monitored areas to test the hypothesis that higher quinolone usage can be linked with higher ARG (antibiotic resistance gene) prevalence.

2. Experimental

2.1. Chemicals and materials

Table 1 shows the selection of the analytes considered in this study with information on their chemical structure, chirality, marketing, use, metabolic and excretion patterns, stereoselective metabolism. Table S1 shows CAS number, molecular formula, molecular weight, log P, pK_a values and supplier information for all targeted analytes. High purity grade standard solutions of achiral analytes were as follows: ciprofloxacin, desethylene-ciprofloxacin, norfloxacin and nalidixic acid. The following analytes were used as racemates: (±)-ofloxacin, (±)-ofloxacin-*N*-oxide, (±)-desmethyl-ofloxacin, (±)-lomefloxacin, (±)-prulifloxacin, (±)-ulifloxacin, (±)-flumequine, (±)-nadifloxacin. Stereoisomerically pure standard solutions used were: *S*-(-)-ofloxacin, also known as levofloxacin, *R,R*-(+)-moxifloxacin, *S,S*-(-)-moxifloxacin and *S,S*-(-)-moxifloxacin-*N*-sulphate with two defined stereocentres and *R*-(+)-besifloxacin. The following deuterated and isotopic analogues of target analytes were used as isotopically-labelled internal standards (ILIS): ciprofloxacin-D_8, (±)-ofloxacin-D_3, (±)-desmethyl-ofloxacin-D_8 and (±)-flumequine$^{13}C_3$. Standard stock solutions were prepared at 1 mg mL^{-1} concentration in acetonitrile for (±)-prulifloxacin, (±)-ulifloxacin, (±)-ofloxacin-D_3 and (±)-flumequine$^{13}C_3$, in water for (±)-lomefloxacin, desethylene-ciprofloxacin, ciprofloxacin-D_8 and (±)-desmethyl-ofloxacin-D_8 and in methanol for the remaining analytes. The elution order of (±)-ofloxacin and (±)-moxifloxacin was determined previously (Castrignano, Kannan et al. 2018). Stock and working solutions of standards were stored at -20° C. A mixture of ILIS was finally prepared from stock solutions at 1 mg L^{-1} by dilution with mobile phase and it was used for spiking the samples. HPLC-grade methanol (MeOH), acetonitrile (ACN), ammonium formate and formic acid (≥96%) were purchased from Sigma Aldrich (UK). Ultrapure water was obtained from a MilliQ system, UK. All glassware was deactivated in order to prevent the adsorption of polar compounds to the hydroxyl sites on the glass surface as described in (Castrignanò, Lubben et al. 2016).

2.2. Wastewater sample collection and storage

24-h composite raw wastewater samples were collected over a week in March 2015 from several wastewater treatment plants across Europe. The sampling protocol used in this study is described elsewhere (Castiglioni, Thomas et al. 2014). Sampling locations were in Norway (Oslo), United Kingdom (Bristol), Denmark (Copenhagen), The Netherlands (Utrecht), Switzerland (Zurich), Italy (Milan) and Spain (Castellón) (Figure S1). Information on population and wastewater flow for cities involved in the study are provided in Table S2. Once collected, wastewater samples were transported to the local laboratory in refrigerated conditions and shipped on ice blocks to the UK within 24 hours. Spiking of ILIS mixture took place on the arrival of these samples (within 24 hours).

2.3. Sample preparation and analysis

2.3.1. Quantification of antibiotics using chiral liquid chromatography coupled with tandem mass spectrometry (chiral HPLC-MS/MS)

Once in the laboratory, 50 mL of wastewater samples were spiked with 50 µL of ILIS mixture at concentration of 1 mg L^{-1} and filtered through GF/F 0.7 µm glass fibre filter (Whatman, UK). Filtrates were then solid-phase extracted by using Oasis HLB cartridges (60 mg, Waters, UK). Before the loading of the samples, these cartridges were conditioned with 3 mL of MeOH and equilibrated with 3 mL of ultrapure water. After the loading phase, a washing step was carried out with 1 mL of ultrapure water, and the analytes were eluted with 4 mL of MeOH into 5 mL silanised glass tubes. The extracts in the glass tubes were then evaporated to dryness under nitrogen flow (5-10 psi) by using a TurboVap evaporator (Caliper, UK). Reconstitution of the extracts was performed by adding 500 µL of the mobile phase, consisting of 10 mM ammonium formate/ MeOH 1:99 v/v with 0.05% formic acid. Before being transferred to polypropylene plastic vials with bonded pre-slit PTFE/Silicone septa (Waters, UK), samples were filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13mm). 20 µL were directly injected into a chiral HPLC-MS/MS. Samples were prepared and analysed in duplicate.

The analysis was undertaken by using a Waters ACQUITY UPLC® system (Waters, Manchester, UK) with a chiral CHIRALCEL® OZ-RH column (5 µm particle size, L × I.D. 15 cm × 2.1 mm, Chiral Technologies, France) connected with a 2.0 mm × 2.0 mm guard filter (Chiral Technologies, France) in the column compartment (temperature set at 30°C). The autosampler was kept at 4°C. The flow rate was 0.1 mL min^{-1} under isocratic conditions. A triple quadrupole mass spectrometer (Xevo TQD, Waters, Manchester, UK) equipped with an ESI was used in positive mode. Data were acquired in MRM mode. Selected MRM transitions, cone voltage (CV) and collision energy (CE) values for each compound were used in accordance with (Castrignano, Kannan et al. 2018) (Table S3). MassLynx 4.1 (Waters, UK) was used to control both systems, the Waters ACQUITY and the Xevo TQD. TargetLynx software (Waters, Manchester, UK) was used for data processing.

2.3.2. Quantification of *qnrS* gene

Wastewater samples were firstly tested on non-selective media plates for proving the suitable volume to be used in a further qualitative test. The test "dry run on non-selective media" was performed by using 100 µL and 200 µL of refrigerated wastewater samples (day 6^{th} and 7^{th}) and plates were then incubated at 37° C overnight (Figure S2). As plates with 100 µL of wastewater incubated provided a distinct bacteria growth, 100 µL of wastewater samples were therefore incubated in cysteine-, lactose- and electrolyte-deficient (CLED) agar plates. CLED agar (Sigma Aldrich, UK) media was prepared in accordance with manufacturer's instructions. ~16 colonies from each plate were isolated and incubated singularly (an example is shown in Figure S3). Every single colony was stocked in cryogenic vials (2mL, Fisherbrand) containing 500 µL of 30% LB/glycerol filter-sterilised and kept in the freezer -80°C as reference. The plates of the incubated wastewater were kept refrigerated (Figures S4-S5).

2.3.2.1 DNA extraction

Triplicate wastewater samples of 1mL each were centrifuged for 5 minutes at 3000 g and the cell pellet was resuspended in 200 µL PBS. 5 µL lysozyme were then added, followed by an incubation of 15' at 37 °C. 200 µL binding buffer and 40 µL proteinase K were added and left in incubation for 10' at 70 °C. DNA extraction was performed in accordance with manufacturer's instructions (High Pure PCR Template Preparation Kit, Roche, Germany). DNA concentrations were determined by Qubit™ fluorometer (Invitrogen™). Measurements of the DNA in the samples were undertaken in parallel with known standard solutions.

2.3.2.2 Target quantification using dPCR

Digital PCR analysis was performed on the QuantStudio® 3D Digital PCR System (Life Technologies, Thermo Fisher Scientific). The digital PCR reaction mixture consisted of QuantStudio® 3D Digital PCR Master Mix, 20X SYBR® Green I dye in TE buffer at pH 8, each primer and DNA sample. The mixture was loaded in a high-density nanofluidic chip to partition the

sample in many independent reactions and sealed. The thermal cycling program was the same reported for qPCR analysis in section 2.3.2.1. AnalysisSuite™ software was used to get quantification of the targeted gene and statistical analysis of the results.

The program was run using thermal cycling conditions. Temperature was first ramped to 95 °C and held for 10 min. It was then lowered to 60 °C for 2 min before increasing to 98 °C for 30 seconds. This cycle between 60 °C and 98 °C was repeated 40 times to allow for efficient gene amplification. The system was then lowered being to 60 °C and held for 2 min, before cooling to room temperature. After cooling, each chip was processed using the QuantStudio 3D Digital PCR system. AnalysisSuite™ software was used to get quantification of the targeted gene and statistical analysis of the results.

2.3.2.3. **Target quantification with qPCR**

The quantification of *qnrS* gene was also performed through real-time quantitative PCR (qPCR) system (StepOnePlus, Applied Biosystems, UK). The following primers (Eurofins Genomics, Germany) were used for specific amplification of *qnrS* gene (Eurofins Genomics, Germany): qnrSrtF11 (GACGTGCTAACTTGCGTGAT) and qnrSrtR11 (TGGCATTGTTGGAAACTTG) (in brackets the sequence 5'> 3' is reported).

The PCR conditions were programmed with an initial denaturation at 95 °C for 10', followed by 40 cycles at the same temperature for 15 seconds and an annealing temperature of 60 °C for a minute. A melt curve was successively performed starting from 65 °C to end up to 95 °C. qPCR reaction was performed in duplicate in a 25 µL volume mixture and conducted in 96 well plates containing 12.5 µL of SYBR Green Master Mix (Applied Biosystems, UK), 0.1 µM of each primer and 5 µL of template DNA. Amplicon cloning was performed by insertion of the PCR product into a plasmid pCR™ 2.1-TOPO® TA vector (Invitrogen™, UK) in the cloning reaction. The following equation (Eq.1) was used for calculating the copy number μL^{-1} as described elsewhere (Rodriguez-Mozaz, Chamorro et al. 2015):

$$\frac{\text{Copy number}}{\mu L} = \frac{\text{Plasmid DNA concentration} \times \text{Avogadro's number}}{\text{Plasmid length} \times 660} \qquad \text{(Eq.1)}$$

where *Plasmid DNA concentration* is expressed in g μL^{-1} and *Plasmid length* in bp. 660 is the average molecular weight of 1 bp (Perini, Casabianca et al. 2011). By ten-fold dilutions of the positive sample, a standard curve was created in order to quantify absolute concentration in European wastewater samples.

Results from qPCR analysis were expressed as Ct (threshold cycle) values, which are relative measurements of the concentration of the target gene. By using the equation from the standard curve, they were then expressed as *qnrS* copies μL^{-1}.

2.4. Calculations

Daily mass loads of fluoroquinolones were calculated by multiplying the concentrations of the analytes expressed in ng L^{-1} by the flow rate (L day^{-1}) and then normalised by the population size of the catchment area (number of people contributing to wastewater analysed). Daily loads of *qnr*S genes were calculated by multiplying the concentrations of the gene expressed in copies L^{-1} by the flow rate (L day^{-1}) and then normalised by the population size of the catchment area.

2.5. Stability study

A stability study was performed by using freshly collected wastewater in dark biotic conditions to verify if the analytes were suitable as WBE biomarkers. Autoclaved duplicate reactors were spiked with a working solution of antibiotics at 1 µg L^{-1} and incubated with wastewater at room temperature and at 4 °C, respectively. The latter temperature set up was used to prove that analytes

were stable in refrigerated conditions (i.e. transport). Other two reactors were used as blank for both settings. Temperature and pH were constantly monitored at every sampling time. 50 mL were collected in duplicate from all reactors at fixed time point (0, 6, 12 and 24 hours) and spiked with an ILIS mixture after sampling before filtration. Samples were prepared as described in Section 2.3.1 and analysed in duplicate by chiral HPLC-MS/MS.

3. Results and Discussion

3.1. European wastewater profiling for fluoroquinolones and their metabolic consumption markers

Several sampling sites across Europe were selected in this study: Oslo, Norway; Bristol, United Kingdom; Copenhagen, Denmark; Utrecht, The Netherlands; Zurich, Switzerland; Milan, Italy and Castellón, Spain (Figure S1). Weekly average concentrations of fluoroquinolones for each city are presented in Table S4. Population-mass loads for the studied antibiotics are shown in Figures 1-2-3. Table S4 provides daily population-normalised mass loads and EF (enantiomeric fraction) values for chiral antibiotics for every day of the week-monitoring campaign. Results from fluoroquinolone stability study of DTRs (Drug Target Residue) are summarised in Table 2. Stability test showed high stability of DTRs. However, further work needs to be undertaken to evaluate stability of analytes in sewers. It also needs to be mentioned that DTR stability was tested only for wastewater samples collected in the UK. One should appreciate microbial and chemical composition of wastewater might change in different locations. Due to the availability of national prescription data only for the UK, Italy and Norway, wastewater –based antibiotics usage estimations were compared with national statistics only in these three countries (Table 3).

3.1.1. Ciprofloxacin

DTRs. Ciprofloxacin and its metabolite desethylene-ciprofloxacin were studied as potential biomarkers of ciprofloxacin use. Ciprofloxacin is an achiral synthetic drug. In humans, 40-50% of consumed ciprofloxacin is excreted unchanged, 2% as desethylene-ciprofloxacin and 4% as sulpho-ciprofloxacin (with antibacterial activity 30 times lower than ciprofloxacin, 7% as oxo-ciprofloxacin with an activity 10-times lower and traces as formylciprofloxacin (http://www.fda.gov/downloads/Drugs/EmergencyPreparedness/BioterrorismandDrugPreparedness/UCM130802.pdf , Bergan, Thorsteinsson et al. 1989)) (Figure S6). Ciprofloxacin is also a metabolite of enrofloxacin, which is a veterinary drug. Metabolism of enrofloxacin leads to excretion of 31% as ciprofloxacin, 5% as oxo-ciprofloxacin and 3% as desethylene-ciprofloxacin (http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014142.pdf).

Population-normalised daily loads. In this study, population-normalised ciprofloxacin loads ranged from a minimum average of 37.6 mg day^{-1} 1000 $people^{-1}$ in Bristol to a maximum value of 409.9 mg day^{-1} 1000 $people^{-1}$ in Castellón. The metabolite loads varied from 9 mg day^{-1} 1000 $people^{-1}$ in Milan to a maximum of 23.9 mg day^{-1} 1000 $people^{-1}$ in Oslo. The highest intra-week variability was found for ciprofloxacin in Zurich, followed by Castellón and Milan, and for desethylene-ciprofloxacin in Oslo and Milan. As expected, most of the countries showed stable mass loads over the sampling week (Figure 1, Table S4). It is important to mention that because of ciprofloxacin's therapeutic use, intra-day and seasonal, and not weekly, variations are usually observed (Coutu, Wyrsch et al. 2013). This seasonal trend is most likely to occur in central and Southern European countries, rather than in Northern countries, where a drop in use during summer is observed because of the effect of temperature. The ratio between parent compound:metabolite ranged between 3:1 and 8:1 for Northern European cities and around 20:1 for Southern cities. According to metabolism data, the ratio indicating human consumption is nearly 22.5:1, thus the loads of ciprofloxacin from Southern cities are expected to be mainly related to consumption.

Drug prescription. In 2015, according to the available official national statistics on drug prescriptions, 5,782 kg of ciprofloxacin were prescribed in England (data elaborated from

Prescription Cost Analysis (PCA) available at (http://www.nhsbsa.nhs.uk/PrescriptionServices/3494.aspx)), 816 kg in Norway (of which 80.5 kg were from Oslo) (http://www.norpd.no) and 26,674 kg in Italy (http://www.agenziafarmaco.gov.it). The excreted quantities of ciprofloxacin and its metabolite were as follows: 2,602 kg of ciprofloxacin and 116 kg of desethylene-ciprofloxacin in England, 367 kg and 16 kg respectively in Norway (36 kg and 2 kg in Oslo) and 12,003 kg and 533 kg in Italy. Considering 45% as the average excretion rate for the parent compound and 2% for the metabolite, the average ciprofloxacin consumption was estimated at 115 mg day^{-1} 1000 $people^{-1}$ in England, 113 mg day^{-1} 1000 $people^{-1}$ in Norway and 596 mg day^{-1} 1000 $people^{-1}$ in Italy (Table 3).

Consumption estimates. The estimates calculated from wastewater analysis were 83.5 and 703.8 mg day^{-1} 1000 $people^{-1}$ in England, 369.9 and 1292.7 mg day^{-1} 1000 $people^{-1}$ in Norway and 772.6 and 487.0 mg day^{-1} 1000 $people^{-1}$ in Italy, using ciprofloxacin and desethylene-ciprofloxacin respectively as DTR. Therefore, wastewater data was in agreement with the official statistics for England and Italy when a tolerance of 30% was applied and when ciprofloxacin was used as DTR. Interestingly, wastewater data indicates higher use of ciprofloxacin in Norway than reported. This is in agreement with another study conducted in Norway in 2010 where high loads of ciprofloxacin denoting 880kg/ year were also reported (Plósz, Leknes et al. 2010). High loads of ciprofloxacin could not be explained, however, Plósz et all suggested that this might be due to transformation of another fluoroquinolone (e.g. enrofloxacin) (Plósz, Leknes et al. 2010). Desethylene-ciprofloxacin is only a minor metabolite, however, it is considered appropriate to utilise it in WBE as a biomarker of ciprofloxacin, especially if applied in conjunction with its parent compound. As both ciprofloxacin and desethylene-ciprofloxacin were found to be stable in wastewater (only <15% decrease was observed at 4 ˚C in 24h) (Table 2), desethylene-ciprofloxacin and parent ciprofloxacin were chosen as biomarkers of ciprofloxacin use. However, unexpectedly high loads of ciprofloxacin in Oslo will require further investigation to fully validate the usage of above DTRs in ciprofloxacin studies. Ciprofloxacin profile from other catchment areas in Norway should be further investigated as differences in spatial distribution of consumption per capita within the country itself might occur.

3.1.2. Ofloxacin

DTRs. The selection of the ofloxacin biomarkers in WBE was based on the evaluation of (±)-ofloxacin and its two minor metabolites: (±)-ofloxacin-*N*-oxide and (±)-desmethyl-ofloxacin (% excretion accounts for 2%, while (±)-ofloxacin-glucuronide along with the parent drug itself account for 80-85%). Disposition of ofloxacin is stereoselective in humans probably due to differences in renal excretion (Okazaki, Kojima et al. 1991). Stereoselective intake of *S*-(-)-ofloxacin is linked to the production of *S*-(-)-metabolites. As in the case of other compounds (Castiglioni, Zuccato et al. 2011), glucuronides could be hydrolysed in wastewater, thus resulting in the formation of the parent compound.

Population-normalised daily loads. In the current study, population-normalised ofloxacin loads ranged from a minimum average value of 4.3 mg day^{-1} 1000 $people^{-1}$ in Utrecht to a maximum value of 727.4 mg day^{-1} 1000 $people^{-1}$ in Milan (Figure 2). The same was found for the metabolites, but with lower mass loads due to their low urinary excretion. In fact, they ranged between 0.4 and 7.4 mg day^{-1} 1000 $people^{-1}$ for ofloxacin-*N*-oxide and between 1.8 and 11.8 mg day^{-1} 1000 $people^{-1}$ for desmethyl-ofloxacin. Intra-week variability of ofloxacin was lower than its metabolites (Table S4).

Previous findings utilising enantiomeric profiling showed that ofloxacin metabolites detected in wastewater originated from human metabolism (Castrignanò E. 2018). In this study, a predominance of the *S*-(-)-ofloxacin loads was observed with respect to *R*-(+)-ofloxacin in northern European cities. In particular, it was found that *S*-(-)-enantiomer was constantly three to four times higher than the *R*- enantiomer throughout the week, respectively in Bristol, in Oslo and in Utrecht. Enantiomerically pure *S*-(-)-ofloxacin was exclusively found in Southern locations, thus indicating usage of enantiomerically pure *S*-(-)-ofloxacin. Furthermore, parent compound:metabolite ratio,

namely ofloxacin:ofloxacin-*N*-oxide ratio, was 10:1 for Northern cities, whilst it was found variable for the other studied sites. Ofloxacin was nearly six times higher than desmethyl-ofloxacin in Oslo and Bristol, whilst it was three times higher in Copenhagen and Utrecht. According to metabolism data, the proposed ratio parent compound:metabolite was 41.2:1 (Table 1). In Castellón and Milan, slightly higher ratios were found, thus suggesting also direct disposal of unused ofloxacin in these cities (Figure 2).

Drug prescription and consumption estimates. According to PCA data in 2015 in England (http://www.nhsbsa.nhs.uk/PrescriptionServices/3494.aspx), 212 kg of (±)-ofloxacin and 120 kg of *S*-(-)-ofloxacin were prescribed. Considering 82.5% as average excretion percentage for the parent compound, the excreted amount was calculated as 87.5 kg as *R*-(+)-ofloxacin and 186.5 kg as *S*-(-)-ofloxacin (this resulted from 87.5 kg excreted from racemic ofloxacin plus 99 kg from enantiopure *S*-(-)-ofloxacin). Therefore, on the basis of the available statistics, its consumption was estimated at 4 mg day^{-1} 1000 people^{-1} as *R*-(+)-ofloxacin and 8 mg day^{-1} 1000 people^{-1} as *S*-(-)-ofloxacin (Table 3). The estimates calculated from wastewater analysis were fully in agreement with the NHS data: 4 mg day^{-1} 1000 people^{-1} as *R*-(+)-ofloxacin and 12 mg day^{-1} 1000 people^{-1} as *S*-(-)-ofloxacin. Regarding the metabolites, 2 and 4 kg were respectively excreted as *R*-(+)- and *S*-(-)-enantiomer. When they were used as ofloxacin DTRs, the estimates from ofloxacin-*N*-oxide were closer to those calculated using ofloxacin as a DTR with respect to those from desmethyl-ofloxacin, but not within 30% of tolerance. In Norway, as a result of prescription of 8.9 and 0.9 kg of ofloxacin as racemate and enantiopure *S*-(-)- form (http://www.norpd.no), respectively, consumption was estimated at 1.2 mg day^{-1} 1000 people^{-1} as *R*-(+)- and 1.4 mg day^{-1} 1000 people^{-1} as *S*-(-)-ofloxacin. However, estimates from wastewater analysis were higher from those calculated from official sources for all considered DTRs. In Italy, prescription data showed that 42234 kg were prescribed as only *S*-(-)-ofloxacin. Therefore, the excreted amounts of *S*-(-)-ofloxacin and its *S*-configured metabolites were estimated to be 34843 kg as parent compound and 845 kg as metabolites. In this case, even if there was an underestimation between estimates from wastewater analysis and those from the official source, data were in agreement in relation to the enantiopure form consumed. In terms of stability, all chosen DTRs were stable after 24 h at 4 ˚C (Table 2) with only *S*-configured ofloxacin and ofloxacin-*N*-oxide being close to 20% of decrease. As in the case of desethylene-ciprofloxacin, ofloxacin-*N*-oxide and desmethyl-ofloxacin, as well as the parent compound proved to be useful DTRs in estimating ofloxacin usage with WBE.

3.1.3. Norfloxacin

DTRs. Norfloxacin is an achiral synthetic fluoroquinolone. 25 to 40% of administered norfloxacin is excreted unchanged in urine (30% as average in faeces within 48 hours) and 5-10% as metabolites within 24-48 hours (http://toxnet.nlm.nih.gov).

Population-normalised daily loads. In wastewater, population-normalised loads were up to 40.2 mg day^{-1} 1000 people^{-1} in Zurich. As in the case of ciprofloxacin and ofloxacin, intra-day variation was observed for norfloxacin by Coutu et al. (2013) with a peak load in wastewater at the first flush in early morning (Coutu, Wyrsch et al. 2013).

Drug prescription. Prescription data showed that only 1.1 kg was dispensed in England in 2015. Considering excretion from urine and faeces, the excreted amount calculated was 0.7 kg. Hence, its consumption was estimated at 0.03 mg day^{-1} 1000 people^{-1} (Table 3).

Consumption estimates. Estimates from wastewater analysis were 1.3 mg day^{-1} 1000 people^{-1} showing a slight disagreement between the two datasets in England. In Norway and Italy data on norfloxacin from wastewater analysis confirmed that it was used even though there was no confirmation from prescription data (Figure 3). To sum up, norfloxacin can be used as a biomarker indicating its usage in WBE as it is stable after 24 h at 4˚C (Table 2).

3.1.4. Nalidixic acid

DTRs. Nalidixic acid is an achiral synthetic quinolone. In humans, only ~2-3% of nalidixic acid is excreted unchanged, 80% is metabolised to an active metabolite 7-hydroxy-nalidixic acid, carboxy metabolite and the inactive conjugates (7-hydroxy-nalidixic acid and nalidixic acid glucuronides) (Moffat, Osselton et al. 2004).

Population-normalised daily loads. In this study, population-normalised loads were up to a maximum value of 2.5 mg day^{-1} 1000 people^{-1} in Oslo (Figure 3). The possible hydrolysis of the glucuronides and, thus, the release of free-nalidixic acid can contribute to loads found in wastewater. As the excretion percentage of these glucuronides is not available, it was assumed that the excretion of glucuronide conjugates of 7-hydroxynalidixic acid and glucuronide conjugates of nalidixic acid were 1:1, therefore estimates were performed considering a total contribution of 40% of the parent compound.

Consumption estimates. Estimates from wastewater analysis were 1.0, 6.3 and 1.5 mg day^{-1} 1000 people^{-1} in England, Norway and Italy using nalidixic acid as DTR (Table 3). However, prescription data from these countries showed that this drug was not dispensed. Considering the low values from estimates, it is possible to assume that both data-sets were in agreement. Nalidixic acid was therefore found to be a good biomarker for its usage also due to its stability in wastewater (Table 2), but other DTRs should be still investigated (i.e. 7-hydroxy-nalidixic acid) to be able to differentiate between consumption and direct disposal of unused drug.

3.1.5. Lomefloxacin

DTRs. (±)-Lomefloxacin is a chiral synthetic fluoroquinolone. Once ingested, 65% is found unchanged in the urine and 9% is excreted as glucuronide (http://www.druglib.com/activeingredient/lomefloxacin/). Its consumption estimates from wastewater analysis considered also the percentage fraction from the glucuronides. Therefore, assuming that lomefloxacin glucuronide is hydrolysed in wastewater, 74% excretion was used for the calculations. To the authors' knowledge, information on its stereoselective metabolism is not available. Unfortunately, under chromatographic conditions used, its enantiomers were not resolved. Therefore, analyses of its loads were intended for (±)-lomefloxacin.

Population-normalised daily loads. In this study, population-normalised loads ranged from a minimum value of 0.1 for Utrecht to a maximum value of 2.6 mg day^{-1} 1000 people^{-1} for Milan (Figure 3).

Consumption estimates. According to national statistics (±)-lomefloxacin was not dispensed in 2015 in England, Norway and Italy. However, wastewater-based consumption estimates indicated usage at 0.7, 1.8 and 3.5 mg day^{-1} 1000 people^{-1} in England, Norway and Italy. Therefore, data from wastewater analysis can potentially provide more accurate data on depicting the usage of such drug than traditional prescription data analysis. As in the case of nalidixic acid, lomefloxacin was found to be stable during the stability study (Table 2) and as a result it can be considered as a suitable biomarker for WBE purposes.

3.1.6. Moxifloxacin

(±)-Moxifloxacin is a synthetic fluoroquinolone that has two chiral centres. It is sold in one stereochemical form of *S,S*-(-)-moxifloxacin. *R,R*-(+)-moxifloxacin is an impurity of the drug (Cruz and Hall 2005), therefore it is unlikely a product of human metabolism. Indeed, *S,S*-(-)-moxifloxacin is excreted unchanged (~20% in urine and 25% in faeces) and as acyl-glucuronide (14% of the dose in urine and moxifloxacin-*N*-sulphate at 35% of the dose in faeces) (Ahmed, Vo et al. 2008, Zhou 2014). In this study, diastereomers of moxifloxacin were separated under selected chromatographic conditions. Hence, it was possible to verify whether *R,R*-moxifloxacin was present in the environmental matrix due to possible microbial conversion of the parent drug. *S,S*-(-)-moxifloxacin and *S,S*-(-)-moxifloxacin-*N*-sulphate were selected as biomarkers of moxifloxacin use.

Population-normalised loads of moxifloxacin and its metabolite were up to a maximum of 21.6 and 149.8 mg day^{-1} 1000 people^{-1} in Castellón with only *S,S*-(-)-enantiomer present (Figure 3). Measured parent compound:metabolite ratios were variable across cities, such as 1:14 for Utrecht, 1:9 for Zurich, 1:7 for Castellón, 1:6 for Copenhagen and 1:2.5 for Milan. In relation to metabolism data (Table 1), the closer ratio was observed for Milan.

According to PCA (http://www.nhsbsa.nhs.uk/PrescriptionServices/3494.aspx), 39.6 kg of *S,S*-(-)-moxifloxacin were dispensed in England in 2015. Therefore, estimates of its consumption were 0.8 mg day^{-1} 1000 people^{-1}. However, neither the parent compound nor its metabolite was found in wastewater samples. Norwegian WBE data were in agreement with national statistics (http://www.norpd.no) as *S,S*-(-)-moxifloxacin was neither prescribed nor detected in wastewater. *S,S*-(-)-Moxifloxacin and the metabolite were found in Italian wastewater samples, leading to consumption estimates of *S,S*-(-)-moxifloxacin of 6.8 and 19.0 mg day^{-1} 1000 people^{-1}, when they were both used as biomarkers. Ratio parent:metabolite was 1:2 and this could be ascribed to degradation of parent compound by microbes in sewage or biofilms in-sewer transport. This was different from what was observed in the case of ciprofloxacin and ofloxacin, where measured ratios of the metabolite to parent compound were slightly higher than expected from human metabolism for some cities but lower for others, indicating that in-sewer degradation for these compounds might be less significant than for moxifloxacin. However, further studies on stability during sewer transport are required to support this hypothesis. As prescription data did not show any dispensation of such drug, both datasets were in disagreement. Moxifloxacin and its metabolite were found to be suitable biomarkers for WBE approach due to high urinary excretion and high stability in wastewater (Table 2).

3.1.7. Prulifloxacin

(±)-Prulifloxacin is a synthetic prodrug sold as racemate for oral administration. It is converted in its active compound, ulifloxacin, by a hepatic enzyme. The chiral centre is not the metabolic site and therefore metabolism *S*-(-)-prulifloxacin is not stereoselective (Yang, Aloysius et al. 2011). Only *S*-(-)-ulifloxacin has bactericidal effects, but the enantiomerically pure *S*-(-)-form is not commercially available yet (YING, Peng et al. 2012). Ulifloxacin is excreted at 17-23% in the urine and 17-29% in the faeces.

In the current study, population-normalised ulifloxacin loads were 22.3 and 1.5 mg day^{-1} 1000 people^{-1} in Milan and Castellón respectively (Figure 3). Enrichment of ulifloxacin with first-eluting enantiomer was observed in Milan, whilst the opposite was observed in Castellón. (±)-Prulifloxacin was expected to be found in wastewater only in the case of direct disposal. Indeed, it was not found in any samples. 4,446 kg of prulifloxacin were prescribed in Italy, that accounted for 1912 kg of excreted ulifloxacin. Estimates from wastewater analysis (52.1 mg day^{-1} 1000 people^{-1}) were slightly higher than those calculated from prescription data (44.1 mg day^{-1} 1000 people^{-1}), suggesting an agreement between two datasets. Both compounds were found to be suitable as biomarkers for prulifloxacin use.

3.1.8. Flumequine

(±)-Flumequine is a racemic drug marketed as a veterinary drug. After enzyme deconjugation, it is excreted as unchanged 81-86% in calves' urine, at 12-17% as 7-hydroxy-flumequine in calves' urine and as glucuronide conjugates (http://www.fao.org/docrep/w8338e/w8338e0a.htm). It undergoes stereoselective metabolism in sheep, cattle and poultry (Besse, Guyonnet et al. 1998).

Population-normalised loads ranged from 0.4 in Copenhagen to 1.2 mg day^{-1} 1000 people^{-1} in Zurich (Figure 3). Enrichment of flumequine with first-eluting enantiomer was detected in all samples from the locations investigated. Its presence in wastewater can be associated with its excretion as a result of animal metabolism (EF>0.5) or its direct disposal (EF=0.5). In wastewater, EF values for flumequine ranged from 0.6 ± 0.0 to 0.9 ± 0.1. In this case, the first option seems to

be the most plausible because of the chiral signature in all the samples (EF>0.5). Flumequine was found to be stable during the stability study (Table 2).

3.1.9. Other quinolones

(±)-Nadifloxacin and *R*-(+)-besifloxacin are discussed in "S1- Other quinolones". As both were not detected at enantiomeric level in composite wastewater samples, their enantiomeric profiling could not be investigated.

3.2. European wastewater profiling for *qnrS* gene and correlation with environmental quinolone prevalence

European wastewater samples were analysed for *qnrS* gene to test the hypothesis that usage of antibiotics might be linked with higher prevalence of antibiotic resistance. *QnrS* gene was selected because of its reduced susceptibility to fluoroquinolones according to Rodriguez-Mozaz et al. (Rodriguez-Mozaz, Chamorro et al. 2015) and Marti et al. (Marti, Variatza et al. 2014). Target *qnrS* quantification was performed using dPCR and qPCR as described in Experimental. The results are shown in Figure 4, whilst a discussion on qualitative results is provided in "S2- Qualitative test in selective media" (Table S5). Interestingly, prevalence of the *qnrS* gene in terms of the copy number per 1L of wastewater was relatively consistent across studied locations. However, when normalised to wastewater flows, an interesting pattern appeared indicating higher prevalence of the *qnrS* gene in terms of 'daily loads' in Milan when compared to other locations. As *qnrS* is not specific to any quinolone in particular, calculations based on the total concentrations, daily loads and the population-normalised loads of all studied (fluoro)quinolones were performed in order to compare prevalence of both quinolone and *qnrS* gene in studied locations (Figure 4). An interesting pattern was observed. The highest daily loads of the *qnrS* gene in Milan corresponded with the highest total quinolone load in Milan proving the hypothesis that higher usage of quinolones is linked with higher prevalence of quinolone resistance genes. Also, Utrecht with the lowest quinolone usage (low daily loads) had also one of the lowest daily loads of the *qnrS* gene. However, similar trend was not observed in Oslo and Bristol. Interestingly, concentrations of quinolones in Castellón's wastewater were observed to be the highest, which could provide a misleading conclusion on high risks from exposure but daily loads revealed that the actual daily burden from quinolones in the catchment area is low (hence, low *qnrS* gene load), albeit still high when normalised to the population size.

ECDC/EFSA/EMA first joint report studied the relationship between national consumption of fluoroquinolones/quinolones and the risk of reduced susceptibility to ciprofloxacin by using *E. coli*, *Salmonella spp.*, *C. coli* and *C. jejuni* as indicators (http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/4006.pdf). According to that, the impact on the contribution of quinolone consumption could be explained as follows:

(i) cross-resistance between quinolones and fluoroquinolones are similarly detected by the use of epidemiological cut-off values for ciprofloxacin resistance;
(ii) ciprofloxacin resistance in *E. coli* is leaded by the selection of quinolones for the first mutation step;
(iii) the dissemination of plasmid-mediated resistance to quinolones mediated by *qnr* genes in *Salmonella spp.* can provide opportunities for co-selection of unrelated antimicrobials.

On the other hand, it was also reported that differences in the occurrence of ciprofloxacin resistance were observed in countries with similar low level of ciprofloxacin consumption from ciprofloxacin resistance in *C. coli* data. The reasons were ascribed to differences in the fluoroquinolone consumption in years previous to this study and in bacteria resistance spreading among countries (http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/4006.pdf). For these reasons, the fact that *qnrS* gene was not so high in terms of copy number in the analysed samples from Italy and Spain with respect to other Northern European cities could be difficult to

explain. In this study the correlation between occurrence of antibiotics and occurrence of their resistance gene was found, but the results should be interpreted with caution, due to limited dataset regarding ARG analysis. This is in partial agreement with Rodriguez-Mozaz et al. (2015) (Rodriguez-Mozaz, Chamorro et al. 2015), where a significant correlation was proved at local level. A multi-variable approach, that considers the contribution of co-selecting factors, such as biocides and heavy metals, along the resistance genes levels, could enable a systematic investigation on such correlations and determine the role played from these factors in the AMR context.

4. Conclusions

Wastewater-based epidemiology (WBE) was applied for the first time in seven cities across Europe with the aim of estimating quinolone consumption via the analysis of human urinary metabolites combined with prevalence of *qnrS* (quinolone resistance) gene in wastewater. The main conclusions are as follows:

1. WBE was proven to be a powerful tool that enabled estimation of (fluoro)quinolone antibiotic consumption/usage over a week-long study across seven European locations. Notably, the occurrence of quinolones in wastewater reflected the spatial trend from estimated quinolones consumption reported by ECDC in 2015 (Table S6).
2. The most comprehensive panel of quinolone biomarkers was considered and the following biomarkers were found to be suitable for WBE applications: ciprofloxacin and desethylene-ciprofloxacin, (±)-ofloxacin with its main metabolites (±)-ofloxacin-*N*-oxide and (±)-desmethyl-ofloxacin, norfloxacin, nalidixic acid, lomefloxacin, moxifloxacin and its metabolite *N*-sulphate, the precursor (±)-prulifloxacin with its active compound (±)-ulifloxacin, (±)-flumequine.
3. The assessment of the parent:metabolite ratio lead to the conclusion that unused ofloxacin is likely directly disposed of through the sewer network in the Southern European cities due to higher parent:metabolite ratio with respect to the estimated metabolism ratio.
4. Enantiomeric profiling of quinolone markers enabled the patterns of drug use to be understood and spatial drug use estimated in near-real time. The exclusive stereoselective use of *S*-(-)-ofloxacin was observed in Southern cities, whilst racemic ofloxacin was more predominant in Northern European cities (due to differences in prescriptions of the drug itself). The consumption of moxifloxacin was demonstrated by the presence of *S,S*-(-)-configured moxifloxacin and *S,S*-(-)-moxifloxacin-*N*-sulphate. Enantiomeric profiling of prulifloxacin showed that only its metabolite, ulifloxacin, was found in Milan and Castellón. Therefore, the presence of ulifloxacin was related to prulifloxacin metabolism and the direct disposal of unused prulifloxacin did not occur in any of the locations monitored. Despite flumequine metabolites not being included in the study, the enrichment of its first-eluting enantiomer in all the samples was attributed to animals' metabolism rather than its direct disposal.
5. European wastewater samples were analysed for *qnrS* gene to test the hypothesis that usage of antibiotics is linked with higher prevalence of antibiotic resistance. The highest daily loads of the *qnrS* gene in Milan corresponded with the highest total quinolone load in Milan proving the hypothesis that higher usage of quinolones is linked with higher prevalence of quinolone resistance genes. Also, Utrecht with the lowest quinolone usage (low daily loads) had also one of the lowest daily loads of the *qnrS* gene. However, similar trend was not observed in Oslo and Bristol. Interestingly, concentrations of quinolones in Castellón's wastewater were observed to be the highest, which could provide a misleading conclusion on high risks from AMR but daily loads revealed that the actual daily burden from quinolones in the catchment area is low (hence, low *qnrS* gene load), albeit still high when normalised to the population size.

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References

Ahmed, S., N. T. Vo, T. Thalhammer, F. Thalhammer, K. B. Gattringer and W. Jager (2008). "Involvement of Mrp2 (Abcc2) in biliary excretion of moxifloxacin and its metabolites in the isolated perfused rat liver." J Pharm Pharmacol **60**(1): 55-62.

Bade, R., L. Bijlsma, J. V. Sancho, J. A. Baz-Lomba, S. Castiglioni, E. Castrignano, A. Causanilles, E. Gracia-Lor, B. Kasprzyk-Hordern, J. Kinyua, A. K. McCall, A. L. N. van Nuijs, C. Ort, B. G. Plosz, P. Ramin, N. I. Rousis, Y. Ryu, K. V. Thomas, P. de Voogt, E. Zuccato and F. Hernandez (2017). "Liquid chromatography-tandem mass spectrometry determination of synthetic cathinones and phenethylamines in influent wastewater of eight European cities." Chemosphere **168**: 1032-1041.

Baz-Lomba, J. A., S. Salvatore, E. Gracia-Lor, R. Bade, S. Castiglioni, E. Castrignano, A. Causanilles, F. Hernandez, B. Kasprzyk-Hordern, J. Kinyua, A. K. McCall, A. van Nuijs, C. Ort, B. G. Plosz, P. Ramin, M. Reid, N. I. Rousis, Y. Ryu, P. de Voogt, J. Bramness and K. Thomas (2016). "Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities." BMC Public Health **16**(1): 1035.

Bergan, T., S. B. Thorsteinsson, R. Rohwedder and H. Scholl (1989). "Elimination of ciprofloxacin and three major metabolites and consequences of reduced renal function." Chemotherapy **35**(6): 393-405.

Besse, S., J. Guyonnet and P. Delatour (1998). "Quantification of flumequine enantiomers in plasma by high-performance liquid chromatography." Journal of veterinary pharmacology and therapeutics **21**(4): 330-332.

Castiglioni, S., I. Senta, A. Borsotti, E. Davoli and E. Zuccato (2014). "A novel approach for monitoring tobacco use in local communities by wastewater analysis." Tob Control.

Castiglioni, S., K. V. Thomas, B. Kasprzyk-Hordern, L. Vandam and P. Griffiths (2014). "Testing wastewater to detect illicit drugs: state of the art, potential and research needs." Sci Total Environ **487**: 613-620.

Castiglioni, S., E. Zuccato and R. Fanelli (2011). Illicit drugs in the environment: occurrence, analysis, and fate using mass spectrometry, John Wiley & Sons.

Castrignano, E., A. M. Kannan, E. J. Feil and B. Kasprzyk-Hordern (2018). "Enantioselective fractionation of fluoroquinolones in the aqueous environment using chiral liquid chromatography coupled with tandem mass spectrometry." Chemosphere **206**: 376-386.

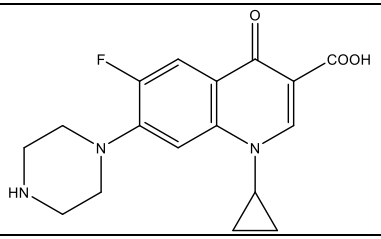
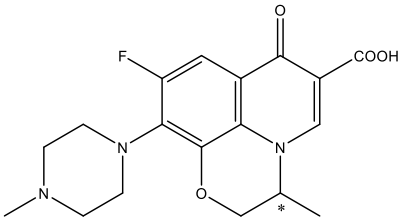
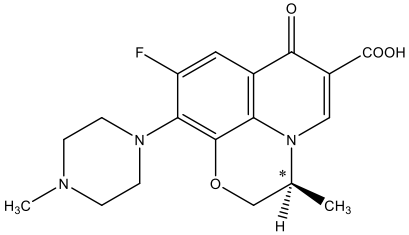
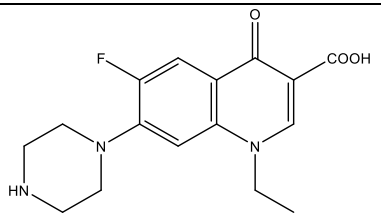
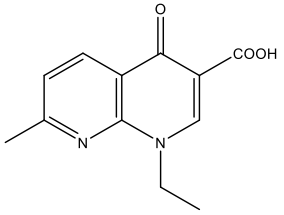
Castrignano, E., A. Lubben and B. Kasprzyk-Hordern (2016). "Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry." Journal of Chromatography A **1438**: 84-99.

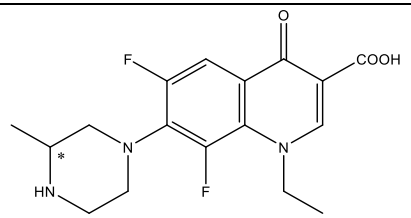
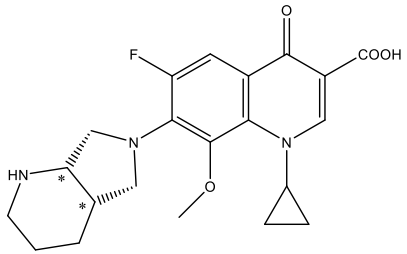
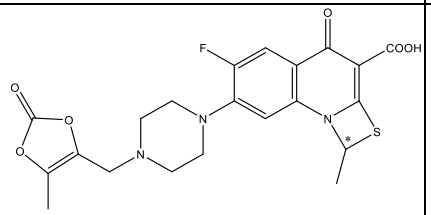
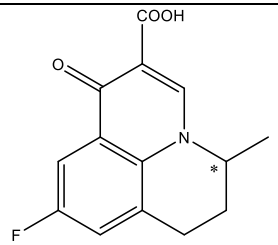
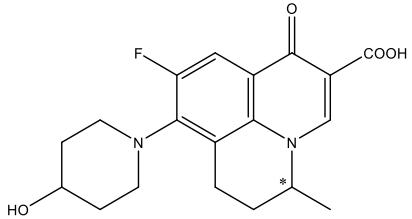
Castrignanò, E., A. Lubben and B. Kasprzyk-Hordern (2016). "Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry." J Chromatogr A **1438**: 84-99.

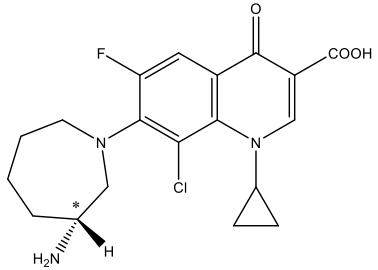
Castrignanò, E., M. Mardal, A. Rydevik, B. Miserez, J. Ramsey, T. Shine, G. D. Pantoș, M. R. Meyer and B. Kasprzyk-Hordern (2017). "A new approach towards biomarker selection in estimation of human exposure to chiral chemicals: a case study of mephedrone." Scientific Reports.
Castrignanò, E., Z. Yang, R. Bade, J. A. Baz-Lomba, S. Castiglioni, A. Causanilles, A. Covaci, E. Gracia-Lor, F. Hernandez, J. Kinyua, A.-K. McCall, A. L. N. van Nuijs, C. Ort, B. G. Plósz, P. Ramin, N. I. Rousis, Y. Ryu, K. V. Thomas, P. de Voogt, E. Zuccato and B. Kasprzyk-Hordern "Enantiomeric profiling of chiral illicit drugs in a pan-European study." Water Research.
Causanilles, A., D. R. Cantillano, E. Emke, R. Bade, J. A. Baz-Lomba, S. Castiglioni, E. Castrignano, E. Gracia-Lor, F. Hernandez, B. Kasprzyk-Hordern, J. Kinyua, A. K. McCall, A. L. N. van Nuijs, B. G. Plosz, P. Ramin, N. I. Rousis, Y. Ryu, K. V. Thomas and P. de Voogt (2018). "Comparison of phosphodiesterase type V inhibitors use in eight European cities through analysis of urban wastewater." Environment International **115**: 279-284.
Coutu, S., V. Wyrsch, H. K. Wynn, L. Rossi and D. A. Barry (2013). "Temporal dynamics of antibiotics in wastewater treatment plant influent." Science of The Total Environment **458–460**: 20-26.
Cruz, L. A. and R. Hall (2005). "Enantiomeric purity assay of moxifloxacin hydrochloride by capillary electrophoresis." Journal of Pharmaceutical and Biomedical Analysis **38**(1): 8-13.
Emke, E., S. Evans, B. Kasprzyk-Hordern and P. de Voogt (2014). "Enantiomer profiling of high loads of amphetamine and MDMA in communal sewage: A Dutch perspective." Science of the Total Environment **487**: 666-672.
Gracia-Lor, E., N. I. Rousis, E. Zuccato, R. Bade, J. A. Baz-Lomba, E. Castrignano, A. Causanilles, F. Hernandez, B. Kasprzyk-Hordern, J. Kinyua, A. K. McCall, A. L. N. van Nuijs, B. G. Plosz, P. Ramin, Y. Ryu, M. M. Santos, K. Thomas, P. de Voogt, Z. G. Yang and S. Castiglioni (2017). "Estimation of caffeine intake from analysis of caffeine metabolites in wastewater." Science of the Total Environment **609**: 1582-1588.
http://toxnet.nlm.nih.gov.
http://www.agenziafarmaco.gov.it.
http://www.druglib.com/activeingredient/lomefloxacin/.
http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/4006.pdf.
http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014142.pdf.
http://www.fao.org/docrep/w8338e/w8338e0a.htm.
http://www.fda.gov/downloads/Drugs/EmergencyPreparedness/BioterrorismandDrugPreparedness/UCM130802.pdf.
http://www.nhsbsa.nhs.uk/PrescriptionServices/3494.aspx.
http://www.norpd.no.
http://www.who.int/foodsafety/cia/en/. (2017). "Highest Priority Critically Important Antimicrobials." Retrieved 03 May 2017, 2017, from http://www.webcitation.org/6umBvwqeb.
Kasprzyk-Hordern, B. and D. R. Baker (2012). "Estimation of community-wide drugs use via stereoselective profiling of sewage." Science of the Total Environment **423**: 142-150.
Kümmerer, K. (2009). "Antibiotics in the aquatic environment - A review - Part I." Chemosphere **75**(4): 417-434.
Lopardo, L., B. Petrie, K. Proctor, J. Youdan, R. Barden and B. Kasprzyk-Hordern (2019). "Estimation of community-wide exposure to bisphenol A via water fingerprinting." Environ Int **125**: 1-8.
Marti, E., E. Variatza and J. Balcázar (2014). "Bacteriophages as a reservoir of extended-spectrum β-lactamase and fluoroquinolone resistance genes in the environment." Clinical Microbiology and Infection **20**(7): O456-O459.
Moffat, A. C., M. D. Osselton, B. Widdop and E. Clarke (2004). Clarke's analysis of drugs and poisons: in pharmaceuticals, body fluids and postmortem material s. Vol. 1, Pharmaceutical Press.
Okazaki, O., C. Kojima, H. Hakusui and M. Nakashima (1991). "Enantioselective disposition of ofloxacin in humans." Antimicrobial Agents and Chemotherapy **35**(10): 2106-2109.
Ort, C., A. L. van Nuijs, J. D. Berset, L. Bijlsma, S. Castiglioni, A. Covaci, P. de Voogt, E. Emke, D. Fatta-Kassinos, P. Griffiths, F. Hernandez, I. Gonzalez-Marino, R. Grabic, B. Kasprzyk-Hordern, N. Mastroianni, A. Meierjohann, T. Nefau, M. Ostman, Y. Pico, I. Racamonde, M. Reid, J. Slobodnik, S. Terzic, N. Thomaidis and K. V. Thomas (2014). "Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis." Addiction **109**(8): 1338-1352.

Perini, F., A. Casabianca, C. Battocchi, S. Accoroni, C. Totti and A. Penna (2011). "New approach using the real-time PCR method for estimation of the toxic marine dinoflagellate Ostreopsis cf. ovata in marine environment." PLoS One **6**(3): e17699.
Plósz, B. G., H. Leknes, H. Liltved and K. V. Thomas (2010). "Diurnal variations in the occurrence and the fate of hormones and antibiotics in activated sludge wastewater treatment in Oslo, Norway." Sci Total Environ **408**(8): 1915-1924.
Polesel, F., H. R. Andersen, S. Trapp and B. G. Plósz (2016). "Removal of Antibiotics in Biological Wastewater Treatment Systems-A Critical Assessment Using the Activated Sludge Modeling Framework for Xenobiotics (ASM-X)." Environ Sci Technol **50**(19): 10316-10334.
Pruden, A., R. Pei, H. Storteboom and K. H. Carlson (2006). "Antibiotic resistance genes as emerging contaminants: studies in northern Colorado." Environmental Science & Technology **40**(23): 7445-7450.
Reid, M. J., L. Derry and K. V. Thomas (2013). "Analysis of new classes of recreational drugs in sewage: Synthetic cannabinoids and amphetamine-like substances." Drug Testing and Analysis: n/a-n/a.
Rodriguez-Mozaz, S., S. Chamorro, E. Marti, B. Huerta, M. Gros, A. Sànchez-Melsió, C. M. Borrego, D. Barceló and J. L. Balcázar (2015). "Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river." Water research **69**: 234-242.
Rousis, N. I., E. Gracia-Lor, E. Zuccato, R. Bade, J. A. Baz-Lomba, E. Castrignano, A. Causanilles, A. Covaci, P. de Voogt, F. Hernandez, B. Kasprzyk-Hordern, J. Kinyua, A. K. McCall, B. G. Plosz, P. Ramin, Y. Ryu, K. V. Thomas, A. van Nuijs, Z. G. Yang and S. Castiglioni (2017). "Wastewater-based epidemiology to assess pan-European pesticide exposure." Water Research **121**: 270-279.
Ryu, Y., E. Gracia-Lor, R. Bade, J. A. Baz-Lomba, J. G. Bramness, S. Castiglioni, E. Castrignano, A. Causanilles, A. Covaci, P. de Voogt, F. Hernandez, B. Kasprzyk-Hordern, J. Kinyua, A.-K. McCall, C. Ort, B. G. Plosz, P. Ramin, N. I. Rousis, M. J. Reid and K. V. Thomas (2016). "Increased levels of the oxidative stress biomarker 8-iso-prostaglandin F-2 alpha in wastewater associated with tobacco use." Scientific Reports **6**.
Thomas, K. V., L. Bijlsma, S. Castiglioni, A. Covaci, E. Emke, R. Grabic, F. Hernandez, S. Karolak, B. Kasprzyk-Hordern, R. H. Lindberg, M. Lopez de Alda, A. Meierjohann, C. Ort, Y. Pico, J. B. Quintana, M. Reid, J. Rieckermann, S. Terzic, A. L. van Nuijs and P. de Voogt (2012). "Comparing illicit drug use in 19 European cities through sewage analysis." Sci Total Environ **432**: 432-439.
Yang, Y.-h., H. Aloysius, D. Inoyama, Y. Chen and L.-q. Hu (2011). "Enzyme-mediated hydrolytic activation of prodrugs." Acta Pharmaceutica Sinica B **1**(3): 143-159.
YING, J., F. Peng, Y. Wang, S. An, J. Liang, B. Liang, Q. Ni and C. Luo (2012). Optically active compound of prulifloxacin for treating infection and preparation method thereof, Google Patents.
Zhang, X.-X., T. Zhang and H. H. Fang (2009). "Antibiotic resistance genes in water environment." Applied microbiology and biotechnology **82**(3): 397-414.
Zhou, X. (2014). Moxifloxacin. Handbook of Metabolic Pathways of Xenobiotics, John Wiley & Sons, Ltd.

Table 1 Selected chiral drug biomarkers (in italics) and their pharmacokinetic data

Drug	Structure	Chirality	Marketing	Use	Metabolite	Excretion	Reference
Ciprofloxacin		No	Synthetic	Human	Parent compound *Desethylene-ciprofloxacin* Sulfo-ciprofloxacin Oxo-ciprofloxacin	40-50% 2% 4%	[1], [2]
(±)-Ofloxacin		Yes, 1*C	Synthetic	Human	Parent compound *Desmethyl-ofloxacin* *Ofloxacin-N-oxide*	In urine over 24-48 h and between 4-8% excreted in faeces Small amount of the dose Small amount of the dose	[3], [4]
						Stereoselective metabolism	[5], [6], [7]
S-(-)-Ofloxacin (L-Ofloxacin)		Yes, 1*C	Synthetic	Human	Parent compound *Desmethyl-levofloxacin* *Levofloxacine-N-oxide*	In urine (80% to 85%) and in faeces (2%) within 24 h 2% of the dose 2% of the dose	[3]
Norfloxacin		No	Synthetic	Human	Parent compound Metabolites	25-40% of the dose is excreted in urine, 30% (range: 10-50%) is excreted in feces within 48 hours 5-10% as metabolites within 24-48 hours	[8]
Nalidixic acid		No	Synthetic	?	Parent compound 7-hydroxynalidixic acid (active) Glucuronide conjugates of 7-hydroxynalidixic acid (inactive) Glucuronide conjugates of nalidixic acid (inactive)	2-3% in the urine About 80% of a dose is excreted in the urine in 8h, mainly as glucuronide conjugates	[3]

					7-carboxy metabolite (inactive)		
(±)-Lomefloxacin		Yes, 1*C	Synthetic	Human	Parent compound Glucuronide	65% in urine 9% No info on stereoselective metabolism (to author's knowledge)	[9], [10]
(±)-Moxifloxacin		Yes, 2*C	Synthetic, sold in one form S,S-(-)-moxifloxacin (shown on the left), its impurity is R,R[a]	Human	Parent compound Moxifloxacin acyl glucuronide *Moxifloxacin-N-sulphate*	~20% in urine and ~25% in feces. 14% of the dose in urine 35% of the dose in faeces No info on stereoselective metabolism (to author's knowledge)	[1], [11], [12]
(±)-Prulifloxacin		Yes, 1 *C	Synthetic prodrug, sold as racemate	Human	*Ulifloxacin* Inactive metabolites	17-23% in the urine and 17-29% in the faeces 7% No stereoselective metabolism	[13] [14]
(±)-Flumequine		Yes, 1*C	Racemic	Veterinary [15]	Parent compound 7-hydroxy-flumequine Glucuronides of flumequine	81-86% in calves urine (after enzyme deconjugation) 12-17% in calves urine (after enzyme deconjugation) Stereoselective metabolism in sheep, cattle and poultry	[16] [17]
(±)-Nadifloxacin		Yes, 1*C	Synthetic	Human	Parent compound Sulphates Glucuronides	0.09% of the administered dose was excreted in the urine over 48 hours, <5% eliminated in the urine, 20% as conjugates No info on stereoselective metabolism (to author's knowledge)	[18], [19]

R-(+)-Besifloxacin		Yes, 1*C	Synthetic, sold in one form only	Human	Parent compound	73% in animal feces, and 23% in animal urine. No appreciable metabolism No info on stereoselective metabolism (to author's knowledge)	[1], [20]

[a] http://www.rxlist.com

Table 2 Stability of targeted compounds in influent wastewater samples stored over a 24h.

Analyte	Stability[a] [%]					
	Raw (unfiltered) wastewater, pH 7.4, stored at 17 ◦C			Raw (unfiltered) wastewater, pH 7.4, stored at 4 ◦C		
	6 h	12 h	24 h	6 h	12 h	24 h
Ciprofloxacin	-30.6 ± 1.6	-41.1 ± 9.4	-39.2 ± 10.5	26.9 ± 3.0	-5.5 ± 0.5	-13.4 ± 2.7
Desethylene-ciprofloxacin	-8.6 ± 0.3	-15.1 ± 6.7	-35.6 ± 1.5	10.0 ± 12.2	-4.9 ± 12.3	6.2 ± 11.1
S-(-)-Ofloxacin (*L*-Ofloxacin)	-5.5 ± 5.0	12.7 ± 4.2	-42.8 ± 4.2	11.5 ± 12.3	8.7 ± 2.4	-19.4 ± 0.6
R-(+)-Ofloxacin	23.1 ± 13.6	45.1 ± 3.4	-34.6 ± 3.4	23.2 ± 0.7	17.1 ± 10.1	-1.3 ± 4.1
Norfloxacin	-22.8 ± 2.0	-32.0 ± 17.3	-24.5 ± 7.8	4.6 ± 1.0	-24.3 ± 5.5	-16.6 ± 8.6
S-(-)-Ofloxacin-*N*-oxide	-43.9 ± 15.3	-91.4 ± 3.1	-98.3 ± 0.7	10.7 ± 1.6	-44.4 ± 4.7	-19.0 ± 12.2
R-(+)-Ofloxacin-*N*-oxide	-22.5 ± 3.2	-90.4 ± 4.3	-97.6 ± 0.6	16.7 ± 12.6	-30.2 ± 9.1	-9.1 ± 10.7
S-(-)-Desmethyl-ofloxacin	0.4 ± 4.9	7.4 ± 1.7	-15.5 ± 18.6	15.1 ± 14.1	10.9 ± 7.0	-1.5 ± 18.6
R-(+)-Desmethyl-ofloxacin	7.3 ± 9.8	11.8 ± 15.4	-4.1 ± 19.1	27.7 ± 2.1	11.7 ± 13.3	10.9 ± 15.4
Nalidixic acid	-15.8 ± 4.6	-30.4 ± 9.8	-42.2 ± 8.0	7.8 ± 16.1	-13.5 ± 13.9	8.3 ± 7.3
Lomefloxacin	1.4 ± 7.8	-19.0 ± 9.9	-33.2 ± 12.7	12.8 ± 13.8	5.3 ± 0.8	7.8 ± 2.8
R,R-Moxifloxacin	-2.1 ± 3.2	-5.3 ± 19.2	-11.9 ± 6.7	4.4 ± 1.6	-1.9 ± 9.2	-5.7 ± 12.1
S,S- Moxifloxacin	10.0 ± 19.1	2.8 ± 15.6	-9.9 ± 3.3	30.2 ± 8.6	5.2 ± 4.0	5.0 ± 13.4
Moxifloxacin-*N*-sulphate	0.4 ± 0.3	1.0 ± 9.4	-41.0 ± 10.1	11.7 ± 0.6	13.9 ± 2.7	5.6 ± 7.7
Prulifloxacin-E1	9.0 ± 6.1	13.5 ± 13.4	-5.4 ± 6.5	26.4 ± 9.0	-2.1 ± 5.5	10.1 ± 18.7
Prulifloxacin-E2	10.0 ± 5.5	19.8 ± 11.7	6.6 ± 4.3	21.8 ± 3.8	3.7 ± 4.7	-9.7 ± 10.2
Ulifloxacin-E1	2.0 ± 0.3	6.3 ± 15.8	-13.1 ± 8.0	-2.9 ± 19.7	6.3 ± 9.0	-9.8 ± 10.4
Ulifloxacin-E2	52.0 ± 0.7	61.4 ± 9.1	55.1 ± 4.4	-5.0 ± 2.7	40.9 ± 12.8	6.5 ± 9.1
Flumequine-E1	-1.2 ± 10.0	-6.4 ± 3.9	-21.6 ± 2.5	1.5 ± 0.0	-4.0 ± 7.7	-8.3 ± 10.1
Flumequine-E2	0.5 ± 16.1	-1.1 ± 0.6	-18.6 ± 2.9	0.1 ± 8.1	-7.2 ± 5.9	-8.6 ± 15.8
Nadifloxacin-E1	-5.5 ± 10.3	-13.6 ± 6.7	-37.1 ± 2.0	24.4 ± 5.1	-2.0 ± 12.9	-14.2 ± 13.9
Nadifloxacin-E2	-5.0 ± 16.4	-6.3 ± 5.0	-31.8 ± 3.0	26.5 ± 8.2	-1.1 ± 8.1	-10.2 ± 15.3
R-(+)-Besifloxacin	-21.0 ± 9.9	-47.8 ± 8.1	-81.3 ± 6.4	1.0 ± 12.8	-20.5 ± 7.5	-9.2 ± 5.2

[a] Expressed as difference in percentage from time-point 0 ± SD

Table 3 Consumption estimates were calculated considering prescriptions data and wastewater (WW) analysis.

Pharmaceuticals	**Total consumption (kg/year)**			**DTR**	**Correction Factor**	**Consumption estimates (mg day^{-1} 1000 people^{-1})**					
						Norway		**England**		**Italy**	
	Norway[a]	**England**[b]	**Italy**[c]			Prescription data (2015)[a]	WW analysis (2015)	NHS data (2015)[b]	WW analysis (2015)	Prescription data (2015)[c]	WW analysis (2015)
Ciprofloxacin	816.3 (as J01MA02), 0.0 (as S01AE03 and S02AA15)	5782.0	26674.0	Ciprofloxacin	2.22	113.0	369.9	115.0	83.5	596.0	772.6
				Desethylene-ciprofloxacin	54.2		1292.7		703.8		487.0
Ofloxacin	8.9 (as J01MA01)	212 as (±)-ofloxacin, 120 as *S*-(-)-ofloxacin	42234 as *S*-(-)-ofloxacin	Ofloxacin	1.21	1.2 as *R*-(+)-ofloxacin, 1.4 as *S*-(-)-ofloxacin	4 as *R*-(+)-ofloxacin and 21 as *S*-(-)-ofloxacin	4 as *R*-(+)-ofloxacin, 8 as *S*-(-)-ofloxacin	4 as *R*-(+)-ofloxacin and 12 as *S*-(-)-ofloxacin	1729 as *S*-(-)-ofloxacin	880.1 as *S*-(-)-ofloxacin
	0.9 (as J01MA12-Levofloxacin)			Ofloxacin-*N*-oxide	47.9		27 as *R*-(+)- and 63 as *S*-(-)-		19.8 as *R*-(+)- and *S*-(-)-		353.8 as *S*-(-)-
				Desmethyl-ofloxacin	52.0		89 as *R*-(+)- and 120 as *S*-(-)-		33.4 as *R*-(+)- and 92.1 as *S*-(-)-		576.4 as *S*-(-)-
Norfloxacin	-	1.1	-	Norfloxacin	1.60	-	3.2	0.03	1.3	-	27.5
Nalidixic acid	0.0 (as J01MB02)	-	-	Nalidixic acid	2.50	-	6.3	-	1.0	-	1.5
Lomefloxacin	-	-	-	Lomefloxacin	1.35	-	1.8	-	0.7	-	3.5
Moxifloxacin	-	39.6	-	Moxifloxacin	2.22	-	-	0.8	-	-	6.8
				Moxifloxacin-*N*-sulphate	2.40		-		-		19.0
Prulifloxacin	-	-	4446	Prulifloxacin	-	-	-	-	-	44.1	-
				Ulifloxacin	2.33		-		-		52.1

[a] Data obtained from the Norwegian Prescription Database (NorPD) at the Norwegian Institute of Public Health (http://www.norpd.no)

[b] Data obtained from the Prescription Cost Analysis for England 2015 by Prescribing & Medicines Team Health and Social Care Information Centre

[c] Data obtained from Agenzia Italiana del Farmaco (http://www.agenziafarmaco.gov.it). In particular, consumption in DDD for detailed pharmaceutical was obtained by DDD/1000 ab die value calculated from the proportion between DDD/1000 ab die of quinolones (3.5) and DDD total of quinolones (77.8 millions)

1. http://www.drugbank.ca/.
2. Mack, G., *Improved high-performance liquid chromatographic determination of ciprofloxacin and its metabolites in human specimens.* J Chromatogr, 1992. **582**(1-2): p. 263-7.
3. Moffat, A.C., et al., *Clarke's analysis of drugs and poisons: in pharmaceuticals, body fluids and postmortem material s. Vol. 1*. 2004: Pharmaceutical Press.
4. Wong, F.A. and S.C. Flor, *The metabolism of ofloxacin in humans.* Drug Metab Dispos, 1990. **18**(6): p. 1103-4.
5. Horstkötter, C. and G. Blaschke, *Stereoselective determination of ofloxacin and its metabolites in human urine by capillary electrophoresis using laser-induced fluorescence detection.* Journal of Chromatography B: Biomedical Sciences and Applications, 2001. **754**(1): p. 169-178.
6. Okazaki, O., et al., *Enantioselective disposition of ofloxacin in humans.* Antimicrobial Agents and Chemotherapy, 1991. **35**(10): p. 2106-2109.
7. Zeng, S., et al., *Stereoselective metabolism of ofloxacin in human.* CHINESE JOURNAL OF PHARMACOLOGY AND TOXICOLOGY, 1995. **9**: p. 87-87.
8. http://toxnet.nlm.nih.gov.
9. http://sitem.herts.ac.uk/aeru/vsdb/Reports/1901.htm.
10. http://www.druglib.com/activeingredient/lomefloxacin/.
11. Ahmed, S., et al., *Involvement of Mrp2 (Abcc2) in biliary excretion of moxifloxacin and its metabolites in the isolated perfused rat liver.* J Pharm Pharmacol, 2008. **60**(1): p. 55-62.
12. Zhou, X., *Moxifloxacin*, in *Handbook of Metabolic Pathways of Xenobiotics*. 2014, John Wiley & Sons, Ltd.
13. Keam, S.J. and C.M. Perry, *Prulifloxacin.* Drugs, 2004. **64**(19): p. 2221-34; discussion 2235-6.
14. Yang, Y.-h., et al., *Enzyme-mediated hydrolytic activation of prodrugs.* Acta Pharmaceutica Sinica B, 2011. **1**(3): p. 143-159.
15. http://www.merckvetmanual.com/mvm/pharmacology/antibacterial_agents/quinolones_including_fluoroquinolones.html.
16. http://www.fao.org/docrep/w8338e/w8338e0a.htm.
17. Besse, S., J. Guyonnet, and P. Delatour, *Quantification of flumequine enantiomers in plasma by high-performance liquid chromatography.* Journal of veterinary pharmacology and therapeutics, 1998. **21**(4): p. 330-332.
18. http://www.ciplamed.com/content/nadibact-creamgel.
19. Finch, R.G., et al., *Antibiotic and chemotherapy*. 2010: Elsevier Health Sciences.
20. http://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/DevelopmentResources/UCM185720.pdf.

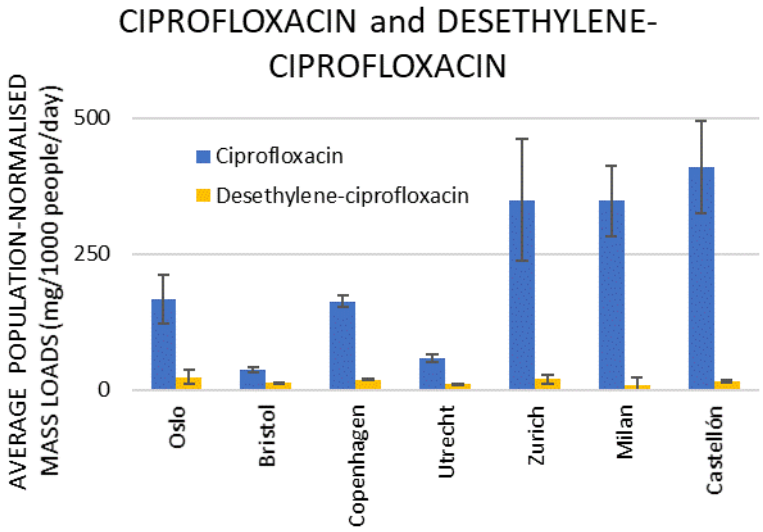


Figure 1 Average population-normalised mass loads for ciprofloxacin and its metabolite.

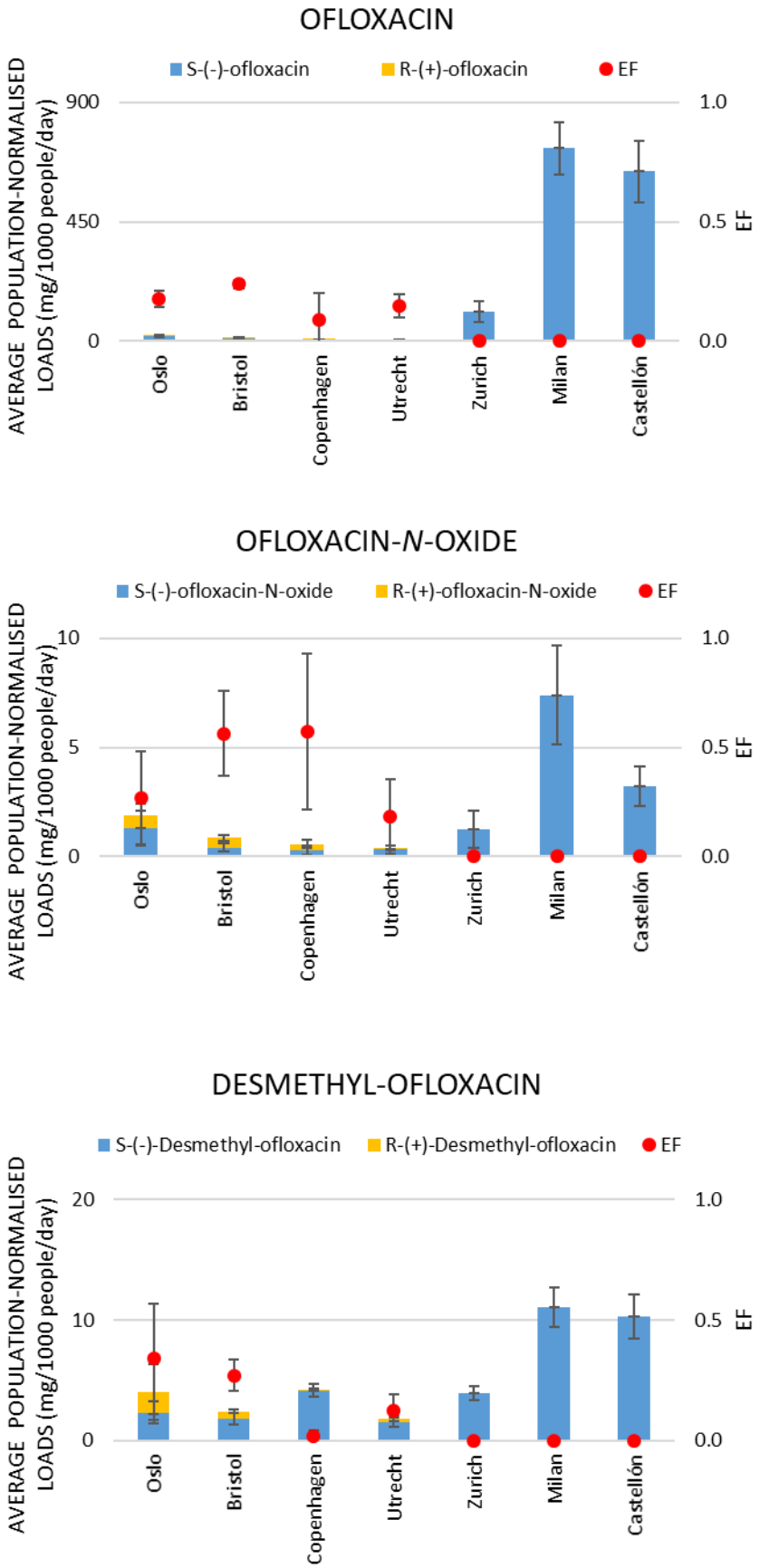


Figure 2 Average population-normalised mass loads for ofloxacin and its metabolites. Mean EFs are shown in the secondary vertical axis.

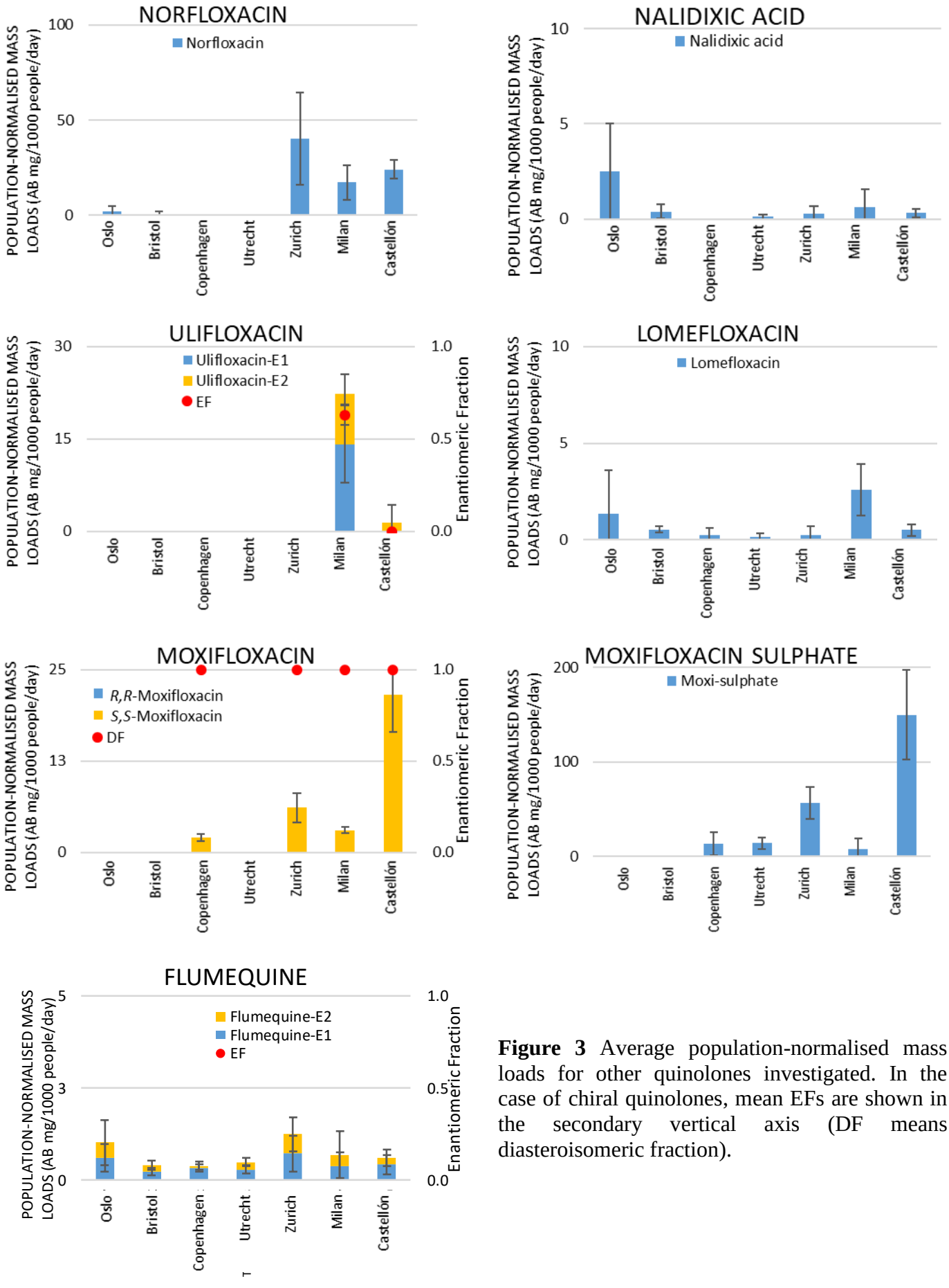


Figure 3 Average population-normalised mass loads for other quinolones investigated. In the case of chiral quinolones, mean EFs are shown in the secondary vertical axis (DF means diasteroisomeric fraction).

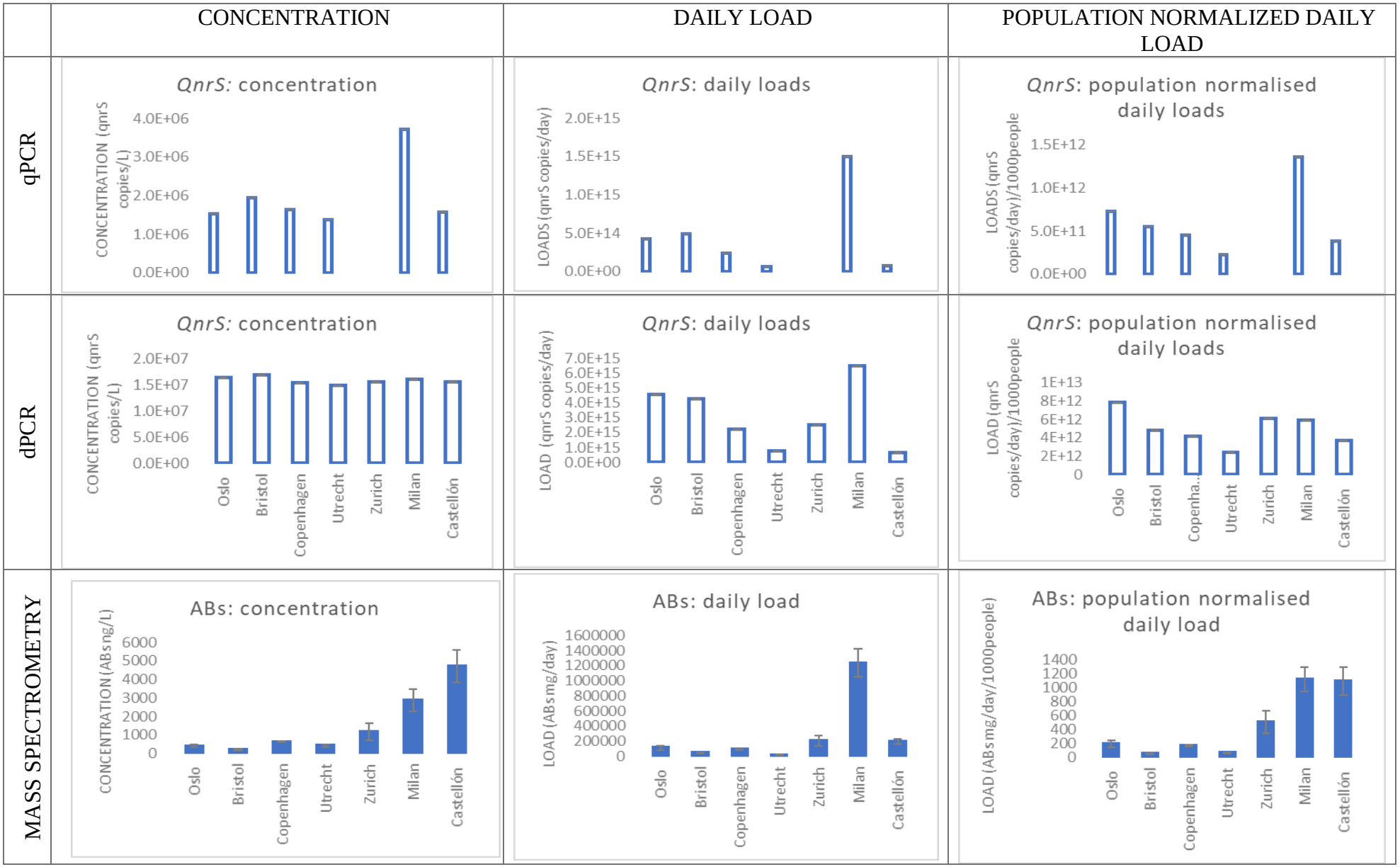


Figure 4 Pan-European monitoring of quinolones (ABs: ciprofloxacin, ofloxacin, lomifloxacin, norfloxacin, nalidixic acid, moxifloxacin, ulifloxacin and flumequine) and *qnrS* gene in wastewater.