Environmental Exposure Assessment of Fluoroquinolone Antibacterial Agents in Sewage, River Water and Soil

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Summary

Fluoroquinolones (FQs) are antibacterial agents used worldwide for the treatment of human and animal infections. In Switzerland, FQs are mainly applied in human medicine (∼ 4 t/year) and after therapeutic use they are excreted, and subsequently enter municipal sewers. Considerations about their environmental relevance are based on their unfavorable ecotoxicity profile and on their possible role on the spread and maintenance of antibacterial resistance among pathogens. FQs have also been identified as causative agents of umuC-gentoxicity in hospital wastewaters. In order to evaluate the environmental hazard posed by FQs, it is important to understand the exposure routes and fate of these bio-active chemicals in the environment. In this study, analytical methods were developed, and the occurrence, behavior and fate of FQs during wastewater treatment, in surface waters and agricultural soils were investigated. Subsequently, an environmental risk assessment was conducted based on field data. Moreover, a toxicity-directed chemical analysis was developed aiming at evaluating the role of FQs in the umuC-genotoxicity of environmental matrices.

Analytical methods for the determination of FQs in aqueous and solid samples were developed. Solid-phase extraction and reversed-phase liquid chromatography with fluorescence detection was used for the analysis of FQs in wastewater effluents and surface waters. FQs in sewage sludges and soils were determined as described for the aqueous samples but preceded by accelerated solvent extraction. Limits of quantification were 45–150 ng/L for wastewater influents, 15–45 ng/L for treated effluents, and 5–17 ng/L for surface waters. For FQs in sewage sludge and soils, limits of quantification were 0.45 mg/kg and 0.18 mg/kg, respectively. Recoveries ranged from 72 to 100 % with an analytical precision of 3 to 15 % (relative standard deviation). Identification of FQs in environmental samples was carried out by considering the chromatographic retention time and the emission fluorescence spectra, and in addition by monitoring characteristic fragments with liquid chromatography tandem mass spectrometry.

Field studies in a regional scale were conducted to assess the environmental exposure of human-use FQs. Ciprofloxacin and norfloxacin,
which represent about 95% of the total FQ consumption in Switzerland, were determined in several environmental compartments. Firstly, the occurrence, behavior and fate during wastewater treatment were investigated by performing a mass balance. Concentrations of FQs in wastewater influents were 255–571 ng/L and 36–106 ng/L in treated effluents with an overall removal from the water stream of 79 to 87% due to mass transfer by sorption to sewage sludge. During anaerobic treatment at the sludge digesters, no significant removal of FQs occurred. Concentrations of FQs in untreated sewage and anaerobically stabilized sludge were 1.4–2.7 mg/kg and 2.1–3.5 mg/kg, respectively. Subsequently, a monitoring of FQ concentrations and a mass balance for the Glatt River was done. Only traces of FQs were found in the river (< 19 ng/L). The mass flow of FQ in the receiving water was lower than was expected from the input of the FQs contained in the treated effluents. It could not be elucidated which transformation process (e.g. sorption, photodegradation) caused the calculated removal (48–66%). Finally, the entry pathway of FQs to agricultural soils after application of sewage sludge was studied. Soil depth profiles indicated that FQ residues persist in the upper layers of sludge-treated soils, with only a limited mobility down the subsoil.

An environmental risk assessment for FQs based on the measured environmental concentrations and toxicity data from the literature was proposed according to European guidelines or draft documents. The calculated risk quotients imply a low risk for a negative impact of FQs to the aquatic environment.

A toxicity-directed chemical analysis based on sequential solid-phase extraction, chromatographic fractionation and umuC-testing was developed to allow a selective bacterial genotoxicity-screening of FQs in several aqueous and solid environmental samples.

With this study, an overview of the occurrence of human-use FQs in the Swiss environment was achieved, and the behavior and the fate of FQs in various aquatic and terrestrial compartment was investigated, providing a significant base for a more profound environmental risk assessment of FQs. In general, this investigation should exemplify the benefit of process-oriented investigations of technical and natural systems, as a basis for realistic environmental exposure and risk assessment of pharmaceuticals.
Zusammenfassung


Analytische Methoden zur Bestimmung von FQ in wässrigen und festen Proben wurden erarbeitet. Festphasenextraktion und Umkehrphasen-Flüssigchromatographie mit Fluoreszenzdetektion wurden angewandt, um FQ in Abwasser und Oberflächengewässern zu messen. FQ in Klärschlamm- und Bodenproben wurden wie für Wasserproben beschrieben bestimmt, nachdem in einem ersten Verfahrensschritt die Feststoffproben zuerst mit 'Accelerated Solvent Extraction' extrahiert worden waren. Die Bestimmungsgrenzen waren 45–150 ng/L für Zulaufe, 15–45 ng/L für Abläufe und 5–17 ng/L für Oberflächengewässer. Für FQ in Klärschlamm und Boden, waren die Bestimmungsgrenzen 0.45 mg/kg und 0.18 mg/kg. Die Wiederfindungsraten lagen zwischen 72 und 100 %, bei einer Präzision von 3–15 % (relative Standardabweichung). Eine Identifizierung der FQ in Umweltproben erfolgte über die chromatographische Retentionzeit und das Emissionfluoreszenzspektrum,
sowie zusätzlich über charakteristische Fragmente in der Tandemmassenspektrometrie.


Eine *Risikoabschätzung* für FQ, basierend auf den gemessenen Umweltkonzentrationen und Toxizitätsdaten aus der Literatur wurde nach EU-Richtlinien oder Richtlinien-Entwürfen vorgeschlagen. Der berechnete Risikoquotient ergab ein geringes akutes Gefährdungspotential für die aquatische Umwelt durch FQ.

Ein *Bioassay-gekoppeltes chemisch-analytisches Verfahren*, basierend auf sequentieller Festphasenextraktion, chromatographischer Fraktionierung und umuC-Test, wurde optimiert, um eine selektive Genotoxizitätsüberwachung der FQ in verschiedenen wässrigen und festen Umweltproben zu ermöglichen.
1

General Introduction
1.1 Environmental Aspects of Pharmaceuticals

The identification and thorough investigation of emerging pollutants becomes fundamental, when a safe and sustainable environment should be preserved. During the last decades, environmental scientists have identified a great number of high volume chemicals applied in agriculture, industry and households, which can enter, disperse and persist in the environment. Recently, pharmaceutical and personal care products have also been recognized as environmental pollutants [1-8]. Several pharmaceuticals from various prescription classes (e.g. antibacterial agents, analgesics, antiepileptic drugs, beta-blockers, bronchodilators, cytostatic drugs, lipid regulators and tranquilizers) have been detected in Austria, Brazil, Canada, Croatia, Denmark, England, Finland, Germany, Greece, Italy, Norway, Spain, Switzerland, The Netherlands, and the USA. Figure 1.1 gives a general outlook of the concentration range of several pharmaceuticals in various aquatic compartments in Europe and North America. Further research is yet needed to overview the magnitude of the environmental occurrence as well as behavior and ultimate fate of many pharmaceuticals after their intended use. High sensitive and selective analytical methods are a prerequisite to determine these compounds in complex matrices at low concentrations as expected in the environment (ng to µg/L range). Such low concentrations are probably not pharmacologically active to humans, however, the ecological impact of a continuous and long-term exposure to complex mixtures of such chemicals (specifically designed to induce biological effects) is currently unknown and highly difficult to assess. Because the application of pharmaceuticals in human and veterinary medicines will continue to increase and diversify, the study of pharmaceuticals in the environment becomes an issue deserving special attention.

1.1.1 Exposure Routes in the Environment: Occurrence and Fate

Pharmaceuticals in the environment may arise from their manufacture, use and disposal. A scheme of the possible exposure routes for pharmaceutical residues in the environment is given in Figure 1.2.
Figure 1.1 Concentration range of various antibacterial agents and other pharmaceutical classes detected in the aquatic environment in Europe and North America. In parentheses are the numbers of compounds detected for each pharmaceutical class [1-8].
Figure 1.2 Possible sources and pathways of human and veterinary pharmaceuticals in the environment after use and disposal.

For human pharmaceuticals the main routes to the environment are expected to be through their use by patients in hospitals or in private homes, and by disposal of unused or out-of-date pharmaceuticals. The latest is yet suspected to be of lesser importance. After their use, pharmaceuticals are excreted with urine and feces, and thus they enter the sewer system. Their behavior and fate once they reach wastewater treatment plants is still mostly unknown. During wastewater treatment, pharmaceuticals can follow one (or a combination) of the three types of behavior: a) bio-transformation or complete mineralization, b) sorption to sewage sludge, and/or b) remain dissolved in treated effluents. The fact that several pharmaceuticals are detected in wastewater effluents and in surface waters located downstream from municipal wastewater treatment plants gives evidence that many of these chemicals are only incompletely removed during wastewater treatment, and hence they are discharged as pollutants into the receiving surface waters [1-8].
Provided that sorption to sludge is an important removal pathway during wastewater treatment, the disposal of the contaminated sludge as fertilizer to agricultural land represents an alternative exposure route for human pharmaceuticals to the environment. Up to date, however, no quantitative data on the presence of pharmaceuticals in sewage sludge is available. Furthermore, the polar nature of the majority of pharmaceuticals can lead to leaching from sludge-treated soils into groundwater or runoff into surface waters. Thus, human pharmaceuticals have been reported in ground water, and generally the source could be traced either to an impact of agricultural areas [9], municipal or industrial wastewater [10] or to a contamination via older landfills over vulnerable aquifers [11]. Only occasionally, pharmaceutical residues have been detected in drinking water [4].

The exposure situation for veterinary pharmaceuticals can be quite different. The application of pharmaceuticals as therapeutics or as growth promoters in livestock and poultry production results in a direct input to soils, mainly through liquid or solid manure [12]. Consequently, ground water might be exposed to veterinary pharmaceutical residues leaching from farmland fertilized with manure. In addition, antibacterial agents are extensively used as feed additives in aquaculture. Their direct application into water has been identified as an important environmental exposure route, since most of the excess ends up in the sediments and surrounding water as reported for many Scandinavian countries [13, 14]. Altogether, veterinary pharmaceuticals (especially antibiotics) have been identified as persistent pollutants in soils treated with manure [12], as well as in sediment cores after medication in fish farms [14].

Before being retrieved from the body, most pharmaceuticals are partially transformed to metabolites, which are generally more water-soluble than the parent compound. Metabolization usually consist of oxidation, reduction or hydrolysis to add reactive functional groups to the molecule (Phase I metabolites), or involve covalent conjugation to polar molecules (e.g. glucuronic acid, sulfate, acetic acid or amino acid) to make the molecule more hydrophilic and better excretable (Phase II metabolites). The subsequent metabolites can have greater or lesser physiologic activity than the parent drugs, and they add to the already complex picture of thousands of highly bio-active chemicals. Therefore, the necessity to
investigate for metabolites is to understand not only the fate and transport of pharmaceuticals, but also their ultimate overall effects on the environment. The occurrence of metabolites has generally not been studied in detail, with the exception of a few specific cases (e.g. clofibric acid, fenofibric acid and salicylic acid) [4, 5]. There is also some evidence that conjugates entering the wastewater treatment may be hydrolyzed to the parent compound [15].

### 1.1.2 Potential Environmental Concern: Effects

Because pharmaceuticals are designed to stimulate a physiological response in humans, plants and animals, potential adverse effects to ecosystems are of great concern. Previous considerations about the environmental relevance of these compounds include for instance: a) eco-(geno)toxic effects on organisms at different trophic levels, b) endocrine disruptor effects on organisms, and c) the spread and maintenance of bacterial resistance to antibiotics. Bacterial genotoxic activity has been detected in hospital wastewater [16, 17], yet seems to undergo significant dilution on passage to wastewater treatment plant, thereby reducing any genotoxic potential to negligible levels [18]. On the contrary, the presence of estrogenic activity in wastewater effluents has been shown to impact on sexual differentiation in fish [19-21]. The induction of bacterial resistance to antibiotics in discharges has also been raised as a potential issue. However, there is no evidence that a significant proportion of resistant organisms in the environment arise as a consequence of such discharges rather than by excretion of resistant organism by man and animals and the spread of resistance by plasmid transfer. Therefore, additional research is warranted before any conclusion about bacterial resistance to antibiotics can be drawn.

A much larger palette of potential adverse effects on ecosystems is expected. However, since mechanisms are not clearly understood prediction seems unfeasible. It has been suggested that the pharmacological mode of action relevant to specific pharmaceuticals should be considered when evaluating their hazard to the environment. However, although pharmaceuticals are usually designed with a specific
mode of action, they can also have numerous effects on non-target receptors/species. The most significant work to date showing subtle effects on non-target species resulting from low concentrations is for the antidepressants fluoxetine and fluvoxamine [22].

The aquatic acute toxicity data available for pharmaceuticals indicate effects usually above the mg/L level, whereas environmental concentrations are orders of magnitude lower at the ng/L or, at most, low µg/L levels (individual pharmaceuticals). Consequently, pharmaceuticals should be in general of no environmental significance when considering acute toxicity in the aquatic environment. A major concern is not necessarily acute effects but rather the manifestation of subtle long-term effects, yet chronic toxicity data is lacking.

The presence of numerous pharmaceuticals sharing a specific mode of action is suspected that could lead to significant effects through additive exposure. Additionally, potential interactive effects may occur from complex mixtures of pharmaceuticals and other pollutants in the environment.

1.1.3 Environmental Risk Assessment

In order to assess the potential environmental risk of pharmaceuticals it is important to have relevant data. This includes environmental monitoring data and information on environmental behavior and fate, as well as ecotoxicity data. Up to date, a comprehensive risk assessment of the vast majority of pharmaceuticals does not exist because of the sparse knowledge on the subject.

In a regulatory framework, chemical companies are asked since 1984 to conduct environmental risk assessment (ERA) for all their new products registered in the European Union, except for pharmaceuticals. However, the recent increasing public concern about the hazards associated with pharmaceutical residues in the environment has lead to the issue of guidance documents by the respective regulatory bodies in the USA and in the European Union. In 1995 a regulatory guideline by the Food and Drug Administration was introduced for new licensed human pharmaceuticals in the USA [23]. In Europe, ecotoxicity data have been required as part of the
safety submission for all new veterinary medicinal products introduced from 1998 on [24]. Discussions whether this legislation may also be expanded to human pharmaceuticals as proposed by the European Agency for the Evaluation of Medicinal Products [25, 26] are still going on.

The guidance to industry on the environmental assessment procedure is seen as a two-phase tier approach: in a first phase, the extent of environmental exposure is estimated as predicted environmental concentrations and it is compulsory for all products. In a second phase the fate and effects of the active residue are assessed in case that the given concentration-threshold values are exceeded. The thresholds set by the regulatory authorities are significantly different between USA and Europe. In the United States, ERA are required for new human-use pharmaceuticals if the predicted environmental concentrations at the point of entry into the aquatic environment is > 1 µg/L. The European discussion paper proposes that an ERA should be required for human-use pharmaceuticals, for which crude predicted concentration in surface waters are greater than 0.01 µg/L. For veterinary pharmaceuticals predicted environmental concentrations of total residue in soil should not exceed 10 µg/kg (> 1 µg/kg for chemicals deriving from industry-related processes). Several scientists have, however, manifested that the proposed thresholds are not scientifically based, because substantial class to class variations in the ability to invoke biological responses exist. A suggested alternative is to set thresholds within different therapeutic classes, although this seems to neither guaranty a complete safe assessment, since pharmaceuticals can act in very different ways, with potentially different biological receptors and chemical potency. Furthermore, not only the parent compound should be the subject for a risk assessment but also the main metabolites (excreted percentage being at least 10% of the applied amount of substance). Some researchers also view the need to assess the impact of human pharmaceuticals on terrestrial ecosystems derived from the widespread practice to dispose sewage sludge to land.

The regulatory ERA is foreseen for new pharmaceuticals, but what about the ones already in use? In an attempt to overview the environmental risk of the current consumption patterns of human pharmaceuticals in Denmark and United Kingdom, two studies have recently been published. For worst case assumptions, an immediate risk for the aquatic environment
was predicted for the analgesics paracetamol, acetylsalicylic acid and ibuprofen in Denmark [27], and additionally for dextropropoxyphen, fluoxetine, oxtetracycline, propanolol, amitriptyline and thioridazine in the United Kingdom [4]. However, when taking account of measured concentrations in the environment such a hazard was no longer demonstrated. Only for 17α-ethinylestradiol a certain risk was predicted when considering chronic toxicity end-points and a low dilution to surface waters [4]. The studies suggest that a notable difference in the types and amounts of pharmaceuticals prescribed in individual countries may be expected, and that the environmental risk between countries is not necessarily comparable. Because of the high number of active substances licensed for use in a country (about 3000 in UK or in Germany), it is important to prioritize pharmaceuticals on the basis of human metabolism and pharmacological activity, possible occurrence, persistence and adverse effects to ecosystems. The ultimate purpose of the environmental risk assessment of pharmaceuticals should be to balance between the significant benefits of pharmaceuticals for human and animal health and the harm to the environment, which should allow the development of appropriate risk management strategies and lead to a sustainable use of pharmaceuticals.

1.2 Fluoroquinolone Antibacterial Agents

Antibiotics are among the most often discussed emerging pollutants, because of their potential role in the spread and maintenance of (multi-) resistance among bacterial pathogens in the environment. On the other side, these compounds are of vital importance for the treatment of infectious diseases in humans and animals, and presently they are among the most widely prescribed groups of pharmaceuticals in human medication. Penicillins, tetracyclines, sulfonamides, and macrolides have been broadly monitored in various environmental aquatic compartments (see Figure 1.1), however much less is known about the occurrence of fluoroquinolones [7, 16, 28].
Fluoroquinolones (FQs) are fully synthetic antibiotics (antibacterial agents) and since their introduction in the market in the 1980s, they have gained substantial popularity. Nowadays, they are considered one of the most valuable antibacterial drug classes, because of their outstanding effectiveness (e.g. broad spectrum of activity and excellent oral bioavailability), and continuous work is being carried out towards the synthesis of new FQs derivatives with improved antibacterial activity [29].

FQs are active against a broad spectrum of pathogenic gram-negative and gram-positive bacteria. Their mode of action is selective and inhibits the activity of the enzyme DNA-gyrase (bacterial topoisomerase II). By inhibition of the unwinding enzyme, the synthesis of DNA and consequently the cell division are also inhibited, leading to cell death. The common core structure of FQ derivatives is a 3-carboxyl-4-oxoquinolone ring system substituted with a fluorine atom at position 6 and a piperazinyl group at position 7 (Figure 1.3). Position 1 can accept a wider range of substituents, the most successful being a cyclopropyl ring.

FQs are ampholytic compounds with pKa values of 5.9 and 6.3 for the carboxylic group and 7.9 and 10.2 for the amino group on the piperazinyl ring and the isoelectric point of the zwitterion is at neutral pH [30]. The maximum octanol/water partition coefficient occurs at the isoelectric point. Additionally, FQs are capable of forming complexes with certain multi-charged cations [31]. These physicochemical properties exert an influence not only on the absorption, transport and elimination of these drugs in the body, but also on the behavior and distribution in the environment. In mammals, FQs are metabolized often only to a limited extent, so a major fraction of the administered dose is excreted unchanged with urine and feces. Most metabolic changes in the FQs are linked to the substituent at position 7, either by: a) dealkylation and oxidation of the amine moiety (< 10%), or b) (reversible) glucuronidation and sulfatation (< 10%) [29]. From the several metabolites, none is excreted in percentage higher than 10% of the applied dose, and in general they show less antibiotic activity than the parent compound [29]. Therefore, it can be anticipated that FQ metabolites should be of minor environmental relevance.

Fluoroquinolones (e.g. ciprofloxacin, norfloxacin, ofloxacin, levofloxacin) have found a wide application in human medicine for the
treatment of urinary and respiratory tract infections, and also gastrointestinal and sexually transmitted diseases. Recently, ciprofloxacin has also become the medicine of choice for the treatment of anthrax infections. In the 1990s, the use of FQs was approved for the treatment and prevention of veterinary diseases in food-producing animals and in
aquacultured fish. Though several FQs were specifically developed for veterinary medicine (e.g. enrofloxacin, danofloxacin, sarafloxacin), almost identical structures of FQs are used for humans and animals. The wide application range and the extensive use and misuse of FQs in veterinary medicine in addition to the misuse of FQs in human medicine raises concern about the possible development of resistant pathogens to this valuable class of pharmaceuticals. Since the approval of FQs for use on poultry and cattle, the incidence of FQ-resistant bacterial infections in humans in the United States raised dramatically, what has been associated to the presence of persistent residues of FQs in edible tissues or milk [30]. Despite calls by regulatory agencies for restriction, the proposed restrictive measures and the establishment of regulatory levels of FQs in veterinary medicine have not yet been widely implemented.

The environmental hazard associated to FQs is not only based on their potential to induce bacterial resistance, but also on their unfavorable genotoxicity and ecotoxicity profile. Hartmann et al. proposed that umuC genotoxicity in hospital wastewater could be mainly induced by FQs, especially by ciprofloxacin [16]. On the other hand, FQs do not show any mutagenic effects in the Ames assay, unless the tester strain contains an excision repair system [17]. Because most studies in eukaryotic cells did not show significant genotoxic effects in vitro or in vivo, it seems that FQs act highly specific on bacterial gyrase rather than eukaryotic topoisomerases [32], so any human hazard should be expected. FQs have also shown to be highly toxic against their target organisms (bacteria) [33], however, effects on non-target organisms, such as algae have also been reported [34]. An environmental risk assessment of ciprofloxacin based on acute aquatic toxicity and predicted environmental concentrations has recently been published by Halling-Sørensen et al. [33], who calls for urgent study on the fate and effects of FQs in the environment. Therefore, to avoid any environmental or human health implications, the occurrence of FQs in the environment should be investigated.

The entry route of FQs into the environment can be either through common wastewater pathways, via manure dispersion in agricultural land or through application in aquaculture. Because in Switzerland, FQs are mainly applied for the treatment of human diseases (~ 4 t/years versus ~ 0.5 t/year veterinary medicine) [35], the major entry route of FQs to the
Swiss environment seems via communal sewage. Ciprofloxacin, a largely prescribed FQ in Switzerland, was determined by Hartmann et al. in hospital wastewater in the range of 3 to 87 µg/L [16], and in wastewater effluents by Alder et al. at the ng/L level [28]. Recently, its occurrence has also been reported in US surface waters by Kolpin et al. [7]. Several authors have evaluated the fate of FQs in various environmental compartments based on laboratory experiments. Using a simple test with wastewater bacteria, Kümmerer et al. found no biodegradation of FQs, indicating that no elimination during wastewater treatment should be expected [36]. Burhenne et al. extensively studied the photodegradation potential of FQs [37, 38], which may be of importance once FQs are released into surface waters. Photodegradation of FQs in aqueous solution was observed, and it occurred in two main steps: a) alterations in the piperazine substitution at position 7, and b) photolytic breakdown of the FQ core structure. However, a decrease in the photodegradation rate of FQs was detected in the presence of humic acids [39], which was explained to be due to the competition between UV-absorbing humic acids and the FQs in absorbing photons. The sorption characteristics of FQs to soils, humic acids and dissolved organic matter have also been investigated. Nowara et al. found that FQ bind strongly to the clay fraction of soils through electrostatic interactions [40]. Electrostatic and hydrophobic interactions have also been invoked in the sorption of FQs to humic acids [39] and to dissolved organic matter [41]. Aerobic biotransformation of the amine moiety of various FQs by soil microorganisms, including bacteria, yeast and molds, has also been described [42-44]. Consequently, once FQs reach the terrestrial ecosystem, biodegradation can be foreseen, though the strong binding to soils may be expected to reduce bioavailability and so allow FQ residues to persist in the environment. Thus, Hektoen et al. found persistent FQ residues in sediments cores after medication in fish farms [14].

Although laboratory experiments are indispensable to predict and understand the behavior of chemicals in the environment, monitoring data and field studies are essential to obtain a comprehensive study of the exposure situation in the environment. Therefore, the occurrence, behavior and fate of FQs at field conditions are of direct interest to properly assess their environmental risk.
1.3 Scope of this Work

The scope of this work was to evaluate the main exposure routes of FQs in the Swiss environment and to investigate the behavior and fate of FQs during wastewater treatment, in surface waters and in agricultural soils. To accomplish this, new analytical methods were developed to allow the determination of FQs in several environmental matrices. Subsequently, an environmental risk assessment according to European guidelines was proposed. Furthermore, an insight on the genotoxicity potential of FQs occurring in environmental samples was intended, by developing a toxicity-directed chemical analysis. Within this dissertation the following studies were performed:

Analytical methods (Chapter 2, 3 and 4). Specific analytical methods are essential for investigating emerging pollutants in the environment. For the determination of FQs reliable methods were developed, validated and applied within this work for the analysis of aqueous and solid samples. Accelerated solvent extraction was selected for the extraction of FQs from sewage sludges and soils. Solid-phase extraction with a mixture of non-polar and cation exchanger sorbents was used as clean-up for extracts from sewage sludge and soils, and as an enrichment and clean-up step for wastewater and surface waters. The analytical method was based on liquid chromatography with fluorescence detection. For identification purposes a liquid chromatography with tandem mass spectrometry method was favored. Limits of quantification were in most cases low enough for the determination of FQs in several environmental matrices.

Field study and mass balance during wastewater treatment (Chapter 4 and 5). In order to study the occurrence, behavior and fate of FQs during wastewater treatment, regional field studies were conducted. An overall removal of FQs during mechanical-biological wastewater treatment was determined for several wastewater treatment facilities. Furthermore, a study on the removal capacity of various treatment stages was investigated in more detail by performing a mass balance for the largest wastewater treatment plant in Switzerland. Mass transfer from wastewater to sewage sludge was also evaluated. Altogether, these studies
revealed the behavior and fate of FQs during wastewater treatment under aerobic and anaerobic conditions.

Field study and mass balance in the Glatt Valley watershed (Chapter 4). The occurrence of FQs after discharge of treated effluents was monitored in three locations at the Glatt River during winter and summer seasons. The mass flows of all treated effluents along the Glatt River (input) and the mass flows at the outlet of the River (output) allowed to assess the overall removal of FQ from the dissolved fraction along a 36-km long river.

Field study and soil depth profiles in experimental plots (Chapter 5). Since during wastewater treatment a considerable amount of FQs sorbed to sewage sludge, the occurrence of FQs in sludge-treated soils was evaluated in experimental plots. The field experiments allowed a first insight on the behavior and fate of FQs in the top and subsoil of sludge-treated land at long term. Preliminary information on the persistence and mobility of FQs in sludge-treated soils was obtained.

Environmental risk assessment in the Glatt Valley watershed (Chapter 4). The environmental concentrations measured for the aquatic environment within this study provided an exposure assessment of FQs to aquatic organisms. This exposure data was contrasted to acute toxicity data from the literature, in order to evaluate the environmental hazard of FQs, according to European guidelines or draft documents. Furthermore, predicted environmental concentrations for the region of Switzerland were calculated and compared with measured concentrations on the Glatt Valley watershed.

Toxicity-directed chemical analysis (Chapter 6). A sequential solid-phase extraction and an analytical methods were developed to allow the genotoxicity testing of FQs. Dose-effect curves for various FQs and for a metabolite were obtained. The cumulative effect of the two environmentally relevant FQs was also investigated. Preliminary results on FQ-genotoxicity of fractionated extracts deriving from several environmental matrices were obtained.
1.4 Literature Cited


Fluoroquinolones (FQs) are among the most important antibacterial agents (synthetic antibiotics) used in human and veterinary medicine. An analytical method based on reversed-phase liquid chromatography with fluorescence detection was developed and validated for the simultaneous determination of nine FQs and the quinolone pipemidic acid in urban wastewater. Aqueous samples were extracted using mixed phase cation exchange disk cartridges, which were subsequently eluted by ammonia solution in methanol. Recoveries were above 80% at an overall precision of better than 10%. Instrumental quantification limits varied between 150 and 450 pg injected. The presented method was successfully applied to quantify FQs in effluents of urban wastewater treatment plants. The two most abundant human-use FQs ciprofloxacin and norfloxacin occurred in primary and tertiary wastewater effluents at concentrations between 249 to 405 ng/L and 45 to 120 ng/L, respectively. The identity of FQs in urban wastewater was confirmed by recording full fluorescence spectra and liquid chromatography directly coupled to tandem mass spectrometry. These results indicate that conventional environmental risk assessment overestimates FQ concentrations in surface waters by 1 to 2 orders of magnitude.
2.1 Introduction

In recent years, public and scientific concern about the relevance of trace amounts of pharmaceuticals that occur in the environment has been continuously increasing [1, 2]. A central aspect is the potential risk for aquatic and/or soil organism associated with the presence of trace concentrations of these active compounds. Of major concern is the potential emergence and spread of drug-resistance due to antibiotics. Environmental risk assessment studies combine the hazard with the likeliness of exposure, basing their evaluation on predicted environmental concentrations (PECs) and laboratory data. However, measured environmental concentrations (MECs) are indispensable to provide accurate data on the real concentrations, likewise details on the fate of pharmaceuticals in different environmental compartments [3-6]. Consequently, the development of selective and sensitive analytical methods for environmental matrices is of great importance to assess properly the respective risks.

Fluoroquinolone antibacterial agents are probably among the most important class of synthetic antibiotics in human and veterinary medicines expanded world-wide. In Switzerland, fluoroquinolones (FQs) contribute to approximately 15% of the total amount of antibiotics used for human medical therapy (ca. 4 tons/year), compared to penicilins (50%), cephalosporins (15%) and macrolides (15%) [7]. The leading FQs for human and animal treatment in Switzerland are shown in Figure 2.1, including the quinolone pipemidic acid, which is of importance in that country [8]. Ciprofloxacin (CIP) and norfloxacin (NOR) are the major human-use FQs in Switzerland, each contributing to around 42-48% of the total domestic FQ consumption. Ofloxacin (OFL) and levofloxacin (LEV) (the latter the pure L-enantiomer of the former racemic mixture OFL) contribute to a lesser extent (ca. 4% each). Since administered FQs are excreted largely unchanged (generally < 25% metabolized) [9], FQs are expected to enter the environment mainly either via human excretion into wastewaters or via dispersion of manure onto agricultural soils.
Several authors have tried to predict the potential environmental fate [10-13] and aquatic bacterial toxicity [13-15] of some FQs, although always based their work on laboratory data and non-experimental PECs. A PEC based environmental risk assessment of CIP has recently been published [16]. Preliminary investigations revealed CIP to occur in urban wastewaters [17], indicating a possible occurrence of other FQs. Many chromatographic methods have been published for the analysis of FQs in biological matrices (reviewed in [18-20]). However, to our knowledge
there is no method available to analyze trace amounts of several FQs in environmental samples. Because of the low concentrations occurring in the environment, enrichment and sample clean-up are indispensable. Cation exchange extraction has proven most suitable for the enrichment of CIP from wastewaters [17] and river waters [21]. However, a comprehensive method to enrich various FQs with reliable recoveries is still awaited.

Here we report on a specific and sensitive liquid chromatography (LC) with fluorescence detection (FLD) method to determine multiple FQs residues in wastewater samples. We have emphasized urban wastewaters, because they are the primary route of entry of human-use Pharmaceuticals into the environment. We focus on the FQs most commonly used in Switzerland (six human- and three veterinarian-use FQs) and the quinolone pipemidic acid. Additionally, tandem mass spectrometry (MS/MS) was used for identification purposes due to its higher specificity. The specific aims of our study were to: (1) achieve satisfactory enrichment for the analytes of interest, (2) develop and validate a specific and sensitive LC-FLD method, (3) establish a confirmatory LC-MS/MS method for identification purposes, and (4) check the power of the new method to analyze urban wastewaters.

2.2 Experimental Section

2.2.1 Chemicals and Materials

Reference compounds were purchased from Sigma-Aldrich unless otherwise stated. Ciprofloxacin (CIP) and enrofloxacin (ENR) were supplied by Bayer AG (Wuppertal and Leverkusen, Germany). Levofloxacin (LEV) was obtained from Aventis Pharma AG (Zurich, Switzerland), danofloxacin (DAN) by the Swiss Federal Veterinary Office (Bern, Switzerland), and fleroxacin (FLE) by Roche Diagnostics GmbH (Mannheim, Germany). Difloxacin (DIF) and tosufloxacin surrogate standard (TOS) were received from Abbott Laboratories (Baar, Switzerland). Standard solutions of 400 µg/mL were prepared in a water: methanol mixture (1:1) containing 0.2% v/v hydrochloric acid and stored at −20°C. Standard solutions were renewed monthly. Working standard
mixtures of 10 µg/mL and 1 µg/mL were prepared in 25 mM orthophosphoric acid, stored at + 4°C, and renewed weekly. Mixed phase cation exchange disk cartridges (MPC, octyl phase and benzenesulfonate mixture, high density 12 µm particle size) were supplied by Varian International AG (Basel, Switzerland). Other extraction materials were: Isolute C18, ENV+ (ICT Internationale Chemie Technik GmbH, Bad Homburg, Germany), Bond Elut CBA, PPL, ENV (Varian International AG, Basel, Switzerland), Oasis HLB (Waters, Rupperswil, Switzerland) and EN Lichrolute (Merck, Dietikon, Switzerland).

All solvents were reagent grade or higher in quality. HPLC-grade water, acetonitrile and methanol (MeOH) were purchased from Scharlau (Barcelona, Spain). ortho-Phosphoric acid (o-H₃PO₄) 85%, ammonia solution 25%, hydrochloric acid (HCl) 32%, and trifluoracetic acid (TFA) were supplied from Merck (Dietikon, Switzerland). Triethylamine and sodium hydroxide solution (puriss) were obtained from Fluka AG (Buchs, Switzerland).

### 2.2.2 Sample Collection

Primary and tertiary wastewater effluents were collected as 24 h-composite samples from different urban wastewater treatment plants around Zurich, Switzerland. Samples were collected in amber glass bottles and immediately filtered through 0.45 µm cellulose nitrate membrane filters (Sartorius GmbH, Göttingen, Germany). Subsequently, samples were set at pH 3 to reduce biological activity and stored in the dark at + 4°C until analysis.

Primary effluents were collected after primary clarification, presenting a pH between 8.0 - 8.5, and a conductivity of 0.9 - 1.4 mS/cm. Tertiary effluents were collected after advanced treatment with contact filtration (quartz), sample characterization was: pH value of 7.1 - 7.9, conductivity between 0.7 - 0.9 mS/cm, oxygen content around 5.7 - 6.9 mg/L and a total organic carbon of 3.4 - 4.9 mg/L.
2.2.3 **Solid-Phase Extraction (SPE)**

FQs were extracted from wastewaters using mixed phase cation exchange (MPC) disk cartridges preconditioned with 2 mL of methanol and 2 mL of water at pH 3. Samples of 50 mL primary and 150 mL tertiary effluent were spiked with 400 ng TOS surrogate standard prior to extraction. Samples at pH 3 were then percolated through the disk cartridge at a flow of approximately 1 mL/min using a vacuum manifold (Supelco, Bellafonte, PA). After extraction, the disk cartridges were vacuum dried for 5 min. Compounds were then eluted with 2.5 mL 5% ammonia solution in 15% MeOH, neutralized using 0.5 mL H₃PO₄ 85%, and immediately analyzed.

The materials C18, C18ec, ENV, ENV+, EN, PPL and Oasis HLB were tested as described above, but using 3 x 2 mL methanol and 3 x 2 mL water at pH 3 for cartridge preconditioning. For the CBA material, sample pH was adjusted to 6.8; cartridge preconditioning was done using 3 x 2 mL methanol and 3 x 2 mL water at pH 6.8.

Accuracy was determined by recovery studies on MPC disk cartridges by spiking wastewater samples. Six replicate analyses were performed using primary and tertiary effluents (50 mL and 150 mL, respectively) spiked with 10 ng of FQ standard mixture plus 400 ng of TOS surrogate standard. Breakthrough on MPC disk cartridges was controlled by extracting 50, 75, and 100 mL of primary effluent, and 150, 200, and 250 mL of tertiary effluent containing native FQs spiked with 400 ng TOS surrogate standard in two stacked MPC disk cartridges.

2.2.4 **Liquid Chromatography – Fluorescence Detection (LC-FLD)**

Separation was performed on an HP-1090 series II liquid chromatograph (LC) equipped with a programmable HP-1100 fluorescence detector (FLD) from Agilent Technologies (Switzerland). The FLD excitation wavelength was 278 nm and emission wavelength, 445 nm, except for OFL/LEV (500 nm). The LC column (250 x 3 mm) and precolumn (20 x 3 mm) were filled with Discovery RP-AmideC16 5 µm particles (Supelco, Buchs, Switzerland). Eluent A was a 25 mM aqueous o-
Chapter 2

H₃PO₄ solution (pH 2.4), and eluent B was acetonitrile. Elution started with 5% B. A 17 min linear gradient to 7% B, followed by a 5 min isocratic elution, and a 13 min linear gradient to 17% B was used for analysis. After washing with 85% acetonitrile for 5 min, the initial conditions were re-established by a 2 min linear gradient, followed by an equilibration time of 10 min. Analyses were performed at a flow rate of 0.7 mL/min and column temperature of 50°C. Sample aliquots of 200 µL were injected using an HP 1090 auto-sampler.

2.2.5 Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)

The LC system consisted of a Perkin Elmer Sciex Series 200 equipped with a quaternary pump, a vacuum degasser, and an auto-sampler. FQs were chromatographed using a Nucleosil RP-C18 column (250 x 2 mm, 5 µm) equipped with an 8 x 3-mm precolumn of the same type (Maier and Nagel GmbH, Düren, Germany). Because eluent A used in the fluorescence method consisted of a phosphoric solution that can interfere with MS analysis, a separation method using a mobile phase of 0.1% TFA aqueous solution (A) and acetonitrile as organic modifier (B) was developed. The gradient was run from 12% to 15% B in 15 min, followed by a washing step of 6 min and equilibration at initial conditions for 15 min. The flow rate was 0.3 mL/min at room temperature, and the injection volume was 20 µL.

MS/MS data were acquired using a Perkin-Elmer Sciex API 365 triple stage quadrupole (Q₁Q₂Q₃) mass spectrometer with electrospray ionization. The analyses were performed in positive ion mode, with a spray voltage of 5.1 kV. Orifice skimmer differences varied from 24 to 36 V and ring electrode voltages from 140 to 200 V. Nitrogen was used as curtain gas with a flow rate of 1 L/min, and as nebulizer gas at an approximate flow rate of 0.7 L/min. The mobile phase flow was split 1:10 in the interface, resulting in 30 µL/min spray into the mass spectrometer. MS/MS parameters were optimized in continuous flow mode. Q₁ was scanned over a range of m/z 100 – 400 amu for precursor ion optimization. The protonated molecular ion [MH]+ of the respective analyte was used as the
precursor ion for subsequent MS/MS experiments. Ion spray voltage, quadrupole, and lens conditions for the nitrogen collision induced dissociation were optimized. Subsequently, product ion spectra were obtained by scanning Q3 over a mass range of m/z 100 - 400 amu.

2.2.6 Identification, Quantification, and Quality Control

Peak identification was performed routinely by comparing fluorescence spectra (fixed excitation wavelength at 278 nm, and scanning emission wavelength from 300 to 500 nm), and retention times of each sample with corresponding reference compounds. Additionally, more specific confirmation using MS/MS spectra was performed as follows: (1) FQs were detected in environmental samples by scanning for the most intense product ion for each FQ investigated, and (2) identification was accomplished separately by multiple reaction monitoring using three to four characteristic transitions for each detected FQ.

Five-point calibration curves were generated for each FQ standard mixture and the TOS surrogate standard acquired at excitation wavelength 278 nm, and emission wavelength 445 nm (except 500 nm for OFL/LEV). Concentrations of FQs in wastewater samples were calculated by comparing the ratios of peak areas for the FQs and TOS surrogate standard in the SPE extracts to that of standard solutions. Instrumental detection limits (IDL) and instrumental quantification limits (IQL) for FQs were calculated first on the basis of the standard deviation (n = 10) of the analysis of a FQ standard mixture of 1 ng on column. IDL and IQL were defined as 3 and 10 times the standard deviation of the FQ measurements, respectively. However, since IQL was not within the linear range, it was defined as the second lower point of the linear range of the calibration curve.

Amber vials were used for preparing and storing standard solutions, as well as during the analytical procedure to avoid photodegradation. Conductivity of each sample was controlled with a conductivity cell (Testo 240, Germany) to assure that tested samples were within the range for which the method was validated (< 1.5 mS/cm). TOS was added as a surrogate standard to all samples before the extraction step to correct for
losses during sample enrichment. Standard solutions were stable for one month at -20°C, as noted by others [22].

Procedural and instrumental blanks were analyzed for each set of 10 samples to control for laboratory contamination and analytical interferences. Duplicate analyses were carried out for each sample. In every set of 10 samples, two samples were spiked with 10 and 20 ng of the FQ standard mixture to control matrix effects on recovery. Instrumental precision of the LC-FLD was assessed using an average of 10 independent measurements of a standard mixture of 10 ng/mL. The precision of the entire procedure for primary and tertiary effluent samples (50 mL and 150 mL, respectively) was determined using 6 replicates each spiked with 10 ng of FQ standard mixture and 400 ng of TOS surrogate standard prior to extraction.

2.3 Results and Discussion

2.3.1 Solid-Phase Extraction with Mixed Phase Cation Exchange Disk Cartridges

Solid-phase extraction using ionic exchangers often has been used for the clean-up of FQs from extracted biological samples [23, 24]. Because of possible disturbances by ionic sample components, ion exchange enrichment is not very well accepted for environmental samples [25]. However, for extracting FQs from wastewater samples, mixed phase cation exchange (MPC) disk cartridges were shown to present the most selective material for clean-up, and offered the best recovery efficiencies.

A variety of other extraction materials was tested: an apolar sorbent (C18), polymeric sorbents (ENV, ENV+, EN, PPL, Oasis HLB), and a weak cation exchanger (CBA). None of these materials offered comparable selectivity and enrichment efficiency for wastewater samples vs. MPC disk cartridges. The weak cation exchanger CBA (carboxylic acid, 40 µm particle size) provided similar selectivity but up to 20% lower extraction efficiency compared to the MPC disk cartridges (12 µm particle size).

The MPC disk cartridge material is a mixed mode silica based sorbent consisting of a special nonpolar octyl phase and a strong cation exchanger.
(benzenesulfonate). This combination of hydrophobic and cation exchange properties results in a more specific interaction with FQs via the aromatic moiety of the FQ core and the charged amino groups of the FQ substituents, respectively. At the working pH 3, FQs predominate in their cationic form, since pKa values for the carboxylic group are between 5.9 and 6.3, and for the amino groups on the piperazine moiety between 7.9 and 10.2 [26]. Additionally, this mixed mode material increases method specificity since the mechanisms in the enrichment step and the subsequent reversed-phase chromatography are operating based on different principles. Furthermore, the small particle size of the MPC disk cartridge material (12 µm) favors the diffusion step involved in the cation exchange enrichment, and therefore, faster kinetics and better analyte retention can be expected [25].

The influence of cationic sample components on extraction efficiency of the MPC disk cartridges was tested by adding 1g EDTA to 50 mL primary effluent spiked with FQ standard mixture and 400 ng TOS surrogate standard. Enrichment efficiency was not significantly altered. The conductivity of the primary and tertiary effluent samples tested was always below 1.5 mS/cm. The presented method was validated for samples not exceeding this value. Higher ion content in sample could induce earlier breakthrough and thus reduce extraction efficiencies of FQs. The impact of sample dilution was also investigated for primary effluents, and the extraction efficiency did not increase after dilution with distilled water (1:1, 1:2 and 1:4).

Recovery of the TOS surrogate standard from wastewater using MPC disk cartridges ranged from 100% for primary effluents to 93% for tertiary effluents (Table 2.1). Recoveries of FQs in 50 mL spiked primary effluent samples and 150 mL tertiary effluent samples ranged from 81% to 96% and from 72 to 97%, respectively. Experiments to determine the breakthrough of FQ indicated that these analytes were quantitatively isolated from 50 mL primary effluent and from 150 mL tertiary effluent by the first of two stacked MPC disk cartridges. Neither FQs nor TOS surrogate standard were detected on the second disk cartridge. When 75 mL primary effluent and 200 mL tertiary effluent were extracted, less than 5% of the amount found in the first disk cartridge was detected in the second MPC disk cartridge. However, when enriching 100 mL primary effluent and 250 mL
Table 2.1 Accuracy and Precision of the Determination of Fluoroquinolones in Wastewater Effluents

<table>
<thead>
<tr>
<th>compound</th>
<th>spiked amount (ng/L)</th>
<th>recovery^b (%)</th>
<th>relative standard deviation^b (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>primary effluent</td>
<td>tertiary effluent</td>
<td>primary effluent</td>
</tr>
<tr>
<td>PIP^a</td>
<td>200</td>
<td>67</td>
<td>89</td>
</tr>
<tr>
<td>FLE^c</td>
<td>200</td>
<td>67</td>
<td>95</td>
</tr>
<tr>
<td>OFL/LEV^d</td>
<td>400</td>
<td>133</td>
<td>81</td>
</tr>
<tr>
<td>NOR^c</td>
<td>200^c</td>
<td>67^c</td>
<td>91</td>
</tr>
<tr>
<td>CIP^e</td>
<td>200^c</td>
<td>67^c</td>
<td>92</td>
</tr>
<tr>
<td>LOM^c</td>
<td>200</td>
<td>67</td>
<td>95</td>
</tr>
<tr>
<td>DAN^c</td>
<td>50</td>
<td>16</td>
<td>88</td>
</tr>
<tr>
<td>ENR^f</td>
<td>200</td>
<td>67</td>
<td>96</td>
</tr>
<tr>
<td>DIF^g</td>
<td>200</td>
<td>67</td>
<td>94</td>
</tr>
<tr>
<td>TOS^d</td>
<td>8 000</td>
<td>2 667</td>
<td>100</td>
</tr>
</tbody>
</table>

^a Acronyms see Figure 2.1. ^b Mean values calculated from six replicate determinations. ^c Spiked amount in addition to the native CIP and NOR in sample. ^d Tosufloxacin (TOS) was used as surrogate standard. ^e Measurement at emission wavelength 445 nm. ^f Measurement at emission wavelength 500 nm.

tertiary effluent, breakthroughs occurred on the order of 20% and 15%, respectively.

The flow rate must be carefully controlled because this turned out to be a critical factor of the extraction method. The slow kinetics involved in ion exchange mechanisms required an accurate control of flow rate [25]. For the present method, the optimum flow rate was 1 mL/min. Several solvent mixtures were tested for the elution of FQs from MPC disk cartridges. A variety of aqueous solutions, containing either ammonia, triethylamine, or sodium hydroxide, at two concentration levels (1% and 5%) were evaluated. Eluent composition did not influence the final recovery rates,
although FQs eluted faster and less eluent volume was needed with increasing base strength. Additionally, methanol content was evaluated for recovery of FQs from disk cartridges, yielding optimal efficiency at 15% MeOH. Finally, a 5% ammonia solution in 15% MeOH was selected as extraction eluent. A 50-mL extracted primary effluent spiked with a standard mixture containing 20 ng of each FQ and 400 ng TOS surrogate standard were enriched to optimize the elution volume. Sequential elution with 5% ammonia solution in 15% MeOH using 1 to 6 mL collected separately were analyzed. The first mL eluted between 75 to 85% of most FQs, except for the more retained FQs (DAN, ENR, DIF and TOS surrogate standard) which were eluted up to 60%. To assure a quantitative elution, 2.5 mL were used as a final elution volume. To increase sensitivity of the presented method, evaporation of the enriched extracts could be included. However evaporation to dryness should be avoided since sorption to glass walls seems to occur.

2.3.2 Liquid Chromatography – Fluorescence Detection (LC-FLD)

The structural similarities of the examined FQs required an efficient separation that was achieved using a RP-AmideC16 column (Figure 2.2). As expected, no separation could be achieved for the enantiomeric forms OFL and LEV, thus, only total amounts of OFL/LEV are reported here. Using a conventional RP-C18 column (Supelco, Buchs, Switzerland) OFL/LEV and NOR co-eluted. Therefore, it can be inferred that the amide functionality of the RP-AmideC16 phase provides a selective hydrogen bonding with the amino group on the piperazine moiety of NOR. Resolution (Rs) [27] obtained for the FQs was always higher than 2, except for the critical pair DAN - ENR (Rs = 1.4) where baseline separation was difficult. Phosphoric acid was used as eluent A in the chromatographic separation, leading a working pH of 2.4. By applying a flat gradient from 5 to 7% acetonitril, most FQs were efficiently separated, although a steeper gradient to 17% acetonitril was needed to elute the more retained DIF and TOS surrogate standard.
Figure 2.2 Liquid chromatogram of a reference mixture of fluoroquinolones (see Figure 2.1 for acronyms). The pair OFL/LEV could not be separated because LEV is the pure L-enantiomer of the racemic mixture OFL. Modified RP-AmidoC16 column at 50°C; fluorescence detection (excitation at 278 nm, emission at 445 nm). The chromatographic conditions are described in the experimental section.

The separation of ionic analytes are influenced by changes in temperature, therefore FQ separation was studied at 30°C, 40°C, and 50°C. Because separation was improved with increasing column temperature, a working temperature of 50°C was used.

Because the inherent fluorescence of the FQs enables very sensitive and specific detection, fluorescence detection (FLD) was selected as the main identification and quantification technique at an excitation wavelength of 278 nm. Most of the FQs included in this study have fluorescence emission maxima at 445 nm, except for the pair OFL/LEV at 500 nm. Therefore, 445 nm was selected as the main analysis wavelength to obtain the maximum sensitivity for most FQs studied, with an extra run at 500 nm included as an alternative method when lower quantification limits for OFL/LEV were needed. Although fluorescence spectra are similar among FQs, their combination with individual retention times allows a quick and more reliable peak identification. Peaks were positively identified when sample and standard fluorescence spectra matched at least 90%.
2.3.3 Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)

Tandem mass spectrometry was selected as an additional identification technique for confirmation purposes. An example of a product ion mass spectrum of CIP with suggested fragments is shown in Figure 2.3.

![Mass spectrum of ciprofloxacin obtained by LC-ESI-MS/MS.](image)

**Figure 2.3** Mass spectrum of ciprofloxacin obtained by LC-ESI-MS/MS. Fragment ions such as \([\text{MH} - \text{CO}_2 - \text{C}_2\text{H}_5\text{N} - \text{C}_3\text{H}_4]^+\) (245 m/z) and \([\text{MH} - \text{H}_2\text{O} - \text{C}_2\text{H}_5\text{N}]^+\) (231 m/z) are characteristic for ciprofloxacin. The loss of \(\text{C}_2\text{H}_5\text{N}\) corresponds to the fragmentation on the piperazine group (Pip) and the loss of \(\text{C}_3\text{H}_4\) to the removal of the cyclopropyl substituent. Injection volume was 20 µL, positive ion mode, spray voltage 5.1 kV, orifice skimmer potential difference 36V, and ring electrode voltage 180 V.

In accordance with the literature [26], two major fragments of the protonated molecular ion \([\text{MH}]^+\) were observed in the multiple reaction monitoring (MRM) analysis mode, the loss of \(\text{H}_2\text{O}\) and \(\text{CO}_2\), both from the carboxylic group. Both primary product ions were found to fragment further by partial loss of the piperazine substituent (Pip) to different extents.
followed by a loss of the ethyl or cyclopropyl groups. In the case of NOR, initial loss of hydrofluoric acid also was observed. Further fragmentation to smaller product ions has been extensively described elsewhere [26, 28], but was not relevant for our purpose.

When using mass spectrometry as a confirmatory method, it is recommended that a minimum of three ions per compound are monitored [29]. For tandem mass spectrometry analysis, some references (e.g. [30]) suggest the necessity for diverse product ions for a high degree of specificity. In our case, apart from the parent ion [MH]$^+$, at least three MRM transitions per FQ investigated were monitored. The principal transitions monitored were: [MH]$^+$ → [MH – H$_2$O]$^+$, [MH]$^+$ → [MH – CO$_2$]$^+$, [MH]$^+$ → [MH – H$_2$O – Pip]$^+$, [MH]$^+$ → [MH – CO$_2$ – Pip]$^+$, [MH]$^+$ → [MH – H$_2$O – HF]$^+$. Losses of H$_2$O and CO$_2$ from the protonated molecular ion are common, but losses on the piperazine substituent in combination with the respective retention time assures a high specificity for FQs, and are therefore ideal for identification purposes.

### 2.3.4 Quantification and Quality Control

Few analytical methods on FQs apply internal standards for quantification [18]. In these cases, the internal standard is either (i) a structural FQ analogue of the studied compound, e.g. PIP [31-33], or DIF [34-36]), or (ii) a quinolone derivative of the investigated FQ [37, 38]. We expected the presence of different human- and veterinarian-use FQs, therefore the choice of a internal standard was restricted to a FQ not used in Switzerland. TOS was selected as surrogate standard, of which any human-use is restricted to Japan [39]. Because of the low natural fluorescence of TOS, any occasional occurrence of TOS in the Swiss aquatic environment should not interfere on its function as surrogate standard. TOS concentrations were added to 8 µg/L and 2.7 µg/L in primary and tertiary effluents, respectively.

Good linearity was observed over 2 orders of magnitude (except 1 order of magnitude for DAN), with correlation factors of $r^2 > 0.999$ (Table 2.2). Because of the large range in linearity, the response factor method was preferred over the least-square method for determining linearity [27]. Using
the least-square method, the slope and correlation coefficient were influenced heavily by data at high concentrations. Using the response factor approach, ratios of detector response (sensitivity) of each FQ versus analyte concentration were calculated. The instrumental quantification limit, IQL (10σ) was below the linear response range. As a result of this, we defined our IQL to be the second lower linear point within the linear part of the calibration curve (Table 2.2).

Table 2.2 Instrumental Quantification Limits (IQL) and Linear Range for the Determination of Fluoroquinolones

<table>
<thead>
<tr>
<th></th>
<th>IQLa (pg)</th>
<th>linear rangeb (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10σ</td>
<td>second lower</td>
</tr>
<tr>
<td></td>
<td></td>
<td>linear point</td>
</tr>
<tr>
<td>PIPc</td>
<td>200</td>
<td>300</td>
</tr>
<tr>
<td>FLEc</td>
<td>100</td>
<td>300</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>DIFc</td>
<td>200</td>
<td>300</td>
</tr>
</tbody>
</table>

aInstrumental quantification limit calculated as absolute amount injected in picograms (injection volume 200 µl). bInstrumental linear range extrapolated by considering recoveries of 100% and using the second lowest linear point (IQL). cMeasurement at emission wavelength 445 nm. dMeasurement at emission wavelength 500 nm.
The instrumental precision of the LC-FLD based on the standard deviation was the following: below 0.03 min for the retention times, 0.1 to 0.5% for peak area, and 0.05 to 0.4 for resolution. The overall precision of the method is indicated by a relative standard deviation generally < 10% (Table 2.1).

2.3.5 Application to Wastewater Samples

The described method was successfully applied to different urban wastewaters around Zurich, Switzerland. Figure 2.4 shows a typical LC-FLD chromatogram obtained from an extracted tertiary effluent sample.

![LC-FLD Chromatogram](image)

**Figure 2.4** LC-FLD result obtained from the extract of a tertiary effluent sample collected at the urban wastewater treatment plant of Dübendorf, Switzerland. Ciprofloxacin (CIP) and norfloxacin (NOR) can be observed. Tosufloxacin (TOS) was used as surrogate standard. Extracted volume was 150 mL, injection volume 200 µL, amido RP-C16 column, fluorescence detection (excitation at 278 nm, emission at 445 nm). The chromatographic conditions are described in the experimental section.
Commonly consumed FQs, CIP and NOR could be identified in primary and tertiary effluents by means of their fluorescence spectra and tandem mass spectrometry analysis. Using the latter technique, OFL/LEV were also occasionally detected in primary and tertiary effluents. However, these two FQs could not be determined by LC-FLD due to co-eluting peaks and weak fluorescence of OFL/LEV. No matrix component interfered with CIP or NOR identification and quantification using LC-FLD.

Determination of FQs in extracts of primary and tertiary effluents revealed concentrations of CIP and NOR between 249 and 405 ng/L and between 45 and 120 ng/L, respectively (Table 2.3).

**Table 2.3 Concentrations of Ciprofloxacin and Norfloxacin in Primary and Tertiary Effluents from Urban Wastewater Treatment Plants in Switzerland**

<table>
<thead>
<tr>
<th>effluent type</th>
<th>treatment plant</th>
<th>concentration$^b$ (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>primary effluent$^a$</td>
<td>Zurich-Glatt</td>
<td>CIP 331 ± 7</td>
</tr>
<tr>
<td></td>
<td>Zurich-Werdhölzli</td>
<td>251 ± 15</td>
</tr>
<tr>
<td></td>
<td>Kloten-Opfikon</td>
<td>405 ± 32</td>
</tr>
<tr>
<td></td>
<td>Dübendorf</td>
<td>249 ± 3</td>
</tr>
<tr>
<td>tertiary effluent$^a$</td>
<td>Zurich-Glatt$^c$</td>
<td>77 ± 5</td>
</tr>
<tr>
<td></td>
<td>Zurich-Werdhölzli</td>
<td>45 ± 3</td>
</tr>
<tr>
<td></td>
<td>Kloten-Opfikon</td>
<td>108 ± 10</td>
</tr>
<tr>
<td></td>
<td>Dübendorf</td>
<td>55 ± 2</td>
</tr>
</tbody>
</table>

$^a$Filtered 24 h-composite samples.  $^b$Mean and standard deviation of duplicate determinations.  $^c$This facility does not have advanced treatment, so values are referred to secondary effluents.

These results are consistent with the preference of CIP and NOR over other human-use FQs in Switzerland. No veterinarian-use FQs, such as ENR, DAN, or DIF were detected in urban wastewaters. Elimination rates
Chapter 2

during wastewater treatment based on measured FQ concentrations in primary and tertiary effluents varied between 70 to 80%, which is in good agreement with the elimination figures obtained in batch biodegradation experiments (60-85%) [10]. These findings clearly show that the load of FQs in wastewaters is reduced considerably during wastewater treatment, but because complete removal is not achieved, residual amounts of the FQs CIP and NOR are emitted into ambient waters. Assuming a dilution factor of 10 from the MECs here obtained for tertiary effluents, concentrations of CIP and NOR in surface water can be estimated around 5 to 12 ng/L. These values are a 1 to 2 orders of magnitude lower than the PEC estimated by conventional risk assessments [13, 16].

2.4 Conclusions

Solid-phase extraction using mixed phase cation exchange disk cartridges, followed by reversed-phase chromatography with fluorescence detection proved to be a new, specific, and quantitative method for the determination of trace amounts of a wide variety of FQ antibacterial agents in wastewater effluents. Enrichment using the mixed phase cation exchange disk cartridges, compared to a wide range of other materials, was shown to be the most appropriate extraction procedure for FQs in wastewaters.

Out of the ten investigated compounds, the FQs ciprofloxacin (CIP) and norfloxacin (NOR), could be determined quantitatively in urban wastewater treatment plant effluents. Both compounds are derived from human-use medication, contributing to around 90% of the domestic FQs consumed in Switzerland. None of the investigated veterinarian-use FQs were detected in urban wastewater, pointing out a different entry route of veterinary drugs into the environment (e.g. via manure dispersion and animal excretion onto soils). The overall removal of FQs from the aqueous phase during wastewater treatment was found to be efficient, though incomplete (between 70 to 80%), allowing trace amounts to be emitted into the receiving waters.

The analytical method described can serve as a monitoring tool to obtain detailed information on the occurrence, behavior and fate of FQ antibacterial agents in the aquatic environment. Furthermore, preceded by
an efficient extraction procedure, the presented method could serve to determine FQ content in sewage sludge. Additionally, newly emerging FQs (e.g. gemifloxacin, moxifloxacin) may be integrated and quantified without major changes needed. Finally, the present method is easy applicable because sample enrichment is simple and fast, the separation is reliable, and detection is highly sensitive.

Acknowledgement

The financial support of Bayer AG (Germany) is gratefully acknowledged. Eva M. Golet was also partly supported by the Swiss Federal Commission for Foreign Students. We would like to acknowledge Bayer AG (Germany), Roche Diagnostics GmbH (Germany), Abbott Laboratories (Switzerland), and Aventis Pharma AG (Switzerland) for providing reference compounds. We thank the following colleagues who have provided helpful comments on the manuscript: Christa McArdell, Michael Berg, Christopher Robinson, Erika Vye, Marc Suter, and Stephan Müller.

2.5 Literature Cited

Analytical Method for Sewage Sludge and Soil

A method for the quantitative determination of human-use fluoroquinolone antibacterial agents (FQs) ciprofloxacin and norfloxacin in sewage sludge and sludge-treated soil samples was developed. The accelerated solvent extraction was optimized with regard to solvents and operational parameters, such as temperature, pressure, and extraction time. A 50 mM aqueous phosphoric acid / acetonitrile mixture (1:1) was found to be optimum in combination with an extraction temperature of 100°C at 100 bar during 60 and 90 min for sewage sludge and sludge-treated soil samples, respectively. A clean-up step using solid-phase extraction substantially improved the selectivity of the method. Overall recovery rates for FQs ranged from 82 to 94% for sewage sludge and from 75 to 92% for sludge-treated soil, with relative standard deviations between 8 and 11%. Limits of quantification were 0.45 and 0.18 mg/kg dry matter for sewage sludge and sludge-treated soils, respectively. The presented method was successfully applied to untreated and anaerobically-digested sewage sludges and sludge-treated soils. Ciprofloxacin and norfloxacin were determined in sewage sludges from several wastewater treatment plants with concentrations ranging from 1.40 to 2.42 mg/kg d.m. Therefore, contrary to what may be expected for human-use pharmaceuticals, FQs may reach the terrestrial environment as indicated by the occurrence of FQs in topsoil samples from experimental soil fields, to which sewage sludge had been applied.
3.1 Introduction

Pharmaceuticals can enter the terrestrial environment either by direct disposal of sewage sludge and liquid manure to soils, or indirectly through animal medication in fish farming, where medicated feed pellets and feces can end up in sediments. Until now, the terrestrial environment has been studied regarding the environmental fate of animal-use pharmaceuticals because of their input into soils [1] and sediments [2-4]. For human-use pharmaceuticals, however, research has tended to follow their occurrence and fate in the aquatic environment [5-7], whereas terrestrial exposure is considered a minor route of pollution [8, 9]. This prioritization of the aquatic environment for human-use pharmaceuticals is a consequence of the polar nature of the majority of pharmaceuticals and metabolites, which have found their way to the environment via discharge of wastewater effluents into surface waters [5, 6].

Fluoroquinolones (FQs) are highly useful antibacterial agents, particularly because of their broad activity spectrum and good oral absorption [10, 11]. They are applied in both human and veterinary medicine, and almost identical structures are used for humans and animals [12, 13]. In Europe and in the United States, FQs were introduced for human-use in the mid-1980s and approved for therapeutic treatment of livestock in the mid-1990s. In Switzerland, FQs are being primarily applied for treating human infectious diseases (about 4 t/year), whereas in veterinary medicine their use is minor (about 0.5 t/year) [14]. Thus, the primary entry route of FQs into the Swiss environment is via human excretion in sewage, as confirmed by the occurrence of the leading human-use FQs ciprofloxacin and norfloxacin in wastewater effluents and surface waters [15]. During municipal wastewater treatment FQs are significantly eliminated from the water stream (79 to 87%) [15], and their fate is likely to be associated with sewage sludge because of their strong sorption properties [16, 18]. Application of sewage sludge to soils may therefore be a potential route for these human pharmaceuticals to enter the terrestrial environment.

Until now, analytical methods for the quantitative determination of FQs in the environment are only available for aqueous samples [19]. Various methods have been described for the extraction of the structurally related
quinolone oxolinic acid from marine and freshwater sediments [4, 20, 21]. However, relatively low recoveries [20] and precision [4] obtained with some of the analytical methods, spurred to the development of new, robust and powerful methods, which are applicable to a wide range of solid environmental matrices. Accelerated solvent extraction [22] (ASE) was selected for this study, because it has proven to be successful for the extraction of various organic pollutants in several solid environmental sample matrices [23-25].

This paper describes an extraction and clean-up procedure for FQs in sewage sludges and sludge-treated soils using ASE followed by solid-phase extraction (SPE), which in combination with previously described separation and detection methods (LC-fluorescence detection) [19] enables selective, reliable, and quantitative determinations. Ciprofloxacin and norfloxacin were chosen as specific FQ analytes, since they are the most prescribed FQs in Switzerland and they have already been detected in the aquatic environment here [15]. To the best of our knowledge, this article is the first to report on the occurrence of human-use pharmaceuticals in sewage sludges and in sludge-treated soils.

3.2 Experimental Section

3.2.1 Chemicals and Materials

Ciprofloxacin (CIP) was obtained from Bayer AG (Wuppertal, Germany), norfloxacin (NOR) from Sigma-Aldrich and tosufloxacin (TOS) from Abbott Laboratories (Baar, Switzerland). Tosufloxacin was used as surrogate standard, of which any human-use is restricted to Japan [26]. More details on standard solution preparation are given in ref [19]. Mixed-phase cation exchange disk cartridges (MPC, octyl phase and benzenesulfonate mixture, high density 12 µm particle size) were supplied by Varian International AG (Basel, Switzerland). All solvents were of reagent grade or higher quality. HPLC-grade water, acetonitrile, methanol, and isopropanol were purchased from Scharlau (Barcelona, Spain). ortho-Phosphoric acid (\(o\)-H\(_3\)PO\(_4\), 85%), and hydrochloric acid (HCl, 32%) were supplied by Merck (Dietikon, Switzerland). Sodium hydroxide (NaOH) and
sodium chloride (NaCl) were purchased from Fluka Chemie AG (Buchs, Switzerland).

3.2.2 Sample Collection

Sewage sludge. Two anaerobically digested sewage sludges and two untreated raw sludges were collected from mechanical-biological wastewater treatment plants near Zurich, Switzerland. The sewage sludge samples were dried at 60°C for 72 h, finely ground (< 0.5 mm), and stored in amber bottles at room temperature.

Sludge-treated soil. Topsoil samples (0–2.5 cm) from experimental sludge-treated fields were taken close to Zurich at two different locations: Wetzikon (7% organic carbon; 38% clay; 23% sand; 27% silt; pH 6.7) and Reckenholz (4% organic carbon; 18% clay; 54% sand; 21% silt; pH 6.9). Soil samples were collected using a steel cylinder 8 and 21 months after a sludge-application rate of 25 t/ha. This corresponds to a 5 times the normal amount that is allowed in Switzerland every third year (e.g. 5 t/ha every 3 years). The soil samples were dried at 40°C, passed through a 0.2 mm sieve, and stored in the dark at room temperature [27].

3.2.3 Extraction, Clean-up and Analysis

Dried samples of 200 mg sewage sludge and 500 mg sludge-treated soil were weighed, transferred into 11 mL steel extraction cells from Dionex, and thoroughly mixed with ~10 g quartz sand. When spike and recovery experiments were performed, spiked samples were allowed to equilibrate overnight before extraction. A Dionex ASE 200 accelerated solvent extractor (Sunnyvale, CA) equipped with a solvent controller was used for extraction. Various aqueous mixtures were tested as extracting solvent, and a 50 mM aqueous phosphoric acid (pH 2.0) and acetonitrile mixture (1:1, v/v) was found to be optimal. The selected operating conditions were set as default and were as follows: extraction temperature, 100°C; extraction pressure, 100 bar; pre-heating period, 5 min; static extraction period, 15 min; extraction volume ~22 mL; solvent flush, 150% of the cell volume;
nitrogen purge, 300 s; and number of extraction cycles, 4 and 6 for sewage sludge and sludge-treated soil samples, respectively. The ASE extracts were transferred to 200 mL volumetric amber flasks, filled up with distilled water, and adjusted with 32% HCl to pH 3.0. The samples were then shaken and spiked with tosufloxacin surrogate standard (TOS-IS), yielding to surrogate standard concentrations of 100 µg/L for sewage sludge and 50 µg/L for sludge-treated soil samples. In addition, the ion content of the diluted extracts was measured with a conductivity cell (Testo 240, Germany), for it could interfere with the clean up [19]. Subsequently, a 10 mL aliquot of the diluted sludge or soil extracts was extracted by mixed-phase cation exchange (MPC) disk cartridge, and followed immediately by liquid chromatography fluorescence detection (LC-FLD) as described in ref [19].

3.2.4 Validation Parameters and Quality Control

To avoid photodegradation [28], amber vials were used for preparing and storing standard solutions, as well as for the entire analytical procedure. Stability of FQs during sample pre-treatment was checked by spiking a fresh sludge sample, which was subsequently allowed to dry at 60°C for 96 h [29]. Extraction cells filled with quartz sand (inert matrix) were spiked with 0.5 µg FQs, extracted at 100 °C and analyzed using the same protocol, including sample clean-up, to confirm that no thermal degradation of FQs occurred during ASE extraction. TOS-IS was added as a surrogate standard to all samples to correct for losses during sample clean-up. Procedural blanks (quartz sand) were extracted for each set of 10 samples to control for laboratory contamination. An instrumental blank for the LC-FLD was run after every fourth sample to assure no carryover during analysis. Duplicate ASE extractions were carried out for each sample. Breakthrough on MPC disk cartridges was investigated by extracting aliquots of 5, 10, 20, 50 mL of the diluted sewage sludge extracts spiked with 100 µg/L TOS-IS in two stacked MPC disk cartridges. Multiple sequential extractions of the same sewage sludge (6 x 15 min) and sludge-treated soil (8 x 15 min) sample were conducted to assure quantitative extraction. The overall method accuracy was determined by
recovery studies of spiked sewage sludge and sludge-treated soil samples at different concentrations; duplicates of spiked sewage sludge at 2.5, 5, 10, 20 mg/kg and spiked soils at 0.25, 0.5, 1, 2 mg/kg were analyzed. The latter experiment was also used for standard addition studies. In such approach, the original concentration in the unspiked sample was obtained after extrapolating from the standard addition calibration to ‘zero spiked concentration’ [30]. Standard addition quantitation was then compared to the concentration obtained using an internal calibration to review method accuracy. The precision of the entire procedure for sewage sludge and sludge-treated soil samples was determined by extracting 6 replicates containing native FQs, and spiked with a TOS-IS surrogate standard prior to clean-up. Limit of detection (LOD) was defined as 3 times the standard deviation \((n = 10)\) of FQ measurements in pure solvents at 330 pg on column. Because the limit of quantification (LOQ) calculated as 10 times the standard deviation was below the linear range, LOQ was set as the second lowest linear point of the calibration curve [19].

3.3 Results and Discussion

3.3.1 Selection of Extraction Technique

Because most methods described for the extraction of the quinolone oxolinic acid from sediments are based on manual or mechanical shake [20, 21], preliminary experiments were focused on the use of ultra-sonication at room temperature. After extracting a same sample for several hours \((8 \times 30\) min), only very low extraction efficiencies were achieved. Accelerated solvent extraction (ASE) was then chosen, because of the stronger extraction power conferred due to enhanced solvent temperature. In addition to the high extraction yield of ASE, its accelerated extraction and the high degree of automation allowed a significantly higher sample throughput.
3.3.2 ASE Extraction

In order to understand the way in which ASE operational parameters affect the extraction of FQs from environmental samples, individual operational variables were considered. At first, various extraction solvent mixtures were tested. Once the optimum solvent mixture was found, the impact of varying temperature, pressure, and extraction time was studied. Finally, other factors were investigated regarding sample preparation prior to ASE extraction, such as sample pre-wetting, swelling agent, and sample size. The development of the extraction procedure was performed with native digested sewage sludge and with sludge-treated soil samples. The obtained results presented as FQ concentration in sample are shown in Tables 3.1 to 3.4.

Extraction solvent. Table 3.1 shows the results of different aqueous mixtures in combination with an organic modifier (acetonitrile, methanol, or isopropanol) regarding the extraction efficiency of FQs from sewage sludge. Pure organic solvents were not tested, since they were already shown to be inefficient for extracting the quinolone oxolinic acid from sediments [4]. Several authors have taken advantage of the increased solubility effects of hot liquid water (subcritical water) to extract organic pollutants from various solids environmental samples [31-33], and its use recently the use of water as an ASE extraction solvent has recently been reported [24-25]. Among the organic modifiers studied in this study, acetonitrile showed better results than methanol or isopropanol. Following, different aqueous/acetonitrile mixtures (1:3, 1:1, 3:1) were studied. The extraction efficiency varied slightly as a function of the aqueous/organic modifier ratio, with the highest recoveries obtained by at a ratio of 1:1.

Because of the zwitterionic character of FQs (pK_{\text{COOH}} = 5.9 - 6.3, pK_{\text{NH2}} = 7.9 - 10.2) [34], the effect of pH on the extraction efficiency of FQs was studied (Table 3.1). Under elevated temperature, strong acids (e.g. hydrochloric acid or nitric acid) oxidated the steel components of the extraction cell [29, 35] so that the use of such acids had to be avoided. Thus, a diluted phosphoric acid (50 mM, pH 2.0) solution was selected as acidic extraction solvent, and a NaOH (0.1 M, pH 11.0) solution as a basic extraction solvent. The change in pH improved the results with maximum recoveries achieved at an acid pH. Because at low pHs the anionic sites of
FQs and the sewage sludge are protonated it could be possible that the electrostatic repulsion between FQs and the sewage sludge surface might partly account for the better extraction efficiencies at acidic pHs. Moreover, the enhanced extraction efficiency observed at high and low pHs could also be due to the increased aqueous solubility of FQs at extreme pHs, reaching a minimum at neutral pH (zwitterionic form) [36]. On the other hand, the fact that hot phosphoric acid has been reported to enhance the extraction of humic acids from soils [37] could also explain the better extraction of FQs, in case that FQs would be associated with the organic carbon fraction of the sample. Based on the presented results (Table 3.1) a 50 mM aqueous phosphoric acid / actonitrile mixture (1:1) was selected as the extraction solvent.

**Table 3.1** ASE Extraction Efficiency of FQ from Sewage Sludge using Various Aqueous Mixtures. In bold: Selected Operating Conditions

<table>
<thead>
<tr>
<th>Extraction solvent and pH</th>
<th>Ciprofloxacin (mg/kg d.m.)</th>
<th>Norfloxacin (mg/kg d.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH = 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM o-phosphoric acid / actonitrile (1:1)</td>
<td>2.44 ± 0.18</td>
<td>2.36 ± 0.10</td>
</tr>
<tr>
<td>50 mM o-phosphoric acid / methanol (1:1)</td>
<td>2.31 ± 0.01</td>
<td>2.21 ± 0.04</td>
</tr>
<tr>
<td>50 mM o-phosphoric acid / isopropanol (1:1)</td>
<td>2.01 ± 0.12</td>
<td>2.22 ± 0.11</td>
</tr>
<tr>
<td><strong>pH = 7</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water / actonitrile (1:1)</td>
<td>1.69 ± 0.12</td>
<td>1.60 ± 0.01</td>
</tr>
<tr>
<td>water / actonitrile (1:3)</td>
<td>1.14 ± 0.10</td>
<td>1.28 ± 0.05</td>
</tr>
<tr>
<td>water / actonitrile (3:1)</td>
<td>1.27 ± 0.06</td>
<td>1.18 ± 0.11</td>
</tr>
<tr>
<td>water / methanol (1:1)</td>
<td>1.63 ± 0.06</td>
<td>1.59 ± 0.07</td>
</tr>
<tr>
<td>water / isopropanol (1:1)</td>
<td>1.53 ± 0.04</td>
<td>1.52 ± 0.07</td>
</tr>
<tr>
<td><strong>pH = 11</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 M NaOH / actonitrile (1:1)</td>
<td>2.19 ± 0.09</td>
<td>2.30 ± 0.08</td>
</tr>
</tbody>
</table>

*Mean and standard deviation of duplicate determinations. ASE operating conditions: 100°C, 100 bar, 4 cycles of 15 min.*
ASE operating parameters. Besides the extraction solvent, three operating parameters govern the extraction efficiency of ASE: temperature, pressure and extraction time. For subcritical water extraction, however, the efficiency seems to depend primarily on the extraction temperature and time, and pressure has only a minor influence [31]. The effect of temperature, pressure and time on the extraction efficiency of FQs from sewage sludge is presented in Table 3.2.

(i) Temperature is expected to have a pronounced effect on the performance of ASE due to the importance of mass transfer kinetics and solubility. We studied the effect of temperature on extraction efficiency of FQs by varying the system oven from 50°C to 150°C at increments of 25 °C in the different experiments. At higher extraction temperatures, it was observed that increasingly darker extracts were obtained, indicating a larger extraction of soluble organic solid matter. From 50 to 100°C the extraction efficiency of FQs was influenced by temperature, most probably due to an increase in extraction kinetics. Contrary, between 100 to 150 °C, the extraction efficiency remained constant, which indicates no influence of temperature on the equilibrium partitioning (ΔH ~ 0). So a working temperature of 100°C was selected to avoid oxidation of the extraction cell that may occur when using phosphoric acid at higher temperatures [29, 35].

(ii) The effect of pressure on extraction efficiency of FQs was investigated at pressures ranging from 50 to 150 bar. Pressure is used to keep the extraction solvents liquid when the solvents are heated above their boiling points. Within the selected ranges, pressure changes showed no significant effects on the extraction efficiency of FQs, and a working pressure of 100 bar was considered to be appropriate (Table 3.2).

(iii) To investigate the effects of extraction time on extraction efficiency, time was varied from 20 to 80 min by prolonging the static time (5 to 20 min) and by augmenting the number of extraction cycles (from 2 to 4 cycles). In the static method, the long exposure to solvent allows the matrix to swell, thus improving the penetration of solvent into the sample interstices and the contact of the solvent with the analyte. On the other hand, by splitting the extraction time from one to more cycles, the introduction of fresh solvent maintains favorable solvent/sample equilibrium, and hence, improving partitioning into the liquid phase.
Table 3.2 Effects of Temperature, Pressure, and Time on the ASE Extraction of FQs from Sewage Sludge
In bold: Selected Operating Conditions

<table>
<thead>
<tr>
<th>compound</th>
<th>50°C</th>
<th>75°C</th>
<th>100°C</th>
<th>125°C</th>
<th>150°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ciprofloxacin</td>
<td>1.49 ± 0.01</td>
<td>2.25 ± 0.23</td>
<td><strong>2.52 ± 0.04</strong></td>
<td>2.58 ± 0.07</td>
<td>2.61 ± 0.15</td>
</tr>
<tr>
<td>norfloxacin</td>
<td>1.46 ± 0.17</td>
<td>2.13 ± 0.14</td>
<td><strong>2.40 ± 0.17</strong></td>
<td>2.45 ± 0.10</td>
<td>2.53 ± 0.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>compound</th>
<th>50 bar</th>
<th>75 bar</th>
<th><strong>100 bar</strong></th>
<th>125 bar</th>
<th>150 bar</th>
</tr>
</thead>
<tbody>
<tr>
<td>ciprofloxacin</td>
<td>2.34 ± 0.12</td>
<td>2.45 ± 0.09</td>
<td><strong>2.51 ± 0.17</strong></td>
<td>2.42 ± 0.09</td>
<td>2.41 ± 0.09</td>
</tr>
<tr>
<td>norfloxacin</td>
<td>2.47 ± 0.04</td>
<td>2.47 ± 0.06</td>
<td><strong>2.50 ± 0.12</strong></td>
<td>2.45 ± 0.10</td>
<td>2.32 ± 0.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>compound</th>
<th>20 min</th>
<th>40 min</th>
<th>40 min</th>
<th>50 min</th>
<th><strong>60 min</strong></th>
<th>80 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>ciprofloxacin</td>
<td>2.23 ± 0.05</td>
<td>2.44 ± 0.14</td>
<td>2.10 ± 0.04</td>
<td>2.30 ± 0.13</td>
<td><strong>2.45 ± 0.17</strong></td>
<td>2.47 ± 0.13</td>
</tr>
<tr>
<td>norfloxacin</td>
<td>2.06 ± 0.08</td>
<td>2.27 ± 0.02</td>
<td>1.91 ± 0.18</td>
<td>2.10 ± 0.20</td>
<td><strong>2.35 ± 0.13</strong></td>
<td>2.38 ± 0.21</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean concentration and standard deviation of duplicate determinations.
In our study, we observed a raise in extraction efficiency of FQs when increasing the static time from 5 to 10 min, but no substantial improvement was observed when prolonging it to 15 or 20 min. Additionally, by increasing the continuous exposure to fresh solvent from 2 to 4 times, the partitioning of the FQs into the mobile phase was enhanced and thus also the extraction efficiency. Although a 10 min extraction performed four times with partial solvent exchange offered optimal extraction efficiency, 4 × 15 min was selected as operating conditions to assure quantitative extraction even for matrices that are difficult to extract.

To evaluate the ability of the method to quantitatively extract FQs from the studied matrices, multiple sequential extractions of a sample were performed. This approach assumes that the final extraction removes all of the native analytes and that no additional analytes are associated with the sample by stronger interactions than the analytes that were already extracted [38]. For the same sample, up to 6 extraction cycles of 15 min for sewage sludge and 8 × 15 min for sludge-treated soils were separately collected and analyzed. The data from this series of experiments are shown in Figure 3.1. Defining the overall recovery of native FQs after multiple sequential extractions as 100%, the recovery for the sewage sludge sample in the first extract varied from 52 and 65%, with an additional 28−32% and 7−14% in the second and third extracts, respectively. For sludge-treated soils, between 31 and 47% of native FQ was recovered in the first extract followed by an additional 14−30% in the second extract, 14−24% in the third, 8−13% in the fourth, 6−9% in the fifth, and 0−3% in the sixth extract. In order to ensure a quantitative extraction, the number of static extraction cycles was set to 4 × 15 min for sewage sludge and to 6 × 15 min for sludge-treated soils. The fact that FQs associated with sewage sludge are easier extractable than those from sludge-treated soil could indicate that the extraction efficiency depends upon the type of matrix. On the other hand, these results could also be attributable to sample aging, so that stronger interactions between FQs and active sites in the sludge-treated soil samples (sludge disposal October 1999) would be expected compared to sewage sludge samples (sampling October 2000).
Figure 3.1 Extraction yields of FQs after multiple sequential extractions of 15 min each with 50 mM phosphoric acid / acetonitrile (1:1) at 100°C and 100 bar. n.d.: not detectable.

Pre-extraction sample preparation. Among other experimental parameters that could affect ASE extraction, we considered sample preparation as the most relevant. To allow a greater exposure surface and hence better diffusion of the extraction solvent into the matrix interstices, a thorough mixture between the sample and the quartz sand in the cell was required. In some studies from the literature, the effect of pre-wetting the
matrix [39] or electrolyte addition [40] resulted in better extraction yields, especially when extracting native residues. In both cases, the long exposure to solvent allowed the matrix to swell, thus improving the penetration of solvent into its interstices and increasing analyte recovery. Moreover, the addition of high concentrations of sodium ions in the second study [40] was reported to allow an interlayer swelling of the clay structure of the sediments. In our case, we investigated the influence of sample pre-wetting and presence of swelling agent, by allowing sewage sludge and sludge treated soil to swell for at least 15 h before extraction either with water or with aqueous solutions containing different amounts of NaCl (pH 5.5). The obtained results are presented in Table 3.3. As can be seen, sample pre-wetting or the presence of swelling agent did not give higher efficiencies in either sewage sludge or soil extractions. On the contrary, the higher the salt content the lower the extraction efficiency, which might be attributed to a higher ionic activity in water due to the presence of $\text{Cl}^-$ ions [36]. Such an approach was therefore not further investigated and the samples were directly ASE extracted, reducing overall-duration of sample preparation.

**Table 3.3 Effect of pre-wetting and the presence of a swelling agent on the ASE Extraction of FQs from Sewage Sludge and Sludge-Treated Soil Samples. In bold: Selected Operating Conditions**

<table>
<thead>
<tr>
<th>pre-wetting swelling agent</th>
<th>sewage sludge$^b$</th>
<th>sludge-treated soil$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ciprofloxacin</td>
<td>norfloxacin</td>
</tr>
<tr>
<td>none</td>
<td>$2.27 \pm 0.09$</td>
<td>$2.19 \pm 0.04$</td>
</tr>
<tr>
<td>water</td>
<td>$2.00 \pm 0.20$</td>
<td>$1.94 \pm 0.03$</td>
</tr>
<tr>
<td>NaCl 0.1 M</td>
<td>$2.21 \pm 0.21$</td>
<td>$2.21 \pm 0.15$</td>
</tr>
<tr>
<td>NaCl 0.3 M</td>
<td>$1.78 \pm 0.20$</td>
<td>$1.76 \pm 0.11$</td>
</tr>
</tbody>
</table>

$^a$Mean concentration and standard deviation of duplicate determinations. $^b$ASE operating conditions: 100°C, 100 bar, 4 cycles of 15 min. $^c$ASE operating conditions: 100°C, 100 bar, 6 cycles of 15 min.
Table 3.4 shows the dependence of extraction efficiency on sample size under optimal ASE conditions. Sample size was varied from 50 to 500 mg for sewage sludge and from 200 to 2000 mg for sludge-treated soil samples. Regardless of sample size, extraction efficiency was essentially identical up to 300 mg sewage sludge and 1000 mg soil. On the contrary, extraction yields decreased significantly for larger sample sizes, especially for 500 mg sewage sludge and 2000 mg soils, suggesting that higher extraction volumes are required when extracting larger sample amounts. The selected working sample size was 200 mg for sewage sludge and 500 mg for sludge-treated soil to allow sufficiently low limits of quantification and a reliable quantitative analysis.

Table 3.4 Effect of Sample Size on ASE Extraction of FQs from Sewage Sludge and Sludge-Treated Soil Samples. In bold: Selected Operating Conditions

<table>
<thead>
<tr>
<th>compound</th>
<th>concentration(^a) (mg/kg d.m.)</th>
<th>sewage sludge(^b)</th>
<th>sludge-treated soil(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 mg</td>
<td>100 mg</td>
<td>200 mg</td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.44 ± 0.08</td>
<td>2.33 ± 0.06</td>
<td>2.42 ± 0.06</td>
</tr>
<tr>
<td>norfloxacin</td>
<td>2.45 ± 0.10</td>
<td>2.49 ± 0.06</td>
<td>2.37 ± 0.07</td>
</tr>
</tbody>
</table>

\(^a\)Mean concentration and standard deviation of duplicate determinations. \(^b\)ASE operating conditions: 100°C, 100 bar, 4 cycles of 15 min. \(^c\)ASE operating conditions: 100°C, 100 bar, 6 cycles of 15 min.
3.3.3 Post-Extraction Sample Preparation and Clean-up

Sample preparation after extraction is considered an integral part of an extraction procedure, in which a simplified procedure is preferred. Nonetheless, to achieve a selective method for FQs a clean-up step was required. The post-extraction sample preparation was optimized by employing the solid-phase extraction already described for FQs in wastewater [19]. Mixed-phase cation exchange (MPC) disk cartridges were shown to be most selective for FQs and to provide the best recovery rates [19], presumably because of a more specific interaction between the non-polar and ionic sites of FQs and the sorbent. The fact that in the present study an organic modifier was used as extraction solvent in the ASE appeared to be a major problem for the clean-up with MPC disc cartridges, to which aqueous samples are preferable. To overcome this, the ~22 mL ASE extract was diluted up to 200 mL with water, so that the organic content was reduced to about 5% acetonitrile / 95% water. Out of the diluted extract, a 10 mL aliquot was then solid-phase extracted with MPC disk cartridges. This dilution approach was favored to a complete evaporation of the acetonitrile fraction, which would need more sample handling, lead to longer post-extraction sample pre-treatment, and include the uncertainty of FQ losses during sample evaporation because of possible sorption to glass.

3.3.4 Method Validation and Quality Control

The optimized ASE-SPE LC-FLD method was validated for both sewage sludge and sludge-treated soil samples (selected conditions summarized in Experimental Section) with the most important parameters presented in Table 3.5.

Thermal degradation studies. Because ASE extractions were performed at elevated temperatures, thermal degradation was of potential concern. Overall average recoveries obtained for spiked FQs on clean quartz sand were 99 ± 3% for CIP and 95% ± 3% for NOR, giving no evidence of thermal degradation.
**Breakthrough experiments.** FQs were quantitatively isolated from aliquots of 5 and 10 mL of diluted ASE extracts of sewage sludge by the first of two stacked MPC disk cartridges. Occasionally, residues of the FQ (<5% of the amount found in the first disk cartridge) were detected in the second cartridge, which could be derived from the remaining ~5% acetonitrile content in the diluted extracts. When enriching aliquots of 20 mL and 50 mL of diluted ASE extracts, breakthrough occurred on the order of 10% and 20%, respectively. Finally, an aliquot of 10 mL of the diluted extracts was used for sample clean-up. Because high ion content in a sample could induce earlier breakthrough and thus reduce extraction efficiencies of FQs, conductivity of diluted extracts was controlled [19]. Conductivity of diluted extracts following pH adjustment was always less than 1.5 mS/cm, thus method validation was only applicable for samples not exceeding this value.

**Accuracy and precision.** Spiked analytes are not exposed to the same matrix active sites as the native pollutants. Therefore, recoveries obtained with spiked samples might be different than with native samples. In our case, however, multiple sequential extraction from sewage sludge and sludge-treated soils confirmed that a quantitative extraction was achieved for native FQs (see Figure 3.1), therefore we adopted such spiking experiments to evaluate the overall method recoveries, including sample clean-up. Overall method recoveries \( (n = 8) \) ranged from 82 to 94% for sewage sludge \( (m_{\text{CIP}} = 89 \pm 4\%, m_{\text{NOR}} = 88 \pm 4\%, m_{\text{TOS-IS}} = 93 \pm 5\%) \), and from 75 to 92% for sludge-treated soil samples \( (m_{\text{CIP}} = 80 \pm 6\% , m_{\text{NOR}} = 84 \pm 5\%, m_{\text{TOS-IS}} = 87 \pm 8\%) \). Such results are comparable to those obtained for wastewater \( (m_{\text{CIP}} = 92 \pm 5\%, m_{\text{NOR}} = 91 \pm 10\%, m_{\text{TOS-IS}} = 100 \pm 5\%) \) [19], and surface water \( (m_{\text{CIP}} = 85 \pm 10\%, m_{\text{NOR}} = 87 \pm 9\%, m_{\text{TOS-IS}} = 97 \pm 6\%) \) [15], thus the FQ losses were associated with the clean-up procedure more than to the ASE extraction. Spiking experiments at different concentrations were also used for standard addition curves, with the aim to ensure the accuracy of the quantified concentrations in native sewage sludge and sludge-treated soils samples. Relative standard variation between internal standard and standard addition quantification varied between 4–6% for values above the LOQ, and up to 15% for concentrations below the LOQ. Relative standard deviations \( (n = 6) \) in the range of 8 to 11% were achieved for extraction, clean-up and analysis of
six replicates of sewage sludge and sludge-treated soils containing native FQs.

**LOD/LOQ and linearity.** Limits of detection and quantification for 200 mg sewage sludge and for 500 mg soil samples are presented in Table 3.5. The linear range of FQ analysis was defined from the LOQ (second lower linear point) until 50 ng on column, corresponding to 75 mg/kg d.m. (dry matter) for sewage sludge and 30 mg/kg d.m. soil samples.

**Table 3.5 Quality Control Parameters**

<table>
<thead>
<tr>
<th>parameter</th>
<th>sewage sludge&lt;sup&gt;e&lt;/sup&gt;</th>
<th>sludge-treated soil&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ciprofloxacin</td>
<td>norfloxacin</td>
<td>ciprofloxacin</td>
</tr>
<tr>
<td>recovery&lt;sup&gt;a&lt;/sup&gt; (n = 8), %</td>
<td>89 ± 4</td>
<td>88 ± 4</td>
</tr>
<tr>
<td>precision&lt;sup&gt;b&lt;/sup&gt; (n = 6), %</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>limit of detection&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.12</td>
<td>0.05</td>
</tr>
<tr>
<td>limit of quantification&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.45</td>
<td>0.18</td>
</tr>
<tr>
<td>linear range&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.45 - 75</td>
<td>0.18 - 30</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean recoveries and standard deviation of n given replicates of spiked sewage sludge or sludge-treated soil sample.  
<sup>b</sup>Given as relative standard deviation of n replicates.  
<sup>c</sup>ASE operating conditions: 100°C, 100 bar, 4 cycles of 15 min.  
<sup>d</sup>ASE operating conditions: 100°C, 100 bar, 6 cycles of 15 min.  
<sup>d</sup>In mg/kg d.m.

### 3.3.5 Determination of FQs in Environmental Samples

Several environmental samples were analyzed in order to assess the applicability and performance of the developed method. The two FQs occurring in the Swiss aquatic environment CIP and NOR were quantified in several sewage sludges taken from municipal treatment plants in the region of Zurich (Table 3.6). Concentrations of FQs in untreated raw sewage were between 1.40 and 2.03 mg/kg d.m. sewage sludge, being in the same range as those found in digested sludge (2.13 to 2.42 mg/kg d.m.).
The relatively high levels in sewage sludge indicate the high affinity of FQs towards solids and favored sorption to sewage sludge during wastewater treatment.

The FQs CIP and NOR also could be determined in two sludge-treated soil samples from experimental fields near Zurich (Table 3.6 and Figure 3.2). The samples represent two different soils with different composition (see Experimental Section) and treated with sludge at a rate of 25 t/ha. Note that the sludge application ratio was 5 times the allowed amount in Switzerland every third year (5t/ha).

Table 3.6 Concentrations of ciprofloxacin and norfloxacin in sewage sludge from different wastewater treatment plants and sludge-treated soil samples from different experimental fields to which sewage sludge had been applied at 25 t/ha

<table>
<thead>
<tr>
<th>sample type</th>
<th>sampling site</th>
<th>ciprofloxacin (mg/kg d.m.)</th>
<th>norfloxacin (mg/kg d.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated raw sludge</td>
<td>Dübendorf</td>
<td>1.40 ± 0.12</td>
<td>1.54 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Zurich-Werdhölzli</td>
<td>2.03 ± 0.20</td>
<td>1.96 ± 0.15</td>
</tr>
<tr>
<td>digested sludge</td>
<td>Kloten-Opfikon</td>
<td>2.42 ± 0.06</td>
<td>2.37 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Zurich-Werdhölzli</td>
<td>2.27 ± 0.20</td>
<td>2.13 ± 0.19</td>
</tr>
<tr>
<td>sludge-treated soil</td>
<td>Wetzikon</td>
<td>0.35 ± 0.04</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>(8 months after application)</td>
<td>Reckenholz</td>
<td>0.40 ± 0.03</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>sludge-treated soil</td>
<td>Wetzikon</td>
<td>0.28 ± 0.01</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>(21 months after application)</td>
<td>Reckenholz</td>
<td>0.27 ± 0.04</td>
<td>0.30 ± 0.01</td>
</tr>
</tbody>
</table>

*a Mean concentration and standard deviation of duplicate determinations. *b ASE conditions: 100°C, 100 bar, 4 cycles of 15 min. *c ASE conditions: 100°C, 100 bar, 6 cycles of 15 min.
Topsoil concentrations 8 months after sludge application ranged from 0.29 to 0.40 mg FQ/kg d.m., and after 21 months from 0.27 to 0.30 mg FQ/kg d.m. Such concentrations are within the range of those found for veterinary pharmaceuticals in soils after liquid-manure dispersion (0.04 - 0.20 mg/kg tetracycline) [1]. Despite no significant reduction in FQ concentration in topsoil layers could be observed in either of the two soils in the 13-month study period, partial FQ degradation could have occurred previous to the first sampling campaign (8 months after sludge application). Interestingly, two additional peaks appear in the more polar fraction of the sludge-treated soil chromatogram, which could well indicate some FQ degradation products (generally more polar than the parent compound). In any case, the results presented here demonstrate that trace amounts of FQs persist (and may well accumulate) in the terrestrial environment subsequent to sludge application.

**Figure 3.2** LC-FLD chromatogram of a topsoil sample (0–2.5 cm) collected 21 months after sludge application from the experimental field at the Reckenholz area. Determined concentrations: 0.27 mg/kg d.m. ciprofloxacin and 0.30 mg/kg d.m. norfloxacin.
3.4 Conclusions

This work presents a new and highly selective ASE-SPE extraction procedure for the determination of FQs in solid environmental samples. Its applicability for extracting FQs from sewage sludge and solid matrices at the µg/kg level is demonstrated, and the suitability of the previously reported LC-FLD method to solid matrix extracts is proven. The ASE extraction efficiency particularly depended on the composition and pH of the solvent and the temperature and extraction time selected.

The here presented results confirmed the hypothesis that FQs become highly enriched in sewage sludge. Additionally, this study demonstrated that FQs reach the terrestrial environment via the disposal of sewage sludge to agricultural soils. Moreover, the persistence of trace amounts of FQs in sludge-treated soils up to several months after application was demonstrated.

The developed method can be a valuable tool for investigating the fate and behavior of FQs during wastewater treatment by performing mass balance studies through the analysis of sludge, and to further assess the occurrence, persistence, and transport of FQs in sludge-treated soils. These approaches are currently being addressed in our laboratory confirming the usefulness of this analytical method for process-oriented field studies [41]. Since both FQs studied showed identical extraction behavior, besides ciprofloxacin and norfloxacin the described method should also be applicable to other human- or veterinary-use FQs (e.g. sarafloxacin, enrofloxacin). Furthermore, applications to other solid environmental matrices such as sediments should be possible without major modifications.

Acknowledgement

The financial support of Bayer AG (Germany) is gratefully acknowledged. We would like to thank Valentin A. Lanz for preliminary experiments and Franz Günter Kari and Christian Schaffner for providing the soil samples. We also thank the following colleagues for reviewing the manuscript: K.-U. Goss, C. McArdell, C. Robinson and T. Schmidt.
Chapter 3

3.5 Literature Cited


Exposure and Risk Assessment in Wastewater and River Water

The mass flows of fluoroquinolone antibacterial agents (FQs) were investigated in the aqueous compartments of the Glatt Valley watershed, a densely populated region in Switzerland. The major human-use FQs consumed in Switzerland, ciprofloxacin (CIP) and norfloxacin (NOR), were determined in municipal wastewater effluents and in the receiving surface water, the Glatt River. Individual concentrations in raw sewage and in final wastewater effluents ranged from 255 to 568 ng/L and from 36 to 106 ng/L, respectively. In the Glatt River, the FQs were present at concentrations below 19 ng/L. The removal of FQs from the water stream during wastewater treatment was between 79 and 87%. During the studied summer period, FQs in the dissolved fraction were significantly reduced downstream in the Glatt River (15-20 h residence time) (66% for CIP, and 48% for NOR). Thus, after to wastewater treatment, transport in rivers causes an additional decrease of residual levels of FQs in the aquatic environment. Refined predicted environmental concentrations for the study area compare favourably with the measured environmental concentrations (MEC) obtained in the monitoring study. Total measured FQ concentrations occurring in the examined aquatic compartments of the Glatt Valley watershed were related to acute ecotoxicity data from the literature. The risk quotients obtained (MEC/PNEC < 1) following the recommendations of the European guidelines or draft documents suggest a low probability for adverse effects of the occurring FQs, either on microbial activity in WWTPs, or on algae, daphnia and fish in surface waters.
4.1 Introduction

The increasing public concern about the hazards associated with pharmaceutical residues in the environment has triggered activities by scientific, industrial, and regulatory bodies to assess the exposure and effects of these bio-active chemicals in different environmental compartments. These activities led to regulatory proposals in the United States [1] and the European Union [2,3]. The goal of these initiatives is to ensure a safe environment for aquatic and terrestrial organisms as well as satisfactory water quality.

The principal entry route of human pharmaceuticals into the environment is via wastewater to receiving surface waters. Several pharmaceuticals have already been detected in wastewater effluents, river waters and groundwaters, where they typically occur in low micrograms to nanograms per liter concentrations (reviewed in refs [4-6]). While such low concentrations probably are not pharmacologically active to humans, there is some evidence that they might be of environmental relevance [7]. Antimicrobials are the most often discussed pharmaceuticals because of their potential role in the spread and maintenance of (multi-)resistance of bacterial pathogens. Penicillins, tetracyclines, sulfonamides, and macrolides have been broadly monitored in various aquatic compartments of the environment [8-12]. However, much less is known about the environmental occurrence and fate of fluoroquinolones [12-13].

Fluoroquinolones (FQs) represent an important class of antibacterial agents that are widely used against a great number of human and animal infectious diseases. One example is ciprofloxacin, which is the medicine of choice for the treatment of anthrax infections. In Switzerland, only small quantities of FQs are used for animals (~ 0.5 t/year). However, a substantial amount of about 4 t of FQs is annually applied in humane medicine [14], which corresponds to approximately 15% of the total antimicrobial agents consumed by humans in Switzerland. The environmental concern of FQs is not only based on their potential to promote antibiotic resistance, but also on their unfavorable ecotoxicity profile [15]. Recent studies [12, 13] have detected the occurrence of the two predominant human-use FQs [ciprofloxacin (CIP) and norfloxacin (NOR); for structures see Figure 4.1] in Swiss wastewater effluents, indicating a continuous discharge of these
antibacterial agents into surface waters. Hence, it is important to survey the occurrence of FQs during wastewater treatment and in surface waters to help evaluate whether the measured concentrations pose a risk to the aquatic environment.

A regional-scale field study was conducted on the Glatt Valley watershed in Switzerland. In this highly populated area, the water quality of the Glatt River is severely impacted by anthropogenic activities. The goals of our study presented here were: (i) to monitor the occurrence of FQs in wastewater and surface water; (ii) to calculate an average removal efficiency during municipal wastewater treatment and quantify FQ inputs into the receiving river; (iii) to determine whether during transport in rivers FQs occurring in the dissolved fraction are removed to any extent; (iv) to evaluate whether regional-scale predicted environmental concentrations fit the studied field situation; and (v) to discuss the environmental risk of trace amounts of FQs in the aquatic compartments studied.

**Figure 4.1** Chemical structures of ciprofloxacin and norfloxacin, the two fluoroquinolone antibacterial agents determined in the Swiss aquatic environment.
4.2 Experimental Section

4.2.1 Description of the Investigated Area

The Glatt Valley watershed (Figure 4.2) is a densely populated region (260 km²; 175,000 inhabitants) in the northern part of Switzerland, including a significant part of the surrounding settlement area of Zurich City. The Glatt River originates at the outlet of the Greifensee and is a tributary to the Rhine River. The river gradient ranges from 1 to 7%, resulting in an average flow velocity of approximately 0.5 m/s. The riverbank is lined with trees to a certain extent so that light conditions in the water column range from shadow to full exposure to sunlight. Water discharge fluctuates in the range of 3–12 m³/s, and the water residence time in the river is 15–20 h, depending on flow velocity [16]. On its journey from the Greifensee to the Rhine River (36 km), the Glatt River receives considerable amounts of treated wastewater effluents from municipal mechanical and biological wastewater treatment plants (WWTPs). These WWTPs are fully nitrifying all year and partially denitrifying. Some of them also have a post-filtration step to reduce suspended solids. The water retention time for the studied WWTP varied from 12 to 20 h. The operative sludge ages ranged from 7 to 16 days. During the study period, the wastewater flow reaching the largest WWTP discharging into the Glatt River, Zurich-Glatt WWTP, was redirected to the Zurich-Werdhölzli WWTP discharging into the Limmat River. The operating capacity of the Zurich-Glatt WWTP was switched from 100% in winter (February 2001) to 20% during the summer (June – August 2001). This change accounted for a reduction of about 30% of the total discharged treated wastewater into the Glatt River. Thus, for the investigated period the overall dilution factor of treated wastewater to river water was 5 and 8 for winter and summer, respectively.

4.2.2 Sampling

One-week composite samples were collected from several locations on the Glatt River during winter (February 5 – March 4, 2001) and summer
(June 25 – July 15, 2001). A more comprehensive sampling campaign was conducted during a 4-day period (August 20 – 23, 2001) aimed at a simultaneous collection of 24-h composite samples from several stations on the Glatt River and of final effluents from all wastewater treatment plants in the Glatt Valley watershed (Figure 4.2). The sampling at the WWTPs was synchronized with the river sampling in such a way that comparable sample sets were obtained. Additionally, several 24-h composites of raw sewage effluents were collected.

Figure 4.2 Map of the Glatt Valley Watershed, Switzerland, showing the sampling locations. Dots indicate the municipal wastewater treatment plants, and the dot diameter is proportional to the corresponding effluent discharges during the study period. Numbers 1–3 indicate the three river sampling stations. Distance downstream from Greifensee is indicated in parentheses.
The first sampling station along the Glatt River was located close to the outflow of the Greifensee, and the second site (Rümlang) was located after the discharge of five out of eight wastewater treatment plants to the Glatt River. The third station Rheinsfelden was located shortly before the Glatt River joins the Rhine River. Wastewater samples of final effluents were taken from all eight WWTPs discharging into the Glatt River.

Samples were collected in amber glass bottles and immediately filtered through 0.45-µm cellulose nitrate membrane filters. Samples were stabilized by lowering the pH to 3 and were stored in the dark at 4°C until analysis. Conductivity measures showed 0.8–1.2 mS/cm for raw sewage, 0.6–1.0 mS/cm for final effluents, and 0.4–0.6 mS/cm for river samples. Water temperature at the river stations ranged from 5 to 8°C and from 15 to 22°C during winter and summer, respectively.

Flow data from the water gauges at the Greifensee outlet, Rümlang and Rheinsfelden were supplied by the Cantonal Office for Waste, Water, Energy and Air of Zurich (AWEL) and the Swiss Federal Office for Water and Geology. River samples collected at the Greifensee outlet and at the Rümlang station were time proportional (every 20 min), while samples from Rheinsfelden station were flow proportional. The water volume discharges from the WWTPs were available by fixed water gauge recordings.

4.2.3 Analytical Methods and Quality Control

Wastewater. FQs were determined in wastewater effluents as described in detail in an earlier publication [13]. Briefly, analytes were concentrated from wastewater samples by solid-phase extraction using mixed-phase cation exchange disk cartridges (MPC), and subsequently measured by high-performance liquid-chromatography with fluorescence detection (excitation at 278 nm and emission at 445 nm). An internal standard procedure using the fluoroquinolone tosufloxacin (TOS) was applied for quantification. The overall precision of the method given as the relative standard deviation (RSD) was generally better than 10%.

Surface water. The previously described method [13] was further developed to allow measurements in surface waters. For nine FQs and the
quinolone pipemidic acid, the following validation parameters were determined, providing the quality control data given in Table 4.1:

(i) *Breakthrough curves* were determined on two stacked MPC disk cartridges by extracting 250, 500, and 750 mL of surface water containing native FQs (Glatt River) and spiked with 200 ng of TOS surrogate standard. FQs could be quantitatively isolated from 250- and 500-mL water samples by the first of two stacked MPC disk cartridges. Neither FQs nor TOS surrogate were detected on the second disk cartridge. However, when enriching 750 mL of surface water, breakthrough occurred in the order of 15%. Thus, a working volume of 500 mL was selected.

(ii) *Accuracy* was evaluated by recovery rates in MPC disk cartridges. Six replicate analyses were performed using a surface water sample not containing FQs (500 mL, Greifensee outlet) and spiked with FQs and 200 ng of TOS surrogate standard. Recovery of the TOS surrogate from surface water using MPC disk cartridges was 97% and for the FQs studied recoveries varied from 73% to 96%.

(iii) The *precision* of the entire procedure for surface water samples (500 mL, Greifensee outlet) was assessed by using six replicates of a surface water sample not containing native FQs and spiked with FQs and 200 ng of TOS surrogate standard prior to extraction. The overall precision of the method was indicated by a RSD lower than 15%.

(iv) The *linear range* was evaluated by measuring duplicates of FQs at 14 different concentrations (from 3 to 3000 ng/L), and defined as in ref [13].

(v) *Limits of detection (LODs) and limits of quantification (LOQs)* were calculated on the basis of the standard deviation ($n = 10$) of the analysis of a FQ standard mixture corresponding to a concentration in sample of 10 ng/L. LOD and LOQ were defined as 3 and 10 times the standard deviation of the FQ measurements, respectively. In the cases that LOQ values were below the linear range, LOQs were defined as the second lowest calibration point of the linear correlation (Table 4.1).
Table 4.1 Accuracy, Precision, Limit of Detection (LOD), Limit of Quantification (LOQ) and Linear Range for the Determination of Nine Fluoroquinolones and the Quinolone Pipemidic Acid in Surface Waters

<table>
<thead>
<tr>
<th>compound</th>
<th>spiked conc. (ng/L)</th>
<th>recovery (%)</th>
<th>RSD (%)</th>
<th>LOD (ng/L)</th>
<th>LOQ and linear range (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pipemidic acid</td>
<td>15</td>
<td>91</td>
<td>9</td>
<td>3.0</td>
<td>10 - 1950</td>
</tr>
<tr>
<td>fleroxacin</td>
<td>15</td>
<td>94</td>
<td>13</td>
<td>2.5</td>
<td>9\textsuperscript{d} - 3000</td>
</tr>
<tr>
<td>ofloxacin/levofloxacin</td>
<td>30</td>
<td>73</td>
<td>15</td>
<td>5.0</td>
<td>17 - 3000</td>
</tr>
<tr>
<td>norfloxacin</td>
<td>15</td>
<td>87</td>
<td>9</td>
<td>2.5</td>
<td>9\textsuperscript{d} - 1950</td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>15</td>
<td>85</td>
<td>10</td>
<td>2.5</td>
<td>9\textsuperscript{d} - 1950</td>
</tr>
<tr>
<td>lomefloxacin</td>
<td>15</td>
<td>76</td>
<td>14</td>
<td>3.0</td>
<td>10 - 2250</td>
</tr>
<tr>
<td>danofloxacin</td>
<td>7.5</td>
<td>95</td>
<td>11</td>
<td>0.5</td>
<td>5\textsuperscript{d} - 150</td>
</tr>
<tr>
<td>enrofloxacine</td>
<td>15</td>
<td>96</td>
<td>11</td>
<td>3.0</td>
<td>10 - 600</td>
</tr>
<tr>
<td>difloxacine</td>
<td>15</td>
<td>84</td>
<td>10</td>
<td>2.5</td>
<td>9\textsuperscript{d} - 1500</td>
</tr>
<tr>
<td>tosufloxacin\textsuperscript{f}</td>
<td>200</td>
<td>97</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mean values calculated from six replicate determinations. \textsuperscript{b}Limit of detection defined as 3 times the standard deviation of low concentrations (10 ng/L). \textsuperscript{c}The lowest value plotted in the given linearity range corresponds to the limit of quantification (10 times the standard deviation). \textsuperscript{d}LOQ defined as the second lowest point of the linearity range, because the value corresponding to the 10 times standard deviation was below the linear range. \textsuperscript{e}Measurement at emission wavelength 445 nm. \textsuperscript{f}Measurement at emission wavelength 500 nm. \textsuperscript{g}Tosufloxacin surrogate standard.
4.3 Results and Discussion

4.3.1 Concentrations and Mass Flows in Wastewater Effluents

Among the investigated compounds (Table 4.1), only the most consumed human-use FQs in Switzerland, CIP and NOR, were quantitatively determined for wastewater effluents (Table 4.2). Over a 4-day sampling period (August 20 – 23, 2001), concentrations of each CIP and NOR ranged in raw sewage and final effluents from 255 to 568 ng/L and from 36 to 106 ng/L, respectively. Concentrations of FQs were quite similar among the studied WWTPs. The FQ concentrations are comparable to previously published results [12, 13], and are up to 1 order of magnitude lower than concentrations found in final effluents for other classes of antibiotics such as macrolides and sulfonamides in Germany [8] and Switzerland [9, 12]. For the WWTP Kloten-Opfikon, raw sewage from two distinct sewers were collected: (i) mixed wastewater originating from the municipality of Kloten and the Zurich airport, and (ii) municipal sewage from the town of Opfikon. The highest FQ concentrations occurred in the mixed wastewater stream, which also received sewage collected from the airplanes.

Mass flows of individual FQs entering via raw sewage from each WWTP ranged from 3.5 to 8.7 g/day, and the amount discharged to the river ranged from 1.3 to less than 0.1 g/day. Relative loading per capita gave average values of 173 ± 33 µg/day CIP and 154 ± 32 µg/day NOR entering each treatment plant. Correspondingly, 32 ± 14 µg/day CIP and 25 ± 9 µg/day NOR were discharged to the Glatt River.

For wastewater treatment plants with a population in the catchment area of greater than 20,000, removal rates were calculated when raw sewage samples were available (Table 4.2). All studied WWTPs presented a similar efficiency with respect to FQ removal, ranging from 79 to 87%. The WWTPs equipped with post-filtration treatment did not yield significantly better removal.
Table 4.2 Concentrations and Mass Flows of CIP and NOR in Wastewater Effluents and Removal Efficiencies

<table>
<thead>
<tr>
<th>WWTP location</th>
<th>population served (1,000 m³/d)</th>
<th>throughput</th>
<th>concentration (ng/L)</th>
<th>mass flow (g/d)</th>
<th>removal efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>raw sewage</td>
<td>final effluent</td>
<td>raw sewage</td>
</tr>
<tr>
<td>1. Fällanden</td>
<td>25 600</td>
<td>9.3</td>
<td>CIP</td>
<td>NOR</td>
<td>CIP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>447 ± 76</td>
<td>435 ± 74</td>
<td>62 ± 8</td>
</tr>
<tr>
<td>2. Basserdorf</td>
<td>14 500</td>
<td>6.0</td>
<td>n.a.</td>
<td>n.a.</td>
<td>66 ± 7</td>
</tr>
<tr>
<td>3. Dübendorf'</td>
<td>35 500</td>
<td>17.1</td>
<td>n.a.</td>
<td>n.a.</td>
<td>67 ± 14</td>
</tr>
<tr>
<td>4. Zurich-Glatt</td>
<td>19 900</td>
<td>12.1</td>
<td>n.a.</td>
<td>n.a.</td>
<td>106 ± 24</td>
</tr>
<tr>
<td>5. Kloten-Optikon'</td>
<td>25 000</td>
<td>9.8'</td>
<td>568± 94</td>
<td>553± 70</td>
<td>67 ± 13</td>
</tr>
<tr>
<td>6. Niederglatt</td>
<td>28 300</td>
<td>15.9</td>
<td>313 ± 93</td>
<td>255 ± 48</td>
<td>68 ± 23</td>
</tr>
<tr>
<td>7. Büllach'</td>
<td>22 000</td>
<td>10.6</td>
<td>445 ± 55</td>
<td>350 ± 78</td>
<td>72 ± 12</td>
</tr>
<tr>
<td>8. Glattfelden</td>
<td>3 500</td>
<td>1.0</td>
<td>n.a.</td>
<td>n.a.</td>
<td>65 ± 11</td>
</tr>
</tbody>
</table>

| mean value    |                                 |            | 434 ± 93   | 388 ± 112     | 72 ± 14    | 57 ± 12       | 5.5 ± 2.2  | 4.9 ± 2.3     | 0.8 ± 0.4  | 0.6 ± 0.3     |

"Mean throughput during the 4-day period studied (August 20 - 23, 2001). bMean of duplicate analysis of 24-h composites of the four-day study period. cRemoval = (Mraw sewage - Mfinal effluent) / Mraw sewage x 100 (%). dFacility equipped with a post-filtration treatment. eEstimated served population in the Zurich airport. fMixed wastewater fraction of municipal sewage from the town of Kloten and sewage from the Zurich airport. gFraction containing only municipal sewage from the town Opfikon. n.a., not analyzed; n.c., not calculated.
The strong sorption properties associated with FQs [17,18] and the persistence observed for CIP regarding biodegradation [19] suggest sorption to sewage sludge as the main removal pathway of FQs from the water stream during wastewater treatment. In fact, additional work in our laboratory indicates a substantial sorption of FQs to sewage sludge [20].

4.3.2 Concentrations and Mass Flows in the Glatt River

The previously published analytical method [13] proved to be suitable not only for FQ determination in wastewater effluents but also in surface waters (see Experimental Section for validation parameters). This emphasizes the usefulness of this analytical method for the determination of FQs in aquatic matrixes. Among the studied compounds (Table 4.1), CIP and NOR were determined at several sections along the Glatt River. The resulting concentrations and mass flows are given in Table 4.3. When detected, concentrations of CIP and NOR ranged from 5 ng/L up to 18 ng/L (LOD 2.5 ng/L; LOQ 9 ng/L), depending upon sampling location and period. In Germany [8] and Switzerland [9,11], when detected in surface waters, macrolide and sulfonamide antibiotics reach concentrations of at least 1 order of magnitude higher than FQs reported here.

To be able to estimate mass flows at the Rheinsfelden River station, occasionally it was necessary to extrapolate from concentrations below the LOQ, but above the LOD. Thus, FQ mass flows at the three sampling sites varied from 2.2 to 10 g/day. The increase in mass flow of FQs along the first river section from Greifensee (1.2 km) to Rümlang (13.2 km) is due to the discharges of final wastewater effluents into the river. However, the decrease in the mass flow of both FQs in the second river section between Rümlang (13.2 km) and Rheinsfelden (35.2 km) can be inferred as the result of elimination processes. The lower mass flows observed in summer (reduction of 33% in Rümlang, and 30% in Rheinsfelden compared to winter values) are likely due to the 30% reduction of total wastewater discharged into the Glatt River (modification of the operating capacity of the Zurich-Glatt WWTP, see Experimental Section for details).
Table 4.3 Concentration and Mass Flow of Individual FQs CIP and NOR at Several Sampling Sites on the Glatt River in Winter and Summer

<table>
<thead>
<tr>
<th>period</th>
<th>river sampling site</th>
<th>distance downstream from source</th>
<th>concentration (ng/L)</th>
<th>mass flow (g/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>range n.</td>
<td>mean</td>
</tr>
<tr>
<td>winter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rümlang</td>
<td>13.2 km</td>
<td>13-18</td>
<td>15 ± 3</td>
</tr>
<tr>
<td></td>
<td>Rheinsfelden</td>
<td>35.2 km</td>
<td>(8) 14</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>summer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rümlang</td>
<td>13.2 km</td>
<td>9-14</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td>Rheinsfelden</td>
<td>35.2 km</td>
<td>(5) 10</td>
<td>(8) ± 2</td>
</tr>
</tbody>
</table>

The decrease in mass flows for the summer time may be explained by the 30% reduction on treated wastewater discharge on the Glatt River as compared to winter (see Experimental Section for details). Mean of duplicate analysis of four consecutive week composites in the respective sampling site location. Mean of duplicate analysis of three consecutive week composites and of 24-h composites during 4 days in August 2001. Values in parentheses correspond to concentrations below the limit of quantification (9 ng/L), but above the limit of detection (2.5 ng/L). n.d. not detected.
To better investigate the decrease in mass flow along the river, a mass balance for CIP and NOR in the Glatt River was calculated based on the 24-h composite sampling conducted during summer (August 20 - 23, 2001). The Glatt River was considered to be a large chemical and biological reactor, in which all inputs and outputs were then evaluated [21]. Human-use pharmaceuticals have been shown to reach surface waters primarily via discharge of treated wastewater effluents [8, 12, 22], thus analyzing all final effluents in the Glatt Valley watershed was considered to account for all significant inputs of FQs in the river. The headwaters of the Glatt River, the Greifensee, proved a negligible source of FQs (see Table 4.3). On the basis of the results obtained for final effluents (Table 4.2), the overall input of the eight WWTPs to the Glatt River was 6.4 g/day CIP and 5.2 g/day NOR. The output mass flow from the Glatt River was determined, based on the results of the sampling at Rheinsfelden station. The total output of CIP and NOR from the Glatt River was 2.2 g/day and 2.7 g/day, respectively. This value was compared to the total input mass flow, yielding an overall removal along the river of 66% and 48% for CIP and NOR, respectively (Figure 4.3). Because water samples were filtered previous to solid-phase extraction, and suspended particles were not analyzed, the observed removal accounts only for FQs occurring in the dissolved fraction of the river. The input sources of FQs were not evenly distributed along the river: about 73% of the wastewater effluents were discharged into the river in the first section upstream of Rümlang (13.2 km). Removal rates calculated at Rümlang station account for only 30% and 8% for CIP and NOR, respectively, indicating that the degree of removal of FQs will depend on the distance between the WWTPs discharging into the river and the sampling site.

The two most significant processes that could reduce FQ concentrations in surface waters are phototransformation and sorption to particles. Other processes, such as biodegradation or gas exchange, are not considered to be relevant. With respect to biodegradation, FQs can be assumed to be relatively persistent, as reported for CIP [19]. The elimination by gas exchange is considered to be unlikely because FQs are ionic compounds with relatively high molecular weight and, thus, have a low tendency to volatilize. As observed for other photolabile compounds [16], favorable conditions for phototransformation exist in the Glatt River.
Figure 4.3 Longitudinal (distance downstream from source) mass flow profiles of ciprofloxacin and norfloxacin in the Glatt Valley Watershed. Average values during a 4-day period (August 20-23, 2001) and respective standard deviations are plotted. The removal rate between cumulative discharge of FQs through treated effluents to the Glatt River and the respective river sampling stations is indicated.
Considering that maximal light intensity occurs during the summer and that only a low seasonal variability of FQ mass flows was observed during this study, a small contribution of phototransformation to the overall removal rate is inferred. In addition, laboratory experiments have demonstrated that phototransformation of FQs into corresponding metabolites decreases in the presence of humic substances [23]. The strong sorption properties of FQs [17, 18] would indicate sorption to river particles and/or biota as the process primarily responsible for the removal of FQs from surface waters. In that case, the sorbed FQs might settle down and accumulate in the sediment of the river, as reported for the feed additive FQ, sarafloxacin, in fish farming sediments [24]. In fact, only limited conclusions can be drawn based on the results of this study concerning processes affecting the fate of FQs in surface waters. A decrease of the FQs mass flows in the dissolved fraction occurred in the 36-km long river, so although WWTPs act as a major barrier for the enter of FQs into the aquatic environment, a substantial removal of FQs can also be expected during transport in rivers.

4.3.3 Comparison of Predicted and Measured Environmental Concentrations

Because of the lack of field data, the calculation of predicted environmental concentrations (PECs) is a commonly accepted approach for environmental risk assessment [3, 25]. Herein we aimed to assess the actual measured environmental concentrations (MECs) in the aquatic compartments of the Glatt Valley watershed using calculated "worst-case" (PEC_{crude}) and more realistic (PEC_{refined}) assumptions for the same regional scenario in Switzerland (Figure 4.4). A steady-state emission of FQs into the environment throughout the year was assumed, as suggested by this study and previous results [12, 13]. Calculations of PECs for FQs in raw sewage, final effluents and surface water were made using the following equation [3]:

\[
\text{PEC (g/L)} = \frac{[A \times (100 - R)]}{(365 \times \text{PVD} \times 100)}
\]

\text{equation 1}
where $A$ is the averaged annual consumption of each FQ in Switzerland; $R$ is the percentage of removal during wastewater treatment; $P$ is the population of Switzerland ($P = 7,164,400$); $V$ is the volume of wastewater per capita per day; and $D$ is the dilution factor of final effluents to receiving waters. Because the volume of wastewater-use varies significantly from country to country, the range given for the European Union countries [3] was used as a minimum and maximum ($V = 0.15 - 0.30 \text{ m}^3 \text{ day}^{-1} \text{ inhabitant}^{-1}$). The dilution factor was set to zero when calculating PECs for raw sewage and final effluent, and the default factor of $D = 10$ [3] was used when calculating PECs in surface waters. For the worst-case calculation of PEC$_{\text{crude}}$, no human metabolism ($A_{\text{CIPcrude}} \sim 2300 \text{ kg/year}$, $A_{\text{NORcrude}} \sim 2020 \text{ kg/year}$ [14]) and no removal during wastewater treatment were assumed ($R_{\text{crude}} = 0\%$). For more realistic assumptions, human metabolism of FQs was considered when calculating the PEC$_{\text{refined}}$. Moreover, only renal FQ excretion was regarded relevant for the aquatic compartment, since FQs strongly sorb to feces [26, 27]. Excretion of the unchanged drug in urine is between 45 and 62% for CIP and from 33 to 48% for NOR [28], which resulted in $A_{\text{CIPrefined}} \sim 1035-1426 \text{ kg/year}$ and $A_{\text{NORrefined}} \sim 667-970 \text{ kg/year}$. Additionally, the actual mean removal rate of FQs during wastewater treatment, as obtained in this study, was applied ($R_{\text{refined}} = 82\%$). Removal in rivers was not considered, since FQ removal only occurs after transport along rivers; therefore, the degree of removal will depend on the length of the river.

Concentration of individual FQs in the different aquatic compartments examined (Figure 4.4) varied as follows: for raw sewage PEC$_{\text{crude}} = 2575-5864 \text{ ng/L}$, PEC$_{\text{refined}} = 850-3635 \text{ ng/L}$ and MEC = 255-568 ng/L; for final effluents PEC$_{\text{crude}} = 2575-5864 \text{ ng/L}$, PEC$_{\text{refined}} = 153-654 \text{ ng/L}$ and MEC = 36-106 ng/L; and for surface waters PEC$_{\text{crude}} = 257-586 \text{ ng/L}$, PEC$_{\text{refined}} = 15-65 \text{ ng/L}$ and MEC = 5-18 ng/L. The results show a comparable range between PEC$_{\text{refined}}$ and MECs obtained for both CIP and NOR in the Glatt Valley watershed, even more if one considers a more realistic wastewater use in Switzerland ($V = 0.45 \text{ m}^3 \text{ day}^{-1} \text{ inhabitant}^{-1}$; [29]). For surface waters, the results are more closely matching, because the real dilution factor from final effluents to the Glatt River is only 5 to 8, instead of 10. The Glatt Valley watershed is therefore considered a worst-case scenario in
Figure 4.4 Comparison between crude and refined predicted environmental concentrations \((PEC_{\text{crude}}\text{ and } PEC_{\text{refined}})\) with measured environmental concentrations \((MEC)\) of ciprofloxacin and norfloxacin obtained from this study in the Glatt Valley Watershed, Switzerland.

Switzerland. In conclusion, FQ concentrations in wastewater and surface water can be predicted reasonably well if human metabolism of the drug and removal efficiency in WWTP are considered, and if regional differences in wastewater use are included in the analysis.
4.3.4 Risk Characterization of the FQ Occurrence in the Glatt Valley Watershed

The exposure data of FQs in the aquatic compartments of the Glatt Valley watershed reported here were related to acute toxicity data for aquatic organisms found in the literature [15, 19]. On the basis of the latter, and according to the European Commission Directive on the Classification, Packaging and Labeling of Dangerous Substances [30], FQ would be classified as very toxic to bacteria ($EC_{50} \leq 1$ mg/L), toxic to algae ($1$ mg/L < $EC_{50} < 10$ mg/L), and harmful to crustacean and fish ($10$ mg/L < $EC_{50} < 100$ mg/L). The risk quotients ($MEC/PNEC$) calculated in the present study for the different aquatic compartments examined are presented in Table 4.4. The environmental risk assessment (ERA) for surface waters was based on the Draft Discussion Paper proposed by the European Agency for the Evaluation of Medical Products (EMEA) [3]. Furthermore, since antimicrobial agents may cause adverse effects on microbial activity in WWTPs, a risk characterization for microorganism was done based on the Technical Guidance Document for New and Existing Substances of the European Commission [25].

Conventional ERA is usually limited to one compound per assessment. However, FQs are a very homogeneous class both structurally as well as in their intended mode of action [31]. To account for potential additive toxicity of FQs [32], total FQ concentrations occurring in the aquatic environment were considered. Because no ecotoxicity data for NOR were available to calculate toxicity equivalence factors [33], a simplified but conservative approach was applied. We compared the sum of the highest reported MECs for CIP and NOR for each of the aquatic compartments examined, with the predicted no effect concentrations (PNECs) obtained for the expected most potent compound, in this case CIP [31]. The risk ratio of FQs in receiving waters was assessed by comparing acute toxicity data of CIP ($EC_{50}$) to organisms of three trophic levels contributing to the food chain (fish, daphnia, algae) [15] as recommended by EMEA [3]. The predicted no effect concentration in surface waters ($PNEC_{surface\,water}$) was obtained after applying a safety factor of 1000 to the lowest $EC_{50}$ value among the three species.
Table 4.4 Data Used to Calculate Risk Quotients (MEC/PNEC) of FQ Antibacterial Agents in Examined Aquatic Compartments of the Glatt Valley Watershed, Switzerland

<table>
<thead>
<tr>
<th></th>
<th>aquatic compartment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>surface water</td>
<td>WWTP</td>
</tr>
<tr>
<td>organism</td>
<td><em>Selenastrum capricornutum</em> (algae)</td>
<td><em>Pseudomonas putida</em> (bacteria)</td>
</tr>
<tr>
<td>endpoint</td>
<td>Growth inhibition</td>
<td>Growth inhibition</td>
</tr>
<tr>
<td>test system</td>
<td>OECD 201 (15)</td>
<td>ISO 17012 (19)</td>
</tr>
<tr>
<td>EC$_{50, CIP}$</td>
<td>3 mg/L</td>
<td>80 µg/L</td>
</tr>
<tr>
<td>safety factor$^a$</td>
<td>1000</td>
<td>10</td>
</tr>
<tr>
<td>predicted no effect concentration (PNEC)</td>
<td>3 µg/L</td>
<td>8 µg/L</td>
</tr>
<tr>
<td>MEC$_{final effluent}$</td>
<td></td>
<td>0.18 µg/L total FQs$^b$</td>
</tr>
<tr>
<td>MEC$<em>{final effluent/PNEC</em>{WWTP}}$</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>MEC$_{Glatt River}$</td>
<td>0.04 µg/L total FQs$^b$</td>
<td></td>
</tr>
<tr>
<td>MEC$<em>{Glatt River/PNEC</em>{surface water}}$</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

$^a$According to the European draft documents [3] and guidelines [25]. $^b$To consider additive toxicity, the sum of the highest CIP and NOR concentrations obtained in final effluents and river water was compared with the PNEC of the most potent component, CIP.
Although conservative and protective, the safety factor of 1000 on short-term data is suggested [3], which should account for uncertainties derived from intra-species variability, inter-species variations in sensitivity, and extrapolation from acute to chronic toxicity. Since the algae species, *Selenastrum capricornutum*, was the most sensitive to the toxicity of CIP (i.e. EC$_{50}$ 3 mg/L) a PNEC$_{\text{surface water}}$ of 3 µg/L was calculated for FQs in surface waters. According to ref [3,25], we did not include microorganisms when evaluating the risk of FQs in surface waters, but they were the species of concern for WWTP assessment [25]. Because FQs occur at higher concentrations in WWTPs than in surface waters, we assume the risk for microorganism in the latter compartment should hence be covered. In agreement with ref [25], we calculated a PNEC$_{\text{WWTP}}$ of 8 µg/L using EC$_{50}$ data for CIP to a relevant bacterial population in WWTPs, *Pseudomonas putida* [19] and after applying a safety factor of 10 for inter-species variability. This value is comparable to the lowest found minimum inhibition concentrations (MICs) for CIP and NOR (MIC$_{90} \geq$ 10 µg/L) [34] without applying further safety factors. A frequently raised criticism of ERA of pharmaceuticals is its lack of specificity, i.e., not reflecting the specific mode of action for which the investigated drug has been designed. In the case of FQs, the mode of action against bacteria is well-known (DNA-gyrase inhibition) [35]. For this specific endpoint, data are available from a bacterial genotoxicity assay (the umuC-test) with the lowest observed effect concentrations being 5 µg/L and 25 µg/L for CIP and NOR, respectively [31]. Although the genetically modified *Salmonella typhimurium* strain used in the umuC-test is not a very relevant species, the data are certainly useful as a safety reference, and perfectly comparable with the calculated PNECs (low µg/L range).

For both aquatic compartments, Glatt River and WWTPs, risk quotients were less than unity despite the use of total FQ concentrations, thus indicating a low likelihood for acute adverse effects either to the aquatic habitat in surface waters, or to the degradation processes during wastewater treatment. In support of this, experimental data showed no evidence for a selection pressure on bacterial populations in surface waters (NOEC$_{\text{CIP}}$ 3 µg/L) [36] or during wastewater treatment (NOEC$_{\text{CIP}}$ 2 µg/L) [37]. Although the risk quotients here calculated seem reasonably protective when concerning FQ mixtures exclusively, data on chronic ecotoxicity and
mixture toxicity of "pharmaceutical-cocktails" would be needed to satisfy scientific criteria [38]. Moreover, the strong sorption properties of FQs to solid matter suggest that the terrestrial environment is a further relevant exposure pathway (i.e., riverbed sediments, or sludge-treated soils). Therefore, to obtain an overall environmental risk characterization, the potential ecotoxicity of FQs to benthic and soil organisms should be covered. Further research is also needed in order to assess the role of FQs on the spread and maintenance of (multi-) resistance among pathogenic bacteria.

Acknowledgement

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4.4 Literature Cited


Exposure Assessment from Sewage to Soil

The behavior of fluoroquinolone antibacterial agents (FQs) during mechanical-biological wastewater treatment was studied by mass flow analysis. In addition, the subsequent fate of FQs in agricultural soils after sludge application was investigated. Concentrations of FQs in wastewater effluents (raw sewage, primary, secondary, and tertiary effluents) were determined using solid-phase extraction with mixed phase cation exchange disk cartridges and reversed-phase liquid-chromatography with fluorescence detection. FQs in suspended solids, sewage sludge (raw, excess, and anaerobically-digested sludge) as well as in sludge-treated soils were determined as described for the aqueous samples but preceded by accelerated solvent extraction. Wastewater treatment resulted in a reduction of the FQ mass flow of 88 to 91 %, with complete mass transfer to sewage sludge. A sludge-wastewater partition coefficient (logK_{d} \sim 4) was calculated at the activated sludge reactors with an hydraulic residence time of about 8 hours. Under methanogenic conditions of the sludge digesters, no significant removal of FQs occured. These results suggest sewage sludge as main reservoir of FQ residues, and outline the importance of sludge management strategies to determine whether most of the human-excreted FQs, make it to the environment. Field experiments of sludge-application to agricultural land confirmed the long-term persistence of trace amounts of FQs in sludge-treated soils and indicated a limited mobility of FQs down to the subsoil.
5.1 Introduction

Municipal wastewater treatment plants play an important role in the life cycle of human-use pharmaceuticals, because they act as point sources to the aquatic environment. If sorption to sewage sludge is the major removal pathway from the wastewater stream, the application of sewage sludge as soil fertilizer represents an additional entry route for human-use pharmaceuticals into the environment. Hence, it is important to understand the behavior of pharmaceuticals as they pass through wastewater treatment plants. Although, for most human-use pharmaceuticals is well-known that the discharge of treated wastewater effluents to surface water accounts as the major entry pathway to the environment [1-3], their behavior during wastewater treatment is still mostly unknown. Some studies have investigated the fate of pharmaceuticals in laboratory-scale sewage treatment plants [4,5] or have involved laboratory testing for biodegradability [1,4,5]. Some data are also available for full-scale wastewater treatment plants on the overall removal of some pharmaceuticals from the aqueous phase [3, 6-8], but still there is a lack of detailed studies on the efficiency of single treatment process on the elimination of pharmaceuticals. Quantitative data on the occurrence of pharmaceuticals in sewage sludge is also scarce.

Fluoroquinolone antibacterial agents (FQs) are probably the only pharmaceuticals with measured concentrations in both wastewater effluents [6] and in sewage sludges [9]. The two most used FQs in Switzerland, ciprofloxacin (CIP) and norfloxacin (NOR), were determined at the ng/L level in Swiss wastewater effluents [6] and at the low mg/kg level in sewage sludges [9]. Because FQs are excreted by the human body in both urine (CIP 45 - 62%, NOR 20 - 80%) and feces (CIP 15 - 25%, NOR 28%) [10-12], the analysis of liquid and solid sewage matrices is indispensable to follow the entry route of FQs to the environment. During wastewater treatment, FQs show to be substantially removed [6]. Based upon laboratory studies [13-15], sorption to sewage sludge appears as the main removal process, yet confirmation of such predictions at full-scale wastewater treatment is desired.

The sludge management strategy ultimately applied will determine the final fate of the FQ residues sorbed to sewage sludge. Although
controversial [16], the application of sewage sludge as fertilizer to agricultural land is still a strategy widely used in several countries. For instance in Switzerland, 42% of the sewage sludge produced in 1999 (about 88,000 tons) were applied on agricultural land [17]. To protect from potential adverse effects of sewage sludge constituents, a revision of the European Union Directive 86/278/EEC is planned to limit the amount of organic micro-pollutants released to the environment by sewage sludge disposal [18], yet pharmaceuticals are not included. A more radical decision has been taken in Switzerland, where the disposal of sewage sludge into agricultural fields will be from January 2003 on forbidden. Nonetheless, as long as such disposal strategy is in practice elsewhere, better knowledge on the fate and effects of chemicals in sludge-treated soils is desired. FQs have already been identified as persistent pollutants in sludge treated soils [9] and sediments [19], so their behavior in the terrestrial environment is of especial interest.

The first part of this paper reports on field measurements in which the analytical methods for the determination of FQs in aqueous [20] and solid [9] sewage matrices were jointly used to perform a mass balance of FQs in a municipal wastewater treatment plant. In a second part, a field investigation was done to gain an insight on the fate of FQs in sludge-treated soils. Finally, the obtained field data were contrasted with literature on FQs sorption and biodegradation to assess the physicochemical and biological processes that may affect the environmental behavior of FQs.

5.2 Experimental Section

5.2.1 Description of the Studied Wastewater Treatment Plant

The largest municipal wastewater treatment plant (WWTP) in Switzerland, Zurich-Werderhölzli, was chosen to determine the mass balance of FQs (Figure 5.1). This WWTP serves approximately a residential population of 275,000 (1998), which due to the commuters to Zurich City can increase to 450,000 persons on working days. The sewage entering the WWTP is first treated mechanically using a screen and a combined grit and
Figure 5.1 Sampling locations at the Zurich-Werdhölzli waste water treatment plant in Switzerland.
fat removal tank followed by primary clarification (residence time 2-3 hours). The primary effluent flows to the activated sludge systems with an influent to return sludge ratio of about 2:1. The wastewater in the activated sludge tanks (pre-denitrification with 72%-aerated volume) and the secondary clarifiers have a combined residence time of approximately 20 hours. The total solid retention time is about 11 days, which means full nitrification throughout the year with about 60% nitrogen removal due to denitrification and biomass incorporation. Additionally, the facility has a flocculation-filtration step. The primary sludge from mechanical treatment and the excess sludge from biological treatment and filtration are mixed and settled in the primary clarifier. The raw sludge is thickened during 2 to 4 days. The supernatant is decanted while the thickened sludge is stabilized in a two-stage anaerobic digestion process, with a total sludge residence time of approximately 30 days.

Samples of 24-h flow proportional composites of raw sewage, primary, secondary, and tertiary effluents were collected during seven days (October 4 to 10, 2000) by means of automated samplers. In addition, on July 8, 2002 a 24-h flow proportional raw sewage sample was taken for the analysis of suspended solids. After collection into dark amber bottles, all samples were immediately transported into the laboratory. There the samples were filtered through 0.45 µm-cellulose nitrate filters (Sartorius GmbH), and conductivity and pH were determined. Water samples were then preserved by acidification to pH 3.0 and stored in the dark at 4°C until analysis. Suspended solids retained in the filter after filtering 500 mL raw sewage were immediately analyzed. Conductivity of water samples ranged from 0.77 to 0.93 mS/cm and from 0.59 to 0.70 mS/cm in the primary and tertiary effluents, respectively. Sample pH ranged from 7.5 to 8.4. Water temperature at the sampling stations were between 18 to 20°C. For the study period the flow of wastewater through the plant varied from 122,000 to 215,000 m³/day.

Grab samples of raw sludge and excess sludge were collected in polypropylene bottles over a 5-day period (October 5, 6, 9 and 10, 2000) and a single raw sludge sample was taken on July 8, 2002. Anaerobically-digested sludges were sampled after the anaerobic digesters on October 5, 2000 and July 8 and 15, 2002. All the sludge samples were immediately dried at 60°C for 72 h, finely ground, and stored in amber bottles at room
temperature. During the study period, the daily average of dry sludge matter was: 40 tons raw sludge, 20 tons excess sludge, and 25 tons of anaerobically-digested sludge.

5.2.2 Description of the Sludge-Treated Experimental Field

In October 1999 an experimental field was set up at free land in Reckenholz in the region of Zurich, Switzerland [21], in which anaerobically-digested sludge from the municipal WWTP in Uster was added to the field at 50 t/ha (10 times the amount allowed in Switzerland every third year, e.g. 5 t/ha/3 years). FQ concentrations in anaerobically-digested sludge from this WWTP showed similar concentrations (e.g. 2.8 ± 0.3 mg/kg CIP and 2.7 ± 0.2 kg/d NOR, July 8, 2002) as other WWTP studied. Soil samples were collected using a steel cylinder 5 and 21 months after sludge-application. Soil physico-chemical characteristics are presented in Table 5.1. Each sample consisted of four aliquots of randomly chosen soil from the same depth of the studied plot: 0-2.5 cm, 2.5-5 cm, 5-7.5 cm, 7.5-10 cm, 10-15 cm and 15-20 cm. Samples were dried at 40°C and passed through a 0.2 mm sieve.

Table 5.1 Physico-chemical Soil Characteristics of the Experimental Plot

<table>
<thead>
<tr>
<th>soil depth (cm)</th>
<th>0-2.5</th>
<th>2.5-5</th>
<th>5-7.5</th>
<th>7.5-10</th>
<th>10-15</th>
<th>15-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>soil composition (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sand</td>
<td>54</td>
<td>58</td>
<td>58</td>
<td>58</td>
<td>59</td>
<td>61</td>
</tr>
<tr>
<td>silt</td>
<td>21</td>
<td>19</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>clay</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>organic matter content</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>pH</td>
<td>6.7</td>
<td>7.1</td>
<td>6.9</td>
<td>7.0</td>
<td>7.6</td>
<td>7.8</td>
</tr>
<tr>
<td>cation exchange capacity (meq/100g)</td>
<td>28</td>
<td>22</td>
<td>21</td>
<td>19</td>
<td>18</td>
<td>16</td>
</tr>
</tbody>
</table>
5.2.3 Analytical Methods

Wastewater samples were analyzed for FQs by solid-phase extraction using mixed phase cation exchange disk cartridges and reversed-phase liquid-chromatography with fluorescence detection as described in ref [20]. The precision of the method for the occurring FQs CIP and NOR in wastewater effluents, indicated by the relative standard deviation, was between 5 to 8%. Recoveries were always higher than 90%. The quantification limits determined in raw sewage and primary effluents were 90 ng/L, and in secondary and tertiary effluents 30 ng/L.

Suspended solids, sewage sludge and sludge-treated soil samples were extracted using accelerated solid extraction followed by solid-phase extraction and reversed-phase liquid-chromatography with fluorescence detection as described earlier [9]. The precision of the method for FQs in sewage sludge and sludge-treated soil samples was between 8 and 11%. Overall recoveries were always > 80%. For sewage sludge and for sludge-treated soil limits of detection were 0.12 and 0.05 mg/kg, and limits of quantification 0.45 mg/kg and 0.18 mg/kg, respectively. For suspended solids similar validation parameters were assumed.

5.3 Results and Discussion

5.3.1 Concentrations and Mass Flows in Wastewater Effluents and Sewage Sludges

Concentrations of the most used FQs in Switzerland, ciprofloxacin (CIP) and norfloxacin (NOR), in wastewater effluents and sewage sludges of Zurich-Werdhölzli WWTP are given in Table 5.2. Over the sampling period, FQ concentrations were comparable, and lay within the range of those previously reported [6, 9]. Concentrations of individual FQs varied in raw sewage from 315 to 571 ng/L and in primary effluents from 272 to 489 ng/L. In samples of secondary effluents, FQ concentrations ranged from 65 to 109 ng/L, and in tertiary effluents from 39 to 87 ng/L. In addition, FQs were measured in suspended solids from the raw sewage giving concentrations of 180 ng/L CIP and 200 ng/L NOR (based on a rate of 150
mg suspended solids per liter raw sewage). For excess, raw and anaerobically-digested sludge the concentration of individual FQs ranged from 2.3 to 2.7 mg/kg, from 1.7 to 2.7 mg/kg, and from 2.3 to 3.5 mg/kg, respectively.

Mass flows of individual FQs were determined for 24-h composite wastewater effluents and grab samples of sewage sludge over the studied period (Figure 5.2). Besides, Table 5.3 gives the average mass flows of each FQ for the various treatment stages. A similar contribution of each FQ to the total FQ mass flow entering the treatment plant as well as during the treatment process was observed. The mass flow of FQs entering the Zurich-Werdhölzli WWTP ranged from 43 to 83 g/d for the raw sewage \( (m_{CIP} = 67 \pm 12 \text{ g/d}; m_{NOR} = 68 \pm 8 \text{ g/d}) \), and was 26 ± 2 g/d for CIP and 29 ± 3 g/d for NOR in suspended solids. Therefore, of the FQs entering the wastewater treatment, about one third is associated to suspended solids. For primary effluents, the mass flow varied from 43 to 71 g/d \( (m_{CIP} = 52 \pm 9 \text{ g/d}; m_{NOR} = 61 \pm 10 \text{ g/d}) \), and for secondary effluents from 8 to 19 g/d \( (m_{CIP} = 15 \pm 2 \text{ g/d}; m_{NOR} = 11 \pm 2 \text{ g/d}) \). The mass flow of individual FQs leaving the Zurich-Werdhölzli WWTP as filtered effluent ranged from 6 to 13 g/d \( (m_{CIP} = 11 \pm 1 \text{ g/d}; m_{NOR} = 8 \pm 1 \text{ g/d}) \). For raw sewage and primary effluent, the mass flow of FQs fluctuated considerably in comparison to those of secondary and tertiary effluent. This could be due to the mixing effect during wastewater treatment, which may contribute to a better homogenization of FQ with longer residence times. Remarkable, however, is the lower mass flow of FQs entering the Zurich-Werdhölzli WWTP on Sunday (Figure 5.2), which corresponds to only 52 to 67% of the mass flow of other weekdays. This trend nicely correlates with the person-traffic movement to Zurich City (residential population 58% of the total population during working days).

The mass flow of FQs associated with excess sludge varied from 46 to 54 g/d, with an average of \( m_{CIP} = 49 \pm 2 \text{ g/d} \) and \( m_{NOR} = 51 \pm 2 \text{ g/d} \); and for raw sludge, the mass flow ranged from 68 to 108 g/d \( (m_{CIP} = 87 \pm 16 \text{ g/d}; m_{NOR} = 82 \pm 9 \text{ g/d}) \). The amount of FQs associated with anaerobically-digested sludge varied from 58 to 88 \( (m_{CIP} = 78 \pm 11 \text{ g/d}; m_{NOR} = 73 \pm 11 \text{ g/d}) \), and was used to determine the mass flow of FQs exported from the wastewater treatment plant as digested sludge. The larger variability
Table 5.2a Ciprofloxacin Concentrations in Wastewater Effluents and Sewage Sludges

<table>
<thead>
<tr>
<th>Date sampled</th>
<th>Sewage discharge (m³/d)</th>
<th>Raw sewage (ng/L)</th>
<th>Primary effluent (ng/L)</th>
<th>Secondary effluent (ng/L)</th>
<th>Tertiary effluent (ng/L)</th>
<th>Excess sludge (ng/L)</th>
<th>Raw sludge (mg/kg)</th>
<th>Digested sludge (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wed 04-Oct-2000</td>
<td>144 955</td>
<td>571 ± 29a</td>
<td>431 ± 28</td>
<td>109 ± 7</td>
<td>76 ± 7</td>
<td>2.4 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>Thu 05-Oct-2000</td>
<td>137 920</td>
<td>511 ± 51</td>
<td>387 ± 2</td>
<td>109 ± 6</td>
<td>87 ± 3</td>
<td>2.6 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Fri 06-Oct-2000</td>
<td>198 720</td>
<td>329 ± 5</td>
<td>272 ± 25</td>
<td>94 ± 6</td>
<td>68 ± 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sat 07-Oct-2000</td>
<td>136 293</td>
<td>469 ± 29</td>
<td>417 ± 36</td>
<td>92 ± 6</td>
<td>65 ± 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sun 08-Oct-2000</td>
<td>121 599</td>
<td>351 ± 10</td>
<td>308 ± 11</td>
<td>90 ± 1</td>
<td>73 ± 6</td>
<td>2.3 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Mon 09-Oct-2000</td>
<td>214 754</td>
<td>315 ± 10</td>
<td>279 ± 12</td>
<td>79 ± 3</td>
<td>61 ± 2</td>
<td>2.5 ± 0.1</td>
<td>2.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Tue 10-Oct-2000</td>
<td>154 910</td>
<td>433 ± 1</td>
<td>276 ± 25</td>
<td>99 ± 9</td>
<td>73 ± 2</td>
<td></td>
<td>2.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Mon 08-Jul-2002</td>
<td>143 760</td>
<td>525 ± 35</td>
<td></td>
<td></td>
<td>2.0 ± 0.1</td>
<td>3.5 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mon 15-Jul-2002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Average ± SDb</td>
<td></td>
<td>438 ± 61</td>
<td>339 ± 65</td>
<td>96 ± 10</td>
<td>72 ± 8</td>
<td>2.5 ± 0.1</td>
<td>2.2 ± 0.4</td>
<td>3.1 ± 0.4</td>
</tr>
</tbody>
</table>

*aMean and standard deviation of duplicate measurements. bOverall mean and standard deviation.
<table>
<thead>
<tr>
<th>Date sampled</th>
<th>Sewage discharge (m³/d)</th>
<th>NORFLOXACIN</th>
<th>Wastewater effluents (ng/L)</th>
<th>Sewage sludges (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sewage</td>
<td>Raw sewage</td>
<td>Primary effluent</td>
</tr>
<tr>
<td>Wed</td>
<td>04-Oct-2000</td>
<td>144 955</td>
<td>494 ± 14³</td>
<td>489 ± 43</td>
</tr>
<tr>
<td>Thu</td>
<td>05-Oct-2000</td>
<td>137 920</td>
<td>515 ± 8</td>
<td>428 ± 5</td>
</tr>
<tr>
<td>Fri</td>
<td>06-Oct-2000</td>
<td>198 720</td>
<td>382 ± 12</td>
<td>327 ± 12</td>
</tr>
<tr>
<td>Sat</td>
<td>07-Oct-2000</td>
<td>136 293</td>
<td>484 ± 29</td>
<td>473 ± 6</td>
</tr>
<tr>
<td>Sun</td>
<td>08-Oct-2000</td>
<td>121 599</td>
<td>429 ± 39</td>
<td>339 ± 6</td>
</tr>
<tr>
<td>Mon</td>
<td>09-Oct-2000</td>
<td>214 754</td>
<td>343 ± 14</td>
<td>327 ± 3</td>
</tr>
<tr>
<td>Tue</td>
<td>10-Oct-2000</td>
<td>154 910</td>
<td>433 ± 14</td>
<td>348 ± 18</td>
</tr>
<tr>
<td>Mon</td>
<td>08-Jul-2002</td>
<td>143 760</td>
<td>435 ± 21</td>
<td></td>
</tr>
<tr>
<td>Mon</td>
<td>15-Jul-2002</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Average ± SD³

| Sewage      | 439 ± 54 | 390 ± 66 | 69 ± 4 | 52 ± 7 | 2.6 ± 0.1 | 2.1 ± 0.2 | 2.9 ± 0.4 |

³Mean and standard deviation of duplicate measurements. ³Overall mean and standard deviation.
Figure 5.2 Mass flows of ciprofloxacin and norfloxacin over the study period in raw sewage, primary effluent, secondary effluent, tertiary effluent, suspended solids excess sludge, raw sludge, and anaerobically-digested sludge.
### Table 5.3 Average Mass Flows (g/d) of FQs During Wastewater Treatment in Zurich-Werdhölzli for the Study Period

<table>
<thead>
<tr>
<th>Mass Flow (g/d)</th>
<th>Ciprofloxacin</th>
<th>Norfloxacin</th>
<th>Total FQs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw sewage</td>
<td>67 ± 12 (35%)</td>
<td>68 ± 8 (36%)</td>
<td>135 ± 17 (100%)</td>
</tr>
<tr>
<td>Suspended solids&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26 ± 2 (14%)</td>
<td>29 ± 3 (15%)</td>
<td>55 ± 5 (43%)</td>
</tr>
<tr>
<td>Primary effluent</td>
<td>52 ± 9 (27%)</td>
<td>61 ± 10 (32%)</td>
<td>113 ± 18 (59%)</td>
</tr>
<tr>
<td>Secondary effluent</td>
<td>15 ± 3 (8%)</td>
<td>11 ± 2 (6%)</td>
<td>26 ± 5 (14%)</td>
</tr>
<tr>
<td>Tertiary effluent</td>
<td>11 ± 2 (6%)</td>
<td>8 ± 1 (4%)</td>
<td>19 ± 3 (10%)</td>
</tr>
<tr>
<td>Excess sludge</td>
<td>49 ± 2 (26%)</td>
<td>51 ± 2 (27%)</td>
<td>100 ± 3 (53%)</td>
</tr>
<tr>
<td>Raw sludge</td>
<td>87 ± 16 (46%)</td>
<td>82 ± 9 (43%)</td>
<td>169 ± 2 (89%)</td>
</tr>
<tr>
<td>Digested sludge</td>
<td>78 ± 11 (41%)</td>
<td>73 ± 11 (38%)</td>
<td>151 ± 10 (79%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percentage of each FQ contributing to the total FQ mass flow entering the waste water treatment plant as raw sewage and suspended solids.  
<sup>b</sup>Mean and standard deviation of duplicate analysis of a single day.

observed for FQ mass flows in raw sludge compared to excess sludge can be again related to the homogeneity of the sample, yet for grab samples. The high mass flow observed for raw sludge, can be attributed to the fact that it accounts not only for the removed fraction from the waterstream during wastewater and but also for the amount of FQs associated to suspended solids. It should be noted that the percentage of FQs excreted as conjugates, which may be hydrolyzed and revert to the parent compound during wastewater treatment, is minor (< 2% of administered dose [11]).

The presented results give support to previous assumptions [6], which regard FQs excreted in urine as the only source of FQs to aqueous pollution.
5.3.2 Behavior during Wastewater Treatment

The similarity between the combined composition of raw sewage and suspended solids (49% CIP, 51% NOR) and that of the human consumption pattern (48% CIP, 42% NOR) suggests that little alteration of the FQ distribution occurs during the transport to the wastewater treatment plant. Likewise, about one third of FQs are excreted in feces [11], which nicely compares to the percentage of FQs found associated with suspended solids. Within the wastewater treatment plant, the behavior and degree of removal for FQs will depend on their physico-chemical properties. Figure 5.3 shows the mass balance of FQs in the Zurich-Werdmühli WWTP, indicating the magnitude of the mass transfer and relative importance of each stage of treatment on the fate of FQs during wastewater treatment. During mechanical treatment, FQs are already removed up to 46 ± 9% of CIP and 35 ± 11% of NOR. The FQs associated to suspended solids are here deposited, and probably FQs in raw sewage might also sorb onto the recirculated excess sludge added at the inlet of the grit removal tank. Yet the main removal of FQs from the wastewater stream occurs during the biological treatment (39 ± 8% CIP, 53 ± 10% NOR). Finally, an additional 4 ± 1% of CIP and 3 ± 2% of NOR is removed in the flocculation-filtration step, most likely due to sorption of FQs to small particles that precipitate during flocculation. Altogether results in an overall removal during wastewater treatment of 88 ± 2% for CIP and 91 ± 1% for NOR. Because the percentage of FQs associated to excess sludge (51 ± 4% CIP, 55 ± 3% NOR) roughly corresponds to the FQ removal during activated sludge treatment and flocculation-filtration (42 ± 9% CIP, 57 ± 11% NOR), the major process relevant for removing FQs seems the sorption to sewage sludge. Likewise, the overall removal of FQs is within the range of recovered FQs in the raw sludge (90 ± 14 % CIP, 89 ± 11 % NOR). This confirms that the observed removal of FQs from the wastewater stream can be attributed to sorption processes and not to biodegradation during activated sludge treatment. Thus, biodegradation appears of minor importance for the elimination of FQs during wastewater treatment plant, supported by the low biodegradability of FQs in laboratory experiments [13, 14]. Although FQ are degraded by direct sunlight-
Figure 5.3a Mass balance of ciprofloxacin through mechanical treatment, biological treatment, floculation-filtration, and anaerobic digestion in Zurich-Werhölzli, Switzerland.
Figure 5.3b Mass balance of norfloxacin through mechanical treatment, biological treatment, floculation-filtration, and anaerobic digestion in Zurich-Werhölzli, Switzerland.
photolysis [22], this process appears of minor significance, most likely because sunlight can not penetrate into deeper water layers as high turbidities are generated by suspended solids.

Under methanogenic (anaerobic) conditions in the sludge digesters FQs seem quite stable. Only a difference of 10% was observed between the FQ mass flow entering the digesters and the anaerobically-digested sludge, which could be attributed to the averaged character of the digested sludge (~30 days residence time in the digester). In addition, anaerobically degradable compounds are commonly eliminated to a considerable extend [23], whereas FQs are still largely found in the anaerobically-digested sludge (80 ± 14% CIP and 78 ± 14% NOR).

5.3.3 Sludge-Wastewater Partitioning

The tendency of a chemical to sorb and accumulate to solids can be assessed based on the octanol-water partition coefficient ($K_{ow}$). However, this specially holds for uncharged molecules, since $K_{ow}$ values can only be used to describe hydrophobic interactions. In that way, uncharged chemicals with log$K_{ow}$ lower than 2.5 are assumed to show a low sorption potential [24]. Because of their zwiterionic character ($pK_{a COOH} = 5.9 - 6.3$, $pK_{a NH2} = 7.9 - 10.2$) and despite their negative $K_{ows}$, FQs exhibit high sorption properties as inferred from the high $K_d$ values obtained in other studies with various solids (see Table 5.4; [14, 15, 25-32]). This can be attributed to the particular sorption mechanism of FQs, which seems to occur mainly by the effects of electrostatic interactions, although hydrophobic forces are apparently also involved [25, 26, 33]. Therefore, to evaluate the sorption properties of FQs, experimental solid-water partition coefficients ($K_d$) with a strict control over pH conditions are indispensable, as suggested by some authors [27, 34].
Table 5.4 Physico-chemical Constants and Solid-water Partition Coefficients (L/kg) of Selected FQs

<table>
<thead>
<tr>
<th></th>
<th>acidity</th>
<th>octanol-water partition coefficient</th>
<th>solid-water partition coefficients</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>pK_a COOH</td>
<td>pK_a NH2</td>
<td>log K_{ow}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>logK_d sludge</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>logK_d DOM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>logK_d soil</td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>5.9^a - 6.1^b</td>
<td>8.7^b - 8.9^a</td>
<td>(-1.1)^f - (-0.9)^g</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.0^l</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.4^a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.6^a</td>
</tr>
<tr>
<td>norfloxacin</td>
<td>6.3^e - 6.4^e</td>
<td>8.4^b - 8.6^a</td>
<td>(-1.0)^f - (-1.0)^g</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.9^l</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.7^a</td>
</tr>
<tr>
<td>trovafloxacin</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.5^k - 3.8^l</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td>gemifloxacin</td>
<td>6.4^c</td>
<td>9.0^c</td>
<td>0.0^h - 0.2^l</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.1^m</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td>sarafloxacin</td>
<td>6.2^d</td>
<td>10.2^d</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.3^o - 4.7^o</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td>enrofloxacin</td>
<td>6.2^d - 6.3^e</td>
<td>8.0^d - 7.7^e</td>
<td>(-1.6)^d - (2.1)^e</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>n.d.</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>2.7^a - 3.7^a</td>
</tr>
</tbody>
</table>

^aRef [28] (experimental); ^bRef [29] (experimental); ^cRef [15] (experimental); ^dRef [30] (calculated at pH 2.8); ^eRef [25] (calculated at pH 9.2); ^fRef [31] (experimental at pH 7.4); ^gRef [32] (experimental mean obtained from values at pH 5.0, 7.0 and 9.0); ^hRef [15] (experimental at pH 4 and pH 11); ^iRef [15] (experimental at pH 7.5); ^jValues obtained in this study (experimental at pH 7.5-8.4); ^kRef [14] (experimental after 8 h); ^lRef [14] (experimental after 96 h); ^mRef [15] (experimental); ^nReviewed in ref [27] (experimental at pH 9.2); ^oReviewed in ref [27] (experimental range from pH 3.0 to 8.0); ^pRef [26] (experimental at pH 5.0; soil composition: 80% sand, 17% silt, 2% clay, 1% organic matter content); ^qRef [26] (experimental at pH 5.0; soil composition: 39% sand, 43% silt, 17% clay, 1% organic matter content); n.d. no data found.

Assuming an equilibrium between the fraction of FQs sorbed on the activated sludge and the fraction of FQs dissolved in wastewater in the sludge reactors (~8 hours hydraulic residence time), we calculated an experimental sludge-wastewater partition coefficient (K_d, sludge-wastewater in kg/L) for FQs according to:

$$K_{d, \text{sludge-wastewater}} = \frac{C_{\text{sludge}}}{C_{\text{wastewater}}}$$ equation 1
where $C_{\text{sludge}}$ corresponds to the FQ concentration in activated sludge ($\mu g/kg$) and $C_{\text{wastewater}}$ to the wastewater concentration in the sludge reactors ($\mu g/L$). The average concentration of FQs measured for excess sludge (originated during biological treatment) was defined as $C_{\text{sludge}}$ and the $C_{\text{wastewater}}$ was the difference between the average concentrations found in primary and secondary effluents. Using this approach, log $K_{d, \text{sludge-wastewater}}$ values of 4.0 and 3.9 L/kg were calculated for CIP and NOR, respectively, at a pH between 7.5 to 8.4. These values are similar to sludge-water sorption coefficients obtained from batch experiments for other FQs such as trovafloxacin [14] and gemifloxacin [15] (see Table 5.4). In ref [14] trovafloxacin sorption to sludge seemed to depend on equilibrium time (test for 8 and 96 h) but not on the solid concentration. The sludge-wastewater partition coefficients reported here fall within the log $K_{d}$ range reported for FQs and dissolved organic matter or soils (see Table 5.4).

### 5.3.4 Fate in Sludge-Treated Soils

The widespread practice of applying sewage sludge as fertilizer to agricultural land brings forward the need to assess the fate of FQs in sludge-treated soils. With that aim, measured environmental concentrations (MECs) were compared to predicted environmental concentrations (PECs), and related with literature data on sorption and biodegradation.

To estimate the exposure of FQs in Swiss agricultural soils, we considered the concentration range obtained for FQs in sewage sludge obtained in this and previous studies (1 to 4 mg/kg) [17], and we assumed an annual disposal of 88,000 tons/year sludge dry matter to Swiss farmland [17]. This lead to an annual application of approximately 88 to 352 kg of each CIP and NOR into agricultural soils. Given the current practice of applying sewage sludge to agricultural soils, in a maximum allowable rate of 5 tons/ha sludge dry matter to farmland during a period of 3 years, the expected loading rate of CIP and NOR to agricultural soils is 5 to 20 g/ha every 3 years. Assuming an even distribution of FQ residues to 5 cm depth, and a soil density of 1.5 g/cm$^3$ [35], an amount in soil of 0.07 to 0.30 mg/kg for each FQ was calculated. This predicted environmental concentration (PEC) is above the so-called 'phase I trigger value’ of 0.01 mg/kg.
recommended for pharmaceutical residues in Europe by the European Commission [35, 36], implying the need of assessing the occurrence and behavior of FQs in sludge-treated soils.

An experimental field study was carried out, with the aim of investigating the behavior and fate of FQs after application of sewage sludge under severe conditions, rather than simulating normal sludge-disposal practice. Figure 5.4 shows concentration versus depth profile of FQs measured in the sludge-treated soil of the experimental site, 5 and 21 months after sludge disposal. The FQs demonstrated some level of persistence over the study period (21 months) with residual soil concentrations at the µg/kg range. During the initial period FQ seem to accumulate at the topsoil, and thereafter only a limited mobility to the subsoil was observed (measurements below the limit of quantification but above the limit of detection). These results are in agreement with the expected immobility of FQs given by the high sorption coefficients into soils (see log $K_{d, \text{soils}}$ Table 5.4). The slight mobility observed could be due to a dissolved organic matter (DOM) facilitated transport in soils [37, 38] (see log $K_{d, \text{DOM}}$ Table 5.4).

The MECs obtained for the experimental plot were compared with estimated PECs, taking into account the sludge application rate into the experimental field (50 t/ha), and the depth range where FQs were determined. For the first period (up to 5 months) FQs were considered evenly distributed in the top 2.5 cm so a PEC range of 1.40 – 6.00 mg/kg for each FQ was calculated. For the second phase (up to 21 months), a PEC range of 0.18 – 0.75 mg/kg was predicted if FQs were evenly distributed in the 20 cm topsoil. The MECs obtained in the top 2.5 cm were 0.45 ± 0.10 mg/kg for CIP and 0.35 ± 0.10 mg/kg for NOR and from 0 to 20 cm depth, the MEC range was between 0.05 and 0.30 mg/kg (Figure 5.4). The fact that the MECs were always significantly lower than the PECs, suggests that within the studied period (0 to 5 months) FQs underwent partial disappearance either due to bio- or photo-transformation. No data on phototransformation of FQs in soils is available in the literature, however, bio-transformation half-lives between 3 and 5 months [39], and mineralization rates up to 27% within 8 weeks [40, 41] have been reported for several groups of soil microorganism.
Figure 5.4 Soil profile of FQ concentrations after 5 and 21 months following sludge-application of 50 t/ha, corresponding to 10 times the amount normally common in Switzerland. Each point correspond to an averaged concentration of the depth range: 0 - 2.5 cm, 2.5 - 5 cm, 5 - 7.5 cm, 7.5 - 10 cm, 10 - 15 cm, 15 - 20 cm. LOD, limit of detection and LOQ, limit of quantification.
Therefore, biodegradation (or photo-transformation) could have taken place in an initial phase, followed by a long-term persistence in the soil. The observed persistence could either be due to: 1) incorporation of FQs into the soil particles, or a more strong sorption, making them less bio-available to microorganisms present in soil; or 2) simply because FQ concentrations have reached the biodegradable concentration threshold. Similar behavior has commonly been reported for pesticides and other micropollutants [42].

The same persistence and limited mobility was observed for FQs when sludge was applied at 5-fold the common amount in Switzerland (data not shown). Therefore, assuming that 1-fold the allowed amount results in the same behavior, concentrations of FQs would still be above the European 'phase I trigger value' of 0.01 mg/kg [17].

In conclusion, our results suggest that if any biodegradation (or photo-transformation) of FQs occurs in soils, it is not complete, and residual FQs persist in agricultural soils. Therefore, the possibility of a continuous increase of FQ concentration with each addition of sludge can not be excluded. On the other hand, the limited mobility of FQs to the subsoil seem to exclude any threat to ground water due to leaching.

**Acknowledgements**

The financial support of Bayer AG (Germany) is gratefully acknowledged. We appreciate the collaboration of the employees of the wastewater treatment plant Zurich-Werdhöhlzli during sample collection, especially B. Beyeler. We very much acknowledge F.G. Kari and C. Schaffner for providing the soil samples. We thank the following colleagues who have provided helpful comments on the manuscript: M. Berg, F.G. Kari, H.P. Kohler, and C. McArdell.
5.4 Literature Cited


Evaluation of the Bacterial Genotoxic Potential of Environmental Samples
6.1 Introduction

The knowledge about the occurrence and fate of pharmaceuticals in the environment after their use in human and veterinary therapy is scarce, but even less is known about their potential adverse effects on ecosystems. The environmental hazard associated with fluoroquinolone antibacterial agents (FQs) is not only caused by their potential to induce and maintain bacterial resistance as for other antibiotics, but also on their unfavorable eco- and genotoxicity profile. FQs have shown to be highly toxic against some bacteria [1, 2]; not unexpectedly, considering that they have been specially designed to act against microorganisms. Less expected however, was the effect on non-target organisms, such as algae, as recently reported in ref [3]. Moreover, as FQs share an identical mode of action, concentration additivity has been invoked for the simultaneous occurrence of various FQs [2]. In agreement to their specific mode of action (inhibition of bacterial gyrase), FQs have also been reported as main inducers of primary DNA-damage (assessed by the genotoxicity test umuC-assay) observed in hospital wastewaters [4, 5]. The umuC assay is based on the SOS response mechanism, which is induced primarily by the occurrence of single stranded DNA. Since gyrase inhibition leads to the formation of single stranded DNA, FQs can activate the SOS repair system and so appear as genotoxic agents. Positive umuC results in hospital wastewater showed to be strongly dependent on the presence of FQs, particularly of ciprofloxacin [4]. Yet hospital wastewater undergoes significant dilution on passage to wastewater treatment plant, thereby reducing any genotoxic potential to negligible levels [6]. While genotoxicity screening of wastewater is broadly used to identify pollution sources in order to reduce the environmental exposure to harmful toxicants, a human hazard posed by the exposure to FQs at environmental concentrations is unlikely, since they show a low DNA-damaging potential in eukaryotic cells [7].

The development of specific analytical methods for the determination of FQs in several environmental matrices [8, 9] and a monitoring campaign in the Swiss environment [10-12] allows considerations to the environmental impact of FQs. The two leading human-use FQs ciprofloxacin (CIP) and norfloxacin (NOR) were determined in different environmental compartments in concentrations at the low ng/L level for
aqueous samples (255 - 571 ng/L in wastewater influents, 36 - 106 ng/L in treated effluents, < 5 - 18 ng/L in river water) and at the low mg/kg range for sewage sludge (1.4 - 3.2 mg/kg). Unfortunately, the impact of the occurrence of such trace amounts of FQs on ecosystems is uncertain, as well as the potential interactive effects occurring from complex mixtures of FQs, other pharmaceuticals, and pollutants in the environment.

Toxicity-directed chemical analysis has been extensively incorporated into environmental monitoring to investigate specific chemicals or classes of chemicals contributing dominantly to the toxicity of a complex mixture. The most common approach is to characterize environmental samples by chemical identification and quantification of target compounds known as potential toxicants (e.g. PCBs, heavy metals) and to evaluate their contribution to the overall toxicity of the sample [13,14]. However, in addition to known pollutants, unknown pollutants may also contribute significantly to the overall toxicity of complex mixtures, so a second approach of toxicity-directed chemical analysis is aiming at screening and identifying such unknown toxicants [15,16]. In both approaches, the methods generally involve either solid-phase extraction (SPE) of organic compounds from water samples or adequate procedures of extraction from solid samples followed by a series of fractionation steps, where fraction toxicity is measured, and chemicals are identified and quantified using appropriate analytical methods. A procedure that combines chromatographic fractionation and genotoxicity testing of water samples, with the aim of detecting known and unknown genotoxins in HPLC chromatographic fractions of water extracts (so called ToxPrint [16]) has been recently described. Because the detection of genotoxicity is carried out by the umuC-assay, the combination of the ToxPrint procedure with the selective analysis of FQs arises as a useful approach to further evaluate the role of FQs as inducers of umuC-genotoxicity in environmental samples. For this purpose, a study was performed aiming at: 1) obtaining concentration-response curves in the umuC-assay for eleven FQs and one metabolite, and assessing the combined genotoxicity of a mixture of the environmental relevant FQs, CIP and NOR; 2) optimizing a (geno)toxicity-directed chemical analysis for FQs in various environmental samples, based on selective isolation by sequential solid-phase extraction (SSPE), the ToxPrint approach, and the umuC-assay; and 3) evaluating the
usefulness of the developed procedure for whole extract screening of treated wastewater effluents and for chromatographic fraction screening of various environmental samples (wastewater effluents, surface waters, sewage sludges and sludge-treated soils).

6.2 Experimental Section

6.2.1 Standards

The standards of the tested quinolones and fluoroquinolones (here all referred as FQs) were obtained from the following sources: nalidixic acid (NAL), pipemidic acid (PIP), norfloxacin (NOR), ofloxacin (OFL) and lomefloxacin (LOM) from Sigma-Aldrich (Buchs, Switzerland); ciprofloxacin (CIP), enrofloxacin (ENR) and desethylene-ciprofloxacin (M1) from Bayer AG (Wuppertal and Leverkusen, Germany), fleroxacin (FLE) from Roche Diagnostics GmbH (Mannheim, Germany), difloxacin (DIF) and tosufloxacin (TOS) from Abbott Laboratories (Baar, Switzerland), and danofloxacin (DAN) from the Swiss Federal Veterinary Office (Bern, Switzerland).

For concentration-response curves in the umuC-assay, stock solutions (30 to 60 µg/mL) were prepared in distilled water with 4% DMSO, and further diluted with distilled water to concentrations between 100 ng/mL to 7.5 µg/mL. For chemical analysis, standard solutions for CIP, NOR and TOS-IS of 400 µg/mL were prepared in a water: methanol mixture (1:1) containing 0.2% v/v hydrochloric acid and stored at –20°C. Standard solutions were renewed monthly. Working standard mixtures of 10 µg/mL and 1 µg/mL were prepared in 25 mM ortho-phosphoric acid, stored at +4°C, and renewed weekly.

6.2.2 Environmental Samples

Representative environmental samples with regard to the occurrence of FQs in various compartments were taken in the region of Zurich,
Switzerland. Concentrations of the occurring FQs CIP and NOR as well as their ratio are similar along the year [11].

**Raw sewage.** A 4-day composite sample was obtained from a combination of 24-hour composites (August 20 to 23, 2001) collected at the entry point of the municipal WWTP Bülach (Switzerland).

**Treated effluents.** A 4-day composite sample was obtained from a mixture of 24-h composites (August 20 to 23, 2001) collected at the outlet of the Bülach WWTP after mechanical, biological and filtration treatment. For whole extract screening, a single sample was formed out of the combination of 24-h composites of treated effluents from four municipal WWTPs (Fällanden, Basserdorf, Dübendorf and Glattpfelden) discharging into the River Glatt.

**River samples.** A 2-week flow proportional composite sample (June 18 to July 1, 2001) was collected at the Rümlang station in the River Glatt, located downstream of the discharge of five municipal WWTPs.

**Sewage sludge.** An anaerobically-digested sewage sludge (30-days composite) and an untreated raw sludge (grab sample) were collected from the mechanical-biological WWTP Zurich-Werdhölzli (October 2000).

**Sludge-treated soil samples** [17]. A topsoil sample (0–2.5 cm) of sludge-treated field was taken from an experimental plot in Wetzikon. The sewage sludge originated from the municipal WWTP Uster. The soil sample was collected 5 months after sludge-application of 25 t/ha, which corresponds to 5 times the normal rate in Switzerland (5t every third year). Each sample consisted of four equal aliquots of randomly chosen soil from the same depth.

Raw sewage, treated effluents and river samples were immediately filtered through 0.45 µm cellulose nitrate membrane filters, stabilized by lowering the pH to 3 to reduce biological activity and stored in the dark at +4°C until analysis. Sewage sludge samples were dried at 60°C, finely ground (< 0.5 mm), and stored in amber bottles. The sludge-treated soil sample was dried at 40°C and passed through a 0.2-mm sieve [17]. Subsequently, 200 mg sewage sludge and sludge-treated soil were extracted by Accelerated Solvent Extraction (ASE) with an aqueous phosphoric acid / acetonitrile mixture (1:1) at 100 °C as described in ref [9]. The obtained extracts were diluted to 200 mL with water, acidified to pH 3 and directly analyzed.
All samples were split in two portions. One portion was chemically analyzed for FQs by a previously described method consisting of off-line SPE and LC-FLD [8]. The second portion was analyzed as follows: for whole extract screening it went through off-line SSPE, evaporation and directly tested for genotoxicity; for chromatographic fraction screening it went through off-line SPE, followed by the ToxPrint approach [16] with a modified analytical separation method, and tested for genotoxicity with the umuC-assay.

6.2.3 Chemical Analysis with SPE LC-FLD

All environmental samples were selectively analyzed for FQs by solid-phase extraction combined with liquid-chromatography coupled to fluorescence detection [8].

Solid-phase extraction (MPC-SPE). Sample volumes of 50 mL raw sewage, 150 mL treated effluent, 500 mL river water, and 10 mL-aliquot of diluted extract of sewage sludge and sludge-treated soil (all at pH 3) were enriched through mixed-phase cation exchange disk cartridges (MPC, octyl phase and benzenesulfonate mixture) supplied by Varian International AG (Basel, Switzerland). Preconditioning of the MPC cartridges was done with 2 mL methanol and 2 mL water at pH 3. Samples were percolated at a flow approximately 1 mL/min. Compounds were then eluted with 2.5 mL 5% ammonia solution in 15% methanol, and the extract was acidified using 0.5 mL phosphoric acid 85%. Recoveries of CIP and NOR in environmental samples using MPC disk cartridges were better than 80%.

Liquid-chromatography with fluorescence detection (LC-FLD). Sample aliquots of 200 µL were injected to an analytical column (250 x 3 mm) and precolumn (20 x 3 mm) filled with Discovery RP-AmideC16 5 µm particles (Supelco, Buchs, Switzerland). Analyses were performed at a flow rate of 0.7 mL/min and column temperature of 50°C. The eluent A was a 25 mM aqueous o-H₃PO₄ solution (pH 2.4), and eluent B was acetonitrile. Elution started with 5% B. A 17-min linear gradient to 7% B, followed by a 5-min isocratic elution, and a 13-min linear gradient to 17% B was used for analysis. After washing with 85% acetonitrile for 5 min, the initial conditions were re-established by a 2-min linear gradient, followed
by an equilibration time of 10 min. The FLD excitation wavelength was 278 nm and emission wavelength, 445 nm. Peak identification was performed routinely by comparing fluorescence spectra (fixed excitation wavelength at 278 nm, and scanning emission wavelength from 300 to 500 nm), and retention times of each sample with the corresponding reference compounds. Quantification was done using the FQ tosufloxacin (TOS-IS) added to the samples before the solid-phase extraction. Overall precision for CIP and NOR was better than 11% (relative standard deviation). Limits of quantification for CIP and NOR were 90 ng/L for wastewater influents, 30 ng/L for treated effluents, 9 ng/L for surface waters, 0.45 mg/kg for sewage sludge and 0.18 mg/kg for soils.

6.2.4 Whole Extract Screening

The sample obtained after the mixture of treated effluents from four WWTPs was selectively enriched for FQs through a sequential solid-phase extraction (SSPE), evaporated to dryness, redissolved with water and spiked at different concentrations with CIP and NOR. TOS-IS surrogate standard was not added since it would interfere in the genotoxicity testing.

Sequential solid-phase extraction (MPC-Oasis-SSPE). Samples of treated effluent (150 mL) containing FQs and ground water samples (150 mL) not containing FQs (and regarded as blanks) were first extracted with the MPC-SPE procedure described in 6.2.3. Two acidic MPC-extracts (corresponding to 300 mL sample) were combined and then diluted up to 100 mL with water (with a final pH of 3), and extracted using a polymeric material Oasis HLB ([poly(divinilbenzene-co-N-vinylpyrrolidone)]) supplied by Waters (Rupperswil, Switzerland). Oasis cartridges were preconditioned with 3 mL methanol and 3 mL water at pH 3 (adjusted with formic acid). After enrichment, elution followed with 3 mL methanol. The MPC-Oasis-extracts were then evaporated to dryness by a gentle nitrogen stream. Duplicates of treated effluent and ground water MPC-Oasis-extracts were reconstituted with 360 μL distilled water. Duplicates of treated effluent and ground water MPC-Oasis-extracts were spiked with 16 ng FQs (corresponding to the same FQ amount in extract, so called 1-fold spiked extracts) and then reconstituted up to 360 μL with distilled water.
Finally, duplicates of treated effluent extracts were spiked with 48 ng FQs (corresponding to 3 times the amount in sample, so called 3-fold spiked extract) and then reconstituted up to 360 µL with distilled water. The reconstituted extracts were then distributed in 96-well plates, and the six 1:2 dilution series were tested with the umuC-assay as described in 6.2.6.

Recoveries of Oasis-extraction were determined by enriching triplicates of treated effluent samples spiked with 10 ng of CIP and NOR, and 400 ng of TOS-IS surrogate standard. Then the eluent was evaporated to dryness and the extracts were reconstituted to 1 mL with water and analyzed with LC-FLD as described in 6.2.3.

### 6.2.5 Chromatographic Fraction Screening

Chromatographic fractions of environmental samples were investigated by sequential solid-phase extraction (SSPE): first an off-line MPC-extraction as described in 6.2.3, and followed by an on-line Oasis-extraction. Next, the extracts were chemically analyzed with LC-DAD, fractionated, evaporated and tested with umuC as described in the ToxPrint procedure [16], but with a modified analytical separation method.

**On-line Oasis-SPE coupled to LC-DAD.** Previous to on-line analysis, the Oasis cartridges were preconditioned with ~10 mL methanol and ~10 mL water at pH 3 (adjusted with formic acid). The off-line MPC-extracts were diluted up to 100 mL with distilled water and with the help of a pump (Kipp, Analytica 9208) percolated through the on-line Oasis at 2 mL/min. The Oasis material was reused for about ten analysis. Elution of FQs from the cartridge occurred by increasing the percentage of organic modifier on the analytical column. Samples were analyzed by liquid-chromatography (Perkin Elmer binary LC-pump) with ultraviolet detection (photodiode-array detector, Water 996). The analytical column (250 x 4.6 mm) was an Inertsil ODS-80A with a precolumn of the same type (GL Sciences Inc.), the eluent A was 0.2 M formic acid (pH 2.0), and eluent B, acetonitrile. The gradient started with 5% B, followed by a sharp increase of organic solvent up to 10% in 0.1 min and decrease to 5% in 0.5 min, used to facilitate elution of FQs from the on-line Oasis cartridge. A 17-min liner gradient to 25% B followed by a 3-min isocratic elution was used for
separation. Afterwards, 100% B was reached in 10 min and kept for 5 min as washing step. The initial conditions were re-established by a 2-min linear gradient, followed by an equilibration time of 10 min. Flow rate was 0.7 mL/min and column temperature 50°C. The DAD-detection was set at 280 nm. Identification was accomplished by comparing ultraviolet spectra (scanning from 190 to 350 nm) and retention times of samples with corresponding reference compounds. Quantification was done by internal calibration with TOS-IS surrogate standard, added previous to SSPE. Limits of quantification of CIP and NOR for LC-DAD were defined as the second lowest calibration point of the linear correlation. Instrumental precision of the LC-DAD was assessed using an average of 5 independent measurements of a standard mixture.

Chromatographic fractionation and evaporation. At the detector outlet, samples were fractionated in 96-well glass plates by a fraction collector Model 202 (Gilson, Analytical Systems Meyvis). The fraction collection time was 1 min/well, collected from the 14th to the 32nd min of the UV-chromatogram; the previous and posterior fractions were discharged. Next, the HPLC-solvent (~0.7 mL/well) was evaporated from the wells to dryness in a warm water-bath at 60 °C under gentle nitrogen stream, and reconstituted with 60 µL distilled water previous to genotoxicity testing. Losses during evaporation in the 96-well plates were evaluated by comparing concentration-response curves of CIP, NOR and the positive control 4-NQO with and without evaporation. Standards were added to 96-well plates in distilled water or in 0.2 M formic acid (pH 2)/acetonitrile (80:20) (eluent composition expected to chromatographically elute FQs from the analytical column), evaporated and redissolved with distilled water to give test concentrations in the well from 3 to 89 ng/mL for CIP, 6 to 185 ng/mL NOR and 4 to 139 ng/mL 4-NQO. The same concentration range of FQs in distilled water was added directly to the 96-well plates and tested with the umuC-assay. Plastic and glass 96-well plates were evaluated.
6.2.6 Genotoxicity Test: umuC-Assay

The umuC-assay was performed in 96-well plates without metabolic activation according to the German standard method [18]. Briefly, a *Salmonella typhimurium* TA1535 carrying a multicopy plasmid pSK1002 with fused umuC-lacZ genes and the gene for resistance to ampicillin was used as the tester strain. The overnight culture was diluted 5-fold with fresh TGA medium and incubated at 37°C until the bacteria reached an exponential bacterial growth. The incubation mixture consisted of the test compound or treated effluent (for whole extract screening) dissolved in water (180 µL), 20 µL 10-fold TGA and 70 µL bacterial culture. For chromatographic fraction screening, some volume modifications to the standard method were included as follows: 60 µL extract dissolved in water, 6.7 µL 10-fold TGA and 23.3 µL bacterial culture. After 2h incubation, the bacterial suspension was diluted 5-fold with warm TGA medium, followed by a subsequent additional incubation period of 2 h. At the end of the treatment and post-treatment incubation the bacterial growth was measured as turbidity ($E_{600}$) and the level of $\beta$-galactosidase activity ($E_{415}$) was assayed by the colorimetric method using ONPG as a substrate. The genotoxic activities were expressed in enzyme induction factors (IF) relative to the water control. Induction factors above 1.5 are scored as sufficient positive results, estimated as minimal concentrations of genotoxins required to produce statistically significant increases from background controls according to ref [18]. Bacterial growth was expressed in percent compared to the water control, which results were also referred as cytotoxicity (% bacterial death). In all experiments, the standard genotoxin 4-nitroquinoline-N-oxide (4-NQO) was used as positive control, and additionally to water, negative controls with DMSO and 10-fold TGA medium were tested. Media, buffers, reagents, equipment and positive controls are described elsewhere [18]. The results are given as mean of duplicate determinations, except for chromatographic fraction experiments where each sample was only tested once.
6.3 Results and Discussion

6.3.1 Concentration-Response Curves

Previous to environmental sample screening, single substance concentration-response curves of several FQs and one metabolite (M1) (Figure 6.1) were studied using the umuC-assay.

![Chemical structures of the studied compounds](image)

Figure 6.1 Chemical structures of the studied compounds: two quinolones (NAL, PIP), nine fluoroquinolones (CIP, DIF, ENR, FLE, LOM, NOR, OFL, DAN, TOS), one CIP metabolite (M1) and the umuC-assay positive control 4-NQO.
Additionally, binary mixtures for the environmental relevant FQs (CIP and NOR) were investigated in a mixture ratio of 1:1. Aqueous solutions of FQs in appropriate concentrations were prepared and tested. The concentration range tested was adjusted to the different potency of the individual FQ concentration-response curves so that the complete effect range was described. The genotoxin 4-NQO was used as a positive control for the umuC-assay.

**Single substance effect.** Concentration-response curves for genotoxicity and bacterial growth were fitted to sigmoid curves using the program *Prism Graph* [19] (Figure 6.2). For genotoxicity fitting the induction factors were set as free parameters \( (y_{\text{min}} = 0; \ y_{\text{max}}) \), whereas for bacterial growth the minimum and maximum growth was fixed \( (y_{\text{min}} = 0\%; \ y_{\text{max}} = 100\%) \). EC\(_{50}\) values were calculated from the sigmoid curve (inflection point), and the lowest observed effect concentration (LOEC) values for genotoxicity were estimated from experimental concentrations showing induction factors around 1.5 (Figure 6.2, Table 6.1). As expected all compounds exhibited genotoxic effects in the umuC-test, even at the very low \( \mu g/L \) level. The LOEC\(_{\text{genotoxicity}} \) of the most active FQs showed DNA damaging effects below 3 \( \mu g/L \) (DIF, ENR, TOS). This means an approximately 20-fold higher umuC-inducing potency in terms of LOEC\(_{\text{genotoxicity}} \) than exhibited by the positive control 4-NQO. The lowest umuC inducing potency was shown by the quinolones PIP (400 \( \mu g/L \)) and NAL (2 000 \( \mu g/L \)). For the environmental relevant CIP and NOR a LOEC\(_{\text{genotoxicity}} \) of about 5 and 25 \( \mu g/L \) was estimated. The lowest EC\(_{50,\text{genotoxicity}} \) value was 11 \( \mu g/L \) for TOS and the maximal 7 935 \( \mu g/L \) for NAL. The metabolite M1 showed a reduced potency with regard to the parent compound CIP with a LOEC\(_{\text{genotoxicity}} \) of around 500 \( \mu g/L \). No sigmoidal fitting was possible for M1 because of insufficient data points to describe the complete effect range. Cytotoxicity (bacterial death) occurred at slightly higher concentrations than genotoxicity, with EC\(_{50,\text{cytotoxicity}} \) from down 66 \( \mu g/L \) for DIF to 15 270 \( \mu g/L \) for NAL.
Chapter 6

- **NAL experimental**
  - NAL sigmoidal fit
  - EC50 = 7.935 µg/L
  - LOEC = 2 000 µg/L

- **PIP experimental**
  - PIP sigmoidal fit
  - EC50 = 3.383 µg/L
  - LOEC = 400 µg/L

- **CIP experimental**
  - CIP sigmoidal fit
  - EC50 = 18 µg/L
  - LOEC = 5 µg/L

- **DIF experimental**
  - DIF sigmoidal fit
  - EC50 = 12 µg/L
  - LOEC < 3 µg/L

- **EC50**
  - NAL = 2 000 µg/L
  - PIP = 3.383 µg/L
  - CIP = 18 µg/L
  - DIF = 12 µg/L

- **Bacterial growth (%)**
  - NAL = 15 270 µg/L
  - PIP = 8 124 µg/L
  - CIP = 66 µg/L
  - DIF = 55 µg/L
**Bacterial Genotoxicity Evaluation**

EC₅₀ = 14 µg/L

LOEC = 3 µg/L

EC₅₀ = 58 µg/L

LOEC = 12 µg/L

EC₅₀ = 147 µg/L

LOEC = 14 µg/L

EC₅₀ = 112 µg/L

LOEC = 25 µg/L

EC₅₀ = 221 µg/L

LOEC = 51 µg/L

EC₅₀ = 295 µg/L

LOEC = experimental

- ENR sigmoidal fit
- FLE sigmoidal fit
- LOM sigmoidal fit
- NOR sigmoidal fit
Figure 6.2 Fitted concentration-response curves of FQ and the positive control 4-NQO in the umuC-assay.
The genotoxic activity observed for FQs in the umuC-assay (using a genetically modified *Salmonella typhimurium* strain), correlated well with the bacterial potency of the drug (minimal inhibition concentration, MIC) towards *Salmonella* spp [20] (Table 6.1). This correlation has already been observed between FQ genotoxicity in the SOS Chromotest with *Escherichia coli* and the MIC [21]. The lowest LOECs and EC$_{50}$ are observed for the newer FQs (second and third generation), whereas first generation quinolones are less potent. In that way, the less potent degradation product of CIP, desethylene-ciprofloxacin (M1) (human and photometabolite) showed a significantly reduced genotoxic activity compared to the parent compound. The herein genotoxicity concentration response curves are comparable to the range reported for toxicity concentration curves obtained with the bioluminiscence inhibition assay with *Vibrio fischeri* [2].

<table>
<thead>
<tr>
<th>Compound</th>
<th>Genotoxicity LOEC</th>
<th>Genotoxicity EC$_{50}$</th>
<th>Cytotoxicity EC$_{50}$</th>
<th>MIC (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First generation (quinolones)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAL</td>
<td>2 000</td>
<td>7 935</td>
<td>15 270</td>
<td>2 000 - 8 000</td>
</tr>
<tr>
<td>PIP</td>
<td>400</td>
<td>3 363</td>
<td>6 812</td>
<td>-</td>
</tr>
<tr>
<td>CIP</td>
<td>5</td>
<td>18</td>
<td>66</td>
<td>10 - 60</td>
</tr>
<tr>
<td>DIF</td>
<td>&lt;3</td>
<td>12</td>
<td>55</td>
<td>-</td>
</tr>
<tr>
<td>ENR</td>
<td>&lt;3</td>
<td>14</td>
<td>51</td>
<td>-</td>
</tr>
<tr>
<td>Second generation (fluoroquinolones)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLE</td>
<td>12</td>
<td>58</td>
<td>221</td>
<td>60 - 250</td>
</tr>
<tr>
<td>LOM</td>
<td>14</td>
<td>52</td>
<td>147</td>
<td>60 - 250</td>
</tr>
<tr>
<td>NOR</td>
<td>25</td>
<td>112</td>
<td>295</td>
<td>30 - 120</td>
</tr>
<tr>
<td>OFL</td>
<td>15</td>
<td>55</td>
<td>211</td>
<td>60 - 120</td>
</tr>
<tr>
<td>Third generation (fluoroquinolones)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAN</td>
<td>10</td>
<td>71</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>TOS</td>
<td>&lt;3</td>
<td>11</td>
<td>76</td>
<td>-</td>
</tr>
<tr>
<td>Metabolite</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>500</td>
<td>&gt;1 500</td>
<td>&gt;1 500</td>
<td>-</td>
</tr>
<tr>
<td>Positive control</td>
<td>4-NQO</td>
<td>60</td>
<td>508</td>
<td>4 951</td>
</tr>
</tbody>
</table>

*Estimated value obtained for experimental concentrations showing genotoxicity at induction factor around 1.5. Calculated using the sigmoid curve obtained using the Prism Graph [19]. 'Minimal inhibition concentration for *Salmonella* spp. [20].
**Effect of mixtures.** Because in the environment various FQs seem to occur simultaneously, the study of mixture genotoxicity and cytotoxicity is of especial interest. The combination effects of a binary mixture of the two environmentally detected FQs CIP and NOR were experimentally investigated and it was compared to theoretically predicted curves. For prediction of combination effects, two basic concepts termed concentration addition and independent action have been proposed [22-24], which define a case of no interaction. In that way, combined effects can be estimated on the basis of known toxicities of the single compounds.

1) The concept of **concentration addition** (Loewe additivity) is based on the idea that the components of a given mixture have a common site of primary action and so every toxicant present adds to the overall toxicity. The concentration addition concept is mathematically expressed as:

\[
\sum_{i=1}^{n} \frac{c_i}{EC_{x_i}} = 1
\]

where \( n \) is the number of mixture components, \( c_i \) gives the concentration of the respective component in the mixture, and \( EC_{x_i} \) denotes the concentration of the \( i \) substance which provokes \( x \% \) effect if applied singly. Every fraction \( c_i / EC_{x_i} \) gives the concentration of a compound in the mixture scaled for its relative potency.

2) In contrast, the concept of **independence action** (Bliss independence) is based on the assumption that the compounds of a given mixture act on different physiological systems within the exposed organisms. The mathematical formulation is as follows:

\[
E(c_{Mix}) = \prod_{i=1}^{n} [1 - E(c_i)]
\]

where \( E(c_{Mix}) \) refers to the predicted effect (scaled from 0 to 100) of an \( n \)-compound mixture, \( c_i \) is again the concentration of the \( i \) compound, and \( E(c_i) \) is the effect of that concentration if the compound is applied singly.
The experimentally determined concentration-response curves (genotoxicity and cytotoxicity) for a mixture of CIP:NOR at the same ratio as occurring in the environment (1:1) are plotted in Figure 6.3.

**Figure 6.3** Mixture genotoxicity and cytotoxicity (bacterial growth) of the environmental relevant FQs, ciprofloxacin (CIP) and norfloxacin (NOR); fitted experimental concentration-response curves, and predicted Loewe additivity and Bliss independence curves.
For genotoxicity curves, both concepts concentration addition and independence action predict a similar response. However, the results indicate that the combined CIP and NOR genotoxicity only follows the predictions till around the EC$_{50}$, which corresponds to a bacterial growth superior as 75%. Above EC$_{50}$ (or for FQ concentrations causing substantial bacterial death) none of both predictions is hold. On the other hand, cytotoxicity curves obtained for CIP and NOR mixture nicely correlates with the concentration addition concept. Because FQs share an identical mechanism of action (the inhibition of bacterial gyrase), the use of an additive concentration model seems reasonable, which has already been proposed by ref [2] using the bioluminiscence inhibition assay with *Vibrio fischeri*. Further studies are needed to elucidate whether competitive or antagonic genotoxicity between CIP and NOR at high concentrations occurs.

### 6.3.2 Sequential Solid-Phase Extraction and Chemical Analysis

Analytical methods based on solid-phase extraction and liquid chromatography with fluorescence detection are available for the selective determination of FQs in various environmental samples [8, 9]. Acidic and basic conditions play an important role on the enrichment and chromatographic separation of FQs which interferes in subsequent biological testing, since neutral conditions are required to not harm the test organism. Therefore, to selectively enrich FQs from environmental matrices and at the same time allow biological testing, a new methodology was developed based on the ToxPrint approach [16]. The proposed method for the genotoxicity-directed chemical analysis for whole extract screening and chromatographic fraction screening is shown in Figure 6.4.

*Sequential solid-phase extraction (MPC-Oasis-SSPE).* Ionic exchangers (Figure 6.5) were shown as the only adequate material for the selective enrichment of FQs, especially for highly polluted samples such as wastewater [8]. Elution of FQs from this material is done using aqueous ammonia in 5% methanol. By introducing an additional extraction step with the polymeric material Oasis HLB (Figure 6.5), the MPC-extract was once more selectively extracted for FQs, the aqueous ammonia solution.
Figure 6.4 Diagram of the methodology developed in this study for whole extract and for chromatographic fraction screening with the umuC-assay based on the ToxPrint approach [16].
was removed and a volatile solvent such as methanol could be used. Because the enrichment factor obtained with MPC cartridges was thought to be not sufficient for biological testing, 2 or 3 MPC-extracts were combined and diluted prior to enrichment with Oasis cartridges. Dilution with water to 20, 100 and 200 mL was studied, and 100 mL gave better recoveries. Enrichment with Oasis of MPC-extracts at neutral pH and acidic pH was evaluated, showing 10% better recoveries with extracts at pH 3. During on-line enrichment, elution occurs with decreasing polarity of the HPLC-eluent. Therefore, to study the dynamic behavior of FQ elution from the on-line Oasis enrichment, the percentage of acetonitrile was gradually increased from 10 to 90% in a mixture with 0.2 M formic acid used for off-line SPE elution. This experiment showed that 20% acetonitrile is sufficient for a quantitative elution of FQs from the Oasis material. Off-line recoveries for treated effluents eluted either with methanol or with 0.2 M formic acid : acetonitrile (80:20) were between 80-100%, with an overall relative standard deviation of 20% for CIP and NOR. The large standard deviation can be partly attributed to the loss of FQs during evaporation previous to chemical analysis.

Figure 6.5 Solid-phase extraction materials used for the selective enrichment of FQs from environmental samples. MPC mixed-mode silica-based non-polar C8 and strong cation exchanger, and Oasis HLB hydrophilicity-lipophilicity balance (styrene divinyl benzene polymer).

Chromatographic separation. The separation was optimized in such a way that a selective determination of FQs was possible either at neutral conditions with water or using aqueous solvents with volatile acids, which can be easily removed by evaporation. Because the available method for
FQs [8] uses a non-volatile acidic solution (phosphoric acid, pH 2.4) as eluent, a new method was required. Separation at neutral conditions with water was not feasible because of co-elution of FQ in the chromatographic column. Therefore, since acidic conditions seem indispensable for a baseline separation of FQs, volatile acidic solutions were evaluated. Finally, 0.2 M formic acid (pH 2.0) in combination with acetonitrile was selected for analysis. The sensitivity of the UV-detection was evaluated. The determined instrumental quantification limit of 2 ng on column was about 10 times higher than the one obtained for LC-FLD [8]. Instrumental reproducibility of the retention time for CIP, NOR and TOS-IS surrogate standard was less than 1% with LC-DAD.

**Evaporation.** The evaporation to dryness showed to be critical for a quantitative genotoxicity-directed chemical analysis of FQs. Comparison between concentration-response curves of directly tested FQs or tested after evaporation showed that for the use of 96-well plastic plates response decreases considerably. For the evaporation of FQs in water, 30-35% of the expected responses were observed, whereas for FQs in 0.2 M formic acid:acetonitrile (80:20) almost no response was observed (~ LOEC_{genotoxicity}). The use of 96-well glass plates improved results considerably: about 65 to 85% of the expected response was observed for FQs in 0.2 M formic acid:acetonitrile (80:20). The behavior of CIP, NOR and 4-NQO was similar for all conditions tested. Note that complete removal of formic acid residues is indispensable, otherwise acid residues may result in bacterial death.

**6.3.3 Whole Extract Screening**

In a first approach, the developed method was applied to determine umuC-genotoxicity of whole extracts of treated wastewater effluents. FQs show a high genotoxic potential in the umuC assay, which lowest effects are observed at the µg/L level (LOEC_{CIP} ~ 5 µg/L, LOEC_{NOR} ~ 25 µg/L). In the umuC-assay, native samples containing FQs at the µg/L level appear genotoxic [4, 5, 25], while for lower concentrations as generally occur in the environment (ng/L level, [10-12]) an enrichment procedure is necessary to allow perceptible effects (Figure 6.6).
A mix-sample of treated effluents with FQ concentration of about 50 ng/L and uncontaminated ground water samples (blanks) were enriched by sequential solid phase extraction (enrichment factor of 1667) and tested with the umuC-assay. Additionally, treated effluent and ground water extracts were spiked with FQs at concentrations near the effect concentration. The spiking was done in extracts previous to the umuC-assay to avoid losses due to the sequential solid-phase extraction and evaporation, and to reduce the effect of matrix characteristics. Particles were observed in the redissolved extract, however, filtration was avoided since it showed to reduce overall response in the umuC-assay (data not shown). Extracts were tested using dilution curves of the same sample in order to account for intermediate concentrations and obtain a better overview of the effect concentration. Test concentrations varied from 1.5 to 45 µg/L for dilution curves of spiked ground water and unspiked treated effluent; from 3 to 90 µg/L for 1-fold spiked treated effluent and from 6 to 180 µg/L for 3-fold spiked treated effluent. The obtained results are presented in Figure 6.7.

Figure 6.6 Concentration range of FQs determined in the Swiss aquatic environment versus the effect concentration range in the umuC-assay.
Figure 6.7 Dilution curves (1/2 from left to right) with tested FQ concentration for whole extract screening of ground water and treated effluent with the umuC-assay. Lines represent bacterial growth and bars genotoxic activity given as induction factors.

The cytotoxic behavior of the spiked ground water extract was similar as for the pure substance and independent of FQ concentration. For high concentrated extracts the bacterial death was higher than 50%, which might explain the its low genotoxicity. Concerning genotoxicity, the observed effect was much lower than the one expected and independent of FQ concentration. Possibly this is due to a suppression effect, even observed for the spiked ground water blanks (reduced about 80% from the one observed for pure substances in distilled water). This genotoxicity inhibition could be partly explained by matrix effects that might inhibit the mechanism of action of either FQs or the β-galactosidase enzyme. When considering the kind of compounds that could be responsible for such inhibition, salts and EDTA might be considered: the first have the ability of replace, and the second to complex, Mg$^{2+}$ essential for the activity of FQs [26] and of the β-galactosidase enzyme [27]. However, because of the highly selective enrichment procedure used, neither salts nor EDTA should
be present in the tested extracts. Although salts could be retained during the cationic/non-polar MPC-extraction, they should not 'survive' the polar/non-polar Oasis-extraction. Furthermore, at the pH of the MPC-extraction EDTA should be mostly uncharged, so escaping enrichment. Another explanation could be that FQs are sorbed to the particles observed in the redissolved extracts and so make them unavailable to the organism. In any case, further investigations are needed to clarify such matrix effects.

6.3.4 Chromatographic Fraction Screening

The developed method was also applied to the determination of umuC-genotoxicity of chromatographic fractions of various environmental samples in the ToxPrint approach [16]. All samples were previously subjected to SPE-LC-FLD analysis and the occurrence of FQs in each sample was demonstrated. CIP and NOR were again unequivocally identified with LC-DAD in the studied samples and their concentrations and corresponding enrichment factors are listed in Table 6.2.

Following fractionation and evaporation, the umuC-assay was carried out to measure the genotoxicity of chromatographic fractions from 14–32 min of the tested extracts. The obtained results are shown in the series of graphics in Figure 6.8 and 6.9, where in contrast to whole extract screening no inhibition of the genotoxicity was observed. The various extracts exhibited similar genotoxicity patterns, and though generally the highest genotoxic response was determined in fractions 18 to 20 min corresponding to FQs, still very significant signals in later fractions were observed. Based on the concentration-response correlation determined for CIP and NOR and the concentration calculated in well (Table 6.2), an estimate of the relative percentage of effect concentration for each FQ was made.

The genotoxic response calculated for NOR (18-19 min fraction) and for CIP (19-20 min) was usually lower than the ones estimated for similar concentrations of pure substances. Despite accounting for a reduction of FQ amount of ~50% during evaporation (see Section 6.3.2), only between 6 and 77% of the expected genotoxic activity was observed. In addition to the difficulty to quantitative define losses during evaporation, other
Table 6.2 Concentrations of Norfloxacin (NOR) and Ciprofloxacin (CIP) for Various Environmental Samples and through the Described Experimental Procedure

<table>
<thead>
<tr>
<th>aqueous sample</th>
<th>sample conc.(^a) (ng/L)</th>
<th>enrich. factor(^b)</th>
<th>calculated conc. in well (µg/L)</th>
<th>expected conc. for the observed effect(^c) (µg/L)</th>
<th>bacterial growth(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>raw sewage</td>
<td>426 NOR</td>
<td>833</td>
<td>354 NOR</td>
<td>127 NOR (36%)</td>
<td>40% NOR</td>
</tr>
<tr>
<td></td>
<td>472 CIP</td>
<td></td>
<td>593 CIP</td>
<td>81 CIP (21%)</td>
<td>41% CIP</td>
</tr>
<tr>
<td>treated effluent</td>
<td>71 NOR</td>
<td>5000</td>
<td>355 NOR</td>
<td>66 NOR (19%)</td>
<td>26% NOR</td>
</tr>
<tr>
<td></td>
<td>87 CIP</td>
<td></td>
<td>435 CIP</td>
<td>63 CIP (15%)</td>
<td>44% CIP</td>
</tr>
<tr>
<td>river water</td>
<td>11 NOR</td>
<td>25000</td>
<td>120 NOR</td>
<td>218 NOR (181%)</td>
<td>40% NOR</td>
</tr>
<tr>
<td></td>
<td>8 CIP</td>
<td></td>
<td>91 CIP</td>
<td>70 CIP (77%)</td>
<td>34% CIP</td>
</tr>
<tr>
<td>solid samples</td>
<td>sample conc.(^a) (ng/L)</td>
<td>enrich. factor(^b)</td>
<td>calculated conc. in well (µg/L)</td>
<td>expected conc. for the observed effect(^c) (µg/L)</td>
<td>bacterial growth(^d)</td>
</tr>
<tr>
<td>untreated sludge</td>
<td>0.9 NOR</td>
<td>333</td>
<td>310 NOR</td>
<td>178 NOR (58%)</td>
<td>48% NOR</td>
</tr>
<tr>
<td></td>
<td>1.0 CIP</td>
<td></td>
<td>345 CIP</td>
<td>21 CIP (6%)</td>
<td>32% CIP</td>
</tr>
<tr>
<td>digested sludge</td>
<td>2.0 NOR</td>
<td>333</td>
<td>675 NOR</td>
<td>136 NOR (20%)</td>
<td>46% NOR</td>
</tr>
<tr>
<td></td>
<td>1.1 CIP</td>
<td></td>
<td>352 CIP</td>
<td>69 CIP (20%)</td>
<td>74% CIP</td>
</tr>
<tr>
<td>sludge-treated soil</td>
<td>0.3 NOR</td>
<td>333</td>
<td>108 NOR</td>
<td>194 NOR (180%)</td>
<td>37% NOR</td>
</tr>
<tr>
<td></td>
<td>0.4 CIP</td>
<td></td>
<td>119 CIP</td>
<td>62 CIP (52%)</td>
<td>48% CIP</td>
</tr>
</tbody>
</table>

\(^a\) Quantification by UV detection (280 nm). \(^b\) Enrichment factor from enriched sample volume to 60 µL extract in well. \(^c\) Expected concentration in the 96-well plate that would show the observed genotoxic effect (calculated based on the slope of the concentration-response curve and assuming 50% losses during evaporation). \(^d\) Observed bacterial growth in the tested chromatographic fractions (18-19 min for NOR, 19-20 min for CIP) of the 96-well. \(^\ast\) Percentage of the genotoxicity effect observed compared to that expected for the FQ concentration in sample.

Complications need to be considered when quantitatively comparing between effect and FQ concentration. First, in most cases CIP and NOR could not be collected in separate fractions, but they were irregularly split in two fractions (18-19 min and 19-20 min), each fraction containing an
undefined amount of CIP and NOR. This irregularities could explain the 180% of the expected effect for the NOR-fraction (18-19 min) in the river water and sludge-treated soil extracts, in case that also CIP was partially collected in that fraction. Furthermore, enrichment factors were increased to account for losses during evaporation and so prevent low responses in the umuC-assay, and thus caused a significant decrease in the bacterial growth, especially in the FQ fractions (< 50%). Therefore, the lower response observed in those fractions could also have been influenced by the visible increase of cytotoxicity, which interferes in a correlation between concentration and induction factors [28]. This effect can be clearly observed in the isolated highly cytotoxic fraction corresponding to the surrogate standard TOS-IS (24-27 min fractions), which was added to the sample at high amounts for quantification purposes.

Besides the genotoxicity observed in the FQ fractions, other fractions gave a positive response in the umuC-assay. Particularly it was in the later eluting fraction of the chromatogram that the most genotoxic compounds occurred, whereas the most polar compounds showing genotoxicity were the FQs. Thus, other compounds with similar enrichment and separation behavior to FQs are responsible for additional genotoxic effects in the tested extracts. However, no distinct peaks were visible in the chromatograms at 280 nm corresponding to the genotoxic response in those fractions (Figure 6.8). Noticeable is that a larger part of the genotoxic response was located in the fraction close to the occurrence of TOS-IS. For the genotoxic responses in the adjacent fractions to TOS-IS it is not clear whether they are caused by the splitting of the TOS-IS into these fractions, or because of the occurrence of other genotoxic compounds. Such compounds could either show a low UV response at 280 nm, or be highly genotoxic, even at low concentrations. At the end of the chromatogram of the tested extracts, the UV-absorbance of the matrix was always very high (Figure 6.8) and though several peaks could be observed with retention times between 29 and 32 min, no significant genotoxic responses were detected in those fractions.
Figure 6.8 Above a typical reverse-phase LC-DAD chromatogram of environmental extracts (analyzed at 280 nm) with 0.2 M formic acid and acetonitrile as organic modifier. Below the umuC-screening of the chromatographic fractions of the extract corresponding to a municipal raw sewage sample (enrichment factor 833). Lines represent bacterial growth and bars genotoxicity expressed as induction factors.
Figure 6.9 Chromatographic fraction screening of various environmental extracts and an uncontaminated ground water regarded as a blank with the umuC-assay. For enrichment factors see Table 6.2. Lines represent bacterial growth and bars genotoxicity expressed as induction factors.
The decrease in bacterial growth observed in fractions 24-27 min corresponds to the surrogate standard TOS-IS added at cytotoxic concentrations.
A comparison of the genotoxicity values obtained for the raw sewage and the treated effluent showed that by comparing the enrichment factors (Table 6.2), raw sewage had 6 times higher toxicity than the treated effluent. Therefore, for the tested extracts, WWTP treatment seems to reduce genotoxicity of the raw sewage. Furthermore, genotoxicity is reduced for river water extracts. No great variation in the genotoxicity pattern was observed for aqueous and solid extracts, yet the slight different genotoxicity profile between untreated and digested sludge is interesting. Genotoxicity profile was also measured for a soil sample with an important contribution on sludge, which agrees with the fact that the applied sludge was from a different WWTP that the ones tested.

6.4 Conclusions

A highly selective toxicity-directed analytical method for the screening of umuC-genotoxicity of FQ in various environmental samples was proposed, based on the combination of sequential solid-phase extraction, the ToxPrint approach [16] and the umuC-assay. Consequently, the method was applied to whole extract screening of treated wastewater effluents and to chromatographic fraction screening of wastewater effluents, surface water, sewage sludge and sludge-treated soil containing FQs. Genotoxic response was strongly suppressed when testing whole extracts of treated effluents as well as for ground water extracts. An explanation could be inhibition of either FQ or β-galactosidase enzyme activity due to matrix effects. On the other hand, the analysis of chromatographic fraction enabled the detection of FQs at the ng/L range. FQs were the major contributors to the genotoxicity found among several chromatographic fractions, however the observed response was lower than the one expected for the analytical determined FQ concentration. A quantitative comparison between concentration and effect was difficult due to the irregular fractionation, the undefined losses during evaporation and the low bacterial growth in the FQ-fractions. Besides FQs, the extracts contained other compounds with similar enrichment and chromatographic separation behavior to FQs (but not visible at 280 nm), which showed genotoxic activity without metabolic activation.
Concentration-response curves for two quinolones and nine fluoroquinolones were obtained with the umuC-assay, which allow to calculate EC$_{50}$ values for genotoxicity and cytotoxicity. Moreover, a notable correlation between bacterial potency (MIC) and umuC-genotoxicity was observed. Likewise, the genotoxic response of the desethylene-ciprofloxacinmetabolite (M1) was significantly less genotoxic than the more active parent compound ciprofloxacin. Furthermore, no-interactive effects of a mixture of the environmental relevant ciprofloxacin and norfloxacin could be ascertained. The combined concentration-response curve seems to follow the concentration addition model, yet only as long as the bacterial growth is not substantially affected.

The described method should be not only applicable to the (geno)toxic evaluation of FQs with the umuC-assay, but also for other toxicity screening tests. The presented method is most likely the only toxicity-directed analysis where FQs can be isolated, since presumably FQs 'escape' common methods that use polar and/or non-polar solid-phase extraction materials.

Acknowledgement

This work was partially done at Kiwa Research and Consultancy, Nieuwegein (The Netherlands), and it would not have been possible without the much-appreciated engagement of Ivana Bobeldijk. The technical assistance of Albert Brandt and Ton Braat for instruction and help during the ToxPrint analysis and the umuC-assay, respectively, is gratefully acknowledged. Beate Escher is especially acknowledged for sharing her expertise in mixture toxicity. Andreas Hartmann is also thanked for continuous interest and support to this work.
6.5 Literature Cited


[19] Motulsky, H. *Prism Graph Pad, Vers. 3.0.a, Graph Pad, San Diego, CA, USA (1994-2000).*


General Conclusions and Outlook
This dissertation aims at contributing to the environmental exposure assessment of an important class of human-use pharmaceuticals: the fluoroquinolone antibacterial agents (FQs). Besides, an environmental risk assessment according to European guidelines and draft documents was proposed. The principal achievements and main conclusions are given in the subsequent paragraphs:

(i) Specific analytical methods were developed for reliable trace determinations of FQs in various aqueous and solid environmental matrices. These methods are suitable for monitoring the occurrence and evaluate the behavior and fate of FQs in various environmental compartments.

(ii) The major exposure routes of FQs into the Swiss environment were identified: a) FQs reach surface waters by discharge of treated wastewater effluents, and b) FQs are transferred to agricultural soils by the application of sewage sludge as fertilizer. The latest is of especial interest, since the impact of human-use pharmaceuticals to terrestrial ecosystems is expected to be of minor significance. These findings also emphasize the impact of wastewater treatment and sludge management strategies as point sources of FQs to the environment.

(iii) With the help of process-oriented studies an overview on the behavior and fate of FQs during wastewater treatment, in surface waters and in sludge-treated soils was obtained. Mass balance studies showed that during wastewater treatment FQs are efficiently removed (79–87%) from the wastewater, with complete mass transfer to sewage sludge. During sludge digestion no significant removal of FQs was observed. On the other hand, during transport in rivers an additional removal from the dissolved fraction can be expected (48–66%). Once FQs reach agricultural soils after sludge application, persistence and limited mobility down the subsoil must be foreseen.

(iv) An environmental risk assessment of the occurrence of FQs in the Swiss aquatic environment was carried out accordingly to the recommendations of European guidelines and draft documents. The risk quotients obtained, suggested a low probability for adverse effects of the occurring FQs, either on microbial activity in WWTPs, or on algae, daphnia and fish in surface waters.
(v) A **toxicity-directed chemical analysis** was proposed to evaluate the role of FQs in the umuC-genotoxicity of environmental samples.

Assuming an equal distribution of the FQ amount annually imported in Switzerland to the whole territory, supposing no metabolism and removal, and based on the monitoring data herein presented, we can estimate that: a) 25–35% of the imported amount enter wastewater treatment as raw sewage, and about 25–40% are accumulated in sewage sludge (the last including the percentage removed from the water stream and the amount excreted bound to feces), b) around 5–10% are discharged in treated effluents, but only 3–6% can be found in the receiving surface waters, and c) of the FQs associated to sewage sludge, 10–17% are applied annually to soils, but less than 3–5% might be detected. These averaged results indicate that a small percentage of FQs imported into Switzerland reach the environment.

The results of this study, suggest that future investigations of the following topics would be of interest:

(i) The monitoring study on FQs in the Glatt River provided only limited information about the processes affecting the fate of FQs in surface waters (e.g. sorption, photodegradation). Because of the low concentrations in surface waters, additional knowledge could be gained by analyzing **river sediments**.

(ii) To confirm the estimated elimination processes, the behavior of FQs during transport in rives and during wastewater treatment could be modeled with the help of computer **simulation programs**.

(iii) The experimental plot studies in agricultural soils provided preliminary data concerning the **fate** of FQs in **sludge-treated soil**. Laboratory soil column experiments could help to obtain more detailed information about FQ transport. Furthermore, transformation processes, such as biodegradation, at the topsoil of sludge-treated soils might be also a topic of interest.

(iii) Further improvement of the presented toxicity-directed chemical analysis should allow a better elucidation of the role of FQs in the umuC-**genotoxicity** of environmental samples.
Acknowledgments

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Curriculum Vitae

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