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IDENTIFICATION OF FLUOROQUINOLONE ANTIBIOTICS AS THE MAIN SOURCE OF *umuC* GENOTOXICITY IN NATIVE HOSPITAL WASTEWATER

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Abstract—Previous work revealed genotoxic effects in the wastewater of a large university hospital using a bacterial short-term genotoxicity assay, based on a *umuC*::*lacZ* fusion gene (*umuC* assay). These studies ruled out disinfectants and detergents as main causative agents of the genotoxic effects. This paper focuses on specific hospital-related drugs as the cause. The ratio of theoretical mean wastewater concentrations (derived from consumption data) and lowest observable effect concentrations of selected pharmaceuticals were used to calculate *umuC* induction probabilities. The fluoroquinolone antibiotics Ciproxin® and Noroxin® showed the highest induction probabilities and exceeded all other investigated drugs by at least one order of magnitude in significance. Antineoplastic drugs, originally thought to be the main effectors, were found to be of marginal significance using the *umuC* assay. These findings were further supported by investigation of urine samples of hospital patients with the *umuC* assay. The determination of ciprofloxacin in native hospital wastewater by reversed-phase high-performance liquid chromatography and fluorescence detection revealed concentrations from 3 to 87 μg/L. *umuC* induction factor and ciprofloxacin concentrations in 16 hospital wastewater samples showed a log-linear correlation ($r^2 = 0.84$, $p < 0.0001$). These results suggest that the previously measured *umuC* genotoxicity in the wastewater of the hospital under investigation is caused mainly by fluoroquinolone antibiotics, especially by ciprofloxacin. On the basis of these findings, the role of the *umuC* assay as a screening tool for wastewater genotoxicity assessment is discussed.

Keywords—Genotoxicity Hospital wastewater Fluoroquinolones *umuC* antineoplastic drugs

INTRODUCTION

In the past several decades, societies have become responsible for developing and maintaining sustainable water management strategies. The goal of these strategies has been to prevent exposure of humans and the aquatic environment to potentially harmful toxicants and to eliminate the disposal of hazardous or persistent chemicals into the water cycle. Among the many hazards posed by toxic chemicals entering aquatic environments, the impact of genotoxic (DNA-damaging) agents on humans and their environments has been of recent concern [1–3]. Evidence exists for genotoxic effects on aquatic biota [4, 5], where a variety of different sources (e.g., industries, manufacturers or landfill leachates) have been identified as emitters of substantial quantities of genotoxic agents via disposal in sewage [for reviews see 6–8]. The identification of chemicals responsible for the genotoxic effects in environmental samples has been a challenging task, met in the past by two main strategies. The most frequently used bioassay-directed strategy uses fractionation (often paralleled by concentration) of genotoxic mixtures, reassaying their biological activity, and finally chemical analysis of the genotoxic fraction(s). This approach often allows a preliminary chemical classification of suspected genotoxins [9]. The second strategy combines calculated predicted environmental concentrations (PEC) of suspicious chemicals in certain compartments, based on emission data and known environmental fate, with their lowest observable effect concentrations (LOECs), based on experimental toxicologic data. Combination of PECs and LOECs provides an estimate of the effect-causing potential of the agent, which can then be verified by correlating biological effects of environmental samples with analytical concentrations of these chemicals [10]. It is obvious that this second strategy requires much more detailed information on the genotoxic mixture under investigation than does the first strategy. On the other hand, more precise conclusions can often be drawn on the chemicals causing genotoxicity in environmental samples.

Here, we report the source identification process in a case study on hospital wastewater genotoxicity using the second strategy. Hospital wastewater is a known source for many potentially genotoxic chemicals, for example, drugs or disinfectants [11–13]. We recently published the finding of genotoxicity in native hospital wastewater by using a bacterial short-term genotoxicity assay, the *umuC* test [14]. In the previous study, the chemicals causing the genotoxic effects were not clearly identified. In this study we present evidence that the formerly detected effects are caused to a large extent by one single class of antibiotic drugs, the fluoroquinolone (FQ) antibiotics. These fully synthetic antibiotics, structural analogues of the parent compound nalidixic acid, exhibit their antimicrobial properties principally by inhibition of the bacterial DNA unwinding enzyme gyrase, thereby inhibiting DNA synthesis and cell division, leading to rapid cell death in susceptible organisms [15,16]. The FQs used in the hospital under investigation were norfloxacin and ciprofloxacin.

MATERIALS AND METHODS

Chemicals and drugs

The following chemicals and drugs were used: Adriblastin® (doxorubicin-HCL, Farmitalia Carlo Erba AG, Zug, Switzer-

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land); ampicillin-Na (Fluka AG, Buchs, Switzerland); Augmentin® ped. (amoxicillin, SmithKline Beecham, Thörishaus, Switzerland); ciprofloxacin-HCl (88.9%) (Bayer AG, Wuppertal, Germany); Ciproxin® iv (ciprofloxacin, Bayer [Schweiz] AG, Zürich, Switzerland); Cymevene® (ganciclovir, Roche Pharma AG, Reinach, Switzerland); Foscavir® (foscarnet-Na, Astra AG, Dietikon, Switzerland); mefenamic acid (Sigma, Buchs, Switzerland); metronidazole (Sigma); mitomycin-C (Syntex AG, Allschwil, Switzerland); 2-nitrophenyl-β-D-galactopyranoside (ONPG) (Böhringer Mannheim AG, Rotkreuz, Switzerland); Norfloxacin (Sigma); paracetamol (Sigma); Pipril® (piperacillin, Cyanamid AG, Adliswil, Switzerland); Tiberal® (ornidazole, Roche Pharma AG, Reinach, Switzerland); Vepesid® (etoposide, Bristol-Myers Squibb AG, Baar, Switzerland); Zinacef® (cefuroxim, Glaxo AG, Schönbühl, Switzerland).

Sampling

Two-liter wastewater samples were collected in polyethylene flasks out of the main sewer of the hospital using a pump collector. Samples were immediately transported to the laboratory, partitioned into aliquots, and either tested directly or refrigerated at −20°C. Samples that were later subjected to high-performance liquid chromatography (HPLC) analysis were filtered (TITAN HPLC syringe filters, 0.45 μm, nylon, 25-mm i.d., Schmidlin AG, Neuheim/Zug, Switzerland; or Sartorius cellulose acetate filters, 0.45 μm, 13-mm i.d., Dr. Vaudaux AG, Schönenbuch, Switzerland) before being assayed by the *umuC* test.

Drug consumption data

Statistical data giving the amount of drug units or chemicals purchased by the hospital for stationary treatment were used to calculate the theoretical mean sewage concentration (TMSC) of active ingredients in the hospital sewage. We assumed a worst-case scenario where no metabolic degradation or inactivation was taken into account. Furthermore, the purchased amount of a chemical or drug was expected to appear quantitatively in the wastewater. To estimate the TMSC for a single chemical, its total annual sales were divided by the mean annual wastewater flow of the hospital (453,000 m^3).

Genotoxicity assay

The *umuC* assay was performed without metabolic activation as described by Giuliani et al. [14], except that the assay volume was 0.5 ml. β-Galactosidase (β-gal) was assayed according to the procedure of Whong et al. [17] with the following modifications: 100 μl of the test mixture was placed in 400 μl of B-buffer and 100 μl of ONPG was added. After incubation for 30 min at 28°C, the reaction was stopped by adding 300 μl of 1 M Na_2CO_3 and the absorbance at 420 nm (A_{420}) was measured. β-Galactosidase activity was calculated using a formula adapted from Miller [18]

$$\beta\text{-gal units} = 1{,}000 \cdot \frac{A_{420}}{t} \cdot V \cdot A_{600}$$

where t = incubation time in min, v = volume in ml, and A_{600} = absorbance at 600 nm. Genotoxic activity, expressed as induction factor (IF), was calculated as the ratio of β-gal units between the test sample and the negative control. Likewise, growth rates were determined by measuring the A_{600} of test samples and negative controls. Growth rates between 80 and 120% were considered to be normal. Genotoxicity data of samples causing more than 50% reduction of bacterial growth were rejected, because they show poor reliability [19].

HPLC

Determination was based on a reversed-phase HPLC system consisting of a gradient pump, an autosampler, and a fluorescence detector operating at an excitation wavelength of 278 nm and an emission wavelength of 445 nm. The HPLC conditions were adapted from Scholl et al. [20]. Briefly, an octadecylsilica column (Nucleosil 100, 5 μm, 125 × 3 mm i.d. with a 8 × 3 mm i.d. precolumn of the same type, Macherey-Nagel, Germany) was operated at room temperature with a flow rate of 0.8 ml/min. The mobile phase (eluent A, pH 2.4) was a 0.02 M KH_2PO_4 and 0.02 M *ortho*-phosphoric acid buffer. The organic modifier (eluent B) was acetonitrile. Elution started isocratically with 98% eluent A for 0.5 min, followed first by a linear gradient to 90% A for 0.5 min and then by a second linear gradient to 75% A in 10 min. After each injection the column was washed for 2 min with 70% B. Initial eluent composition was reestablished by a 3-min linear gradient, followed by an equilibration time of 6 min.

Quantification was carried out by using ciprofloxacin-HCl (88.9% purity) as an external standard (Fig. 1A). The correlation between peak areas and concentrations was determined by linear regression and showed typically $r^2 = 0.999$. An instrument detection limit of 0.5 μg/L for ciprofloxacin was estimated (signal to noise ratio [S/N] = 10). The precision in hospital wastewaters (relative standard deviation) was 0.8% at 45 μg/L and 4.7% at 5 μg/L ($n = 7$). Recovery rates were 104.2 ± 1.9% and 101.7 ± 1.0% for hospital wastewater samples spiked with 10 μg/L and 50 μg/L ciprofloxacin, respectively ($n = 3$).

RESULTS

Calculation of umuC *induction probabilities (IP)*

To find possible sources for the previously described *umuC* effects [14], we used a three-step strategy, with the results summarized in Table 1. In the first step we used hospital data on drug purchases to calculate total consumption rates of pharmaceuticals with a known or suspected genotoxic potential. Mean values of a period of 3 years (1992–1994) were calculated. These rates were then divided by the mean annual wastewater flow, which resulted in a TMSC of the respective chemical. The calculated concentrations ranged from 0.02 μg/L for the cytostatic drugs mitomycin-C and bleomycin to 201 μg/L for the broad-spectrum antibiotic drug amoxicillin. The two gyrase inhibitors actually in use at the investigated hospital showed calculated sewage concentrations of 14.5 μg/L (ciprofloxacin) and 6.2 μg/L (norfloxacin).

The second step was the assessment of the LOECs of selected chemicals in the *umuC* assay. According to the previous findings that wastewater samples typically showed no reduction of bacterial growth [14], we only considered chemicals that exhibited a genotoxic effect at concentrations where bacterial growth was not, or was only slightly, reduced ($A_{600} \geq 80\%$). Because both disinfectants and detergents did not meet these criteria, we focused on pharmaceuticals. The minimal genotoxicity effect level was defined by an IF of 2, according to the criteria proposed by Whong et al. [17]. The LOECs found ranged from 0.005 mg/L for ciprofloxacin to more than 26 mg/L for fluorouracil (see Table 1).

In addition to the antineoplastic and antibiotic drugs listed

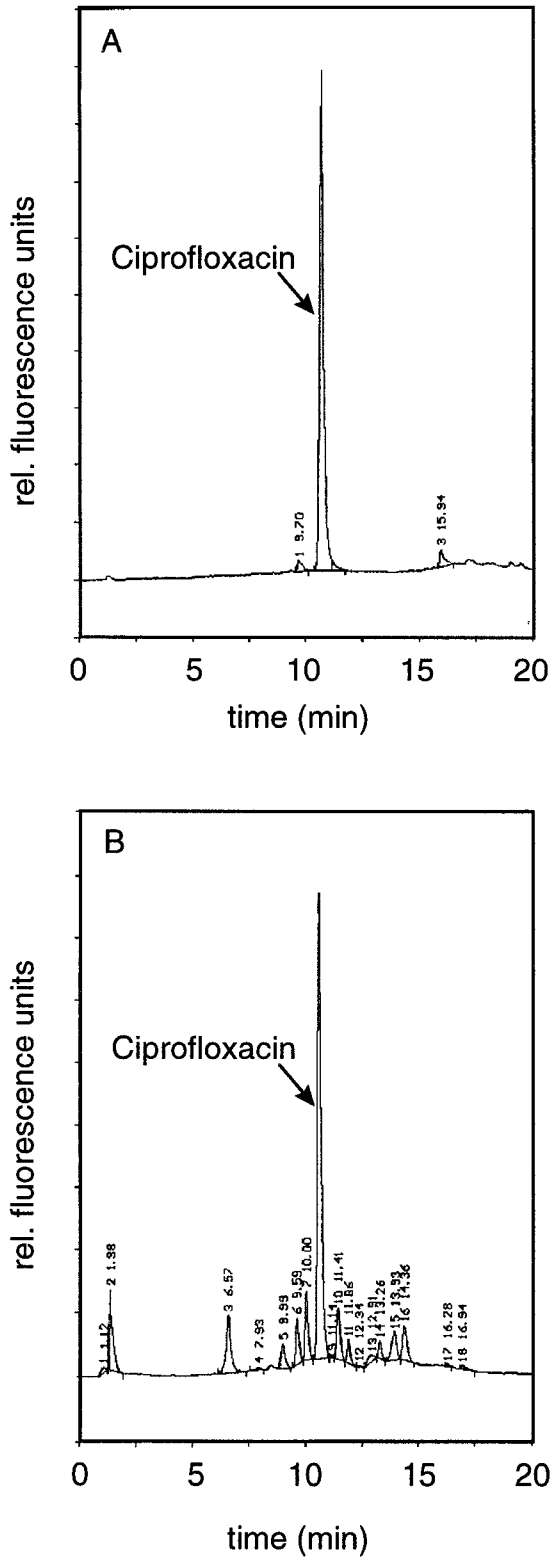

Fig. 1. High-performance liquid chromatography chromatograms of (**A**) the reference compound ciprofloxacin in 0.1 M *ortho*-phosphoric acid buffer and (**B**) a hospital wastewater sample containing 87 μg/L of ciprofloxacin.

in Table 1, other drugs were considered to be potential effect-causing agents. However, when the following drugs were subjected to the *umuC* assay up to the doses indicated in parentheses, at least two orders of magnitude higher than the respective TMSCs, no genotoxic potential was found or bacterial growth was reduced by more than 50% (data not shown): the nonsteroidal antiinflammatory drugs paracetamol (1,000 mg/L) and mefenamic acid (100 mg/L), the antiviral drugs foscarnet-Na (50 mg/L) and ganciclovir (250 mg/L), and the antibiotics cefuroxim (a cephalosporine, 10 mg/L) and piperacillin (an aminobenzylpenicillin, 100 mg/L).

Table 1. Theoretical mean sewage concentrations (TMSCs), lowest observable effect concentrations (LOECs), and *umuC* induction probabilities (IP) of selected hospital-specific chemicals. The IP is defined as the TMSC to LOEC ratio

Drug (active ingredient)	Mean annual consumption (1992–1994) (g)	TMSC (μg/L)	LOEC (*umuC* IF = 2)[a] (mg/L)	*umuC* IP
Etoposide	220	0.49	25	1.96×10^{-5}
Adriamycin	37	0.09	2.5	3.60×10^{-5}
Cisplatin	31	0.09	1.25	7.20×10^{-5}
Fluorouracil	921	2.03	>26	7.81×10^{-5}
Metronidazol	2,827	6.2	50	1.24×10^{-4}
Dacarbazin	85	0.19	>1	1.90×10^{-4}
Bleomycin	6	0.02	0.05	4.00×10^{-4}
Mitomycin C	4	0.02	0.02	1.00×10^{-3}
Ornidazol	3,748	8.3	5	1.67×10^{-3}
Amoxicillin	91,161	201	20	1.01×10^{-2}
Norfloxacin	3,152	6.2	0.025	2.48×10^{-1}
Ciprofloxacin	6,124	14.5	0.005	2.90×10^{0}

[a] IF = induction factor.

The third step of our approach consisted of the calculation of a *umuC* IP for the selected wastewater chemicals, given by the ratio of TMSC and LOEC of the chemical. The IP can be regarded as a rough estimate of a chemical's mean probability to induce the *umuC* system under the worst-case assumptions described above (see Materials and Methods). An IP of 1 means that the average *umuC* induction factor of all sewage grab samples collected throughout a year would be 2. The IPs differed within five orders of magnitude (Table 1): etoposide, an inhibitor of mammalian topoisomerase II had the lowest IP (1.96×10^{-5}), whereas ciprofloxacin, an FQ antibiotic, showed an IP of 2.9. The FQ antibiotics ciprofloxacin and norfloxacin exceeded all the other chemicals tested in significance to the observed genotoxicity by at least one order of magnitude.

Analysis of urine samples

To obtain further information about the potential sources of genotoxicity in hospital wastewater, urine samples of patients treated with antibiotic or antineoplastic drugs were analyzed by the *umuC* assay. We found that urine samples of four patients receiving FQ antibiotics induced the *umuC* system even after 200-fold dilution (norfloxacin, 1 case) or 2,000- to 20,000-fold dilution (ciprofloxacin, 3 cases). In contrast, urine samples from three patients receiving either cisplatin, dacarbazine, or bleomycin/cisplatin/etoposide therapies were genotoxic only when assayed undiluted or, at the most, four times diluted. Urine entering the hospital's sewage system was assumed diluted 100- to 5,000-fold, depending on its volume and the present wastewater flow. These results clearly show that the effect-causing potential of urine containing FQ antibiotics exceeds that of urine containing antineoplastic drugs by several orders of magnitude.

Chemical analysis of ciprofloxacin in hospital wastewater

To investigate concentrations of ciprofloxacin in hospital wastewater samples, HPLC with fluorescence detection

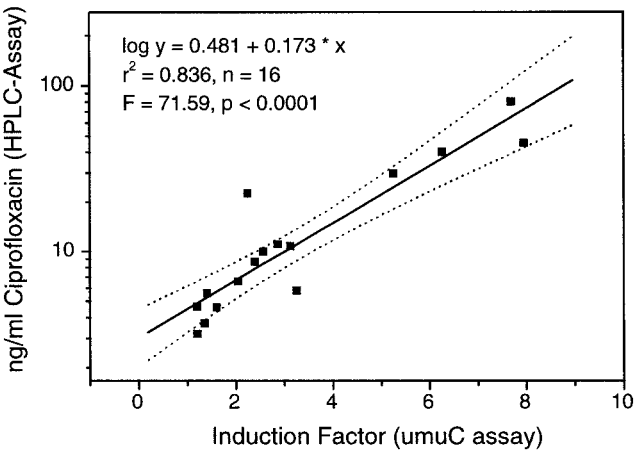

Fig. 2. Log-linear correlation of high-performance liquid chromatography-based concentrations of ciprofloxacin in hospital wastewater samples with their *umuC* induction factor ($n = 16$). Analysis of variance revealed an F ratio of 71.59 with an associated probability level of $p < 0.0001$. Dashed lines indicate the 95% confidence limits of the regression model.

(HPLC-FD) was used. Ciprofloxacin could be detected in hospital wastewater samples without any enrichment. Concentrations varied from 3 to 87 μg/L. Figure 1B shows the chromatogram of a typical hospital wastewater sample. To further assess the impact of ciprofloxacin in hospital wastewater samples on *umuC* induction, induction factors and ciprofloxacin concentrations of 16 sewage samples were compared (Fig. 2). We found a log-linear correlation with $r^2 = 0.84$, supporting the hypothesis that ciprofloxacin contributes strongly to the genotoxicity effects found by *umuC* screening.

DISCUSSION

The results presented herein suggest that FQ antibiotics, especially ciprofloxacin, account for most of the genotoxic effects detected in grab samples of hospital wastewater by the *umuC* assay. Supporting evidence is threefold: First, the comparison of TMSCs of drugs and their LOECs in the *umuC* assay favored FQ antibiotics over all other investigated drugs as causative agents by at least one order of magnitude. Second, we found strong genotoxic effects in the *umuC* assay when testing urine samples of patients receiving Ciproxin. Urine samples induced the *umuC* system in dilutions up to 1:20,000. Third, a strong correlation ($r^2 = 0.84$) between the *umuC* IF and HPLC-determined ciprofloxacin concentrations found in 16 native hospital wastewater samples further supports our conclusion, namely that FQ antibiotics contribute strongly to the earlier described *umuC* effects.

The applied drug emission approach allowed a rough estimate of TMSCs in hospital wastewater. However, concentrations in grab samples of hospital sewage are expected to differ considerably from these calculated concentrations. These differences can mainly be attributed to the complete neglect of metabolic processes that would normally reduce the concentration of native components, and secondarily can be contributed to ignoring fluctuations in concentration due to discontinuous emission patterns and varying wastewater flow. Nevertheless, for ciprofloxacin, expected to remain about 70% unmetabolized following its passage through the body [21], a reasonably precise prediction of drug concentration in hospital sewage was achieved. The predicted TMSC of ciprofloxacin was 14.5 μg/L. Sewage grab sample concentrations determined by HPLC-FD ranged from 3 to 87 μg/L. The predicted IP for ciprofloxacin was 2.9 (see Table 1), whereas the mean *umuC* IF of 851 grab samples taken at the investigated hospital from 1991 to 1992 was 1.5 [14]. The mean IF of new grab sample series is expected to correlate even better because ciprofloxacin consumption rates at this hospital increased during recent years (e.g., by 12% from 1992 to 1994, data not shown). In general, the remarkably good correlation supports the use of the simple worst-case emission model. The TMSCs of chemicals subjected to higher metabolic degradation rates, however, might be overestimated as a consequence of the worst-case approach.

Strong evidence that ciprofloxacin and norfloxacin caused most of the observed *umuC* effects in hospital wastewater was obtained by testing the urine samples of selected patients using the *umuC* assay. These patients received antibiotic or antineoplastic drugs. The results clearly showed that urine samples containing the antineoplastic agents cisplatin, dacarbazine, bleomycin, or etoposide became nongenotoxic at low dilution factors, which were at least 25 times lower than the average dilution of urine typically released into the hospital's sewage system. These findings rule out cytostatic drugs as the main causative agents of genotoxicity detected by *umuC* screening. In contrast, urine samples containing FQ antibiotics showed *umuC* genotoxicity, even when diluted at least 100-fold, similar to realistic dilution conditions.

Ciprofloxacin was not the only FQ antibiotic applied at the investigated hospital. The other FQ in use, norfloxacin, showed a five times higher LOEC (25 vs 5 μg/L) and its TMSC was less than one half of that of ciprofloxacin (6.2 vs 14.5 μg/L), yielding an approximately 10 times lower *umuC* IP for norfloxacin. This led to the decision not to analyze FQ compounds other than ciprofloxacin. Hospital wastewater, like most industrial or municipal sewages, is a complex mixture of a vast amount of chemicals. Many of these chemicals, principally disinfectants and DNA-reactive drugs, are potential human genotoxins. A positive genotoxicity signal in the *umuC* assay has therefore to be considered a possible relevant indicator for an existing human health risk. Our previous results [14] showed that disinfectants are unlikely to be responsible for the established *umuC* effects. Data presented herein now support evidence that antineoplastic drugs used in human anticancer therapy can also be ruled out as main causative agents. In the light of the present findings, the risk of an acute human genotoxic hazard caused by hospital sewage becomes markedly reduced. All studies that investigated eukaryotic genotoxic effects of ciprofloxacin in vitro or in vivo reported significant genotoxic effects only in concentrations one or several orders of magnitude above the highest concentrations we found in hospital wastewater [22–25]. Furthermore, none of the available data suggest a carcinogenic potential of ciprofloxacin [24,25]. Prokaryotic genotoxicity tests occasionally tend to have a low prediction power for human genotoxicity, for example, for heavy metals [6]. Because we had suspected cytostatic drugs to contribute to a human genotoxic risk in hospital wastewater, one might argue that the *umuC* assay could yield false negative results by underestimating cytostatic drugs in general and heavy metal compounds (e.g., cisplatin) in particular. However, studies determining the concentrations of the cytostatic drugs bleomycin and cisplatin in hospital and municipal sewage streams reported concentrations in the low parts per billion to parts per trillion range (10^3 to 10^4 times lower than serum levels of cancer patients) [26,27]. This renders

acute genotoxic or mutagenic effects towards mammalian organisms unlikely.

Further investigations will be necessary to monitor the fate of FQ gyrase inhibitors in the aquatic environment and to assess the ecological impact posed by the release of these antibiotics into public wastewater. With regard to the effects of FQs, the most probable impaired organisms are prokaryotes. Microorganisms in the activated sludge of sewage treatment plants will come into contact with substantial amounts of the drug. The environmental fate of FQs will largely depend on the biotransformation potential of these organisms and on the sorption properties of FQs. Unpublished data of the manufacturer [28] and our ongoing experiments suggest a low biodegradability of ciprofloxacin. For norfloxacin, strong binding to human fecal material has been described [29]. However, the extent and the mechanisms of FQ sorption under environmental conditions remain unclear at present, mainly due to both their lipophobic and amphoteric nature. Our findings that neither nylon nor cellulose acetate filtering (0.45 μm) reduced genotoxic effects significantly (data not shown) indicate a rather hydrophilic postemission behavior of FQs in hospital wastewater. This would suggest that FQs resistant to biodegradation in the sewage treatment plant would remain in the aqueous phase rather than readily adsorb to sludge or sediment.

Furthermore, our results demand a critical discussion of the *umuC* assay as a routine wastewater screening test for genotoxicity. In Germany, for example, the *umuC* test is currently under discussion as an integral part of routine industrial wastewater surveillance. The *umuC* test, along with other bacterial short-term genotoxicity assays based on the SOS response mechanism (e.g., SOS chromotest [30]), has frequently been used for the screening of complex environmental samples from different origins [17,31–36]. Substantial evidence exists that these tests are valuable tools for the rapid detection of potentially hazardous genotoxicity in environmental samples. The question now arises whether the high sensitivity of the *umuC* test to detect FQ antibiotics (which show low mammalian genotoxicity) reduces its applicability as a wastewater genotoxicity screening tool. From the viewpoint of mammalian genotoxic risk assessment, the present results could be interpreted as false positives (error type I [37]). An important conclusion implied by this study is that caution is needed when hospital sewage is being investigated by the *umuC* test or an equivalent SOS genotoxicity assay. Positive genotoxic results should not be interpreted further before analytical data of FQ antibiotic concentrations of the respective wastewater stream are available. Because hospitals in Switzerland, for example, consumed on average only 16% of all FQ antibiotics used for human health applications between 1990 and 1994 (Information Medical Statistics Ltd., Switzerland, personal communication), one cannot exclude that ciprofloxacin and other FQs could be present in nonhospital sewage streams (e.g., municipal wastewater). More analytical data will be needed to further assess the occurrence of FQ antibiotics in different sewage streams.

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