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Occurrence and Fate of the Cytostatic Drugs Cyclophosphamide and Ifosfamide in Wastewater and Surface Waters†

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The two oxazaphosphorine compounds cyclophosphamide and ifosfamide are important cytostatic drugs used in the chemotherapy of cancer and in the treatment of autoimmune diseases. Their mechanism of action, involving metabolic activation and unspecific alkylation of nucleophilic compounds, accounts for genotoxic effects described in the literature and is reason for environmental concern. The occurrence and fate of cyclophosphamide and ifosfamide were studied in wastewater treatment plants (WWTPs) and surface waters in Switzerland, using a highly sensitive analytical method based on solid-phase extraction and liquid chromatography tandem mass spectrometry.
The compounds were detected in untreated and treated wastewater at concentrations of <0.3−11 ng/L, which corresponded well with concentrations predicted from consumption data and typical renal excretion rates. Weekly loads determined in influent and effluent wastewater were comparable and suggested a high persistence in WWTPs. Furthermore, no degradation was observed in activated sludge incubation experiments within 24 h at concentrations of ∼100 ng/L. Processes that may be relevant for elimination in natural waterbodies were studied with a set of incubation experiments in the laboratory. After extrapolation to natural conditions in surface waters, a slow dark-chemical degradation (half-lives on the order of years) is the most important transformation process. Degradation by photochemically formed $HO^{\bullet}$ radicals may be of some relevance only in shallow, clear, and nitrate-rich waterbodies but could be further exploited for elimination of these compounds by advanced oxidation processes, i.e., in a treatment of hospital wastewater. In surface waters, concentrations ranged from ≤50 to 170 pg/L and were thus several orders of magnitude lower than the levels at which acute ecotoxicological effects have been reported in the literature (mg/L range). However, due to a lack of studies on chronic effects on aquatic organisms and data on occurrence and effects of metabolites, a final risk assessment cannot be made.

Introduction

The occurrence of pharmaceuticals in surface waters and groundwater has attracted increasing attention (*1*−*4*). Pharmaceuticals are designed to exhibit biological activity in humans and may, in principle, have adverse effects on aquatic organisms. Compounds with a very potent mechanism of action such as cytostatic drugs are of particular environmental concern, even though consumption rates and expected concentrations in the environment may be comparatively low.

Cytostatic drugs inhibit growth and division of tumor and other fast-growing cells, for example, by alkylation of DNA. Important alkylating cytostatic drugs are the two oxazaphosphorines cyclophosphamide and ifosfamide (Figure 1), which are used in the chemotherapy of various forms of cancer (bronchial, breast, and ovarian cancer, lymphomas, leukemias, etc.), in the treatment of autoimmune diseases (e.g., rheumatoid arthritis), and as immunosuppressants after organ transplantations (e.g., bone marrow transplantations (*5*)). The compounds are prodrugs that are metabolically activated by a cytochrome P_{450} enzyme system to the corresponding oxazaphosphorine mustards, the actually alkylating compounds (Figure 1). This mechanism of action also accounts for adverse effects such as mutagenic, carcinogenic, teratogenic, and embryotoxic effects described in the literature (cited in refs *2* and *6*−*8*). Such adverse effects and impacts on reproduction, immune system, etc., in principle, may also be expected to occur in higher aquatic organisms such as fish.

Precise data on the consumption of oxazaphosphorines could be obtained for Switzerland, where 55 kg cyclophosphamide and 12 kg ifosfamide were consumed in 2002 (population, 7.3 million, ref *9*). The pharmaceuticals are administered in different treatment schemes, inpatient or outpatient, at typical dosages of ∼1 g cyclophosphamide and 2 g ifosfamide per treatment (*5*). Oxazaphosphorines are not completely metabolized in the body. Typical renal excretion rates of the parent compounds are ∼13% and 15%, respectively (*5*).

The compounds thus reach the aquatic environment via hospital or domestic wastewater and wastewater treatment plants (WWTPs). Cyclophosphamide and ifosfamide have been analyzed in wastewater at concentrations in the ng/L range (*6*, *10*, *11*), whereas in natural waters the compounds have not been detected, due to a lack of analytical methods with adequate sensitivity (*11*). In this paper, we present an analytical method, based on solid-phase extraction and liquid chromatography tandem mass spectrometry, that allowed quantification of cyclophosphamide and ifosfamide not only in wastewater but also in surface waters down to sub-ng/L concentrations.

Earlier studies had shown a high persistence of the two oxazaphosphorines in WWTPs (*6*, *7*, *10*, *12*), but these studies had been conducted at very high concentrations at which cytotoxic effects on degrading microorganisms may not be excluded, potentially leading to false negative results. In the present study, the biodegradability of cyclophosphamide and ifosfamide in activated sludge was investigated at ng/L concentrations. Further laboratory incubation experiments were performed with lake water, without or with exposure to artificial sunlight, to study potential dark-chemical or photochemical degradation of the compounds.

Expected concentrations of cyclophosphamide and ifosfamide in wastewater and surface waters were then estimated from consumption data and hydrological data, considering

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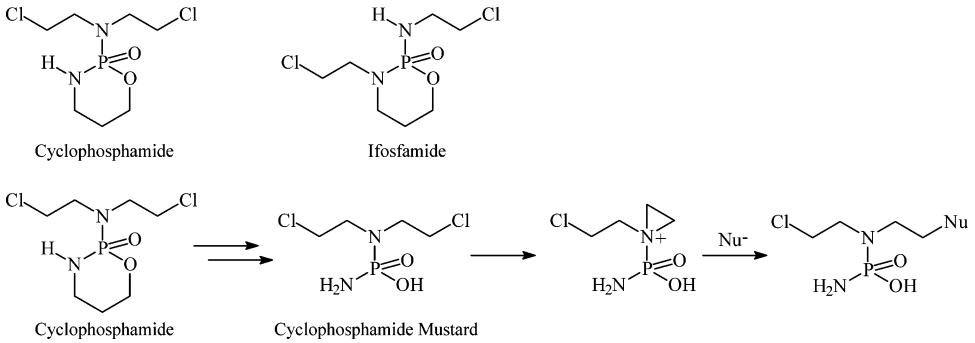

FIGURE 1. Structures of the two oxazaphosphorines cyclophosphamide and ifosfamide, metabolic activation to the corresponding oxazaphosphorine mustard, and alkylation of nucleophilic compounds (Nu).

published renal excretion rates and all relevant elimination processes. These predicted concentrations were compared with measured concentrations. The exposure situation was thus assessed in two independent ways. Finally, ecotoxicological data, as far as available from the literature, were compared with exposure data to evaluate possible risks for aquatic organisms.

Experimental Section

Chemicals. Ifosfamide (3-(2-chloroethyl)-2-(2-chloroethyl)-amino-tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide; quality, reference standard) was obtained from USP, Rockville, MD, cyclophosphamide monohydrate (2-[bis-(2-chloroethyl)-amino]-tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide; purity of cyclophosphamide, >94%), caffeine anhydrous (>99%), pyridine (>99.8%), isopropanol (≥99.8%), and sodium azide (≥99.0%) were from Fluka, Buchs, Switzerland, 4-chlorobenzoic acid (99%) and 4-nitroacetophenone (98%) were from Aldrich, Steinheim, Germany, potassium nitrate (>99%) was from Merck, Darmstadt, Germany, Suwannee River Fulvic Acid (SRFA, type standard) was from the International Humic Substances Society, and $^{13}C_3$-labeled caffeine was from Cambridge Isotope Laboratories, Cambridge, MA. Water used as the solvent after solid-phase extraction and as eluent in liquid chromatography−mass spectrometry was purified over several sorption cartridges of a Seralpur PRO90CN system from Labtec Services, Wohlen, Switzerland.

Water Samples. Wastewater samples were obtained from three WWTPs located in the region of Zurich, Switzerland (Zurich Werdhölzli, Männedorf, and Wädenswil; Supporting Information, Figure S1; note that there are additional WWTPs located around Lake Zurich). These installations operate with 3 or 4 stages, a mechanical, biological (activated sludge), and chemical treatment (phosphate precipitation by iron salts, no chlorination), and subsequent sand filtration (*13*). Influent and effluent wastewaters were collected flow-proportionally during 24 h and stored at ∼4 °C. In the lab, consecutive 24 h samples were combined to flow-proportional 4−7 day composite samples, which were extracted within the next few days. Wastewater from the effluent of the primary sedimentation basin of WWTP Wädenswil was used for recovery experiments (see below).

Surface water was sampled from Lake Zurich at Wädenswil and at its outflow in the city of Zurich, the origin of the river Limmat (grab samples, taken at 0−1 m depths), and from the Limmat ∼6 km downstream of the discharge from WWTP Zurich (24 h flow-proportional samples; Supporting Information, Figure S1). Consecutive 24 h river samples were combined to flow-proportional 6−7 day composite samples. Water from Lake Zurich taken at Wädenswil was used for recovery experiments, and a "fossil" groundwater (Aqui, Zurich) was analyzed as a blank sample. Water samples were stored at ∼4 °C and extracted within a few days.

Solid-Phase Extraction. Extraction was done with reusable columns containing ∼10 mL of a macroporous polystyrene divinylbenzene adsorbent (Bio-Beads SM-2, 20−50 mesh, Bio-Rad Laboratories, Hercules, CA). Separate adsorbent columns were used for wastewater and surface water samples to prevent cross-contamination. The samples (volumes, ∼1 L) were fortified with an internal standard, $^{13}C_3$-caffeine (0.052 ng/μL), to spike levels of ∼10.4 ng/L (wastewater) and ∼2.6 ng/L (natural waters) and were then passed through the solid-phase extraction (SPE) columns at ∼10 mL/min. The analytes were recovered with 5 mL of methanol, which also removed residual water from the SPE material, and then with 10 mL of dichloromethane. The combined eluates were shaken vigorously, and the phases were allowed to separate. The dichloromethane phase was transferred into a glass vial. Additional volumes of 10 and 5 mL of dichloromethane were passed through the column, partitioned with the methanolic phase, and transferred to the same vial. After complete evaporation of dichloromethane at room temperature under a gentle draught of air, the residues were taken up in 1 mL of water. (Note that some suspended material was formed, which did not affect the analysis of the target compounds.)

Liquid Chromatography Tandem Mass Spectrometry. Cyclophosphamide and ifosfamide were separated on a XTerra RP_{18} column (2.1 mm × 50 mm, particle size, 3.5 μm, from Waters, Milford, MA) using an Agilent 1100 Series high-performance liquid chromatography (HPLC) system (binary pump, microvacuum degasser, Palo Alto, CA) and a LC PAL autosampler (CTC Analytics, Zwingen, Switzerland). The LC conditions were as follows: linear gradient from 100% H_2O/0.1% formic acid to 100% methanol/0.1% formic acid within 10 min, followed by an isocratic phase of 2 min; flow rate, 0.25 mL/min; injection volume, 100 μL.

The HPLC column was connected to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a turbo ion spray (TIS) source, operated in positive mode (ion spray voltage, 5 kV, 400 °C) and, for trace analysis, multireaction monitoring (MRM) with the following ion transitions: cyclophosphamide, m/z 261 → 140 with a collision energy of 32 eV (and for confirmatory purposes m/z 263 → 142, 32 eV); ifosfamide, m/z 261 → 154, 31 eV (m/z 261 → 182, 25 eV); and $^{13}C_3$-caffeine, m/z 198 → 140, 28 eV. For cyclophosphamide and ifosfamide, the most important fragmentation reaction under the selected conditions was the cleavage of the N−P bond leading to the formation of the ions m/z 140 and 120 (starting from protonated $^{35}Cl_2$-cyclophosphamide, m/z 261) and m/z 78 and 182 (from protonated $^{35}Cl_2$-ifosfamide, m/z 261). Typical product ion spectra are shown in Figure S2 (Supporting Information). The base peak with m/z 154 in the spectrum of ifosfamide possibly resulted from the loss of C_2H_4 from the ion m/z 182 with internal rearrangement involving a Cl shift (Supporting Information, Figures S2c and S2d).

Because of matrix-dependent ion suppression (formation of Na cluster ions, mainly in wastewater extracts), quantification was done with standard addition. For that, the extract was divided into three aliquots of 300 μL, which were fortified with 0, 10, and 20 μL of an aqueous solution containing 0.12 ng/μL cyclophosphamide and 0.15 ng/μL ifosfamide. Concentrations were determined from plots of peak area ratios relative to the internal standard, $^{13}C_3$-caffeine, versus added amount of the oxazaphosphorines. The response was linear in all matrices ($r^2 > 0.99$).

Activated Sludge Incubations. The biodegradation of cyclophosphamide and ifosfamide was investigated in activated sludge under laboratory conditions. For that, 1.5 L of untreated wastewater from the primary sedimentation basin of WWTP Jona-Rapperswil (population served, 25 000) were mixed with 0.6 L of return sludge, thus in a similar ratio as under typical operating conditions. The suspension was stirred at room temperature and aerated with water-saturated, compressed air through a glass frit (flow, ~0.2 L/min). One incubation experiment was performed at a concentration level of 90 ng/L cyclophosphamide and 120 ng/L ifosfamide, which allowed direct analysis with liquid chromatography tandem mass spectrometry (LC-MS−MS) without SPE. In a second incubation, a ten times higher concentration was used to follow potential degradation of the compounds more easily. After an incubation time of 4.5 h, 1 mL of an aqueous caffeine solution (44 μg/mL) was added to check the biological activity of the sludge under laboratory conditions. Caffeine is readily biodegradable in activated sludge (*14*). Periodically, samples were removed, filtered (0.45 μm ChromafilPET-45/25, Macherey-Nagel, Düren, Germany), and analyzed by LC-MS−MS as described above.

Light and Dark Incubations in Lake Water. Twelve incubation experiments were conducted to characterize the degradation behavior of cyclophosphamide and ifosfamide in lake water. The purposes of the individual experiments (light/dark, with/without addition of reagents, reference compounds, actinometer) are explained in the Results and Discussion section.

Water for incubation experiments was sampled from Lake Zurich at Wädenswil on February 2, 2005, at ~10 cm in depth. Some chemical parameters of the lake water, relevant for formation or degradation of transient photooxidants, are the following: pH 8.08, 2.51 mM alkalinity, 1.6 mg/L dissolved organic carbon, 0.7 mg N/L nitrate, 2.0 μg N/L nitrite, and <0.1 mg/L iron.

Initial concentrations of cyclophosphamide, ifosfamide, 4-chlorobenzoate, 4-nitroacetophenone, pyridine, and additional reagents in the different incubation solutions are indicated in Table S1 (Supporting Information). Care was taken to avoid the addition of organic solvents such as methanol from stock solutions. Light-sensitive solutions were prepared in the dark. The pH decrease by addition of SRFA was compensated with 0.1 M NaOH.

Quartz glass test tubes (diameter, 12 mm) were then filled with 25 mL of the incubation solutions and capped with glass stoppers to prevent water−air exchange. Potential evaporation of water was checked by regular weighing but found not to be relevant. The test tubes were illuminated with actinic lamps for up to 69 days (four tubular low-pressure mercury-vapor fluorescent lamps, type TL 40W/05, Philips). These lamps emit UV radiation between 300 and 460 nm with a maximum at 365 nm. The spectral UV irradiance is comparable to that of 24 h averaged sunlight at 50° N in July under clear sky conditions. The photochemical experiments were performed in an environmental chamber at ~22 °C. Dark controls were incubated at ~20 °C. Periodically, 1 mL aliquots were removed, fortified with 1.3 ng/25 μL of $^{13}C_3$-caffeine as the internal standard, and analyzed as described above.

4-Chlorobenzoic acid and 4-nitroacetophenone were analyzed by HPLC with a diode array detector (pump, Jasco PU-980; autosampler, Jasco AS-1555; diode array detector, MD-1510) under following conditions: column, LiChrospher 100-5 RP C_{18}, 4 mm × 250 mm, particle size 5 μm (Macherey-Nagel, Düren, Germany); eluent, acetonitrile/water/phosphoric acid 50:50:0.1, isocratic conditions; injection volume, 20 μL; flow rate, 1 mL/min; detection wavelengths, 240 nm (4-chlorobenzoic acid) or 288 nm (4-nitroacetophenone).

The photolysis of the actinometer 4-nitroacetophenone (experiment 10, Supporting Information, Table S1 (*15*)) followed first-order kinetics with a rate constant of 0.05 day^{-1}, confirming constant light conditions throughout the incubation studies, whereas in the dark control (experiment 12), only a slow degradation was observed with a rate constant of ~0.002 day^{-1}.

Results and Discussion

Recoveries and Limits of Detection of the Analytical Procedure. Typical LC-MS−MS chromatograms of cyclophosphamide and ifosfamide are shown in Figure 2 (untreated wastewater) and Figure 3 (spiked groundwater). Recoveries ranged from 74% to 94% for cyclophosphamide and from 75% to 102% for ifosfamide and were acceptable for all matrices and fortification levels (0.25−30 ng/L, Table 1).

The sensitivity of the analytical method depended on the type of matrix (wastewater vs surface water, ion suppression, see Experimental Section) but also on the actual plate number of the HPLC column, which decreased somewhat over time. Therefore, in Table 1, concentration ranges are indicated for the resulting limits of detection (LODs). The analytical procedure allowed the detection of cyclophosphamide and ifosfamide down to concentrations of ~0.02 ng/L in natural water samples and ~0.2−0.3 ng/L in wastewater samples and was thus at least 1−2 orders of magnitude more sensitive than previously published methods (*16*, *17*) and our early attempts to analyze the compounds with gas chromatography mass spectrometry (GC-MS) after SPE and trifluoroacetylation, with LODs in the range of 1−4 ng/L cyclophosphamide and 1−20 ng/L ifosfamide (*18*). No oxazaphosphorines were detected in blank samples (fossil groundwater).

High Persistence of Cyclophosphamide and Ifosfamide in WWTPs. The biodegradability of cyclophosphamide and ifosfamide was previously investigated by other research groups with various tests according to Organization for Economic Cooperation and Development (OECD) guidelines, and neither ready, nor inherent biodegradability tests, nor confirmatory tests showed any degradation (*6*, *7*, *10*, *12*). However, these studies were conducted at very high concentration levels (up to 750 mg/L) at which cytotoxic effects on degrading microorganisms may not be excluded, potentially leading to false negative results. Therefore, batch incubation studies with activated sludge were performed at much lower concentrations that could, under worst-case conditions, actually occur in WWTPs (experiments at ~100 ng/L and ~1 μg/L).

Even at these lower concentration levels, however, no degradation of cyclophosphamide or ifosfamide could be observed within 24 h, thus confirming the results of the above-mentioned OECD tests. The reference compound caffeine, in contrast, was completely degraded within 1 h of incubation, indicating that the activated sludge was biologically active under laboratory conditions.

Further elimination processes in WWTPs such as volatilization or sorption to sewage sludge were considered to be negligible because of the low Henry's law constant (6.9×10^{-11} atm L/mol, calculated value for cyclophosphamide (*19*)) and the low octanol−water partition coefficient (log K_{ow}, 0.97 for cyclophosphamide (*19*)), respectively. Analyses of the two

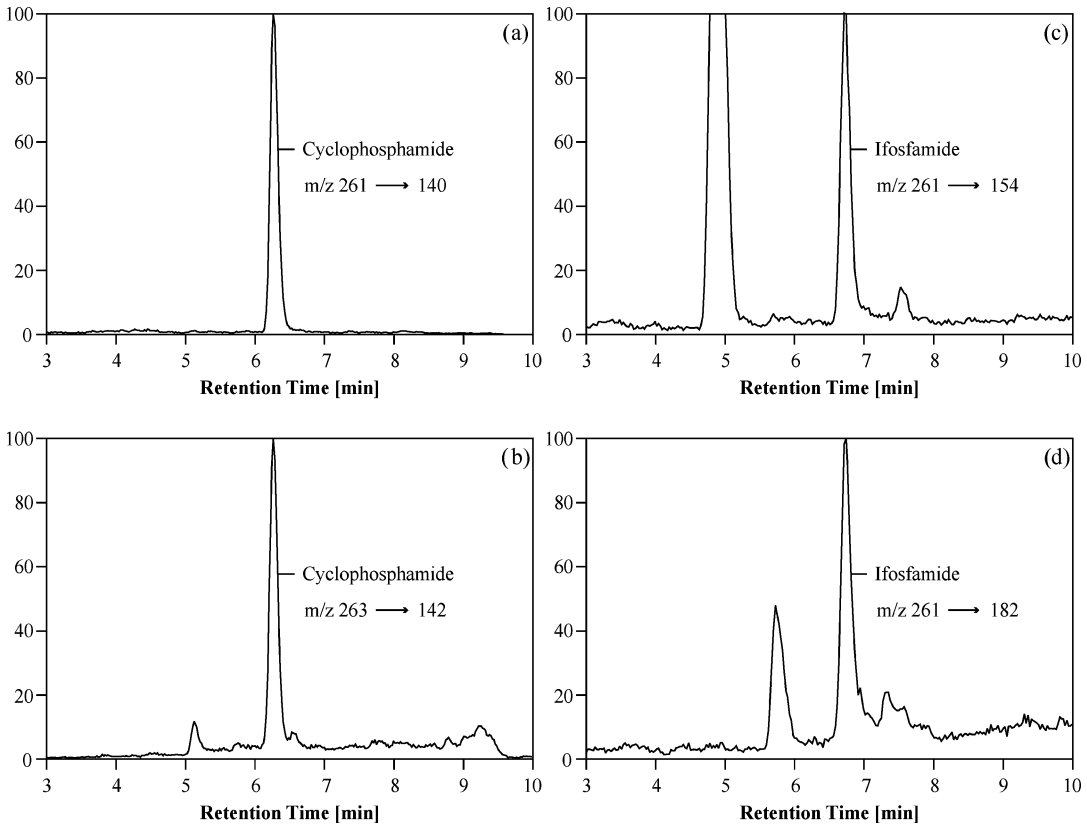

FIGURE 2. LC-MS−MS-MRM chromatograms showing the elution of (a and b) 6 ng/L cyclophosphamide and (c and d) 3 ng/L spiked ifosfamide in a sample of untreated wastewater from WWTP Wädenswil. MRM transitions: *m/z* 261 → 140 for quantification of cyclophosphamide, *m/z* 263 → 142 for confirmatory purposes; *m/z* 261 → 154 (and 261 → 182) for ifosfamide. Signal intensities are normalized to 100%.

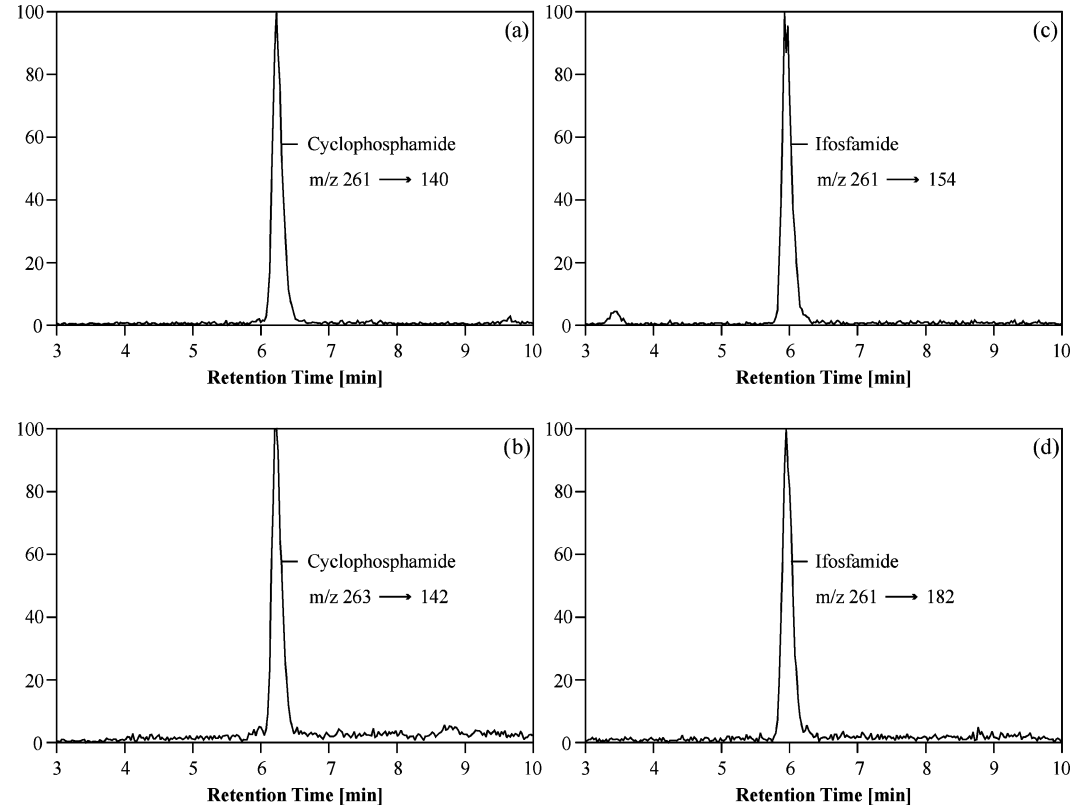

FIGURE 3. LC-MS−MS-MRM chromatograms showing the elution of (a and b) 0.25 ng/L cyclophosphamide and (c and d) 0.30 ng/L ifosfamide in a spiked groundwater sample. MRM transitions: *m/z* 261 → 140 for quantification of cyclophosphamide, *m/z* 263 → 142 for confirmatory purposes; *m/z* 261 → 154 (and 261 → 182) for ifosfamide. Signal intensities are normalized to 100%.

TABLE 1. Recoveries and Limits of Detection (LODs, Signal/Noise ≈ 3) of Cyclophosphamide and Ifosfamide in Groundwater, Lake Water, and Treated and Untreated Wastewater

matrix	fortification level (ng/L)	recovery cyclophosphamide (%)	recovery ifosfamide (%)	LOD cyclophosphamide (ng/L)	LOD ifosfamide (ng/L)
groundwater	0.25−0.30[a]	81	81	0.02−0.1	0.02−0.1
lake water	2.5−30[b]	90−94	90−102	0.02−0.1	0.02−0.1
treated wastewater				0.3	0.3−0.4
untreated wastewater	2.5−30[c]	74−88	75−93	0.2−1	0.3−2

[a] No cyclophosphamide and ifosfamide were detected in unspiked "fossil" groundwater (<0.02 ng/L). [b] Unspiked lake water already contained ~0.07 ng/L cyclophosphamide, which was considered in the calculation of the recovery. [c] Unspiked wastewater already contained 6 ng/L cyclophosphamide, which was considered in the calculation of the recovery.

TABLE 2. Pseudo-First-Order Rate Constants (day^{-1}) for the Degradation of Cyclophosphamide (CP), Ifosfamide (IF), 4-Chlorobenzoate (4-CB), and 4-Nitroacetophenone (PNAP) in Lake Water and Deionized Water with and without Addition of Nitrate, Isopropanol, Suwannee River Fulvic Acid (SRFA), or Sodium Azide[a]

	matrix	degradation processes[b]	CP	IF	4-CB	PNAP
		UV Incubations				
1	lake water	chem, photo	0.016 ± 0.005	0.005 ± 0.003		
2	lake water + NO_3^-	chem, photo with increased HO•	0.022 ± 0.005	0.014 ± 0.005		
3	lake water + isopropanol	chem, photo without HO•	0.012 ± 0.005	0.000 (±) 0.003		
4	lake water + SRFA	chem, photo with increased $^1O_2$, *DOM, less HO•	0.015 ± 0.003	0.005 ± 0.003		
5	lake water + NaN_3	chem, photo with less $^1O_2$	0.014 ± 0.003	0.002 ± 0.002		
6	lake water	(chem), photo primarily by HO•			0.009 ± 0.002	
7	lake water + NO_3^-	(chem), photo with increased HO•			0.013 ± 0.002	
8	lake water + isopropanol	(chem), photo without HO•			0.001 (±) 0.002	
9	lake water + SRFA	(chem), photo with increased $^1O_2$, *DOM, less HO•			0.004 ± 0.002	
10	deionized water	(chem), direct photolysis				0.050 ± 0.003
		Dark Incubations				
11	lake water	chem	0.009 ± 0.004	0.000(±)0.002		
12	deionized water	(chem)				0.002 ± 0.001

[a] For experimental conditions, see text and Supporting Information, Table S1. Errors indicate 95% confidence intervals. [b] Biodegradation is possible in lake water but unlikely (see text): chem = dark-chemical degradation; photo = photochemical degradation, i.e., direct photolysis or indirect photochemical degradation by HO• radicals, singlet oxygen ($^1O_2$), or excited triplet states of dissolved organic matter (*DOM).

oxazaphosphorines in WWTPs finally confirmed their high persistence. The concentrations and loads measured in untreated and treated wastewater were comparable (see below).

Behavior in Surface Waters: Design of Incubation Experiments. Considering the results of the activated sludge incubation studies, it can be assumed that in surface waters biological degradation is also negligible and, for the above-stated reasons, volatilization and sorption/sedimentation are likewise irrelevant elimination processes. However, abiotic degradation may be of some importance in natural waters with higher residence time, i.e., in standing or slowly flowing water bodies. Furthermore, photochemical degradation may play a role in surface waters.

Direct photolysis can be excluded as well, because cyclophosphamide and ifosfamide show negligible absorption in the range of tropospheric sunlight (decadic molar absorption coefficients in distilled water $<1\ M^{-1}\ cm^{-1}$ at wavelengths >290 nm, data not shown). Photochemical degradation may, however, be possible by reaction with transient photooxidants such as HO• radicals, singlet oxygen ($^1O_2$), or excited triplet states of dissolved organic matter (*DOM).

Incubation experiments therefore were designed to distinguish dark-chemical degradation from indirect photochemical degradation by different photooxidants. Kinetic experiments were performed with lake water, with or without exposure to artificial sunlight, and with or without the addition of selected reagents that increase or decrease the concentrations of transient photooxidants (Table 2). Further incubations were carried out with the reference compound 4-chlorobenzoate that specifically reacts with HO• radicals (*20*).

Slow Dark-Chemical Degradation of Cyclophosphamide. In lake water incubated under dark conditions, cyclophosphamide degraded slowly with a half-life of ~80 days (experiment 11, Table 2). This dark-chemical degradation apparently followed first-order kinetics with a rate constant of 0.009 ± 0.004 day^{-1} (error indicates 95% confidence interval, Table 2). The concentration of ifosfamide, however, did not decrease noticeably.

The observed half-life for the dark-chemical degradation of cyclophosphamide is consistent with literature data, where half-lives of 45−121 days were estimated (extrapolated values, incubation times only 17 days, pH 3.4−8.6, 20 °C (*21*)). For ifosfamide, a half-life of ~620 days was derived (pH 5, 25 °C) from experiments at higher temperatures (*22*).

Indirect Photochemical Degradation by HO Radicals. In irradiated lake water (experiment 1), cyclophosphamide and ifosfamide were degraded faster than in the dark with half-lives of ~44 and ~144 days, respectively (Figure 4, Table 2). It therefore was concluded that transient photooxidants were involved in the degradation of the two oxazaphosphorines.

HO• radicals, for example, react rather unspecifically with many organic compounds. They are formed primarily by the photolysis of nitrate, nitrite, and certain iron species, whereas dissolved organic carbon, carbonate, and bicarbonate are important HO• scavengers (*23*). In experiments 2 and 3, the concentration of HO• radicals was thus specifically increased by addition of nitrate or decreased by addition of isopropanol, respectively. In fact, for cyclophosphamide and ifosfamide,

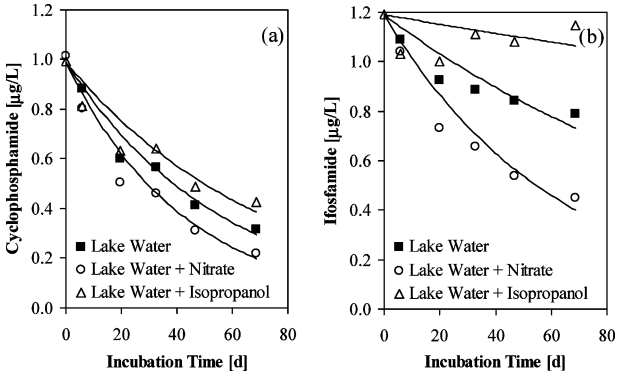

FIGURE 4. Influence of nitrate and isopropanol on the photochemical degradation of (a) cyclophosphamide and (b) ifosfamide in lake water. Curves indicate fits according to pseudo-first-order kinetics. For experimental conditions, see text and Supporting Information, Table S1.

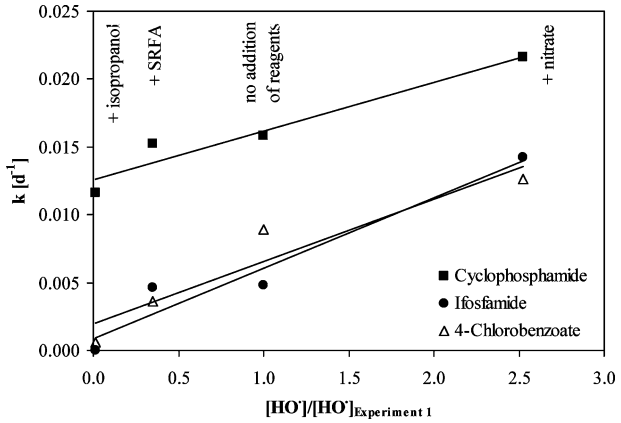

FIGURE 5. Correlations between pseudo-first-order rate constants for the degradation of cyclophosphamide, ifosfamide, and 4-chlorobenzoate and estimated HO• concentrations relative to that in lake water without addition of reagents (experiment 1).

a faster degradation was observed at higher nitrate concentrations, and a slower degradation was found in the presence of isopropanol (Figure 4), indicating that HO• radicals are important in the photochemical degradation of these compounds.

The corresponding rate constants (Table 2) correlated with the HO• concentrations that were estimated according to empirical formulas given in refs *23* and *24* (Figure 5). As expected, such a correlation was also observed for the reference compound 4-chlorobenzoate (experiments 6−9). The increase of the rate constant as a function of the HO• concentration was similar for cyclophosphamide, ifosfamide, and 4-chlorobenzoate (slopes in Figure 5), which means that corresponding second-order rate constants for the reaction with HO• radicals were comparable. For 4-chlorobenzoate, an almost diffusion-controlled reaction was reported in the literature with a rate constant of $5 \times 10^9\ M^{-1}\ s^{-1}$ in water (*25*).

The three regression lines, however, differed with respect to the intercept, i.e., the rate constant in the absence of HO• radicals (experiments 3 and 8, Figure 5). Whereas for 4-chlorobenzoate and ifosfamide no measurable dissipation could be observed in the absence of HO• radicals, cyclophosphamide degraded with a half-life of ~60 days, primarily by dark-chemical degradation (see above) and possibly by reaction with other transient photooxidants.

Fulvic acids may act as HO• scavengers and as source of $^1O_2$ or *DOM, but the degradation rates of the two oxazaphosphorines changed only marginally upon addition of SRFA (experiment 4, Table 2). Furthermore, only a small effect was observed in the presence of NaN_3 (experiment 5), which was added to decrease the concentration of $^1O_2$ (*26*).

HO• radicals thus seem to be the most important photooxidants of cyclophosphamide and ifosfamide in lake water, but will be of minor relevance for their overall dissipation behavior in surface waters as discussed in the following paragraph. However, degradation by HO• radicals could be further exploited for elimination of these compounds by advanced oxidation processes (*27*), i.e., in a treatment of hospital wastewater.

Extrapolation to Surface Waters. Although exposure to artificial sunlight induced a faster degradation of cyclophosphamide and ifosfamide under laboratory conditions, indirect photochemical degradation is of low importance in surface waters, because UV light, responsible for the formation of HO• radicals (~320 nm), typically penetrates only the topmost few centimeters. A shallow, clear, and nitrate-rich waterbody may be an exception.

Dark-chemical degradation is thus expected to be the dominant degradation process in most surface waters. Corresponding temperature-corrected half-lives in surface waters of temperate zones with average temperatures below 10 °C are estimated at ~400−800 days for cyclophosphamide and ~10−20 years for ifosfamide (considering activation energies of ~100 and 118 kJ/mol, respectively (*21*, *22*)).

In streams and rivers, dark-chemical degradation will, therefore, be a negligible process as the compounds are simply eliminated by water exchange. In lakes and ponds, dark-chemical degradation may be relevant for the overall dissipation, depending on the mean water residence time in the waterbody. For example, dark-chemical degradation of cyclophosphamide is expected to be equally or even more important in lakes with a water residence time of >1 year, whereas dark-chemical degradation of ifosfamide may only be relevant in lakes with a residence time of >10 years.

Predicted Concentrations in WWTPs and Surface Waters. Concentrations of cyclophosphamide and ifosfamide in wastewater were estimated based on annual consumption data for Switzerland (in 2002, 55 and 12 kg per year by a population of 7.3 million, respectively (*9*)), considering an average water consumption of 500 L person^{-1} day^{-1}, typical renal excretion rates of 13% and 15%, respectively (*5*), and assuming that the compounds are not eliminated in the sewer system. With that, average concentrations of ~5.4 ng/L cyclophosphamide and ~1.4 ng/L ifosfamide were predicted in untreated and treated wastewater. In WWTPs with a high fraction of hospital wastewater (e.g., in Männedorf, one of the investigated sites, see below) and depending on the actual number of chemotherapy treatments, temporary concentrations of up to ~100 ng/L may be expected (realistic worst-case situation).

Due to further dilution and dark-chemical degradation, the concentrations of oxazaphosphorines in surface waters will be even lower than those in WWTPs, typically in the sub-ng/L range. For example, for Lake Zurich, average concentrations of 0.07−0.08 ng/L cyclophosphamide and 0.03 ng/L ifosfamide are predicted (volume of the lake, 3.36 km^3; mean water residence time, 1.2 years; population in catchment area, 330 000, cited in ref *14*). In a worst-case situation, where wastewater with comparatively high concentrations of oxazaphosphorines is discharged to a small receiving waterbody, concentrations of a few ng/L may be possible.

Selection of WWTPs. Cyclophosphamide and ifosfamide were analyzed in three WWTPs located in the region of Zurich, Switzerland (Supporting Information, Figure S1). WWTP Männedorf was selected because wastewater from a hospital with a relatively large oncology division is treated in this plant and because there is comparatively little dilution of the hospital wastewater by domestic wastewater of only

TABLE 3. Concentrations and Loads of Cyclophosphamide and Ifosfamide in WWTPs, Canton of Zurich, Switzerland.

			cyclophosphamide				ifosfamide			
			influent		effluent		influent		effluent	
WWTP	sampling date	throughput (m^3 day^{-1})	conc. (ng/L)	load (g/day)	conc. (ng/L)	load (g/day)	conc. (ng/L)	load (g/day)	conc. (ng/L)	load (g/day)
Männedorf[a]	Sept 20−23, 2002[c]	16 500	∼4	∼0.06	∼2	∼0.04	<15		<2	
	Sept 24−27, 2002[d]	14 900	11	0.16	10	0.15	<15		<2	
Wädenswil[b]	July 6, 2004[e]	*e*	6	*e*	*f*	*f*	<0.3	*e*	*f*	*f*
Zurich[b]	Mar 29-Apr 3, 2005	194 000	5	0.97	4	0.72	5	0.91	6	1.22
	Apr 18−24, 2005	316 000	2.0	0.64	2.1	0.67	∼1.4	∼0.45	1.7	0.54

[a] Analysis by GC-MS. [b] Analysis by LC-MS−MS. [c] No chemotherapy treatments in hospital of Männedorf. [d] Chemotherapy treatments with 2.7 g of cyclophosphamide in hospital of Männedorf between September 24 and 26, 2002. [e] Grab sample. [f] No effluent sample taken.

∼9000 inhabitants (*28*). WWTP Männedorf was thus expected to represent a realistic worst-case situation. Furthermore, in Männedorf, precise information was available on the actual consumption of the two oxazaphosphorines in the hospital. Wastewater samples were taken during a period with and without chemotherapy treatments in hospital.

Further analyses were done at WWTP Zurich, where wastewater from several hospitals with oncology is treated. These wastewaters, however, are largely diluted by domestic wastewater of ∼370 000 inhabitants (*28*). Therefore, WWTP Zurich rather represents a typical situation for urban areas. The actual consumption of oxazaphosphorines in the numerous hospitals in Zurich could not be obtained.

Finally, a wastewater sample was taken at WWTP Wädenswil, a city with ∼19 000 inhabitants (*28*), but without oncology in the local hospital.

Occurrence in WWTPs. Cyclophosphamide was detected in all wastewater samples at concentrations between 2 and 11 ng/L (Table 3). Concentrations and loads determined in untreated wastewater were similar to those in treated wastewater, consistent with the lack of degradation observed in the activated sludge incubation experiments.

During a 4 day period without chemotherapy treatments in the hospital of Männedorf, cyclophosphamide could, nevertheless, be detected in the local WWTP, at concentrations of ∼4 ng/L in untreated wastewater. This finding can be explained by its use in ambulant chemotherapy as well as by its use as an immonosuppressive drug.

On the following days, three patients were treated with 0.9 g of cyclophosphamide in the hospital of Männedorf. As expected, higher concentrations of 11 ng/L could now be measured in influent wastewater. The total load of cyclophosphamide passing the WWTP during the 4 days of sampling amounted to ∼0.6 g (Table 3). Subtracting the load that was recorded during 4 days without chemotherapy treatments in hospital (from nonpoint sources, ∼0.2 g), it could be estimated that ∼0.4 g of cyclophosphamide was from hospital wastewater. This was about 14% of the administered amount (2.7 g) and corresponded well with typical renal excretion rates of ∼13% (*5*).

The samples from WWTP Männedorf were taken during a time with heavy rainfall, so the wastewater was diluted substantially (∼4× dry weather throughput). In a dry weather situation with the same loads of cyclophosphamide, concentrations would have been up to about 40 ng/L and thus close to the above-predicted worst-case concentration.

From WWTP Zurich, composite samples of influent and effluent wastewater were analyzed twice for oxazaphosphorines. The wastewater throughput during the first sampling campaign (194 000 m^3/day) corresponded to typical operating conditions (220 000 m^3/day (*28*)), whereas considerably more wastewater was treated during the second sampling (316 000 m^3/day). Consequently, the concentration of cyclophosphamide was lower in the second sample (2.0 ng/L in the influent, Table 3), about half that in the first sample (5 ng/L), whereas the loads were comparable (0.97 and 0.64 g/day, respectively). The measured concentrations were very close to those predicted from consumption and excretion data (∼5.4 ng/L, see above).

Ifosfamide was found in WWTP Zurich at concentrations of ∼1.4−5 ng/L in influent and 1.7−6 ng/L in effluent wastewater, respectively (Table 3), which was somewhat higher, but nevertheless in good agreement with the predicted average concentration of 1.4 ng/L. However, the compound could not be detected in WWTP Männedorf (due to lower sensitivity of the GC-MS method used) nor in WWTP Wädenswil (<0.3 ng/L, no hospital with oncology).

The concentrations of cyclophosphamide and ifosfamide measured in these Swiss WWTPs tended to be somewhat lower than those reported in the literature for German WWTPs (<6−143 ng/L cyclophosphamide (*6*, *11*), <6−43 ng/L ifosfamide (*10*, *11*)). The consumption of the two oxazaphosphorines in Germany (population, 82 million) was estimated at 200−400 kg per year, but it is not clear whether this number refers to the individual oxazaphosphorines or to both (cited in ref *29*).

Occurrence in Surface Waters. Cyclophosphamide could be detected in all surface waters investigated. In Lake Zurich and in the River Limmat at the outflow of Lake Zurich (Supporting Information, Figure S1), concentrations were close the limit of detection (∼0.05−0.07 ng/L), whereas somewhat higher concentrations were found in the Limmat downstream of the discharge from WWTP Zurich (0.15−0.17 ng/L, Table 4). The analytical procedure was also sufficient to detect ifosfamide in the river Limmat below WWTP Zurich (∼0.08−0.14 ng/L) but not in Lake Zurich and its outflow (<0.05 ng/L).

As in WWTPs, the concentration of cyclophosphamide in Lake Zurich could be predicted with high accuracy (0.07−0.08 ng/L, see above), confirming that the above-stated assumptions were reasonable. The nondetection of ifosfamide in Lake Zurich was also consistent with its predicted concentration of 0.03 ng/L.

In another study, the two oxazaphosphorines could not be detected in German surface waters due to the lower sensitivity of the method used (LOD, 10 ng/L (*11*)). The only study found in the literature, which reported up to 10 ng/L of cyclophosphamide in an Italian river, unfortunately did not give precise information on the analytical procedure and the sampling site (*30*).

Mass Balance for River Limmat. Samples from the River Limmat up- and downstream of the discharge from WWTP Zurich were taken at the same time as effluent samples from WWTP Zurich, which allowed a simple mass balance to be set up for this river. In the first sampling campaign, the load of cyclophosphamide in the Limmat increased from ∼0.4 g/day at the outflow of Lake Zurich to 1.3 g/day below WWTP

TABLE 4. Concentrations and Loads of Cyclophosphamide and Ifosfamide in Lake Zurich and the River Limmat, Canton of Zurich, Switzerland[a]

			cyclophosphamide		ifosfamide	
lake/river	sampling date	throughput (m^3 s^{-1})	concentration (ng/L)	load (g/day)	concentration (ng/L)	load (g/day)
Lake Zurich[b]	July 13, 2004		~0.07		<0.05	
Limmat at outflow of Lake Zurich[b]	Apr 05, 2005	93	~0.05	~0.4	<0.05	<0.4
	Apr 22, 2005	120	~0.06	~0.7	<0.05	<0.5
Limmat downstream WWTP Zurich[b]	Mar 29-Apr 03, 2005	102	0.15	1.3	~0.14	~1.3
	Apr 18−24, 2005	138	0.17	2.0	~0.08	~0.9

[a] For a map with sampling sites, see Supporting Information, Figure S1. [b] Analysis by LC-MS−MS.

Zurich (Table 4). During this time, 0.7 g/day cyclophosphamide was discharged with treated wastewater to the river (Table 3). The error of this simple mass balance is thus less than 20%, which is not much considering the error of the flow-proportional sampling and the higher analytical error at concentrations close to the limit of detection. Corresponding mass balances for cyclophosphamide during the second sampling and for ifosfamide during both sampling campaigns were consistent as well.

Comparison of Exposure with Available Effect Concentrations. In comparison to other pharmaceuticals, oxazaphosphorines are used in rather low quantities. Consequently, their concentrations in surface waters are typically in the pg/L range only, despite their high persistence. These exposure concentrations are thus several orders of magnitude lower than the concentrations at which acute ecotoxicological effects have been reported in the literature.

For ifosfamide, an EC_{50} value of 162 mg/L was determined for *Daphnia magna* (OECD 202, 48 h, cited in ref *31*), and a LC_{50} value of >1000 mg/L for the fish species *Salmo gairdneri* (OECD 203, 96 h, ref *31*). In mutagenicity tests (sister chromatid exchange and micronucleus tests) with cyclophosphamide on the fish species *Astyanax bimaculatus* and *Anguilla anguilla*, effects were observed in the mg/L range (*32*−*34*).

The potential for bioaccumulation is supposed to be low because of the low octanol−water partition coefficients (log K_{ow}, 0.97 for cyclophosphamide (*19*)). However, studies on chronic effects on aquatic organisms, such as full-life-cycle or multigeneration studies, tests on reproduction and immunosuppression, etc., were not found in the literature. Furthermore, no data have been published on the occurrence and effects of oxazaphosphorine metabolites. For example, for the highly active, alkylating mustards (Figure 1), no reference compounds were available, so no analytical method could be developed. A qualitative LC-MS−MS method for carboxy- and keto-cyclophosphamide, both not cytostatically active, could be developed based on the urine of a patient, but the metabolites could not be detected in environmental samples (for details, see ref *18*). Therefore, a final risk assessment for cyclophosphamide and ifosfamide and their metabolites cannot be made.

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Supporting Information Available

Initial concentrations of reagents in incubation experiments with lake water and deionized water, a map with sampling sites, ESI product ion mass spectra of cylcophosphamide and ifosfamide. This material is available free of charge via the Internet at http://pubs.acs.org.

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