Chemical and toxicological evaluation of transformation products during advanced oxidation processes
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ABSTRACT
The entry of pharmaceuticals into the water cycle from sewage treatment plants is of growing concern because environmental effects are evident at trace levels. Ozonation, UV- and UV/H2O2-treatment were tested as an additional step in waste water treatment because they have been proven to be effective in eliminating aqueous organic contaminants. The pharmaceuticals carbamazepine, ciprofloxacin, diclofenac, metoprolol and sulfamethoxazole as well as the personal care products galaxolide and tonalide were investigated in terms of degradation efficiency and byproduct formation in consideration of toxic effects. The substances were largely removed from treatment plant effluent by ozonation, UV- and UV/H2O2-treatment. Transformation products were detected in all tested treatment processes. Accompanying analysis showed no genotoxic, cytotoxic or estrogenic potential for the investigated compounds after oxidative treatment of real waste waters. The results indicate that by-product formation from ozonation and advanced oxidation processes does not have any negative environmental impact.

Key words | musks, ozone, pharmaceuticals, toxicity, UV/H2O2, waste water treatment

INTRODUCTION
A central goal of environmental research is the removal of organic micropollutants from the water cycle, according to the European Water Framework Directive (WRRL 2000). Pharmaceuticals are of great concern because they have been recognized as potentially hazardous to the aquatic ecosystem. Introduction into water bodies occurs mainly via municipal waste water since their removal in conventional waste water treatment plants (WWTPs) is insufficient. Up to μg/L loads have been detected in the effluents. Ozonation, UV- and UV/H2O2-treatment have been shown to be effective in eliminating aqueous organic contaminants (Joss et al. 2008). These easily implementable waste water treatment technologies are promising methods for preventing residual concentrations of active compounds in the water cycle. They are capable of transforming biologically recalcitrant compounds, as well as those poorly rejected by activated carbon (Le-Minh et al. 2010). Application of full-scale ozonation has already achieved satisfactory removal rates (Hollender et al. 2009).

UV-oxidation is based on the formation of hydroxyl radicals, which are powerful oxidants reacting unselectively with all water constituents. The efficiency of the process depends on the water matrix containing differing amounts of constituents and dissolved organic carbon (DOC) (von Sonntag 2008). An acceleration of the reaction can be achieved by the addition of hydrogen peroxide, which serves as a radical source. Hydroxyl radical formation also occurs in waste water treatment with ozone but the contribution to degradation is of minor importance. The direct ozone reaction is the primary degradation mechanism for most organic contaminants in waste water with selective reaction mechanisms (Lee & von Gunten 2010; Lee et al. 2013).

Although most micropollutants can be removed from waste water during oxidative treatment processes, technological conditions as well as economic aspects, impede complete mineralization to H2O and CO2. This results in the formation of oxidation by-products in remarkable...
concentrations (von Gunten 2003). In fact, the degradation behaviour of various micropollutants is understood in great detail (Rosal et al. 2010; Janzen et al. 2011; Lekkerkerker-Teunissen et al. 2012; Santiago-Morales et al. 2012).

But only a few toxicological evaluations are available for transformation products. Hence, a toxicological assessment is essential for the applicability and total efficiency of the oxidative treatment. Based on occurrence and reported environmental effects, the following compounds were selected for the study: The sulfonamide antibiotic sulfamethoxazole is frequently found in high concentrations of up to 2 μg/L in WWTP effluent. Oxidative treatment has been correlated with toxic effects (Dirany et al. 2011). The antibacterial agent ciprofloxacin has been reported to have an increased genotoxic potential after UV irradiation (Garcia-Käuffer et al. 2012). WWTP concentrations of metoprolol, the most commonly used betablocker, range from 0.2–4 μg/L. Presence in high concentrations has been shown to be responsible for photosynthesis inhibition. Oxidative treatment might reduce this potential as structural changes in human metabolism already cause a loss in the specific mode of action (Escher et al. 2006). The antiepileptic drug carbamazepine is found in similar high concentrations in effluents (0.8–6 μg/L). The high persistence leads to an accumulation in groundwater. Diclofenac is a regularly prescribed analgesic in Germany. Insufficient degradation in WWTPs leads to effluent concentrations of 0.8–2 μg/L. The musks, galaxolide and tonalide, occur in treatment plant effluents in concentrations of 1–6 and 0.4–3 μg/L, respectively. Galaxolide, as well as tonalide, could be correlated to antagonistic effects towards the estrogen receptor beta (ERβ) (van den Burg et al. 2008; Simmons et al. 2010). Tonalide has been characterized with a predicted no-effect concentration of 3.5 μg/L, which is only slightly above concentrations detected in the environment (Balk & Ford 1999).

In typical effluents, most single compounds are below effect concentrations, though combination effects can occur, enhancing the environmental risk. Transformation products are supposed be less toxic and have a higher bioavailability than their parent compounds. However, this assumption still needs approval through toxicological evaluation. Furthermore, a transfer of investigations to real waste waters is required. Therefore, different oxidative treatment processes have been applied to WWTP effluent. Degradation efficiency, formation of transformation products and toxic effects were investigated.

## METHODS

### Materials

High-performance liquid chromatography (HPLC), ultra liquid chromatography/mass spectrometry (ULC/MS) grade water and methanol were purchased from Biosolve (Valkenswaard, The Netherlands). Carbamazepine, ciprofloxacin, diclofenac, sulfamethoxazole, formic acid, hydrogen peroxide and catalase from Aspergillus niger were purchased from Sigma-Aldrich (Taufkirchen, Germany) in the highest quality. Tonalide was provided by Lothaer Streleck GmbH & Co. KG (Hamburg, Germany) and galaxolide was received from International Flavors & Fragrances (Hilversum, The Netherlands). WWTP effluent for laboratory experiments was provided by the local treatment plant, Duisburg-Vierlinden, Germany. On-site pilot scale experiments were conducted at the treatment plant, Bottrop, Germany. Both treatment plants are equipped with a preceding denitrification process. The sludge retention time amounts to 15 and 19 days, respectively.

Chinese hamster ovary cells (CHO-9) (ECACC, Salisbury, United Kingdom) were used for the cytotoxicity and genotoxicity testing, whereas the human ductal epithelial tumor cell line (T47D) (Biodetection Systems, The Netherlands) was used for the estrogenicity testing.

### Oxidation experiments

Ozone, UV- and UV/H2O2 laboratory and pilot scale experiments were carried out in pure water spiked with one compound, effluent and spiked effluent to investigate degradation efficiency, toxic effects and formation of by-products. In a 1 L laboratory scale, a 15 W Hg-LP UV lamp (Herneus Noblelight, Hanau, Germany) was applied in a 1 L reservoir. The water was pumped through the plant by a flexible tube pump (Multifix constant M 838, Alfred Schwinherr KG, Schwäbisch-Gmünd, Germany), using fluoropolymeric tubes. A water cooling unit was integrated to maintain the temperature at 30 C throughout the experiment. Filling and sampling were carried out at the reservoir. An illustration of the plant is published by Tuerk et al. (2010). UV oxidation was investigated alone and with 0.3 g/L H2O2. Peroxide residuals in the samples were removed with 250 μL of catalase (c = 0.4 KU/mL) to avoid false positive results during the toxicity tests.

Ozonation was carried out in batch-mode by adding concentrated ozone water produced with a COM-CD-HF 2 ozone...
generator (Anseros, Tübingen, Germany) to the samples. The concentration of ozone in the water was determined by measuring the absorption at 260 nm. After mixing, the samples were left until the ozone was completely consumed.

UV/H2O2 oxidation was also investigated in continuous flow scale experiments at the waste water treatment plant Duisburg-Vierlinden. An IBL uviblox®WPT system equipped with 2 × 4 kW UV lamps (IBL, Heidelberg, Germany) and a magnetic dosing pump type BT4a0220 (ProMinent Dosiertechnik GmbH, Heidelberg, Germany) was embedded after secondary sedimentation.

Analytical methods

All samples were prepared by filtration with Chromafil RC 0.45 μm syringe filters (Machery-Nagel, Düren, Germany) and subsequently measured.

Galaxolide and tonalide were analyzed with a Trace GC Ultra coupled to a DSQ Mass spectrometer (Thermo Scientific, Dreieich, Germany). Chromatographic separation was carried out using a DB-5MS column (J&W Scientific, Folsom, USA, 15 m × 0.25 mm, 0.25 μm). Samples were completely transferred to the GC-MS with a programmed temperature vaporizer cooled injection system. The injection volume was 1 μL. Mass spectrometric quantification was performed in selected ion monitoring (SIM) mode.

Quantitative analysis of the pharmaceuticals was performed with an LC 20 HPLC system (Shimadzu, Duisburg, Germany) coupled to a hybrid mass spectrometer (Q Trap 3200, AB Sciex, Darmstadt, Germany) in multiple reaction monitoring (MRM) mode with the two most intense mass transitions. Transformation products were analyzed with information dependent acquisition (IDA) experiments in an examination range of m/z = 50–500.

Toxicity measurements

Cytotoxic effects in CHO cells were tested with the MTT-test (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a colorimetric assay based on the cleavage of the MTT tetrazolium salt into a formazan, using 50 μM monomethylarsenous acid (MMA III) as a positive control. The absorption at 595 nm was used for the quantification of cell viability as a measure for cytotoxicity.

Genotoxicity was also tested with CHO cells with the Alkaline Comet Assay according to Ostling & Johanson (1984), determining the degree of DNA damage (single and double strand breaks, alkali labile sites). A charge-coupled device (CCD) camera attached to a fluorescence microscope (Leica DMBL, Leitz GmbH & Co KG, Wetzlar, Germany) and the Comet Assay 4 Software (Perceptive Instruments, Suffolk, United Kingdom) were used for the interpretation of the Olive tail moment.

The estrogenicity of the samples was investigated with the ER Calux, a reporter gene assay using an estrogen responsive element (ERE) which was coupled to a luciferase gene in T47D cells (BioDetection Systems, Amsterdam, The Netherlands). A Tecan GENios plate reader was used for quantification of the luminescence and absorption. For all methods, the cells were exposed for 24 h to the water samples.

Each experiment was carried out in triplicate and the mean values as well as the standard deviation (MTT-test) or the standard error of mean (Comet Assay) are displayed. Statistical analysis of the alkaline comet assay was performed using the Mann–Whitney U-test.

RESULTS AND DISCUSSION

Effluent characterization

The WWTP effluents in Duisburg-Vierlinden underlay accompanying analyses of pH and the dissolved organic carbon (DOC) content. The pH ranged between 7 and 8.5. The DOC amounted to 7 ± 1 mg/L. Nitrite levels ranged from < 0.005 to 0.5 mg/L. The investigated micropollutants were frequently found as shown in Table 1.

Effluent from the WWTP Bottrop was similar to Duisburg-Vierlinden in terms of nitrite level (< 0.04 mg/L) and pH (6.8–7.3). The average total organic carbon (TOC) content was 10.6 mg/L.

Galaxolide

Janzen et al. have already determined reaction kinetics and transformation products in water purification with ozone (Janzen et al. 2011). The main transformation product of galaxolide (HHCB) is HHCB lactone, which is more stable against ozone than the parent compound. Based on these studies, the toxic effects of galaxolide and HHCB lactone were determined. No estrogenic effects occurred up to a concentration of 50 μg/L (data not shown). Figure 1 shows that neither galaxolide nor HHCB lactone are significantly cytotoxic and genotoxic up to 50 μg/L. The toxicity tests were also applied to UV/H2O2 oxidized samples initially containing 0.1 mg/L galaxolide in pure water; 30 min oxidative treatment caused no cytotoxic effects (data not shown).
Tonalide

The treatment of tonalide with ozone and UV/H2O2 results in a number of stable transformation products (Janzen et al., 2014). Toxic effects were investigated in pure water with a starting concentration of 0.1 mg/L. Ozonation lead to neither cytotoxic nor genotoxic effects. These tests were also negative for UV/H2O2 with commonly applied exposure times. However, after applying UV/H2O2 for 20 min, CHO cell growth was reduced to 74%, indicating weak cytotoxic effects.

Pharmaceuticals

Transformation product formation was scrutinized in laboratory scale with spiked WWTP effluent. The main degradation mechanisms of carbamazepine oxidation are well-known (McDowell et al., 2005; Marco-Urrea et al., 2010). Carbamazepine samples of UV and UV/H2O2 treatment in pure water, as well as ozonated samples in WWTP effluent, were tested for cytotoxicity and genotoxicity up to a concentration of 10 μg/L. The results exhibited no effects.

Ciprofloxacin offers several points of oxidative attack. Previous studies described formation of transformation products (Hu et al., 2007; Calza et al., 2008; An et al., 2010). Experiments with ozone confirmed the following transformation products in positive ionization mode: m/z = 228, 263, 268, 308, 348, 360 and in negative mode: m/z = 304, 332. Additional by-products with m/z = 115 in positive and m/z = 376 in negative mode were detected. During UV and UV/H2O2 application, the products m/z = 263, 268, 348 in positive and m/z = 166, 286, 290, 304, 332 in negative mode, were confirmed. In addition, the product m/z = 376 (negative mode) was detected. Initial concentrations of 1.4 mg/L during ozonation and 0.1 mg/L during UV and UV/H2O2 caused neither cytotoxic nor genotoxic effects.
Degradation mechanisms of diclofenac are described for various advanced oxidation processes and treatment with ozone (Sein et al. 2008). Due to the extensive research already published, the investigation was limited to toxicity studies. Diclofenac concentrations up to 10 μg/L did not show any cytotoxic and genotoxic effects. The treatment with ozone, UV and UV/H2O2 also resulted in negative tests.

Ozonation and UV/H2O2 application achieved fast metoprolol degradation. The occurrence of the main oxidation by-products during UV/H2O2 is illustrated in Figure 2. The products m/z = 134 and 240 evolve from reactions with ozone and hydroxyl radicals as previously reported (Song et al. 2008; Benner & Ternes 2009), m/z = 236 has been detected in radiolytic degradation (Slegers et al. 2006), whereas the product m/z = 308 has not been mentioned before. All transformation products are less susceptible to degradation by UV/H2O2 than metoprolol. However, they occur in lower concentrations and are suspected to have a higher bioavailability than metoprolol. Cytotoxic and genotoxic tests were negative for metoprolol as well as for the oxidative treated samples.

Sulfamethoxazole degradation has been studied for various oxidative processes. Nine products are described after ozone, and five after UV treatment (Zhou & Moore 1994; Abellan et al. 2008). Only one of the products occurred in both processes. Several further products have been detected in other advanced oxidation processes (Marciocha et al. 2009; Trovo et al. 2009). In our studies the following transformation products were formed: m/z = 221, 215 in positive ionization and m/z = 266, 156, 181 in negative mode. None of these products are in accordance with literature data, which might depend on exact process conditions. Sulfamethoxazole is not cytotoxic or genotoxic in the tested concentration range up to 1.4 mg/L. Furthermore, after ozonation, UV and UV/H2O2 oxidation the toxicity tests did not show any effects.

**WWTP effluent samples**

The efficiency of the ozonation process was also tested in laboratory scale with effluent containing typical concentrations (see Table 1) of the pharmaceuticals. An application of 5 mg/L ozone resulted in good removal rates of 91% for carbamazepine, 84% for diclofenac, 89% for metoprolol and 85% for sulfamethoxazole, respectively. Ciprofloxacin was not detected after ozonation. Figure 3 shows that the cytotoxicity and genotoxicity of the unspiked WWTP effluent are below effective concentrations before and after ozone, UV and UV/H2O2 treatment.
Pilot scale application on-site

In order to transfer the results to environmental conditions, comparative studies were performed in a pilot scale continuous flow system installed at the WWTP Bottrop. Degradation efficiencies of UV and UV/H₂O₂ were investigated with different flow rates. According to trend, the lower the flow rate the higher the removal rates are. At 10 m³/h, the exposure time is too short to achieve a complete compound removal. However, the addition of H₂O₂ accelerates the removal rates. The optimized adjustment is a flow rate of 6 m³/h + 58 mg/L H₂O₂, achieving an elimination rate of above 80% for all compounds except ciprofloxacin, which was below the limit of detection.

CONCLUSIONS

Experiments with single compounds in high concentrations provide useful information about general behavior during oxidative treatment. All investigated micropollutants show good removal rates during treatment with ozone and UV/H₂O₂. Formation of oxidation by-products was analyzed. Several well-known transformation products and processes could be confirmed. Further transformation products were detected for galaxolide, ciprofloxacin, metoprolol and sulfamethoxazole. The evaluation of toxic effects was performed in consideration of environmental concentrations. Application of ozone and advanced oxidation processes in waste water treatment do not seem to have any negative environmental impact. Moreover, high concentrations of the investigated compounds do not exhibit any cytotoxic or genotoxic potential.

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