The Fate of Antibiotics and Anticancer Drugs in the Aquatic Environment - Evaluating the Photolysis of Ciprofloxacin and Monitoring the Course of its Genotoxicity by a Combination of Experimental and *In Silico* Testing

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PREFACE

This work is based on the results and conclusions of the following articles. To provide a better overview, bold Roman numerals are assigned to each article, which can be found in the text. As presented in the framework paper, they read as follows:

[I]
I. Michael, M.I. Vasquez, E. Hapeshi, T. Haddad, E. Baginska, K. Kümmerer, and D. Fatta-Kassinos (2014):
Metabolites and transformation products of pharmaceuticals in the aquatic environment as contaminants of emerging concern.
In: D.A. Lambropoulou and L.M.L. Nollet (Eds.), Transformation Products of Emerging Contaminants in the Environment. Analysis, Processes, Occurrence, Effects and Risks, vol. 2, pp. 413–458.
JOHN WILEY AND SONS LTD, Chichester, United Kingdom.

[II]
T. Haddad, E. Baginska, and K. Kümmerer (2015):
Transformation products of antibiotic and cytostatic drugs in the aquatic cycle that result from effluent treatment and abiotic/biotic reactions in the environment: An increasing challenge calling for higher emphasis on measures at the beginning of the pipe.
WATER RESEARCH, vol. 72, pp. 75–126.

[III]
T. Haddad and K. Kümmerer (2014):
Characterization of photo-transformation products of the antibiotic drug Ciprofloxacin with liquid chromatography–tandem mass spectrometry in combination with accurate mass determination using an LTQ-Orbitrap.
CHEMOSPHERE, vol. 115, pp. 40–46.

[IV]
M. Garcia-Käufer, T. Haddad, M. Bergheim, R. Gminski, P. Gupta, N. Mathur, K. Kümmerer, and V. Mersch-Sundermann (2012):
Genotoxic effect of ciprofloxacin during photolytic decomposition monitored by the *in vitro* micronucleus test (MNvit) in HepG2 cells.
ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH, vol. 19, pp. 1719–1727.

[V]
A.P. Toolaram, T. Haddad, C. Leder, and K. Kümmerer (2016):
Initial hazard screening for genotoxicity of photo-transformation products of ciprofloxacin by applying a combination of experimental and *in-silico* testing.
ENVIRONMENTAL POLLUTION, vol. 211, pp. 148–156.

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List of Symbols and Abbreviations

AOPs	Advanced oxidation processes
CAS-RN	Registry number in chemical abstracts service
CI	Combination index
CIP	Ciprofloxacin
ESI	Electrospray ionization
IR	Infrared spectroscopy
min	Minute
MN	Micronucleus
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^n	Multistage tandem mass spectrometry
NMR	Nuclear magnetic resonance
NPOC	Non-purgeable organic carbon
QSAR	Quantitative structure activity relationship(s)
RDB	Ring and double-bond
TP	Transformation product
UV	Ultraviolet
WWTP	Wastewater treatment plant

ZUSAMMENFASSUNG

Auf den Einsatz von Arzneimitteln folgt meist ein nur unvollständiger Abbau der Wirkstoffe im Körper von Mensch oder Tier. Somit werden die entsprechenden Ausgangssubstanzen und ihre Metabolite ausgeschieden und gelangen in das Abwasser. In der aquatischen Umwelt und/oder während der Abwasserbehandlung kommt es dann sehr häufig nicht zu einer vollständigen Mineralisierung, sondern zur Bildung mehr oder weniger stabiler Transformationsprodukte (TPs). Entsprechende Studien konzentrieren sich größtenteils auf die stattfindende Abbaukinetik ursprünglicher Wirkstoffe, ohne dabei auf die tatsächlichen chemischen Strukturen oder die Menge der entstehenden TPs einzugehen. Lediglich in neuesten Studien zeichnet sich ein Trend ab, auch diese Informationen zu präsentieren. Da Medikamente maßgeblich zur Kontamination der Umwelt beitragen, ist es weiterhin von außerordentlicher Wichtigkeit, nicht nur die Transformationsprodukte selbst, sondern auch ihre diesbezügliche potenzielle Wirkung zu benennen. Transformationsprodukte können unter Umständen dieselben oder gar toxischere Auswirkungen auf Organismen haben als ihre Muttersubstanz.

Die entsprechenden Hauptziele dieser Arbeit lassen sich in zwei Teile gliedern: Der erste Teil befasst sich mit den Umweltauswirkungen, die von Arzneimitteln, Metaboliten und ihren TPs ausgehen können, und präsentiert dann für eine Auswahl von Medikamenten eine bewertende Zusammenfassung der bereits publizierten Informationen zu ihren jeweiligen Transformationsprodukten. Der zweite Teil umfasst weiterführende Untersuchungen zu dem Antibiotikum Ciprofloxacin (CIP), nämlich: a) eine Bewertung der Effektivität des photolytischen Abbauprozesses im Labor, b) die Identifizierung von Abbauprodukten und c) eine vorläufige Einschätzung potenzieller Auswirkungen auf die Umwelt (z.B. bakterielle Toxizität, Mutagenität und Genotoxizität mit experimentellen Systemen sowie computerbasierten Methoden, wie "quantitative structure activity relationships" (QSAR)).

Diese Arbeit basiert auf den Ergebnissen und Schlussfolgerungen der fünf im Anhang aufgeführten Artikel.

Mit Bezug auf den ersten Teil der Zielsetzung und zur thematischen Eingrenzung wurde eine umfangreiche Literaturrecherche durchgeführt. Sie konzentrierte sich auf die Arzneimittelgruppen der Antibiotika und Zytostatika. Für die recherchierten Medikamente wurden die bereits publizierte Daten über ihre jeweiligen TPs und deren Bildung unter verschiedenen Reaktionen zusammengeführt. Im Hinblick auf den zweiten Teil der Zielsetzung wurden eigene Forschungsergebnisse erhoben. CIP wurde einer Photolyse mit

einer UV- und einer Xenon-Lampe unterzogen. Es wurde analysiert, wie umfangreich der entsprechende Abbau von CIP geschieht, welche Folgeprodukte gegebenenfalls entstehen bzw. welche davon zumindest vorläufig identifizierbar sind und welche biologische Auswirkung sie haben.

Die anfängliche Literaturrecherche hat insgesamt zu 294 Transformationsprodukten geführt, welche für 15 Wirkstoffe in ihrer chemischen Struktur bereits identifiziert wurden. Von diesen verfügten 158 Produkte bereits über eine zugeordnete "registry number in chemical abstracts service" (CAS-RN). Bezüglich des experimentellen Teils konnten nach der Photolyse von CIP mittels LC-MS/MS, u.a. hochauflösende MS-Techniken, 11 TPs identifiziert werden. Der Teilabbau erfolgte vor allem durch Defluorierung, Addition von Hydroxylgruppen oder Abbau des Piperazin-Rings. Während der Photolyse zeigte das Gemisch aus CIP und seiner TPs im "Ames Test" keine Mutagenität und im Mikronukleus-Test keine Genotoxizität. Anders indizierte der *umuC*-Test durchaus Genotoxizität, die jedoch mit zunehmender Bestrahlungszeit abnahm und somit eher auf den Abbau von CIP zurückzuführen war. Die angewandten computerbasierten Methoden sagten Mutagenität für einige TPs voraus und nicht für CIP selbst. Dies steht im Gegensatz zu dem Ergebnis des "Ames Test", der keine Mutagenität für das Gesamtgemisch aus TPs und CIP indizierte. Es liegt die Vermutung nahe, dass diese Form der Toxizität in den experimentellen Tests nicht indiziert wurde aufgrund einer antagonistischen Wechselwirkung der Transformationsprodukte oder einer zu geringen Konzentration dieser Produkte in der Mischung.

Die Vielzahl der gefundenen Transformationsprodukte aus der Literaturrecherche und die hier vorgelegten Ergebnisse der photolytischen Behandlung von Ciprofloxacin demonstrieren, dass die technischen Methoden zur Abwasserreinigung und Trinkwasseraufbereitung zwar die Ausgangsstoffe größtenteils entfernen, jedoch nicht vollständig mineralisieren. Die Ergebnisse zeigen auch, dass je nach Art der Randbedingungen unterschiedliche TPs gebildet werden, die abbaustabil sein und zu Folgeproblemen führen können. Für eine umfangreichere Risikoabschätzung ist die Durchführung von weiteren Toxizitätstests erforderlich.

Aus Sicht der Nachhaltigkeit ergibt sich als wesentliche Implikation, dass bei der Trinkwasseraufbereitung mittels UV-Bestrahlung auch TPs unbekannte Toxizität und Struktur entstehen könne und dass die Art der TPs und die Bildung ihrer Kinetik von den jeweiligen Bedingungen abhängen und somit keinen allgemeinen Empfehlungen abgegeben werden können. Daraus folgt weiterhin, dass bereits bei der Herstellung von Wirkstoffen die

Bioabbaubarkeit der Strukturen und somit die Umweltfreundlichkeit berücksichtigen werden müssen, um die Problematik gezielt angehen zu können. Die Schadensbekämpfung sollte somit nicht allein der Abwasserbehandlung obliegen, sondern die Bildung von stabilen und mitunter schädlichen TPs kann von Anfang an gemindert werden.

SUMMARY

After being administrated to humans or animals, pharmaceuticals may be metabolized by a variety of mechanisms and pathways within the body. Once these compounds and/or their metabolites are excreted, they may undergo degradation in the aquatic environment. Unfortunately, a rapid and complete mineralization cannot always be guaranteed, whereas relatively stable transformation products (TPs) may be formed. The largest part of older studies focused on investigation of the elimination kinetics of parent compounds without considering the amount and chemical structure of individual TPs. Only recently, there is an increasing trend to deliver such information. Nevertheless, since drugs are defined as significant environmental pollutants, it is not only important to elucidate their TPs, but also necessary to investigate whether these formed compounds preserve the same mode of action as the parent compound or are even more toxic.

Thus, two main objectives of this thesis can be formulated. Firstly, to highlight the concern originated by metabolites and transformation products of pharmaceuticals that contaminate the environment. Hereby, the already-published knowledge on TPs within a certain selection of drugs is assessed to exemplify the number and quality of the existing information on their TPs. Secondly, to particularly investigate the fate of the antibiotic ciprofloxacin (CIP). This is done by (a) evaluating the suitability and sustainability of the photolytic decomposition as an advanced water treatment technique, (b) monitoring the course of genotoxicity of the irradiated mixtures using a battery of genotoxicity and cytoxicity *in vitro* assays, and (c) considering the potential genotoxicity for CIP's individual TPs by the employment of *in silico* approaches using quantitative structure activity relationships (QSAR) models.

This thesis based on the results and conclusions of five articles, which can be found in the appendix.

A systematic literature review was conducted on the current state of knowledge on pharmaceuticals and its derivatives in the environment. Two groups, namely antibiotics and anticancer drugs, were considered more closely with respect to the availability of chemical structures for their TPs. Furthermore, the photodegradation of CIP as well as a preliminary toxicity assessment of its identified TPs were investigated in three research papers.

An extensive review with a table at its core shows the existing data on 158 TPs, which already have an assigned registry number in chemical abstracts service (CAS-RN), was presented. In total, 294 TPs, identified with chemical structures in the literature, were found for 15 compounds out of the 21 that were selected as target compounds.

Eleven TPs, created from CIP, were identified by high-performance liquid chromatography/high-resolution multiple-stage mass spectrometry. It was detected that the transformation of CIP mainly occurred through substitution of fluorine, defluorination, hydroxylation of the quinolone core and the breakdown of the piperazine ring. Some of the identified TPs of CIP were predicted as genotoxic by QSAR analysis, while the experimental testing for a few genotoxic and cytotoxic endpoints showed that the potential of the resultant mixtures could be primarily dependent on the concentration of residual CIP. In contrast, irradiation mixtures were neither mutagenic in the Ames Test nor genotoxic in the *in vitro* Micronucleus Test. It is possible that the effect of the TPs was masked by antagonistic mixture interactions and/or they were not formed at effectively concentrations. Nevertheless, all of the identified TPs of CIP still retained the core quinolone moiety, which is responsible for the biological activity. Thus, a more comprehensive assessment, encompassing more genotoxic endpoints, chemical analysis characterization and exposure analyses, needs to be conducted.

Information available on TPs demonstrates that already slight changes in treatment conditions and processes result in the formation of different TPs. Nevertheless, most of the transformation products could neither be identified nor fully assessed regarding their toxicity. This, in turn, presents a major challenge for the identification and assessment of TPs. Hence, from a practical and sustainability point of view, limiting the input of pharmaceuticals into effluents as well as improving their (bio)degradability and elimination behavior, instead of only relying on advanced effluent treatments, is urgently needed. Solutions that focus on this "beginning of the pipe" approach should minimize the adverse effects of parent compounds by reducing the formation of TPs and their entrance into the natural environment.

1. INTRODUCTION AND MOTIVATION

After being administrated to humans or animals, pharmaceutically active compounds may be metabolized by a variety of mechanisms and pathways within the body. The rate and extent of these processes are specific to each compound and may sometimes even be different between species. In the case of human pharmaceuticals, once these compounds and/or their metabolites are excreted, they reach wastewater treatment plants (WWTPs), if such plants are in place, or directly reach surface water (Halling-Sørensen et al., 1998; Ternes, 1998; Zwiener, 2007; Ding and He, 2010).

In WWTPs, pharmaceuticals and metabolites may undergo an additional transformation, for instance, by activated sludge during aerobic wastewater treatments or anaerobic digestion of sludge, which results in new molecules, i.e. bio-transformation products (Ternes et al., 2002; Kagle et al. 2009; Längin et al., 2009; Trautwein and Kümmerer, 2011). However, biotic processes can induce a limited degree of transformation, taking into account the fact that pharmaceutical compounds have a designed resistance to biodegradation (Kümmerer et al., 2004; Fatta-Kassinos et al., 2011).

Hydrolysis and other abiotic oxidation reactions may also play a role in the transformation of pharmaceuticals in WWTPs (Burhenne et al., 1997; Thiele- Bruhn, 2003; Kümmerer, 2009a). Together with WWTPs effluents, the mixture of pharmaceuticals, bio-transformation products and metabolites enters surface water bodies. Once pharmaceuticals are discharged into the aquatic environment, they can undergo photolysis caused by exposure to the direct sunlight. As a result of this reaction, photo-transformation products are formed (Liberti and Notarnicola, 1999; Meneses et al., 2010; Fatta-Kassinos et al., 2011; Mahmoud and Kümmerer, 2012). Furthermore, UV treatment is also very common for water treatment in order to disinfect drinking water (Canonica et al., 2008). Additionally, the so-called advanced oxidation methods are under discussion for the treatment of wastewater and WWTP effluents in order to improve the removal rate of micro-pollutants such as pharmaceuticals. Processes used for these purposes are for example chlorination and advanced oxidation processes (AOPs), such as H_2O_2/UV, O_3/UV, TiO_2/UV, Fenton, and photo-Fenton (Legrini et al., 1993; Zwiener, 2007; Magdeburg et al., 2014).

Upon biotic or abiotic partial degradation of pharmaceuticals and/or their metabolites during wastewater treatment or in the environment, new molecules with different properties, known as transformation products (TPs), may be formed (Ravina et al., 2002; Längin et al., 2008; Trautwein and Kümmerer, 2012; Mahmoud et al., 2013).

Abiotic environmental factors (e.g. temperature, sunlight, salinity, pH) can make a significant contribution to the type and degree of transformation of pharmaceuticals that are then released into the environment through the discharge of the treated effluent. The TPs can be further transported and distributed in surface water or groundwater. Even an additional degradation or transformation cannot be excluded. All of these processes do not often completely mineralize the pharmaceuticals and can therefore frequently produce TPs (Kosjek and Heath, 2008; Radjenović et al., 2009; Richardson and Ternes, 2014; Trautwein et al., 2014).

Understanding the transformation of pharmaceuticals is essential for accurately determining their ultimate environmental fate, conducting accurate risk assessments and improving their removal. Thus, identification of products formed by incomplete degradation, e.g. in biodegradation experiments or photolysis studies, has become the most challenging step in environmental analysis (Krauss et al., 2010; Kosjek and Heath, 2011; Richardson and Ternes, 2014). The formed TPs are indeed new chemicals, for which standards are currently not available. Thus, analytical methods that combine high separation efficiency with a maximum of molecular structural information are required. Recent advances in mass spectrometric instrumentation (such as tandem quadrupole or Q-trap mass analyzers and, only recently, high resolution mass spectrometry) are highly valuable tools that allow a deeper insight into the transformation processes of pharmaceutical compounds (Calza et al., 2008; Dewitte et al., 2008; Sirtori et al., 2010; López et al., 2014). However, the presence of several compounds in the sample of a treatment makes the interpretation of spectra challenging. Furthermore, such expensive machines are not often available and low concentration of TP presents an additional challenge.

The TPs may still exhibit the same mode of action as the parent compound (i.e. the active moiety remains intact during transformation), which means that they could also have a toxicological effect on non-target organisms in environmental systems, either aquatic or terrestrial (Fatta-Kassinos et al., 2011; Boxall et al., 2004). As the TPs usually occur in a mixture together with the parent compound, their contribution to the overall effect cannot be neglected. Toxicity tests with various microorganisms have indicated that chronic exposure to pharmaceuticals and their TPs is more critical than acute exposure (Ferrari et al., 2004; Lindberg et al., 2007; Kümmerer, 2009a).

As a wide variety of pharmaceutically active compounds has been presented in the environment, the wisest approach is to restrict the focus on a certain selection of pharmaceuticals taking into account the ones that most likely pose a significant hazard to both

environment and human health. In this work, the examples of selected pharmaceuticals and their TPs were investigated to provide a picture of what this knowledge might tell us. Two groups, comprising antibiotics and anticancer drugs, were considered. Among the wide amount of such substances, a selection of 21 compounds was made.

Anticancer drugs can be cytotoxic, genotoxic, mutagenic and teratogenic, and it is generally accepted that no threshold of safety can be given for some of them. Moreover, this group of pharmaceuticals has been studied very little (Steger-Hartmann et al., 1997; Allwood et al., 2002; Johnson et al., 2008; Zounkova et al., 2010; Kümmerer et al., 2014). Concerning antibiotics, they are of great interest not only because of their high usage, but also because of their direct influence on bacterial communities. They might impact geochemical cycles and soil fertility, and could possibly contribute to resistance development of potential human pathogens (Daughton and Ternes, 1999; Kümmerer, 2009a; Kümmerer, 2009b).

To assess the quality of data on pharmaceutical TPs regarding these two groups as water contaminants, a comprehensive review on scientific literature and available data regarding the availability of chemical structures for each compound, was conducted. On the basis of these results, the antibiotic agent ciprofloxacin (CIP) became the central focus of this thesis and has, thus, been investigated in more detail resulting in three research papers. To obtain more information on the potential risks of CIP to environmental and human health, the genotoxicity and mutagenicity of CIP and its photo TPs were studied experimentally. On the other hand, various *in silico* software that used quantitative structure activity relationship (QSAR) models were applied for predicting the effects on a number of cytotoxicity endpoints.

CIP is a broad-spectrum antibiotic that can be found in the environment at µg/L concentration level and below. In the aquatic environment, the fate of CIP is potentially governed by several mechanisms such as photodegradation, adsorption and biotransformation (Cardoza et al., 2005). However, with respect to the last process, CIP was reported as not being readily biodegradable and therefore the corresponding transformation is not expected to be the major removal pathway (Al-Ahmad et al., 1999; Kümmerer et al., 2000). In contrast, photodegradation can be a possible process of CIP removal prior to and upon its release to the environment. Several *in vitro* genotoxicity assays have shown that the irradiated mixtures containing TPs and CIP may be mutagenic in the Ames Test and genotoxic to several cell lines including mouse lymphoma, human hepatoma HepG2 cell, and Jurkat cell (Chételat et al., 1996; Sánchez et al., 2005). However, Paul et al. (2010) showed that UV irradiation can attenuate the cytotoxicity of CIP. Even though CIP is a known *umuC* inducer, none of these

studies have monitored the changes in genotoxicity of treated CIP using this test. Nevertheless, QSAR predictions have shown that some TPs may be capable of inducing the *umuC* gene at lower concentrations than CIP (Li et al., 2014).

This thesis is made of five articles and a framework paper, which contains this introduction and core parts that elucidate the internal coherence of the articles, the aspects studied in the articles, the methods employed and the results. The appendix B contains the reprints of publications from a book as well as peer reviewed journals, on which this work is based.

2. AIMS AND OBJECTIVES

The main objectives of this thesis read as follows:

- Assessment of the knowledge on TPs regarding the drugs of interest in order to exemplify the number and quality of information published on TPs.
- Evaluation of suitability and sustainability of photolytic decomposition as an advanced treatment technique with regard to the antibiotic agent CIP.
- Monitoring the course of genotoxicity of CIP and its TPs formed during photolysis using a battery of genotoxicity and cytoxicity *in vitro* assays.
- Assessing the potential genotoxicity for CIP's individual TPs by the employment of *in silico* approaches using QSAR models.

3. RESEARCH APPROACH

In order to meet the above-mentioned objectives, the following work tasks were addressed and resulted in five research papers.

The first part, papers **I** and **II**, reviews the current state-of-knowledge on pharmaceuticals and their derivatives, which are released into the environment. In paper **I**, examples of research documenting the existence, identification and occurrence of the main pharmaceutical metabolites in the environment were described in detail. Attention was given to the main transformation products of pharmaceuticals identified in wastewater during biodegradation experiments. A summary of photochemical studies, including the identification of TPs and/or the evaluation of their biological effects during the application of photolysis, was also provided.

Paper **II** is an extensive literature research conducted to investigate whether TPs of selected target compounds have been found during different types of treatment processes for effluents

and water. If that was the case, information on their respective chemical structure were collected. Two groups, namely antibiotics and anticancer drugs, were considered. Among the wide amount of such substances, a selection of 21 compounds was made based on available knowledge in terms of potential effects, occurrence, persistence, and consumption. These compounds are representatives of different families of compounds within each therapeutic group.

The second part, papers **III** – **V**, focuses on the investigation of photodegradation of CIP as well as preliminary toxicity assessment of its identified TPs. In paper **III**, photodegradation of CIP in aqueous solution using UV as well as xenon lamps, which strives to simulate solar radiation, was studied. The TPs generated from CIP were initially analyzed by an ion trap in the MS, MS/MS and MS^3 modes. These data were used to elucidate and clarify the structures of the degradation products. Furthermore, the proposed products were confirmed by accurate mass measurement and empirical formula calculation for the molecular ions of TPs using LTQ Orbitrap XL mass spectrometer. The data obtained from structure elucidation, along with abundance profile of TPs during photo experiments, were used to construct the supposed transformation pathways of CIP and its TPs under both UV and xenon irradiation.

In the next step, papers **IV** and **V**, the genotoxicity and cytotoxicity of CIP as well as its mixture of TPs following photolysis was investigated as CIP was not completely mineralized within the photo treatments. While in **IV**, the WST-1 assay and the *in vitro* micronucleus (MNvit) assay in HepG2 cells were employed, a comprehensive genotoxicity study on CIP and its TPs was addressed in **V**. A battery of assays (i.e. the Ames Test using the strains TA98 and TA100, *umuC* Test using strain TA1535 psk 1002, and *in vitro* micronucleus assay using flow cytometry (CHO-K1)) was applied to investigate the genotoxicity of whole mixtures. Further, a combination of statistical models such as Case Ultra and Leadscope (Roberts et al., 2000) and rule-based models like Oasis Catalogic (Laboratory of Mathematical Chemistry, 2012) was utilized to predict selected genotoxicity endpoints of the individual TP. The simplified molecular input line entry specification (SMILES) code was used to introduce molecular formula of TP into various computer-based QSAR models for predicting the effects on a number of cytotoxicity endpoints. Furthermore, the combination index (CI) analysis of residual CIP in the irradiated mixtures was performed for the *umuC* Test. It represents an assay detecting the mechanism of genotoxicity and mutagenicity of CIP so that it can be determined how well photolysis is capable of reducing the *umuC* induction.

4. RESULTS AND DISCUSSION

Paper **I** can be defined as a search on pharmaceuticals and their derivatives present in the environment. Herein, many examples of research, documenting the existence of human metabolites for drugs such as ibuprofen, acetylsalicylic acid, carbamazepine and clofibrate in various environmental compartments, were addressed. Pharmaceuticals like diclofenac, chlorpromazine and trimethoprim are among many others, which have been found to be biologically transformed under wastewater treatment conditions, were also reported therein. However, little is known concerning the specific routes and mechanisms through which environmental bacteria degrade these compounds. With regards to pharmaceuticals' degradation, it appears that hydroxylation, isomerization, dehalogenation, dealkylation, cyclization, decarboxylation, dimerization and ring opening are among the most frequently observed transformation mechanisms of parent compounds. There is a general lack of data with respect to the chronic effects of pharmaceutical metabolites and TPs in the environment, making it difficult to refine and optimize the existing models so that they become more accurate. Moreover, it is also unknown which organisms and endpoints are relevant for the pharmaceuticals biological potency as well as their TPs testing.

An extensive review with a table at its core shows the existing data on 158 TPs for a selection of compounds from the antibiotics and anticancer groups, was presented and discussed in **II**. In total, 294 TPs, identified with chemical structures in the literature, were found for 15 compounds out of the 21 that were selected as target compounds from the group of antibiotics and antineoplastics. 158 of them had an assigned registry number in chemical abstracts service (CAS-RN), whereas 136 have not yet been registered in the CAS system. However, not only one TP may be the result of different reactions e.g. photodegradation, biodegradation or AOP. It is possible that numerous different TPs are formed within one type of treatment, applied under different conditions. Structures of TPs were found to be more often reported for antibiotics than for anticancer drugs. On the one hand, this could be related to the high occurrence of antibiotics in the environment, due to their high consumption, and the high interest in these compounds because of their contribution to resistance. On the other hand, the reason could lie in the fact that only a few studies were done to assess the environmental impact of anticancer compounds as they are less well-known and used at much lower amounts. The most TPs were found for the two antibiotics ciprofloxacin and trimethoprim. Only a minority of studies on the assessment of the ecotoxicological potency of TPs included long-term toxicity on non-target organisms.

The results, as shown in **III**, indicate that CIP achieved primary elimination after 128 min of UV irradiation under standard lab conditions. However, complete mineralization of CIP and its TPs did not occur as the total non-purgeable organic carbon (NPOC) remained almost constant over the full irradiation time. Eleven TPs for CIP were identified within 128 min of irradiation. The xenon lamp irradiation did not result in any additional TPs compared to the UV lamp. Nevertheless, the kinetics of formation of TPs was different between UV and xenon lamps. The molecular formulas of the detected TPs, based on data derived from MS^n, were confirmed by accurate mass analysis, ring and double-bond (RDB) values as well as mass deviation, matching the corresponding values of CIP. The transformations of CIP were found to occur mainly through substitution of fluorine, defluorination, hydroxylation of the quinolone core and the breakdown of the piperazine ring. All TPs still retained the core quinolone structure, which is responsible for the biological activity.

Papers **IV** and **V** deal with the effects of UV irradiation of ciprofloxacin solution on toxicity by applying various mutagenicity and genotoxicity tests *in vitro* as well as *in silico* systems. The experimental results, as shown in paper **IV**, demonstrate that CIP and its transformation products were not cytotoxic towards HepG2 cells. A concentration-dependent increase of micronucleus (MN) frequencies was observed for the parent compound CIP (low observed effect level, 1.2 µmol L^{-1}). Furthermore, CIP and the irradiated samples were found to be genotoxic with a significant increase relative to the parent compound after 32 min ($P < 0.05$). A significant reduction of genotoxicity was found after 2h of irradiation ($P < 0.05$).

Irradiation mixtures were neither mutagenic in the Ames Test nor genotoxic in the *in vitro* MN Test, as found in paper **V**. The *umuC* gene was induced in the irradiated mixtures but further analysis using CI showed that this may most likely be attributed to the presence of CIP. Therefore, the TPs in these irradiated mixtures did not significantly contribute to the SOS repair response induction. It is possible that the effect of the TPs was masked by antagonistic mixture interactions and/or they were not formed at effectively concentrations. Nevertheless, QSAR predictions suggested that the TPs may be capable of inducing chromosome aberration and mammalian mutation. Unlike CIP, some TPs were predicted to cause bacterial mutation and MN *in vivo*. However, the Leadscope model predicted that like CIP, all TPs may cause *in vitro* mammalian mutation. QSAR estimations by different models all have their respective weaknesses and strengths and therefore, predictions may be different. Nevertheless, since the TPs retained much of the quinolone moiety, they would have the potential for interference with bacterial DNA just like CIP. Yet, the alterations of the substituents can affect the affinity of TPs to DNA binding and ultimately their potency. The

formation of stable TPs can be expected in wastewater treatment and in surface water. Certainly, additional TPs were formed that have not been detected by the applied analytical methods. These products could be non-ionisable under the condition of ESI selected herein. Another possibility is that they may get lost during the chromatographic analysis.

Overall, investigations on TPs of pharmaceuticals were mostly done at lab-scale. The formed transformation products within the reactors were often identified by means of chromatographic and mass spectrometric instruments. However, the confirmation of structures of TPs can only be done in comparison to a synthesized reference standard. In case no standard is available, complementary techniques such as NMR and IR spectroscopy should be applied. For an accurate study of environmental samples, where TPs are in trace and affected by the complexity of matrix, advanced sensitivity and accuracy of analytical methods is required to remove uncertainty in elucidation of structures. It is important to keep in mind that such a task is time-consuming and very expensive.

In general, the growing number of elucidated TPs is rationalized by ineffective removal treatments. Information available on TPs demonstrates that a slight change in treatment conditions and processes leads to the formation of different TPs. This, in turn, makes it difficult to select the right conditions for effluent treatment and it also presents a big challenge for the identification and assessment of TPs.

5. CONCLUDING REMARKS AND FUTURE OUTLOOK

The degradation of pharmaceutically active compounds in the environment does not always lead to rapid and complete mineralization. Instead, relatively stable TPs may be formed in the course of the degradation process. The largest part of older studies focused on the investigation of the elimination kinetics of parent compounds without presenting any information on neither the chemical structure of individual TPs formed nor their amount. However, only recently, there is an increasing trend to deliver such information. Since drugs are considered as important environmental pollutants, it is not only important to elucidate their TPs in environmental media, but it should also be investigated whether the TPs preserve the same mode of action as the parent compound or are even more toxic.

The combination of the experimental results and the predicted data by *in silico* approaches gave valuable understandings of the environmental fate, behavior and risk of the target compound, i.e. ciprofloxacin, and its correspondingly formed TPs. The battery of genotoxicity assays employed in the recent research only covered a few endpoints with a few cell lines or

bacterial strains. While this may provide an initial risk characterization of the particular mixtures, QSAR data have predicted that genotoxic and mutagenic risk related to a few TPs, which might follow the photolysis of CIP. Therefore, a more comprehensive assessment encompassing more genotoxic endpoints, chemical analysis characterization (e.g. detection, isolation) and exposure analyses (including biodegradation studies) is needed to explore and determine their respective environmental risks. However, direct testing of selected TPs, using other cell lines that are known to induce MN by CIP and using lower dilution factors should be investigated before it can be excluded that the mixture of TPs are not genotoxic to mammalian cells.

Today, the presence of TPs in the aquatic environment and the fact that they can actually pose a higher risk to environmental and human health than their parent is accepted. Investigations aiming at answering questions about the pharmaceuticals' transformation process, their by- and end-products as well as their significance, are very costly and time-consuming if not impossible. Additionally, a severe risk of drowning in much unrelated and non-assessable data, both from a scientific and from a technical treatment-related point of view, is expected.

Limiting the input of pharmaceuticals into effluents as well as improving their (bio)degradability and elimination behavior, instead of only relying on advanced effluent treatments, is urgently needed. Solutions that focus on this "beginning of the pipe" approach should minimize the adverse effects of parent compounds by reducing formation of TPs and their entrance into the natural environment.

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APPENDIX A

OVERVIEW OF ARTICLES INCLUDED IN THIS CUMULATIVE THESIS

I avouch that all information given in this appendix is true in each instance and overall.

Tarek Haddad

AUTHORS' CONTRIBUTIONS TO THE ARTICLES AND ARTICLES PUBLICATION STATUS

In accordance with the (Richtlinie zur kumulativen Dissertation – Dr. rer. nat. der Fakultät Nachhaltigkeit, Leuphana Universität Lüneburg, 24.02.2012) in the following termed "the guideline".

Title of thesis: The fate of antibiotics and anticancer drugs in the aquatic environment - Evaluating the photolysis of ciprofloxacin and monitoring the course of its genotoxicity by a combination of experimental and *in silico* testing.

This thesis is a cumulative dissertation based on core five articles. Table 1 gives an overview of the articles with declaration of the portion of my individual scientific contribution to each study, publication status of each manuscript and information on the chosen publication media. In addition, titles, places and dates of presentations of related results at academic conferences are given. Furthermore, the specific scientific contribution of each co-author in preparing the article is shown in the declaration of authorship (Table 2).

Table 1. Overview of the articles in this thesis with publication status of each

Article*	Title	Specific Contribution of all authors	Relative importance of own contribution **	WF	Publication status	Publication medium (IF/Q/R)	Conference contributions
I	Metabolites and transformation products of pharmaceuticals in the aquatic environment as contaminants of emerging concern	IM, MV, EH, TH, EB, KK, DK	Co-author with important contribution [Wichtiger Anteil]	0.5	Chapter published (2014) in: Transformation Products of Emerging Contaminants in the Environment. Analysis, processes, occurrence, effects and risks DOI: 10.1002/9781118339558.ch14	Chapter in editorial reviewed Book (John Wiley and Sons Ltd, Chichester, UK)	

II	Transformation products of antibiotic and cytostatic drugs in the aquatic cycle that result from effluent treatment and abiotic/biotic reactions in the environment: An increasing challenge calling for higher emphasis on measures at the beginning of the pipe	TH, EB, KK	Co-author with equal contribution [Gleicher Anteil]	1.0	Journal article published (2015) in Water Research DOI: 10.1016/j.watres.2014.12.042	International peer-reviewed Journal (5.528/Q1/9)	SETAC 2011
III	Characterization of photo-transformation products of the antibiotic drug Ciprofloxacin with liquid chromatography–tandem mass spectrometry in combination with accurate mass determination using an LTQ-Orbitrap	TH, KK	Co-author with predominant contribution [Überwiegender Anteil]	1.0	Journal article published (2014) in Chemosphere DOI: 10.1016/j.chemosphere.2014.02.013	International peer-reviewed Journal (3.340/Q1/39)	ANAKON 2011
IV	Genotoxic effect of ciprofloxacin during photolytic decomposition monitored by the *in vitro* micronucleus test (MNvit) in HepG2 cells	MK, TH, MB, RG, PG, NM, KK, VS	Co-author with important contribution [Wichtiger Anteil]	0.5	Journal article published (2011) in Environmental Science and Pollution Research DOI: 10.1007/s11356-011-0686-y	International peer-reviewed Journal (2.828/Q1/54)	
V	Initial hazard screening for genotoxicity of photo-transformation products of ciprofloxacin by applying a combination of experimental and *in-silico* testing	AT, TH, CL, KK	Co-author with equal contribution [Gleicher Anteil]	1.0	Journal article published (2015) in Environmental Pollution DOI: 10.1016/j.envpol.2015.12.040	International peer-reviewed Journal (4.143/Q1/17)	SETAC 2013 and PHARMAS2013
			Sum	**4.0**			
			Required	3.0			

WF = Weighting Factor according to §14 of the guideline.

IF = The 2014 Impact Factor based upon Thomson Reuters (Journal Citation Reports® 2015), http://admin-apps.webofknowledge.com/JCR/JCR

Q = Quartile, R = Journal Rank; in the category of ENVIRONMENTAL SCIENCES for the year 2014 based upon Thomson Reuters (Journal Citation Reports® 2015); total Journals in that category are 223 (accessed 13.03.16).

* Articles order according to content as presented in the framework paper.

** According to §12 of the guideline in relation to the contributions of other co-authors.

Conference contributions (acronym, society, date, venue):

ANAKON 2011, International conference on analytical chemistry, 22-25 March 2011, Zurich, Switzerland.

SEATC 2011, Society of Environmental Toxicology and Chemistry - North America 32nd Annual Meeting, 13-17 November 2011, Boston, MA, USA.

SEATC 2013, Society of Environmental Toxicology and Chemistry - North America 34th Annual Meeting, 17-21 November 2013, Nashville, TN, USA.

PHARMAS 2013, International conference entitled "Pharmaceutical Products in the Environment: is there a problem?", 03-04 May 2013, Nimes, France.

DECLARATION OF AUTHORSHIP

Table 2. Authors' contributions to the articles (according to §12 of the guideline)

	Article I	Article II	Article III	Article IV	Article IV
Conception of research approach	DK, IM	EB, KK, TH	KK, TH	KK, MK, PG, RG	AT, TH, CL
Development of research methods	DK, EB, EH, IM, KK, MV, TH	EB, KK, TH	KK, TH	MK, PG, RG,TH	AT, CL, TH
Data collection and data preparation	EB, EH, IM, MV, TH	EB, TH	TH	MK, MB, PG, TH,	AT, CL, TH
Execution of research	EB, EH, IM, MV, TH	EB, TH	TH	IM, MV, EH, TH, EB	AT, TH
Analysis/interpretation of data	DK, EB, EH, IM, KK, MV, TH	EB, KK, TH	KK, TH	MB, MK, KK, NM, PG, RG, TH	AT, CL, TH
Writing of the manuscript	EB, EH, IM, MV, TH	EB, TH	TH	MB, MK, PG, TH	AT, TH
Internal revision of manuscript	DK, KK	EB, KK, TH	KK, TH	KK, MK, RG, VS	AT, CL, KK

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Appendix B

Reprints of Articles

The present thesis is based on the following five articles; they are organized in the manner as presented in the framework paper and are referred to by bold Roman numerals in the text.

ARTICLE

I

Metabolites and Transformation Products of Pharmaceuticals in the Aquatic Environment as Contaminants of Emerging Concern

Reprinted from Publication: Irene Michael, Marlen Ines Vasquez, Evroula Hapeshi, Tarek Haddad, Ewelina Baginska, Klaus Kümmerer, Despo Fatta-Kassinos (2014). Metabolites and transformation products of pharmaceuticals in the aquatic environment as contaminants of emerging concern. In: Dimitra A. Lambropoulou and Leo M. L. Nollet (Eds.), Transformation Products of Emerging Contaminants in the Environment. Analysis, Processes, Occurrence, Effects and Risks. John Wiley and Sons Ltd, pp. 413–458. DOI:10.1002/9781118339558.ch14. © 2014 John Wiley and Sons Ltd.

14

Metabolites and Transformation Products of Pharmaceuticals in the Aquatic Environment as Contaminants of Emerging Concern

Irene Michael[1,2], Marlen Ines Vasquez[1,2], Evroula Hapeshi[1,2], Tarek Haddad[3], Ewelina Baginska[3], Klaus Kümmerer[3] and Despo Fatta-Kassinos[1,2]

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14.1 Introduction

The widespread detection of pharmaceuticals in environmental samples, as a result of the latest advances in analytical tools and focused field surveys, has led to great concern over the potential risks associated with their consecutive release and persistence in the natural ecosystems [1]. Once in the human body, the ingested pharmaceutical molecule undergoes a set of biochemical reactions and is then excreted as a mixture of the parent compound and its metabolites [2]. After excretion of such mixtures into the sewage systems and their further introduction into the urban wastewater treatment plants (UWTPs), both parent compounds and metabolites can undergo structural changes by a variety of processes [3,4]. These processes can be biotic (biodegradation, mainly by bacteria and fungi) and non-biotic or abiotic (e.g., hydrolysis and photolysis) [5].

Biotic processes can induce a limited degree of transformation, taking into account the fact that pharmaceutical compounds have a designed resistance to biodegradation [6,7].

Transformation Products of Emerging Contaminants in the Environment: Analysis, Processes, Occurrence, Effects and Risks, First Edition. Edited by Dimitra A. Lambropoulou and Leo M. L. Nollet.
© 2014 John Wiley & Sons, Ltd. Published 2014 by John Wiley & Sons, Ltd.

Upon biotic or abiotic partial degradation of pharmaceuticals and/or their metabolites during wastewater treatment, new chemical entities with different properties, known as transformation products (TPs), may be formed [5]. Abiotic environmental factors (e.g., temperature, sunlight, salinity, pH) can make a significant contribution to the transformation of pharmaceuticals which then enter the environment through the disposal of the treated effluent. The TPs can be further transported and distributed in surface water or groundwater and possibly further degraded/transformed.

Direct and indirect photolysis can be considered as the primary pathway for their abiotic transformation in various environmental systems [8]. While direct photolysis is caused by direct absorption of solar light by the substrates, the indirect photolysis involves naturally occurring photosensitizers, like dissolved organic matter (DOM), which under solar irradiation can generate strong oxidant species such as hydroxyl radicals ($HO^•$) and singlet oxygen ($^1O_2$). Structural transformations may also be a result of the application of technological processes, such as effluent treatment by advanced oxidation processes (AOPs). AOPs are quite efficient novel methods for water and wastewater treatment [9–11]. These processes involve the use and generation of powerful transitory species, mainly but not exclusively $HO^•$.

Identification of the TPs present in environmental samples is a difficult task, especially because of the possible differences in concentration at which they are generated and extracted during the solid phase extraction method. This task represents an important analytical challenge because the TPs formed are new chemical entities for which standards are currently not available. Thus, analytical methods that combine high separation efficiency with a maximum of molecular structural information are required. Recent advances in mass spectrometric instrumentation (such as tandem quadrupole or Q-trap mass analyzers and, only recently, high resolution mass spectrometry) have provided the environmental scientists with highly valuable tools to gain deeper insight into the transformation processes of pharmaceutical compounds present in complex environmental matrices [2].

Understanding the transformation of pharmaceuticals is essential for accurately determining their ultimate environmental fate, conducting accurate risk assessments and improving their removal. The TPs may preserve the same mode of action as the parent compound (i.e., the active moiety remains intact during transformation) and elicit a toxicological effect on non-target organisms in environmental systems, either aquatic or terrestrial [6,12]. As the TPs usually occur in a mixture with the parent compound, their contribution to the overall effect cannot be neglected [13].

TPs can differ from the parent compounds in two ways: in their bioconcentration (toxicokinetics) and/or their mode of toxic action (toxicodynamics). In rare cases, transformation can create new toxicophores that can lead to higher toxicity by a similar or dissimilar mode of action [14]. Toxicity tests with various microorganisms have indicated that chronic exposure to pharmaceuticals and their TPs is more critical than acute exposure, because the latter does not reflect the real environmental conditions, where organisms are continuously exposed to pharmaceutical residues at sub-therapeutic levels ($ng\,L^{-1}$–$\mu g\,L^{-1}$) [15]. The concern also comes over the quality and safety of drinking water, since environmental waters are often used as a source and several studies have reported the occurrence of pharmaceuticals in drinking water [16].

The possibility that pharmaceuticals can cause negative effects on aquatic organisms has been widely discussed and documented in the scientific literature [17–19]. However, very few reports are available with regard to the potential toxic effects of their derivates (metabolites and TPs) [6,20]. As more evidence is amassed, regulatory agencies are increasingly taking the view that it would be beneficial to take a precautionary approach and try to

regulate and reduce pathways by which these TPs enter the environment, without waiting for the science to catch up [21].

This chapter is primarily focused on the identification and occurrence of the main pharmaceutical metabolites in the environment. It also aims to present some relevant information on the main TPs formed during the microbial degradation and the application of photo-driven AOPs, along with the principal reaction types and transformation mechanisms. However, the purpose of this chapter is not to provide a complete literature review on this topic, but rather to highlight some examples documenting the importance of the elucidation of TPs and the evaluation of their ecotoxicological potency, the aim being to bridge the various knowledge gaps associated with these issues. It also intends to provide insight into what future directions might be taken to help scientists in this challenging task of enhancing the available data on the fate, behavior, and ecotoxicity of pharmaceutical residues in the environment.

14.2 Human Metabolites in the Aquatic Environment

After achieving systemic circulation, most drugs are subjected to transformation in the human body, with the liver being the major site of metabolism. These conversions frequently entail the loss of pharmacological activity and an increase in hydrophilicity, thereby promoting elimination [2,22]. The metabolism of drugs in the human body can be divided into two stages: phase I, which involves oxidation, reduction or hydrolysis, and phase II in which metabolism encompasses conjunction reactions [14]. In phase I, various enzymes convert lipophilic organic molecules to more polar compounds by introducing reactive functional groups into the molecule. Phase II typically involves conjunction of the compound with either sugars (glucuronidation) or peptides to improve solubility and facilitate excretion [23]. The metabolites formed during phase II have been observed to be readily deconjugated during biological wastewater treatment to reform the active substance or its corresponding phase I metabolite [23].

Although many studies indicate that pharmaceutical contaminants can exhibit long-term ecological risks [6,24–26], many of the investigations regarding risk assessment have only considered the ecotoxicity of the parent drug, with very little attention given to the potential contributions of their metabolites.

Because the metabolites formed during the drugs' transformations in the human body are generally more hydrophilic, they can be readily excreted in urine and end up in urban wastewater treatment plants (UWTPs). After passing through wastewater treatment, metabolites are released directly into the environment. Table 14.1 provides some examples of research documenting the existence of human metabolites in various environmental compartments.

For example, the hydroxylated and carboxylated metabolites of the anti-inflammatory drug ibuprofen (IBF) have been detected in water samples, with hydroxy-IBF being the major component in sewage ($1.27\,\mathrm{mg\,L^{-1}}$), whereas carboxy-IBF was dominant in seawater samples from Tromsø-Sound ($7.0\,\mathrm{ng\,L^{-1}}$) in Norway, into which sewage was discharged [45]. The aforementioned metabolites of IBF were also detected in UWTP effluent ($0.005–0.43\,\mu\mathrm{g\,L^{-1}}$) and in the corresponding receiving river water ($<0.001\,\mu\mathrm{g\,L^{-1}}$) in Sweden [54].

In a study conducted in Germany, three metabolites of the analgesic drug phenazone have been identified in groundwater samples but were mostly removed during the conventional drinking water treatment process, except 1-acetyl-1-methyl-2-dimethyl-oxamoyl-2-phenylhydrazide (AMDOPH), which was detected at a concentration of $0.9\,\mu\mathrm{g\,L^{-1}}$ [58]. Additional investigations were also carried out to assess the toxicological relevance of the metabolite AMDOPH, being the dominating residue in drinking water. It was proved that there is no

Table 14.1 *Pharmaceutical metabolites of human origin detected in the environment.*

Parent compound	Human metabolites detected in the environment	Matrix[a]	Reference
Acetylsalicylic acid	Salicylic acid	influent	[27]
		influent/effluent	[28]
		groundwater	[29]
		effluent, river water, lake water	[30]
		influent/effluent	[31]
		river water	[20]
	Gentisic acid	influent	[27]
Amitriptyline	Nortriptyline	influent	[32]
Atorvastatin	*o*-hydroxy atorvastatin	drinking water	[33]
		effluent	[34]
	p-hydroxy atorvastatin	drinking water	[33]
		effluent	[34]
Carbamazepine	2-hydroxycarbamazepine	influent/effluent	[35]
		influent/effluent, biosolids	[36]
		river water	[20]
	3-hydroxycarbamazepine	influent/effluent	[35]
		influent/effluent, biosolids	[36]
		river water	[20]
	10,11-dihydro-10,11-epoxycarbamazepine	influent/effluent	[35]
		influent/effluent, biosolids	[36]
		river water	[37]
		effluent	[34]
		river water	[20]
	10,11-dihydro-10,11-dihydroxycarbamazepine	influent/effluent, river water	[35]
		influent/effluent, biosolids	[36]
		influent/effluent	[38]
	10,11-dihydro-10-hydroxycarbamazepine	influent/effluent	[35]
		influent/effluent, biosolids	[36]
Clofibrate	Clofibric acid	influent	[39]
		groundwater	[40]
		effluent	[41]
		effluent	[42]
		effluent	[43]
		influent/effluent	[44]
		river water, lake water	[45]
		influent	[46]
		influent	[47]
		river water	[48]
		river water	[20]
Cocaine	Benzoylecgonine	influent	[49]
Erythromycin	Erythromycin-H_2O	seawater	[50]
		seawater	[51]
		influent	[44]
		pharmaceutical effluent, hospital effluent, river water	[52]

Table 14.1 *(Continued)*

Parent compound	Human metabolites detected in the environment	Matrix[a]	Reference
Fluoxetine	Norfluoxetine	drinking water	[33]
		influent/effluent	[53]
		influent/effluent	[32]
Ibuprofen	Hydroxy-ibuprofen	influent/effluent, seawater	[45]
		influent/effluent, river water	[54]
	Carboxy-ibuprofen	influent/effluent, seawater	[45]
		influent/effluent, river water	[54]
		influent/effluent, river water	[55]
Methadone	2-ethylene-1,5-dimethyl-3,3-diphenylpyrrolidine	influent/effluent	[56]
Methylphenidate	Ritalinic acid	river water	[57]
Phenazone	1-acetyl-1-methyl-2-dimethyl-oxamoyl-2-phenylhydrazine	groundwater, drinking water	[40]
	dimethyloxalamide acid-(*N'*-methyl-*N*-phenyl)-hydrazide	groundwater	[58]
	1-acetyl-1-methyl-2-phenylhydrazide	groundwater	[58]
Sertraline	Desmethylsertraline	influent/effluent	[32]
	Desmethylvenlafaxine		
Sulfamethoxazole	N_4-acetylsulfamethoxazole	influent/effluent	[59]
		influent/effluent	[60]
		river water	[20]

[a] Influent and effluent → urban wastewater.

harm to humans by life-time consumption of drinking water containing AMDOPH at the maximum concentrations found in Berlin drinking water, since these concentrations are clearly below the recommended concentration level (3 $\mu g\,L^{-1}$) according to the German Federal Environmental Protection Agency.

Another drug that produces metabolites which may potentially exhibit ecotoxicity in the environment is acetylsalicylic acid (ACA). ACA is rapidly metabolized to salicylic acid (salicylate) and other compounds, including gentisic acid, salicyluric acid and glucuronide conjugates [61]. Salicylic acid was detectable in the influent of an UWTP in Germany, at concentrations up to 54 $\mu g\,L^{-1}$, while the latter and gentisic acid were both detected in the effluent and river streams at low $\mu g\,L^{-1}$ concentrations [27]. Salicylic acid was also observed to be present in low $ng\,L^{-1}$ concentrations in groundwater near a septic system in Ontario [29], and receiving waters of three watersheds in Nova Scotia, Canada [28]. In the ecotoxicological tests, salicylic acid showed no effects on algae, ciliates, and BF-2 cells (cell cultures of the bluegill sunfish), even in the highest test concentrations. In the case of *D. magna* and *V. fischeri*, the EC_{50} values were 118 and 90 $mg\,L^{-1}$, respectively. The zebrafish embryos were the most sensitive test organism, with an LC_{50} of 37 $mg\,L^{-1}$ [62]. Gentisic acid exhibited also acute and chronic toxic effects on *D. magna* and *D. longispina* [63].

Carbamazepine (CBZ), an antiepileptic drug, is extensively metabolized in the liver and one of its major metabolites is 2-hydroxycarbamazepine, which has previously been detected as a urinary metabolite excreted by rats and humans along with its further metabolized product,

2-hydroxyiminostilbene [64]. Five hydroxylated CBZ metabolites (10,11-dihydro-10,11-epoxy-carbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine, 10,11-dihydro-10-hydroxycarbamazepine, 2-hydroxycarbamazepine and 3-hydroxycarbamazepine), have been detected in sewage wastewater influent, effluent, and biosolids [35,36,38]. These metabolites have been recognized to occur in higher concentration in wastewater than the parent compound.

The toxicity of amitriptyline and imipramine metabolites (desmethyl-, didesmethyl-, and N-oxide) towards the protozoan *S. ambiguum* and the crustacean *T. platyurus* was assessed by Nałęcz-Jawecki [65]. Interestingly, it was demonstrated that the toxicity of the mixture of metabolites to the tested species was higher than the predicted value calculated from the concentrations of the drugs and their N-desmethylated derivatives in the sample.

The biologically active clofibrate metabolite, clofibric acid, was detected in urban wastewater effluent at concentration levels ranging from $30\,ng\,L^{-1}$ [43] in Spain, $0.5\,ng\,L^{-1}$ in Italy [41] and $47\,ng\,L^{-1}$ in Taiwan [44].

A notable example of conjugated drug metabolites is the sulfamethoxazole's acetylated metabolite. This can be one explanation for the observed discrepancies in the mass balance of this drug, with higher concentrations of the parent compound being found in UWTPs effluents and the environment than in the influent [66]. N_4-acetylsulfamethoxazole usually accounts for more than 50% of an administered dose in human excretion and can occur in UWTPs influents at concentrations 2.5–3.5 times higher than the concentrations of the parent compound [67]. In addition, glucuronide conjugates of diclofenac (DFC) metabolites may also be present in aqueous systems due to their high water solubility [68]. Furthermore, glucuronide and sulfate conjugated hormones can be hydrolyzed in sewage, thus increasing the contribution of the parent drugs in the wastewater [69].

Here it is noted, that the European Medicines Agency (EMEA) has set guidelines for reporting total concentrations of drugs (parent compound and metabolites) that are being excreted in the aquatic environment. An approach, based on two phases, has also been developed to predict the ecological risk and effects associated with metabolites entering the environment at a concentration higher than 10% of the parent compound [70].

The importance of identifying and evaluating the effects of pharmaceutical metabolites in the environment cannot be underestimated. Persistent pharmaceutical metabolites require consideration for risk assessment because the effects resulting from exposure to a mixture of parent compound and its metabolites may be quite different from what could be observed during a bioassay test using only a single compound.

14.3 Biotransformation Products in the Aquatic Environment

In some cases, pharmaceuticals and their human metabolites can be microbially transformed in UWTPs (biotic processes), which are designed potentially to reduce the release of the organic load into the aquatic environment. The biological treatment of wastewater, which encompasses the activated sludge process as the most widely used one in UWTPs, involves the transformation of dissolved and suspended organic contaminants by microorganisms (mainly bacteria and protozoa flora). Biotransformation could be the result of either catabolism (use of the compound as a carbon and energy source) or co-metabolism (coincidental transformation of the compound without use as a carbon or energy source) [71,72]. Only a limited amount of work has been addressed to drugs that may be biotransformed in the environment. In this section, attention is given to the main biotransformation products (bio-TPs) of pharmaceuticals identified in wastewater during biodegradation experiments (Table 14.2).

Table 14.2 *The main pharmaceutical bio-TPs detected in UWTPs and during laboratory biodegradation experiments.*

Parent compound	Biotransformation products	Biodegradation experiment	Reference
17-β estradiol	Estrone 17-β estradiol-17-glucuronide 17-β estradiol-3-glucuronide	Aerobic *in vitro* batch experiment with activated sludge	[73]
	Estrone	Conventional wastewater treatment	[74]
Acyclovir	Carboxy-acyclovir	Laboratory experiments with activated sludge	[75]
Atenolol	Atenololic acid	Batch reactor with activated sludge under aerobic conditions and laboratory scale membrane bioreactor	[76]
Azithromycin	phosphorylated azithromycin	Membrane bioreactor	[77]
Bezafibrate	4-chlorobenzoic acid	Membrane bioreactor	[78]
Carbamazepine	10,11-dihydro-10,11-dihydroxycarbamazepine 2-hydroxycarbamazepine	Conventional wastewater treatment	[35]
Chlorpromazine	((3-(2-chloro-10H-phenothiazin-10-yl)propyl)(methyl)amino)methylacetate hydroxylated phenothiazine N-(3-(2-chloro-10H-phenothiazin-10-yl)propyl)-N-methylformamide	Zahn-Wellens test	[79]
	Promazine	Anaerobic biodegradation	
Ciprofloxacin	succinyl ciprofloxacin 7- [(carboxymethyl)amino]-1- cyclopropyl 6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid	Membrane bioreactor	[77]
Ciprofloxacin	*N*-acetylciprofloxacin	Batch experiments with isolated *E. coli* strain	[80]
Diatrizoate	2,4,6-triiodo-3,5-diamino-benzonic acid	Zahn–Wellens test; Labolatory scale Sewage treatment plant	[81] [82]
Diclofenac	*p*-benzoquinoneimine	Fixed bed bioreactor under aerobic conditions	[83]
	2,6-dichlorophenyl)-1,3-dihydro-2*H*-indol-2-one 2-((2,6-dichlorophenyl)amino)benzyl alcohol 2-((2,6-dichlorophenyl)amino)benzyl alcohol methyl ether	Small-scale pilot plant with activated sludge	[84]

(*continued*)

Table 14.2 *(Continued)*

Parent compound	Biotransformation products	Biodegradation experiment	Reference
Dimethylaminophenazone	1-acetyl-1-methyl-2-phenylhydrazide Acetoaminoantipyrine Ormylaminoantipyrine 1-acetyl-1-methyl-2-dimethyloxamoyl-2-phenylhydrazide	Batch experiments with biologically active filter material	[85]
Glibenclamide	Glibenclamide hydroxide	Batch reactor with activated sludge under aerobic conditions and laboratory scale membrane bioreactor	[76]
Ibuprofen	Hydroxy-ibuprofen Carboxy-ibuprofen Carboxy-hydratropic acid	Bench scale biofilm reactors and *in vitro* batch experiment with activated sludge	[86]
	Hydroxy-ibuprofen Carboxy-ibuprofen	Semi-batch river biofilm reactor	[87]
	Hydroxy-ibuprofen Carboxy-ibuprofen	Subsurface flow constructed wetlands	[88]
	1-Hydroxy-ibuprofen 2-Hydroxy-ibuprofen 1,2-Dihydroxy-ibuprofen	Batch experiments with the white-rot fungi *Trametes versicolor*	[89]
Iopromide	Carboxyiopromide Dicarboxylated-iopromide Bis-dehydroxy-iopromide	*In vitro* batch experiment with nitrifying activated sludge	[90]
	5-amino-*N*,*N*′-bis(2,3-dihydroxypropyl)-2,4,6-trrodo-*N*-methylisophthalamide	Laboratory sewage treatment plant	[91]
Ketoprofen	3-(hydroxy-carboxy-methyl) hydratropic acid 3-(keto-carboxy-methyl) hydratropic acid	*In vitro* batch experiment with activate sludge	[78]
Mestranol	17-a ethinylestradiol	Aerobic *in vitro* batch experiment with activated sludge	[73]
Metformin	Guanylurea	Fixed bed-bioreactor	[92]
		Closed bottle test; Manometric respiratory test; Zahn–Wellens test	[93]

Naproxen	Desmethyl-naproxen	Membrane bioreactor	[78]
	5-methoxy-2-naphthyl)ethan-1-ol	Small-scale pilot plant with activated sludge	[84]
	Desmethyl-naproxen and its conjugate	*In vitro* batch experiment with three *Cunninghamella* sp.	[94]
Norfloxacin	succinylnorfloxacin 7-[(carboxymethyl) amino]-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylicacid	Membrane bioreactor	[77]
Norfloxacin	*N*-acetylnorfloxsacin	Batch experiments with isolated *E. coli* strain	[80]
Norfloxacin	*N*-acetylnorfloxsacin	Batch experiments with *Mycobacteria*	[95]
Phenazone	1,5-dimethyl-1,2-dehydro-3-pyrazolone	Batch experiments with biologically active filter material	[85]
Propyphenazone	4-(2-methylethyl)-1,5-dimethyl-1,2-dehydro-3-pyrazolone	Batch experiments with biologically active filter material	[85]
Roxitromycin	phosphorylated roxithromycin	Membrane bioreactor	[77]
Sulfamethazine	Formyl-sulfamethazine Sulfamethazine desulfonated N_4-glucosulfamethazine Desaminosulfamethazine N_4-hydroxy-sulfamethazine N_1-hydroxy-sulfamethazine	*In vivo* and enzymatic degradation experiments	[96]
Sulfamethoxazole	Hydroxy-*N*-(5-methyl-1,2-oxazol-3-yl)benzene-1-sulfonamide	Batch experiment with *Rhodococcus rhodochrous*	[97]
Trimethoprim	α-hydroxy-trimethoprim Hydroxylated trimethoprim	*In vitro* batch experiment with nitrifying activated sludge	[98]
Trimethoprim	(5-(3,4,5-trimethoxybenzyl) pyrimidine-2,4-diamine,5-hydroxyl) (5-(1-carboxyl,1-methoxy, 5-methoxy 1-,4-pentene) pyrimidine-2,4-diamine, 5-hydroxyl)	Lab-scale bioreactors	[99]
Verapamil	2-(3,4-dimethoxyphenyl)-2-isopropyl-5-(methylamino)pentane nitrile	Zahn–Wellens test	[100]

Quintana *et al.* [78] investigated the microbial degradation pathway of anti-inflammatory drugs and blood-lipid regulators (i.e., bezafibrate (BZF), naproxen (NPR), ibuprofen (IBF), and ketoprofen (KET)) during laboratory biodegradation tests, demonstrating that they were degraded only co-metabolically. The first step in microbial BZF degradation appears to be the hydrolytic cleavage of the amide bond, generating the degradable 4-chlorobenzoic acid. Two isomers of hydroxy-IBF were detected as the main bio-TPs of IBF, while cleavage was considered as the primary step in the microbial degradation of NPR, yielding desmethyl-NPR. In contrast, KET was the only compound biotransformed in the reactor, yielding two metabolites (3-[hydroxy-carboxy-methyl]-hydratropic acid) and its oxidative form (3-[keto-carboxymethyl]-hydratropic acid).

Three bio-TPs of IBF (hydroxy-IBF, carboxy-IBF, and carboxy-hydratropic acid) were identified in biodegradation experiments with activated sludge in both biofilm and a batch reactor [86] which were, however, identical to human metabolites detected in UWTPs influents at higher concentrations than IBF [55,101]. The experiments revealed hydroxy-IBF as the major bio-TP under oxic conditions, and carboxy-hydratropic acid under anoxic conditions. Carboxy-IBF on the other hand, was found under oxic and anoxic conditions only in the batch experiments. Together, the bio-TPs did not account for more than 10% of the initial concentration of the parent compound.

Laboratory studies indicated that IBF bio-TPs (hydroxy- and carboxy-IBF) were identified and readily degraded in a river biofilm reactor [87]. The authors pointed out two main differences between the metabolism of IBF in humans and the environment. Carboxy-IBF appears later than hydroxy-IBF and tends to persist in humans. In contrast, in the biofilm reactors the appearance and degradation of these metabolites occurred in the opposite order. In addition, IBF was found to occur as two isomeric forms; one of these isomers is the pharmacologically active substance, readily metabolized by humans, whereas, in environmental samples, this isomer is the most prominent form.

The aforementioned IBF bio-TPs were analyzed in selected effluent samples from two subsurface flow constructed wetlands (SSFCWs) [88]. SSFCWs are designed to remove suspended solids and organic matter from wastewater but there is little information on the effect of the characteristics of organic matter on the removal efficiency of specific contaminants. Nevertheless, they only accounted for approximately 5% of the recorded IBF degradation. In addition, the presence of carboxy-IBF and hydroxy-IBF in the influents and effluents of a pilot SSFCW used to treat urban wastewater at a similar IBF concentration [102], supports the observation that biodegradation intermediates do not accumulate in the wetlands.

A major bio-TP of diclofenac (DFC) occurred transiently and was identified to be *p*-benzoquinoneimine (derivative of 50-hydroxy-DFC), in a fixed-bed column bioreactor under aerobic conditions [83]. Abiotic adsorption to the biofilm was shown to determine the subsequent fate of this reactive product of 5-hydroxydiclofenac (aut-)oxidation. The apparent lack of a degradative potential for this compound, as well as the failure to detect an enrichment of DFC-depleting microbial activity, indicate a co-metabolic nature of DFC transformation. 40-hydroxy-DFC, the favored TP of eukaryotic DFC metabolism, was not identified.

In another study, the biodegradability of two estrogens (17β-estradiol and mestranol) was examined in an aerobic batch reactor with activated sludge from a UWTP [73]. The experiments revealed that 17β-estradiol was oxidized to estrone while mestranol was transformed in small amountss into 17α-ethinylestradiol. Furthermore, two glucuronides of 17β-estradiol (17β-estradiol-17-glucuronide and 17β-estradiol-3-glucuronide) were detected.

The biotransformation of the two antiviral drugs, acyclovir (ACV) and penciclovir (PCV), during activated sludge treatment was investigated by Prasse *et al.* [75]. Structural elucidation of the formed bio-TPs showed that transformation only took place at the side chain, leaving the guanine moiety unaltered. The oxidation of the primary hydroxy group in ACV resulted in the formation of carboxy-ACV, while PCV transformation was more diverse with several enzymatic reactions taking place, such as the oxidation of the terminal hydroxy groups and β-oxidation followed by the acetate cleavage.

Terzic *et al.* [77] investigated the biotransformation of various groups of antibiotics: fluoroquinolones (nofroxacin and ciprofloxacin) and macrolides (azithromycin and roxitromycin), sulfonamides (sulfadiazine, sulfathiazole, sulfapyridine, and sulfamethoxazole), and trimethoprim in an MBR (membrane bioreactor) inoculated with activated sludge and spiked with a mixture of these pharmaceuticals. The analysis of the influent and effluent led to the potential identification of six different TPs belonging to fluoroquinolones and macrolides. The bio-TPs of roxithromycin and azithromycin were formed by phosphorylation of the desosamine moiety. Transformation of fluoroquinolones yielded two types of products: conjugates, formed by succinylation of the piperazine ring, and smaller metabolites, formed by an oxidative break-up of piperazine moiety to form the 7-[(2-carboxymethyl)amino] group. It was also pointed out that some of these transformations were part of antibiotic inactivation mechanisms of sludge microorganisms to lower the negative influence of these compounds.

Jung *et al.* [80] found two major TPs of the two fluoroquinolones ciprofloxacin and norfloxacin. Transformation was investigated during biodegradation experiments with an isolated strain of *E. coli.* Flasks with growth medium and antibiotics were inoculated with selected samples from wastewater treatment plant *E. coli* strain. Out of 22 bacterial isolates capable of growth in the presence of norfloxacin, only *E. coli* LR09 strain was able to transform ciprofloxacin and norfloxacin by N-acetylation, producing one major metabolite *N*-acetylciprofloxacin and *N*-acetylnorfloxacin. *N*-Acetylnorfloxacin was also found as a bio-TP by Adjei *et al.* [95] as a product of metabolism of *Mycobacteria*.

In a study performed by Eichhorn *et al.* [98]; a structural elucidation of the antibiotic trimethoprim (TMP) bio-TPs, which were produced by nitrifying activated sludge bacteria in a small-scale laboratory batch reactor, was reported. Two microbial TPs were detected. The first corresponded to α-hydroxy-TMP, which had been described as one of the major human metabolites, while for the second product a twofold oxidation of the aromatic ring within the diaminopyrimidine substructure was postulated. Here it is noted that the second product identified in this work had not been reported in higher organisms, and thus its formation is hypothesized to be strictly confined to the microbial community in nitrifying activated sludge.

Biological transformation of TMP was also investigated by Yi *et al.* [99]. TMP removal was assessed in two lab-scale bioreactors: a nitrifying bioreactor and a heterotrophic bioreactor. Two new bio-TPs were identified under these test conditions. First, after a hydroxylation reaction (5-(3,4,5-trimethoxybenzyl) pyrimidine-2,4-diamine,5-hydroxyl) and secondly, after cleavage of the trimethobenzyl ring (5-(1-carboxyl,1-methoxy, 5-methoxy 1-,4-pentene) pyrimidine-2,4-diamine, 5-hydroxyl).

Regarding the biotransformation of the iodinated X-ray contrast media, Kormos *et al.* [103] reported the occurrence of four non-ionic iodinated X-ray contrast media (iohexol, iomeprol, iopamidol and iopromide) bio-TPs in different aqueous matrices (treated wastewater, surface water, groundwater, and drinking water). Twenty six bio-TPs were

detected in wastewater effluents while a significant change in the pattern of the formed products was observed after bank filtration and groundwater infiltration under aerobic conditions.

Additionally, Pérez *et al.* [104] investigated the aerobic biodegradability of iopromide and identified its bio-TPs in bioreactors containing mixed liquor from either the conventional activated sludge or the nitrifying activated sludge of a UWTP that uses a two-stage biological treatment process. Iopromide bio-TPs revealed that the iodinated ring remained intact and that minor transformations in its structure occurred during biodegradation. Three bio-TPs produced by the oxidation of the primary alcohols (forming carboxylates) on the side chains of iopromide were identified during the conventional activated sludge treatment. Furthermore, one product formed by dehydroxylation at the two side chains was identified in the nitrifying activated sludge.

Biodegradation of diatrizoate, ionic iodinated contrast medium, was investigated by Kalsch [82], in three different test systems: sewage sludge, river water and river water with river sediment. Findings of this study showed that in test systems with river water and river water with sediment, diatrizoate undergoes biotransformation by deacetylation. Two TPs were found with one biotransformation end product: 2,4,6-triiodo-3,5-diamino-benzonic acid. What is more, in systems with sewage sludge diatrizoate showed poor sorption, which suggests that the substance will probably not be retained in the sewage treatment plant. These findings were later confirmed by Haiß and Kümmerer [81]. In the following study two systems of biodegradation testing were applied, both using activated sludge as the bacteria source. A laboratory scale sewage treatment plant (flow through simulation test) and a Zahn–Wellens test (batch screening test). Only in the Zahn–Wellens test was diatrizoate eliminated and the above described TP formed.

Two studies report the biodegradation of metformin (MET), an antidiabetic drug, during wastewater treatment [92,93]. A recently published article identified guanylurea as the only recalcitrant, aerobic, bacterial TP of MET, but effluent concentrations of guanylurea could not completely account for the removed fraction of MET [93]. Information about the ecotoxicological relevance of guanylurea is scarce. Guanylurea showed no toxic effects on the bacterial community in a manometric respiratory test at a concentration of $11.9\,mg\,L^{-1}$. Trautwein and Kümmerer [93] detected only $1.9\,\mu g\,L^{-1}$ guanylurea in the effluent of the UWTP Forchheim (Southwest Germany), although MET was present at higher concentration ($57\,\mu g\,L^{-1}$) in the influent. The direct transformation of MET into guanylurea was also investigated by Scheurer *et al.* [92] using a batch scale fixed-bed bioreactor (FBR).

The biodegradation of the β-blocker atenolol (ATE) and the hypoglycaemic agent glibenclamide (GLB) has been reported by Radjenović *et al.* [105]. The biodegradation tests were performed in batch reactors under aerobic conditions, using as inoculum sewage sludge from a conventional activated sludge treatment (CAS) and a laboratory-scale membrane bioreactor (MBR). The biodegradation pathways of ATE and GLB were identical when degraded during MBR and CAS treatment. In the case of ATE, bacterial hydrolysis of the amide bond led to the same primary bio-TP, atenololic acid, while the biodegradation of GLB by activated sludge proceeded via bacterial hydroxylation of the cyclohexyl ring, which resulted in the formation of glibenclamide hydroxide.

The aerobic biodegradation and biotransformation of the calcium channel antagonist (a cardiovascular drug) verapamil was investigated by Trautwein *et al.* [100]. From two biodegradation tests differing mainly in bacterial density only in one of them was removal of verapamil and formation of a bio-TP determined. During the biodegradation test with activated sludge, verapamil was transformed into the dead-end product 2-(3,4-

dimethoxyphenyl)-2-isopropyl-5-(methylamino)pentane nitrile. This bio-TP is also known to be one of the products in the human metabolism of verapamil.

Codeine, an opium alkaloid, was transformed in aerobic batch experiments with activated sludge into several TPs [106]. Biological oxidation of codeine led to the formation of the α,β-unsaturated ketone codeine, which was considered as the precursor for further abiotic and biotic transformation due to its high chemical activity.

Trautwain and Kümmerer [79] studied the biodegradation of the tricyclic antipsychotic drug chlorpromazine (CPR) in four different biodegradation tests namely the closed bottle test (CBT), the manometric respirometry test (MRT) the Zahn–Wellens test (ZWT), and the Anaerobic degradation test (ANAD). Only in ZWT and ANAD were TPs which could be linked to biotic processes formed. In ZWT three TPs were identified. As a result of an enzymatic monooxygenation, followed by acetylation, ((3-(2-chloro-10H-phenothiazin-10-yl) propyl)(methyl)amino)methylacetate was identified, while a complete degradation of the CPR side chain resulted in a single hydroxylated phenothiazine. The third bio-TP identified was *N*-(3-(2-chloro-10*H*-phenothiazin-10-yl)propyl)-*N*-methylformamide as a result of bacterial N-demethylation, followed by carboxylation. In ANAD, due to bacterial anaerobic dehalogenation, promazine (another drug from the phenothiazine family) was formed. However, according to the authors it is not certain whether it is a product of biotic or abiotic transformation since the test was conducted without sterile control.

Although, as discussed above, some pharmaceuticals are biologically transformed under wastewater treatment conditions, little is known concerning the specific routes and mechanisms though which environmental bacteria degrade these compounds.

14.4 Transformation of Pharmaceuticals During Photolysis and Advanced Oxidation Processes

14.4.1 Photolysis

Photolytic degradation can occur in the aquatic environment either through the direct exposure to sunlight or through the implementation of an ultraviolet (UV) disinfection process which is increasingly finding application at the UWTPs. Photolytic degradation can be either direct or indirect. In direct photolysis, the target contaminant absorbs a solar photon which leads to a break-up of the molecule. In an indirect photolysis mechanism, naturally occurring molecules in the system, such as dissolved organic matter (DOM), act as sensitizing species, generating strong reactive agents, for example, singlet oxygen ($^1O_2$), hydroxyl radicals ($HO^\bullet$) or alkyl peroxyl radicals ($^\bullet OOR$), and hydrate electrons under solar radiation [6,107]. A summary of photochemical studies including identification of TPs and/or evaluation of their biological effects during the application of photolysis is provided in Table 14.3.

Diclofenac (DFC) has been extensively studied for its photodegradation in water and wastewater. Direct photolysis has been described as the predominant transformation pathway for its elimination under environmental conditions, while some photo-transformation products (photo-TPs) have been identified as responsible for its toxicity [8,137].

The degradation of DFC and the subsequent formation of three photo-TPs have been investigated by Buser *et al.* [101,109]. It was estimated that more than 90% of DFC was eliminated in a lake, most likely by photolytic degradation. The two photo-TPs correspond to the methyl esters of carbazole-1-acetic acid and its 8-chloro derivative, respectively,

Table 14.3 *Summary of photochemical studies including identification of TPs and/or evaluation of their biological effects during the application of photolysis.*

Parent compound	Light source	Water matrix	Tested microorganism	Biological effects (toxicity/antimicrobial activity)	Reference
Diclofenac	*Natural sunlight*	*Demineralized water* Reconstructed standard freshwater	–	–	[108]
	Natural sunlight	Aqueous solution	–	–	[109]
	Natural sunlight	Milli-Q water	*S. vacuolatus*	Initial solution (23 mg L^{-1}) → inhibition: 14.6% After 53 h → inhibition: 93% After 144 h → inhibition: 70%	[110]
	UVA (125 W)	Aqueous phosphate buffer solution/ Methanol	–	–	[111]
	Simulated sunlight ($\lambda = 290$–800 nm)	Bidistilled water	*S. vacuolatus*	EC_{50}(DFC) = 4.8 mg L^{-1} EC_{50}(CPAB*) = 48 mg L^{-1} The EC_{50} of CPAB was a factor of 10 lower than that for DCF, due to the higher hydrophobicity of CPAB (log $K_{ow} = 3.62$) compared with DCF (log $K_{ow} = 2.04$) at pH 7.0. (*) CPAB = 2-[2(chlorophenyl)amino]benzaldehyde	[112]
Naproxen	UVA (125 W)	Aqueous solution	–	–	[113]
	Simulated sunlight (150 W)	Distilled water/ Drinking water	*D. magna* *V. fischeri*	Photo-TP 1b → $EC_{50} = 59.44$ mg L^{-1} (*D. magna*); $EC_{50} = 42.95$ mg L^{-1} (*V. fischeri*)	[114]

			Photo-TP 1d → $EC_{50} = 12.61$ mg L^{-1} (*D. magna*); $EC_{50} = 20.61$ mg L^{-1} (*V. fischeri*) Photo-TP 1e → $EC_{50} = 13.65$ mg L^{-1} (*D. magna*); $EC_{50} = 16.17$ mg L^{-1} (*V. fischeri*) Photo-TP 1g → $EC_{50} = 10.51$ mg L^{-1} (*D. magna*); nd (*V. fischeri*) Photo-TP 3 → $EC_{50} = 6.43$ mg L^{-1} (*D. magna*); $EC_{50} = 30.41$ mg L^{-1} (*V. fischeri*) Photo-TP 4a → *nd* (*D. magna*); *nd* (*V. fischeri*) Photo-TP 4b → $EC_{50} = 50$ mg L^{-1} (*D. magna*); *nd* (*V. fischeri*)	
Simulated sunlight (150 W)	Drinking water	*B. calyciflorus* *T. platyurus* *C. dubia* SOS-Chromotest Ames Fluctation Test	Acute toxicity Initial solution → $LC_{50} = 62.48$ mg L^{-1} (*B. calyciflorus*); $LC_{50} = 84.09$ mg L^{-1} (*T. platyurus*); $EC_{50} = 66.37$ mg L^{-1} (*C. dubia*) Photo-TP 1 → $LC_{50} = 9.45$ mg L^{-1} (*B. calyciflorus*); $LC_{50} = 8.23$ mg L^{-1} (*T. platyurus*); $EC_{50} = 16.70$ mg L^{-1} (*C. dubia*) Photo-TP 2 → $LC_{50} = 11.37$ mg L^{-1} (*B. calyciflorus*); $LC_{50} = 5.30$ mg L^{-1} (*T. platyurus*); $EC_{50} = 10.09$ mg L^{-1} (*C. dubia*) Photo-TP 3 → $LC_{50} = 14.46$ mg L^{-1} (*B. calyciflorus*); $LC_{50} = 14.01$ mg L^{-1} (*T. platyurus*); $EC_{50} = 16.49$ mg L^{-1} (*C. dubia*) Photo-TP 4 → $LC_{50} = 4.51$ mg L^{-1} (*B. calyciflorus*); $LC_{50} = 11.63$ mg L^{-1} (*T. platyurus*); $EC_{50} = 6.30$ mg L^{-1} (*C. dubia*) Chronic toxicity Initial solution → $LC_{50} = 31.82$ (*B. calyciflorus*); $LC_{50} = 0.56$ mg L^{-1} (*T. platyurus*); $EC_{50} = 0.33$ mg L^{-1} (*C. dubia*)	[115]

(*continued*)

Table 14.3 (*Continued*)

Parent compound	Light source	Water matrix	Tested microorganism	Biological effects (toxicity/antimicrobial activity)	Reference
				Photo-TP 1 → LC_{50} = 3.86 (*B. calyciflorus*); LC_{50} = 0.46 mg L^{-1} (*T. platyurus*); EC_{50} = 0.10 mg L^{-1} (*C. dubia*) Photo-TP 2 → LC_{50} = 1.9 (*B. calyciflorus*); LC_{50} = 0.45 mg L^{-1} (*T. platyurus*); EC_{50} = 0.026 mg L^{-1} (*C. dubia*) Photo-TP 3 → LC_{50} = 6.86 (*B. calyciflorus*); LC_{50} = 0.25 mg L^{-1} (*T. platyurus*); EC_{50} = 1.06 mg L^{-1} (*C. dubia*) Photo-TP 4 → LC_{50} = *na* (*B. calyciflorus*); LC_{50} = 0.67 mg L^{-1} (*T. platyurus*); EC_{50} = 0.062 mg L^{-1} (*C. dubia*) SOS-Chromotest and Ames Fluctation Test → no mutagenic/genotoxic effect	
Ibuprofen	UV	Methanol	Red-blood cell ysis test Enzyme leakage	Benzylic alcohol (l-(4 isobutylpheny1)-ethanol) (>1 mM) → toxic to both human red blood cells and fibroblasts l-(4 isobutylphenyl acetophenone) → toxic to the cultured fibroblast The other photo-TPs and IBF were non-toxic.	[116]
Ketoprofen	Simulated sunlight (507.5 W m^{-2}; λ = 300–80 nm)	Freshwater Seawater	–	–	[117]
	UVA (7 W, λ_{max} = 254 nm)	Acetonitrile: water (1:1)	–	–	[118]
4-methylaminoantipyrine, 4-formylaminoantipyrine, 4-acetylaminoantipyrine	Simulated sunlight	Synthetic seawater Synthetic freshwater Milli-Q water	*D. magna*	Initial solution (10 mg L^{-1}) → inhibition: 0–20% (24 h); 5–40% (48 h) After photolysis → inhibition: 27–55% (24 h); 60–85 (48 h)	[119]

Carbamazepine	UV	Milli-Q water	–	–	[120]
Atorvastatin, Carbamazepine, Levofloxacin, Sulfamethoxazole	Simulated sunlight ($765\,W\,m^{-2}$)	Deionized water Synthetic field water solutions	–	–	[121]
Sulfamethoxazole	UV ($\lambda > 290\,nm$)	Aqueous solution	–	–	[122]
Trimethoprim	Simulated sunlight ($250\,W\,m^{-2}$)	Demineralized water Simulated seawater	*V. fischeri*	Initial solution ($20\,mg\,L^{-1}$) → no effect After 49 h (aqueous solution) → inhibition: 13% After 49 h (simulated water) → inhibition: 4%	[123]
Metoclopramide	UV fluorescent lamps	Deionized water	–	–	[124]
Flupentixol	UV fluorescent lamps	Deionized water	–	–	[125]
Propranolol, atenolol, metoprolol	Hanau Suntest, filtered 1 kW Xenon arc lamp: 290–800 nm	Deionized water	–	–	[126]
Propranolol	Hanau Suntest CPS, 1.1 kW Xenon arc lamp	Natural surface water	–	–	[127]
Propranolol, Metronidazole	UV-254 mercury lamp (UV-C, 30 W) UV-365 black light mercury lamp (UVA, 20 W).	Deionized water	*Allium*	Initial solution (MET) → $IC_{50} = 102.5\,mg\,L^{-1}$ Initial solution (PRO) → $IC_{50} = 39.8\,mg\,L^{-1}$ Irradiated solution (MET) → $IC_{50} = 86.7\,mg\,L^{-1}$ Irradiated solution (PRO) → $IC_{50} = 83.5\,mg\,L^{-1}$	[128]

(continued)

Table 14.3 (*Continued*)

Parent compound	Light source	Water matrix	Tested microorganism	Biological effects (toxicity/antimicrobial activity)	Reference
Tetracycline	Medium mercury lamp (500 W)	Milli-Q water	*V. fischeri*	Initial solution (20 mg L^{-1}) → inhibition: 33% Irradiated solution → inhibition: 61% (150 min); 64% (300 min)	[129]
Ciprofloxacin	Medium-pressure mercury lamp (150 W)	Milli-Q water	*V. fischeri*	Initial solution (6 mg L^{-1}) → no effect Irradiated solution → no effect	[130]
Difloxacin, sarafloxacin	Simulated sunlight (Xenon lamp, 500 W m^2, $\lambda = 290$–800 nm)	Milli-Q water	*S. aureus* *K. pneumoniae* *P. aeruginosa* *E. coli*	Initial solution (DIF): MIC ($\mu g\ L^{-1}$) = 0.05 (*K. pneumoniae*); 0.5 (*E. coli*); 0.5 (*S. aureus*); 10 (*P. aeruginosa*) The concentration of DIF fell below the MIC (for *S. aureus* and *E. coli*) between the 3rd and the 4th hour of irradiation and for *K. pneumoniae* after 8 h. Initial solution (SARA): MIC ($\mu g\ L^{-1}$) = 0.01 (*K. pneumoniae*); 2.0 (*E. coli*); 2.0 (*S. aureus*); 10.0 (*P. aeruginosa*) The concentration of SARA fell below the MIC after 3 h of irradiation (for *S. aureus* and *E. coli*) and for *K. pneumoniae* after 48 h.	[131]
Bezafibrate, gemfibrozil, fenofibrate, fenofibric acid	150 W solar simulator equipped with Xenon lamp	Distilled water and sewage	–	–	[132]
Hydrochlorothiazide	Natural sunlight Simulated sunlight (Xenon lamp, 150 W)	Distilled water and sewage	–	–	[133]
Tamoxifen	Natural sunlight	Milli-Q water	*B. calyciflorus* *T. platyurus*	Acute toxicity	[134]

Simulated sunlight (150 W) high-pressure mercury lamp (500 W, $\lambda > 300$ nm)	*D. magna* *C. dubia*	Initial solution → $LC_{50} = 0.97$ mg L^{-1} (*B. calyciflorus*); $LC_{50} = 0.40$ mg L^{-1} (*T. platyurus*); $EC_{50} = 1.53$ mg L^{-1} (*D. magna*) Photo-TP 2 → $LC_{50} = 1.07$ mg L^{-1} (*B. calyciflorus*); $LC_{50} = 0.47$ mg L^{-1} (*T. platyurus*); $EC_{50} = 1.74$ mg L^{-1} (*D. magna*) Photo-TP 3 → $LC_{50} = 0.95$ mg L^{-1} (*B. calyciflorus*); $LC_{50} = 0.94$ mg L^{-1} (*T. platyurus*); $EC_{50} = 2.82$ mg L^{-1} (*D. magna*) Photo-TP 4 → $LC_{50} = 1.06$ mg L^{-1} (*B. calyciflorus*); $LC_{50} = 1.59$ mg L^{-1} (*T. platyurus*); no effect (*D. magna*) Photo-TP 5 → $LC_{50} = 1.31$ mg L^{-1} (*B. calyciflorus*); $LC_{50} = 1.28$ mg L^{-1} (*T. platyurus*); $EC_{50} = 3.27$ mg L^{-1} (*D. magna*) Irradiated solution → $LC_{50} = 17.79$ mg L^{-1} (*B. calyciflorus*); $LC_{50} = 15.97$ mg L^{-1} (*T. platyurus*); no effect (*D. magna*) Chronic toxicity Initial solution → $EC_{50} = 0.25$ mg L^{-1} (*B. calyciflorus*); $EC_{50} = 0.81$ .g L^{-1} (*C. dubia*) Photo-TP 2 → $EC_{50} = 0.26$ mg L^{-1} (*B. calyciflorus*); $EC_{50} = 0.41$ g L^{-1} (*C. dubia*) Photo-TP 3 → $EC_{50} = 0.156$ mg L^{-1} (*B. calyciflorus*); $EC_{50} = 2.8$ g L^{-1} (*C. dubia*) Photo-TP 4 → $EC_{50} = 0.123$ mg L^{-1} (*B. calyciflorus*); $EC_{50} = 0.77$ g L^{-1} (*C. dubia*) Photo-TP 5 → $EC_{50} = 0.125$ mg L^{-1} (*B. calyciflorus*); $EC_{50} = 0.89$ g L^{-1} (*C. dubia*) Irradiated solution → $EC_{50} = 15.58$ mg L^{-1} (*B. calyciflorus*); $EC_{50} = 9.6$ g L^{-1} (*C. dubia*)

(continued)

Table 14.3 (Continued)

Parent compound	Light source	Water matrix	Tested microorganism	Biological effects (toxicity/antimicrobial activity)	Reference
Bezafibrate, Fenofibrate, Gemfibrozil	Simulated sunlight (Xenon lamp, 150 W)	Distilled water	*V. fischeri* *B. calyciflorus* *T. platyurus* *D. magna* *C. dubia* *P. subcapitata*) Ames Test SOS-Chromotest	Acute toxicity Initial solution (BZF) → nd (*V. fischeri*); LC_{50} = 60.91 mg L^{-1} (*B. calyciflorus*); LC_{50} = 39.69 mg L^{-1} (*T. platyurus*); LC_{50} = 100.08 mg L^{-1} (*D. magna*); EC_{50} = 75.79 mg L^{-1} (*C. dubia*) Photo-TP 1 (BZF) → nd (*V. fischeri*); nd (*B. calyciflorus*); nd (*T. platyurus*); nd (*D. magna*); EC_{50} = 77.11 mg L^{-1} (*C. dubia*) Photo-TP 2 (BZF) → EC_{50} = 37.24 mg L^{-1} (*V. fischeri*); LC_{50} = 109.32 mg L^{-1} (*B. calyciflorus*); LC_{50} = 45.96 mg L^{-1} (*T. platyurus*); nd (*D. magna*); EC_{50} = 90.57 mg L^{-1} (*C. dubia*) Initial solution (FNF) → nd (*V. fischeri*); LC_{50} = 64.97 mg L^{-1} (*B. calyciflorus*); nd (*T. latyurus*); LC_{50} = 50.12 mg L^{-1} (*D. magna*); nd (*C. dubia*) Photo-TP 1 (FNF) → EC_{50} = 22.16 mg L^{-1} (*V. fischeri*); LC_{50} = 46.29 mg L^{-1} (*B. calyciflorus*); LC_{50} = 27.16 mg L^{-1} (*T. platyurus*); LC_{50} = 17.68 mg L^{-1} (*D. magna*); EC_{50} = 42.24 mg L^{-1} (*C. dubia*) Initial solution (GMF) → EC_{50} = 85.74 mg L^{-1} (*V. fischeri*); LC_{50} = 77.30 mg L^{-1} (*B. calyciflorus*); LC_{50} = 161.05 mg L^{-1} (*T. platyurus*); LC_{50} = 74.30 mg L^{-1} (*D. magna*); nd (*C. dubia*) Photo-TP 1 (GMF) → nd (*V. fischeri*); nd (*B. calyciflorus*); nd (*T. platyurus*); nd (*D. magna*); nd(*C. dubia*)	[135]

Chronic toxicity

Initial solution (BZF) → nd (*P. subcapitata*); $EC_{50} = 0.44$ (*B. calyciflorus*); $EC_{50} = 0.13\ mg\ L^{-1}$ (*C. dubia*)

Photo-TP 1 (BZF) → nd (*P. subcapitata*); $EC_{50} = 1.44$ (*B. calyciflorus*); $EC_{50} = 1.49\ mg\ L^{-1}$ (*C. dubia*)

Photo-TP 2 (BZF) → nd (*P. subcapitata*); $EC_{50} = 7.36$ (*B. calyciflorus*); $EC_{50} = 7.35\ mg\ L^{-1}$ (*C. dubia*)

Initial solution (FNF) → $EC_{50} = 19.84\ mg\ L^{-1}$ (*P. subcapitata*); $EC_{50} = 1.44$ (*B. calyciflorus*); $EC_{50} = 0.76\ mg\ L^{-1}$ (*C. dubia*)

Photo-TP 1 (FNF) → $EC_{50} = 14.14\ mg\ L^{-1}$ (*P. subcapitata*); $EC_{50} = 1.73$ (*B. calyciflorus*); $EC_{50} = 0.92\ mg\ L^{-1}$ (*C. dubia*)

Initial solution (GMF) → $EC_{50} = 15.19\ mg\ L^{-1}$ (*P. subcapitata*); $EC_{50} = 0.44$ (*B. calyciflorus*); $EC_{50} = 0.53\ mg\ L^{-1}$ (*C. dubia*)

Photo-TP 1 (GMF) → $EC_{50} = 71.49\ mg\ L^{-1}$ (*P. subcapitata*); $EC_{50} = 0.36$ (*B. calyciflorus*); $EC_{50} = 0.43\ mg\ L^{-1}$ (*C. dubia*)

Mutagenicity (Ames test)

Initial solution (BZF) → mMR = 1.58 (TA98); mMR = 1.10 (TA100)

Photo-TP 1 (BZF) → mMR = 1.64 (TA98); mMR = 1.79 (TA100)

Photo-TP 2 (BZF)) → mMR = 1.38 (TA98); mMR = 0.34 (TA100)

Initial solution (FNF) → mMR = 10.70 (TA98); mMR = 0.93 (TA100)

Photo-TP 1 (FNF) → mMR = 11.13 (TA98); mMR = 0.96 (TA100)

(continued)

Table 14.3 (Continued)

Parent compound	Light source	Water matrix	Tested microorganism	Biological effects (toxicity/antimicrobial activity)	Reference
				Initial solution (GMF) → mMR = 1.46 (TA98); mMR = 1.17 (TA100) Photo-TP 1 (GMF) → mMR = 121 (TA98); mMR = 21.90 (TA100) *Genotoxicity (SOS-Chromotest)* Initial solution (BZF) → mIF = 3.63 Photo-TP 1 (BZF) → mIF = 1.56 Photo-TP 2 (BZF)) → mIF = 2.12 Initial solution (FNF) → mIF = 2.87 Photo-TP 1 (FNF) → mIF = 1.63 Initial solution (GMF) → mIF = 2.61 Photo-TP 1 (GMF) → mIF = 2.45	
Ciprofloxacin	Medium-pressure mercury lamp (150 W)	Milli-Q water	Micronucleus test in HepG2 cells	Initial solution (0 min) → MN frequency = 24.33 MN/1000 BNC Irradiated solution (32 min) → A significant increase of MN frequency relative to the parent compound Irradiated solution (128 min) → A significant reduction of MN frequency relative to the parent compound	[130]
Captopril	Medium-pressure mercury lamp (150 W)	Milli-Q water	–	–	[136]
Chlorpromazine	Simulated sunlight (Xenon lamp, TXE150)	Milli-Q water	–	–	[79]

Notes: na: not available; nd: not detected; EC_{50}: half maximal effective concentration; IC_{50}: half maximal inhibitory concentration; LC_{50}: half maximal lethal concentration; MIC: minimal inhibitory concentration; mMR: maximum (mutagenic ratio) = number of revertants/plate compared to the control; mIF:maximum (induction factor) = R/R_0.

previously identified as photo-TPs of DFC in aqueous phosphate buffer solution or methanol during irradiation with UVA light [111].

A tentative transformation pathway of DFC has also been proposed by Agüera *et al.* [108]. Thirteen photo-TPs were identified, indicating that photolysis of DFC occurred by two main routes. One is a consequence of the initial photocyclization of DFC into carbazole derivatives, while the other route goes through the initial decarboxylation of DFC and further oxidation of the alkyl chain. The primary photo-TP identified was the 8-chloro-9*H*-carbazole-1-yl-acetic acid, followed mainly by the degradation of the alkyl chain and, to a lesser extent, by hydroxy substitution of chlorine to form the 8-hydroxy-9,9a-dihydro-4a*H*-carbazol-1-yl) acetic acid, a highly reactive product that can be the precursor of dimers. It should be noted that the dehalogenation of the chlorocarbazole moiety has played only a minor role in DCF phototransformation, the chlorine atom being present in most of the photo-TPs detected. The photocyclization of DFC has been reported elsewhere [137–139].

DFC photo-TPs and their phytotoxicity after exposure to natural midsummer sunlight were evaluated in the study of Schmitt-Jansen *et al.* [110]. The photo-TPs clearly correlated with toxicity enhancement over time towards *S. vacuolatus*. Inhibition of algal reproduction of the initial DFC solution was in the $mg\,L^{-1}$ range, indicating no specific toxicity of DFC towards *S. vacuolatus*. Phytotoxicity increased after 3.5 h of exposure to sunlight and showed a maximum of sixfold toxicity after 53 h. Schulze *et al.* [112] also identified 2-[(2-chlorophenyl)amino] benzaldehyde (CPAB) as a photo-TP of DFC that has an enhanced acute toxicity to *S. vacuolatus* compared to the parent compound, probably due to its higher lipophilicity. This compound seems to be at least as persistent as the parent DCF, as shown by Schmitt-Jansen *et al.* [110].

Moore and Chappuis [113] investigated the photolysis of naproxen (NPR) in the presence and absence of molecular oxygen. The TPs from photolysis of NPR can be rationalized on the basis of the primary radical species, formed by decarboxylation, reacting with O_2 in aerated solutions. Under aerobic conditions, the initial product was an unstable peroxy intermediate, breaking down in water to 1-(6-methoxy-2-naphthyl) ethanol, which in turn was oxidized to 2-acetyl-6-methoxynaphthalene. The latter product was present in minor amounts from the beginning of the photolytic treatment, and could have been formed directly by disproportionation of the peroxy intermediate [140].

Isidori *et al.* [115] reported that photo-TPs of NPR were 7–16 times more toxic in acute and chronic bioassays of algae, rotifers, and microcrustaceans, whereas no genotoxic and mutagenic effects were found. The aforementioned study was based on isolated and identified photo-TPs. The photo-TPs obtained by solar simulated irradiation, were significantly more toxic than the parent compound for the three organisms tested (*B. calyciflorus*, *T. platyurus* and *C. dubia*). From the data reported on the chronic toxicity, it was possible to note that NPR and its photo-TPs were bioactive at low concentrations, mainly for the primary consumers *B. calyciflorus* and *C. dubia*. Algae showed toxicity values two orders of magnitude lower than rotifers and crustaceans. Neither the parent compound nor its photo-TPs were able to induce *E. coli* activation response during the SOS-Chromotest. In fact, the results did not reveal an association between the compound's concentration and induction factor and the tested concentrations did not produce an induction factor higher than 2. Also, the Ames Fluctuation test (*S. typhimurium*) results did not show statistically significant differences from the negative control, both with and without metabolic activation.

Irradiation of NPR in an aqueous medium (distilled and drinking water) generated seven photo-TPs [114]. The formation of the TPs could be explained by the conversion of the

carboxylate group to a carboxyl radical by photoionization, which could be followed by decarboxylation to produce the corresponding benzyl radical. The formed radical can abstract a hydrogen atom from a suitable donor present in the reaction medium, resulting in the ethyl derivative, or can react with molecular oxygen leading to the formation of an alcohol or ketone group. Their results indicate that some photo-TPs were more toxic than NPR. For *D. magna*, the tested photo-TPs, except for the two dimers, exhibited higher toxicity than the parent compound with lower EC_{50} values. It is noteworthy that these dimers showed different toxicity, thus evidencing the role of a stereostructure–activity relationship.

Contrary to the previously discussed anti-inflammatory drug, IBF was transformed only minimally via direct UV-light, whereas indirect photolysis mediated by radicals was considered as the primary degradation pathway [116]. The most significant primary photodegradation mechanism was the cleavage of the C—C bond α to the carboxy group. Subsequent secondary processes (hydrogen abstraction, dimerization, incorporation of methanol, or reaction with oxygen) might account for the formation of the different photo-TPs. The isolated photo-TPs (which do not involve incorporation of methanol) have induced cytotoxic effects according to the two classical biological *in vitro* assay systems: the red blood cell lysis test and enzyme leakage. One of the isolated photo-TPs, benzylic alcohol (l-(4 isobutylphenyl)-ethanol), was found to be toxic to both human red blood cells and fibroblasts while l-(4 isobutylphenyl acetophenone) was also toxic to the cultured fibroblast. The other photo-TPs and IBF were apparently non-toxic at the highest concentration assayed.

Ketoprofen (KET) was also rapidly degraded in various environmental matrices [117]. The photodegradation pathway described in this study involves a decarboxylation step followed by hydrogen abstraction to form 3-ethylphenyl methanone and a further reaction with O_2 and afterwards with $HO^{\bullet}$ to produce 3-(1-hydroxyethyl)phenyl) methanone. Then, demethylation and subsequent cleavage of the two rings occurred, leading to the formation of more labile structures (benzophenone and 1-phenylethanone) that are easily removed by biotic or abiotic pathways. It is important to mention that some photo-TPs were more persistent than the parent compound.

According to Nakajima *et al.* [118]; KET was decomposed by the UV irradiation, generating two photo-TPs which were identified as 2,3-bis-(3-benzophenyl)butane and 3-acethylbenzophenone.

The direct photolysis of metronidazole (MET) and propranolol (PRO) by means of UV-C and UV-A radiation was studied by Dantas *et al.* [128]. The direct UV photolysis of MET probably occurred first on the ring with posterior attack of functional groups containing nitrogen whereas PRO seemed to undergo UV photolysis on the amino group. Regarding the toxicity profile, direct UV-C photolysis of MET promoted an increase in toxicity for *Allium* roots growing, indicating that the increase of irradiation time can promote the degradation of more toxic by-products. On the other hand, PRO direct UV-C photolysis promoted the reduction of more than 50% of the initial toxicity.

The aquatic persistence of carbamazepine (CBZ) has been suggested to be limited due to the photochemical reactions in surface waters [121,141,142]. A major photodegradation intermediate of CBZ is acridine (a stable azaarene compound with known mutagenic and carcinogenic activity) while hydroxylated/oxidized compounds were formed in the presence of hydroxyl radicals [120]. Direct photolysis of CBZ proceeded through two main routes. Hydration of the C10–C11 double bond is a first minor pathway, leading to 10-hydroxycarbamazepine. The other compounds were formed via a main photodegradation pathway that involves a ring contraction process, as already suggested by Vogna *et al.* [143]. Ring

contraction would possibly involve the formation of carbamazepine-9-carboxaldehyde, resulting from a hydroxylation step at the 10 position. The latter TP might be evolved in three different ways: (i) further hydroxylation with loss of the —$CONH_2$ group leading to hydroxyacridine-9-carboxaldehyde and acridone, to a lesser extent; (ii) simultaneous loss of the carboxyaldehyde and the —$CONH_2$ groups yielding acridine; and (iii) the dimerization which includes a simultaneous reduction of the —$CONH_2$ lateral chain.

Trimethoprim (TMP) was found to be highly stable to direct photolysis [123]. During this process a large number of intermediates were generated, in some cases more persistent than TMP. Hydroxylation, demethylation and cleavage of the original drug molecule were the main degradation mechanisms during the photolytic experiments. A hydroxyl radical conducted mechanism was observed during TMP photolysis in deionized water, induced by the formation of a ketone photosensitizer. The main intermediate identified was a ketone derivative (trimethoxybenzoylpyrimidine), which was proved to be a photosensitizer of TMP degradation. After a long exposure time, the intermediates generated during direct photolysis in distilled water showed a very discrete increase in *V. fischeri* inhibition. In simulated seawater, only 4% of *V. fischeri* inhibition was observed at the end of photolysis.

Difloxacin (DIF) photolysis in water under stimulated natural sunlight conditions was studied by Kusari *et al.* [131]. Difloxacin was found to degrade primarily to sarafloxacin (SARA). It is clearly evident that DIF and SARA undergo rapid degradation in water in the presence of sunlight. However, their photolytic products also exhibit considerable activity against both Gram-positive and Gram-negative bacteria. It is extremely interesting to note that even without isolation and structural elucidation of the individual photolysis products, their potential anti-bacterial efficacies and their burden to the aquatic environment could be assessed. This corroborates to the previously documented results on DIF [144–146]. From the results obtained, it appears that the anti-bacterial action of DIF and its photo-TPs in the water samples was most pronounced on the Gram negative bacterium *K. pneumoniae*, followed by the Gram-positive *S. aureus*, as also reported by Sunderland *et al.* [147].

In the study of Jiao *et al.* [129] the photolytic behavior of tetracycline (TC) and the toxicity of its degradation products were investigated. Two main intermediates with *m/z* of 398 and 413.9 were generated during photolysis. It is worth noting that the naphthol ring of TC remained intact during photolysis. The toxicity of the TPs was evaluated using the luminescent bacterium *V. fischeri*, and the results revealed that the toxicity increased with irradiation.

Analysis of the photo-TPs of ciprofloxacin (CIP) from the study of Vasconcelos *et al.* [130] showed that only a substitution/loss of the fluorine atom, a decarboxylation reaction and partial break of the piperazine moiety occurred. The identified compounds were very similar to CIP. Their findings indicate that the total toxicity of the photo-TPs formed towards *V. fischeri* has been reduced. Moreover, these photo-TPs were not biodegradable in the closed bottle test.

The genotoxic potential of CIP and of its photo-TPs was recently studied by Garcia-Käufer *et al.* [148]. This study was performed in the human-derived hepatoma cell line (HepG2) using the *in vitro* micronucleus (MNvit) assay. The frequency of induced micronuclei by irradiated solutions showed a tendency to increase after treatment with samples obtained between 16 and 64 min of irradiation compared with the non-irradiated sample. These results demonstrated that irradiated samples of CIP are able to exert heritable genotoxic effects on human liver cells *in vitro*. A study performed by Vasquez *et al.* [149]; using ofloxacin (OFL) and its irradiated solutions after photo(cata)lytic treatment reached similar

results, indicating that stable photo-TPs able to cause genotoxicity were formed during the first 32 min of irradiation. This property was reduced as the irradiation time increased. These products were less toxic than OFL to chronic ecotoxicity tests using *V. fischeri* and *P. putida* when the growth was monitored as an endpoint. However, the bioluminescence inhibition of *V. fischeri* had the same trend as the induction of micronuclei, indicating that the transformation products seem to affect the metabolism of the studied bacteria. The biodegradation potential of these photo-TPs was also investigated and they were found to be not readily biodegradable. However, in the presence of a readily biodegradable carbon source (sodium acetate), the biodegradation percentage increased drastically for some of the photolytically treated samples [150].

The presence of persistent and toxic photo-TPs of three relevant metabolites of the analgesic and antipyretic drug dipyrone, 4-methylaminoantipyrine (4-MAA), 4-acetylaminoantipyrine (4-AAA), 4-formylaminoantipyrine (4-FAA) were identified by Gómez *et al.* [151]. The most abundant photo-TP identified in the case of 4-MAA and 4-AAA was *N*-phenylacetamide. 4-FAA showed a high tendency to form dimer products, highly persistent under the assayed conditions. In parallel, acute toxicity tests applied to the irradiated solutions showed an increase in toxicity on *D. magna* during the photodegradation of these compounds as a consequence of the formation of toxic photo-TPs.

Transformation of the anti-cancer drug tamoxifen in water under sunlight irradiation was studied by DellaGreca *et al.* [134]. The main photo-TPs, which have been identified by spectroscopic methods, were characterized as cis-isomer, phenanthrenes and ketone derivatives of the parent compound. Isomerization and cyclization were considered as the main photoinduced reactions of the studied drug. In the same work, the toxic properties of the active pharmaceutical and its TPs were also investigated on *B. calyciflorus* and *T. platyurus*, showing acute effects with LC_{50} values ranging from 0.95 to 1.31 mg L^{-1} and 0.40 to 1.59 mg L^{-1}, respectively. No significant differences among EC_{50} values were determined for chronic toxic effects caused by both tamoxifen and its photo-TPs (0.81 g L^{-1} and 0.41 to 2.8 g L^{-1}, respectively) on *C. dubia*. In parallel, the irradiated solutions showed the lowest effects both in acute and chronic toxicity tests [134].

Toxic and genotoxic effects of three lipid regulators, belonging to the fibrates class, and their isolated photo-TPs were investigated by Isidori *et al.* [135]. Using simulated solar irradiation, bezafibrate (BZF), fenofibrate (FNF) and gemfibrozil (GMF) transformed into several photoproducts. Acute toxitiy data demonstrated that these drugs had a limited effect on the tested organism; $L(E)C_{50}$ values ranged from 39.69 to 161.05 mg L^{-1}. Among all the compounds tested, photo-TP of FNF showed the highest acute effect for all the organisms utilized in bioassays; EC_{50} values ranged from 17.68 to 42.24 mg L^{-1}. Chronic exposure to these compounds caused inhibition of growth population on *B. calyciflorus* and *C. dubia*; EC_{50} values ranged from 0.13 to 7.36 mg L^{-1}. In contrast, *P. subcapitata* seemed to be slightly affected by this class of pharmaceuticals. No mutagenic potential was found for BZF, its photo-TPs and GMF. On the other hand, the Photo-TP of GMF was bioactive both to the Ames test (TA98 and TA100) and SOS-Chromotest.

A recent study on the photo-degradation of captopril (CP) was performed in aqueous solution by irradiation with a medium pressure Hg-lamp [136]. Using LC-MS/MS, *m/z* of 266 has been assumed to be the molecular mass peak of 1-(3-sulfo- 2-methyl-1-oxopropyl)-proline. This structure was suggested by the authors as the only detected photo-TP of CP. Further testing on this TP showed no ready biodegradability under conditions of low bacterial density, the situation in surface water, in the Closed Bottle test.

In the study of Trautwein and Kümmerer [100] irradiation of the neuroleptic drug chlorpromazine (CPR) with a xenon arc lamp led to a huge number of photoproducts. 57 photo-TPs have been separated chromatographically and for 28 of them LC–MS^n experiments of higher order were performed. The molecular structures were given for the three most abundant ions with *m/z* of 317.3, 335.4 and 301.1 as 2-hydroxypromazine sulfoxide, CPR sulfoxide and 2-hydroxypromazine, respectively.

14.4.2 Advanced Oxidation Processes (AOPs)

Table 14.4 presents recent studies applying AOPs for the degradation of various pharmaceuticals in water and wastewater. The study of Coelho *et al.* [152] suggests that oxidation of DCF by ozone, under the experimental conditions used, mainly proceeded by hydroxylation reactions and cleavage of the C—N bond. Decarboxylation, cyclization and ring opening reactions in the phenylacetic acid moiety also occurred in the further steps of the degradation process. The main intermediates formed during ozonation were 2-[2,6-dichlorophenyl) amino]-5-hydroxyphenylacetic acid (D1) and 2,6-dichloro-4-hydroxybenzenamine (D8).

As set out in a previous paper [90], important differences were also observed with respect to the DFC photo-Fenton treatment. Eighteen TPs were identified during photo-Fenton treatment in two tentative transformation routes. The main one was based on the initial hydroxylation of the phenylacetic acid moiety in the C-4 position and subsequent formation of a quinone imine derivative that was the starting point for further multistep degradation involving hydroxylation, decarboxylation, and oxidation reactions. An alternative route was based on the transient preservation of the biphenyl amino moiety that underwent a similar oxidative process of C—N bond cleavage.

Ozonation of DFC solutions led to the formation of the 5-hydroxyl derivative (D1) as the main product, as proposed by Vogna *et al.* [143]. D1 was found in greatest abundance in the early stages of the process, indicating that DCF degradation is preferentially initiated by the hydroxylation of the phenylacetic ring in C5. In the study of Hofmann *et al.* [167], hydroxylation and cleavage of the NH-bridge were determined as the initial reactions, followed by dehalogenation and stepwise ring opening of the aromatic rings.

A comparative study for the TiO_2 photocatalytic treatment of IBF, DCF and NPR under different operational conditions (catalyst loading, temperature and dissolved oxygen concentration) has been reported by Méndez-Arriaga *et al.* [179]. The authors suggested that oxidant attack of the $HO^•$ on the isobutyl chain or/and propanoic moiety of IBF is the first step of the degradation process, followed by a second, less predominant step of the demethylation or decarboxylation giving rise to other different organic acids such as propionic, formic or hydroxypropionic acid. In the later study of Méndez-Arriaga *et al.* [180], the main by-products observed consisted mostly of the decarboxylated and the hydroxylated by-products of IBF.

The photocatalytic degradation of acetaminophen in TiO_2 suspended solution was studied by Zhang *et al.* [166]. Hydroxylation was considered as a main initial degradation step. However, as hydroquinone was the main detected intermediate, it was concluded that the *ipso*-substitution should be the main channel for the reaction with hydroxyl radical. The main intermediates, hydroquinone or 1,4-benzoquinone, could be attacked by the hydroxyl radical to form hydroxylation products.

In the study of Trovó *et al.* [154]; a sulfamethoxazole (SMX) degradation pathway during the photo-Fenton treatment in distilled water was proposed. Two initial transformation

Table 14.4 Summary of photochemical studies including identification of TPs and/or evaluation of their biological effects during the application of AOPs.

Parent compound	Water matrix	Tested microorganism	Biological effect (toxicity/antimicrobial activity)	Reference
		HOMOGENEOUS PHOTOCATALYSIS (Fenton/Fenton-like)		
Amoxicillin	Distilled water	*V. fischeri* *D. magna*	Initial solution inhibition (50 mg L^{-1}) → 30% (*V. fischeri*); 65% (*D. magna*) Treated samples (*V. fischeri*) → no significant changes Treated samples inhibition (*D. magna*) → $(5\text{–}100\%)_{[FeSO4]}$; $(70\text{–}95\%)_{[FeOx]}$	[153]
Sulfamethoxazole	Distilled water Seawater	*V. fischeri* *D. magna*	Initial solution (50 mg L^{-1}) inhibition → <50% (*V. fischeri*); 85% (*D. magna*) $[H_2O_2] = 210$ mg L^{-1} → ~80% (*V. fischeri*); ~65% (*D. magna*) Seawater: toxicity increased from 15 to 86% ($[H_2O_2] = 120$ mg L^{-1})	[154]
Nalidixic acid	Demineralized water Simulated industrial effluent Saline water	–	–	[155]
Trimethoprim	Demineralized water Simulated natural freshwater Simulated wastewater Real effluent	*V. fischeri*	TMP by-products → no toxic effects The variation in bacterial inhibition was attributed to the oxidation intermediates from real effluent organic compounds	[156]
Diclofenac	Distilled water	–	–	[90]
Clofibric acid	Milli-Q water	–	–	[157]
Ibuprofen	Milli-Q water	–	–	[158]
Atenolol	Distilled water Synthetic urban wastewater	–	–	[159]

Sulfanilamide, sulfacetamide, sulfathiazole, sulfamethoxazole, sulfadiazine	Aqueous solution	–	–	[160]
Ranitidine	Distilled water	–	–	[161]
Triclosan, riclocarban	Acetonitrile–water mixture	–	–	[157]
Clarithromycin, roxithromycin	River water	–	–	[162]
Flumequine, nalidixic acid	Distilled water	*V. fischeri*	Initial solutions (20 mg L^{-1}) inhibition → 70–90% Treated samples inhibition → < 50%	[163]
		HETEROGENEOUS PHOTOCATALYSIS (TiO_2)		
Sulfamethoxazole	Milli-Q water	×		[164]
Salbutamol	Milli-Q water	*V. fischeri*	Initial solution (15 mg L^{-1}) inhibition → 4% Treated samples inhibition → $(33, 54, <1\%)_{[5,\ 15,\ >60\ min]}$	[165]
Acetaminophen	Bidistilled water	–	–	[166]
Diclofenac	Milli-Q water	–	–	[167]
Diclofenac	Milli-Q water	*V. fischeri*	Initial solution (15 mg L^{-1}) inhibition → 24% Treated samples inhibition → $(38, 72, <1\%)_{[5,\ 20,\ >90\ min]}$	[168]
Ibuprofen	Milli-Q water	*V. fischeri*	Initial solution (200 mg L^{-1}) inhibition → ~78% Treated samples maximum inhibition → $(\sim 90\%)_{[120\ min]}$ with 40 mg L^{-1} O_2; $(92\%)_{[>240\ min]}$ (non-saturated conditions)	[169]
Trimethoprim	Demineralized water Simulated seawater	*V. fischeri*	Initial TMP solution (20 mg L^{-1}) → no effect After 49 h (aqueous solution) → Inhibition = 33% After 49 h (simulated water) → Inhibition = 54%	[123]
Oxolinic acid	Milli-Q water	*V. fischeri* *E.coli*	$Inhibition_{(V.\ fischeri)}$ = 70% (60 min) $Inhibition_{(E.\ coli)}$ = 100% (30 min)	[170]
Bezafibrate	Milli-Q water	–	–	[171]
Clofibric acid	Milli-Q water	–	–	[142]

(continued)

Table 14.4 (Continued)

Parent compound	Water matrix	Tested microorganism	Biological effect (toxicity/antimicrobial activity)	Reference
Ranitidine	Distilled water	–	–	[161]
Ciprofloxacin	Deionized water	*E. coli*	The process reduces the antibacterial activity of ciprofloxacin solutions with increasing irradiation	[172]
Flumequine, nalidixic acid	Distilled water	*V. fischeri*	Initial solutions (20 mg L^{-1}) inhibition → 70–90% Treated samples inhibition → < 55%	[163]
OZONATION				
Sulfamethoxazole	Milli-Q water	*D. magna* *P. subcapitata*	Immobilization$_{(D.\ magna)}$ → 50% (O_3 dose = 0.2 mM) Inhibition$_{(P.\ subcapitata)}$ → 90% (O_3 dose = 0.02–0.05 mM)	[173]
Diclofenac	Milli-Q water	*V. fischeri*	Inhibition → 50% (15 min)	[152]
Diclofenac	Bidistilled water	–	–	[143]
Trimethoprim	Distilled water Secondary wastewater effluent	*D. magna*	No acute toxicity was noted and no statistically significant growth inhibition	[174]
Roxithromycin	Distilled water Secondary wastewater effluent	*D. magna*	No acute toxicity was noted and no statistically significant growth inhibition	[174]
Bezafibrate	Milli-Q water	*V. fischeri*	Initial solution inhibition → 80% Treated samples inhibition → lower than that of the initial solution (>105 min)	[128]
Clofibric acid	Milli-Q water	*V. fischeri* *D. magna*	Initial solution (100 mg L^{-1}) → EC_{50} = 258 mg L^{-1} (*V. fischeri*); EC_{50} = 91 mg L^{-1} (*D. magna*) The identified oxidation products with higher toxicity were hydroquinone and 4-chlorophenol The toxicity was higher for *D. magna* than for *V. fischeri* High toxicity during the initial stages of ozonation both for *V. fischeri* and *D. magna* Lower toxicity at pH 3 than at pH 5 The products of catalytic runs exhibited considerably lower toxicity	[175]

Paracetamol	Aqueous solution	–		[8]
UV/H_2O_2				
Carbamazepine	Bidistilled water	–	–	[176]
Fenofibric acid	Distilled water	*P. subcapitata*	The increase of toxicity observed at high irradiation times was probably the consequence of the formation of ring-opening products.	[177]
Oxytetracycline, Doxycycline, Ciprofloxacin	Buffered ultrapure water	*V. fischeri*	Initial solution (OTC) → $EC_{50} = 70\ mg\ L^{-1}$ Initial solution (DTC) → $EC_{50} = 32\ mg\ L^{-1}$ Initial solution (CIP) → $EC_{50} = 2.856\ g\ L^{-1}$ Treared samples: (UV) → an increased toxic effect with the decay of the target compounds (UV/H_2O_2) → the toxicity increased first, and then decreased to no measurable toxicity to the applied species.	[178]

Notes: na: not available; EC_{50}: half maximal effective concentration.

pathways were proposed, involving the attack of HO$^•$ either on the benzenic or isoxazole aromatic rings. However, based on the SMX photo-TPs identified, the isoxazole ring was found to be more susceptible to HO$^•$ radical attack than the benzene ring, which persists in most of the intermediates identified. Despite 45% mineralization in seawater, toxicity increased from 16 to 86%, as shown by *V. fischeri* bioassays, which suggests that the intermediates generated in seawater were different from those in distilled water.

The main SMX transformation routes identified in the work of Gómez-Ramos *et al.* [181] were the hydroxylation of the benzene ring, the oxidation of the amino group on the benzene ring, the oxidation of the methyl group in the isoxazole ring and the double bond of the isoxazole ring, and S—N bond cleavage. Six ozonation by-products have been identified, the most abundant of which was the product of S—N bond cleavage. The toxicity on *D. magna* and *P. subcapitata* revealed the formation of toxic by-products during the early stages of reaction when there was still SMX in solution, and the persistence of an important residual toxicity for *P. subcapitata* after the total depletion of SMX.

Flumequine (FLU) and nalidixic acid (NXA) photo-TPs formed during the application of TiO_2 photocatalytic process were evaluated by Sirtori *et al.* [163]. Up to 14 intermediates were identified during the degradation of FLU. The opening of the quinolone structure by electrophilic attack of the HO$^•$ on the double bond of the heterocyclic ring was suggested as the main route of degradation. Defluorination has also been demonstrated to be a significant photodegradation mechanism, as has been reported in previous studies for fluoroquinolones [182]. The photocatalytic degradation of NXA followed a degradation pathway similar to that of FLU. In all cases, photocatalytic processes were associated with a reduction in toxicity, as evaluated by *V. fischeri* bioassay. Girlando *et al.* [183] used a photocatalytic system with TiO_2 in suspension to evaluate the degradation of the antibiotic oxolinic acid (OA). Two main TPs were elucidated: 5-ethyl-7-hydroxy[1,3]dioxolo[4,5-g]quinolin-8 (5*H*)-one (compound A) and 5-vinyl[1,3]dioxolo[4,5-g]quinolin-8(5*H*)-one (compound B). The results indicated also that the oxidation products that remain in solution after the degradation of OA and the compounds A and B are not biologically active against *E. coli*. The results suggested that the OA intermediates have a much lower toxicity on *V. fischeri* compared with the original compound. Indeed, after 60 min of treatment the initial toxicity was reduced by approximately 70%, indicating that the formed TPs are considerably less toxic against the tested bacteria.

The transformation pathways of amoxicillin (AMX) were suggested by Trovó *et al.* [153] including the opening of the four-membered β-lactamic ring and further oxidations of the methyl group to aldehyde and/or hydroxylation of the benzoic ring. Three possible pathways for AMX photo-Fenton degradation were proposed: (i) hydroxylation, (ii) the opening of the four-membered b-lactam ring that yields the stereoisomers of penicilloic acid and a series of derivatives which do not have the β-lactam ring; and (iii) decarboxylation. Although AMX was more rapidly degraded in the presence of ferrioxalate, the sample presented significant toxicity during all the treatment due to the presence of oxalate. Using $FeSO_4$, significant decrease of toxicity to *D. magna* was observed after 90 min irradiation (from 65 to 5%), when 53% of the TOC was removed.

Several parallel AMX transformation pathways were also proposed by Hu *et al.* [164]; which involved attack of hydroxyl radical on the aniline ring, sulfonamide bond, or heterocyclic ring, followed by cleavage of the sulfonamide bond. In pathway A, addition on the aromatic ring by hydroxyl radical forms a hydroxycyclohexadienyl radical, from which another hydroxyl radical can abstract another hydrogen to form the non-radical hydroxy-

substituted intermediate 1. Hydroxyl radical attack on the sulfonamide bond results in the cleavage of the S—N bond. Subsequent abstraction of a hydrogen by the cleaved nitrogen results in the formation of intermediate 3 (pathways A and B), sulfanilic acid (pathways B and C), and hydroxysulfanilic acid (pathway A). Pathway C is initiated by formation of the dihydroxysubstitute intermediate 2. Hydroxyl radical addition to the open carbon position of the isoxazole ring results in the formation of a tertiary carbon-centered radical.

Sirtori *et al.* [123] investigated the TiO_2 photocatalysis of TMP in distilled water. It was found that TMP undergoes the same hydroxylation and demethylation reactions already discussed for photolysis, generating the same or similar intermediates. One of them has previously been reported as a TP in nitrifying activated sludge [98]. Solar TiO_2 photocatalysis showed only a small increase in the inhibition of *V. fischeri*. At completion of the degradation process in distilled water, *V. fischeri* inhibition was 33%, while for simulated seawater, *V. fischeri* inhibition was 54%, that is, the intermediates were moderately toxic for the organism tested.

In a recent study of Michael *et al.* [156]; the number of identified TPs of TMP revealed the complexity involved in the solar Fenton process and proposed the existence of various transformation routes resulting in multi-step and interconnected pathways. The matrix composition affected the formation of TPs, suggesting that the hydroxyl radicals generated during the process were scavenged by anions and organics present in the solution; however, hydroxylation, demethylation and cleavage reactions were observed in all matrices. It should be noted that most of the photoproducts generated during photocatalysis maintained the two-ring TMP structure and major changes occurred in the trimethoxybenzyl moiety. The intermediate TPs generated during the oxidation of the parent compound did not exhibit any toxic effects to *V. fischeri* while the variation in bacterial inhibition was attributed to the oxidation intermediates from real effluent organics. The reaction routes proposed, although sharing certain similarities, differ from others previously reported in the literature. TMP follows similar, but not identical, reaction pathways in TiO_2-photocatalytic experiments, as reported by Sirtori *et al.* [123], and γ-radiolysis, as reported by Luo *et al.* [184].

In the study of Radjenovic *et al.* [174]; five TPs of ROX and four TPs of TMP were identified during ozonation. The most intense fragment ion observed was *m/z* 679, formed by the loss of 4-methoxy-4,6-dimethyl-tetrahydropyran-2,5-diol (cladinose moiety), whereas further expulsion of water and 3-dimethylamino- 3,4,6-trideoxyhexose (desosamine moiety) generated the fragment ions *m/z* 661 and 522, respectively. For the test solutions containing final ozonation products of TMP and ROX no acute toxicity was noted and no statistically significant growth inhibition. Transformation pathways of fenofibric acid (FA) were proposed for UV and UV/H_2O_2 processes which primarily involved the decarboxylation of FA before yielding 4-chloro-4-(1-hydroxy-1-methylethyl) benzophenone and other minor products, predominantly chlorinated [177]. The toxicity of the UV irradiated samples was determined for the 72 h growth of *P. subcapitata*, which was high even after the total depletion of FA. This was attributed to the chlorinated aromatic products that dominated reaction mixtures at intermediate reaction times. There were low values for the toxicity of UV/H_2O_2 treated samples in which fenofibric acid was completely depleted. However, a degree of toxicity reappeared in the highly irradiated mixtures, probably as a consequence of the chlorinated reaction products.

Investigations on the intermediates of bezafibrate (BZF) suggest that the hydroxyl radical attack occurs on the phenoxy ring of the BZF before the ring opening, which produced many cycle opening products in the course of the reaction [171]. The cleavage of the ether and

amide bonds via positive holes was also determined. Chlorobenzoic acid, chlorobenzaldehyde and chlorobenzamide were found as TPs at prolonged irradiation times.

In a study performed by Paul *et al.* [172]; the heterogeneous photocatalytic degradation of ciprofloxacin (CIP) was examined. Their results suggested that piperazine ring transformations generally proceed first by ring cleavage, followed by loss of the secondary amine nitrogen. Microbiological assays with a reference *E. coli* strain indicated that the antimicrobial potency of CIP solutions tracks closely with the undegraded CIP concentration during photolytic or photocatalytic reactions. Similar results were obtained in previous study of Paul *et al.* [185].

Rosal *et al.* [175] investigated the formation of 4-chlorophenol, hydroquinone, 4-chlorocatechol, and 2-hydroxyisobutyric acid as the main by-products of clofibric acid. Sirés *et al.* [186] studied also the oxidation of clofibric acid using the Fenton treatment and proposed a reaction pathway in which clofibric acid was first oxidized to 4-chlorophenol, which was further oxidized either to *p*-benzoquinone via hydroquinone or to 4-chlorocatechol. These products were subsequently degraded to tartronic, maleic and fumaric acids, which were quickly converted into oxalic acid. The latter acid was also obtained from the oxidation of 2-hydroxyisobutyric acid, initially generated when 4-chlorophenol was formed. The formation of 4-chlorocatechol from 4-chlorophenol may take place as a result of a direct ozone attack or by the selective attack of hydroxyl radical on the ortho-position of 4-chlorophenol. Doll and Frimmel [142] investigated the formation of the photo-TPs of clofibric acid and proposed the same pathway, together with a parallel dechlorination reaction which supposedly yielded 2-(4-hydroxyphenoxy)-isobutyric acid. The results of toxicity bioassays on samples taken during the runs showed a significant increase in toxicity during the initial stages of ozonation for both *V. fischeri* and *D. magna* tests. The accumulation of ring-opened acidic structures from clofibric acid was the most likely origin of the increased toxicity of treated samples.

Ozonation of the atriviral drug acyclovir (ACV) and its main bio-TP carboxy-acyclovir (carxoxy-ACV) was investigated by Prasse *et al.* [187]. A single oxidation product was identified as *N*-(4-carbamoyl-2-imino-5-oxoimidazolidin)-formamido-*N*-methoxyacetic acid (COFA) which was characterized as very toxic to *V. fischeri* in contrast to carboxy-ACV which revealed no toxic effects.

The toxicity changes with degradation of three antibiotics, oxytetracycline (OTC), doxycycline (DTC) and ciprofloxacin (CIP) by UV and UV/H_2O_2 were investigated by Yuan *et al.* [178]. The luminescent bacterium test (*Vibrio fisheri*) was applied to the samples before and after exposure to the process at different time intervals. The oxidation of three antibiotics has been clarified into two stages. The first stage was the degradation of the parent compounds, causing increase in the toxicity of the by-products. This indicated that the character structures still preserve the characteristic structure of the parent compounds. In the second stage these more toxic TPs were further converted into non-toxic TPs. Moreover, the degradation efficiency of both degradation processes was monitored as a function of UV photon flux ($mJ\,cm^{-2}$). Results showed that detoxification was much faster than mineralization for the tested antibiotics. The authors concluded that the optimal time for the degradation of pollutants in UV/H_2O_2 process would be determined by parent compound degradation and toxicity changes.

14.5 Conclusions and Outlook

Although the toxicity towards humans associated with pharmaceutical metabolites is mostly known because it has been determined during several years of clinical trials of each drug,

knowledge of the environmental fate and ecological toxicity of these compounds when present in the environment is scarce. It is therefore necessary to prioritize which metabolites are important for toxicity testing and risk assessment.

Hydroxylation, isomerization, dehalogenation, dealkylation, cyclization, decarboxylation, dimerization and ring opening are among the most frequently observed transformation mechanisms of pharmaceuticals. The breakdown pathway is of course related to the characteristics of the specific compound under investigation. The degradation of pharmaceuticals in the environment does not always lead to rapid and complete mineralization. Instead, in the course of the degradation process, relatively stable TPs may be formed. Since drugs are considered as important environmental pollutants, it is not only important to elucidate their TPs in environmental media, but also it is deemed necessary to investigate whether the TPs preserve the same mode of action as the parent compound or are even more toxic.

It is recommended that environmental monitoring for the most commonly used pharmaceuticals or groups of pharmaceuticals should be undertaken as priority and detailed toxicity testing utilizing a wide variety of test organisms and compounds should be performed using the observed concentration levels. Data with regard to the chronic effects of pharmaceutical metabolites and TPs in the environment is lacking, making it difficult to do the necessary refinements on existing models to improve accuracy. Moreover, it is also unknown yet which organisms and which endpoints are relevant for the pharmaceuticals biological potency testing. Therefore, it is critical that advances in analytical methods allowing detection of very low levels of environmental contaminants be mirrored by an increase in chronic exposure data collection for both pharmaceuticals and their metabolites and TPs.

Even though the state-of-the-art is progressing rapidly, considerable additional work needs to be done to ensure that the environmental fate of pharmaceuticals and the appropriate endpoints of the aquatic life and ecosystems issues are properly understood.

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ARTICLE

II

Transformation Products of Antibiotic and Cytostatic Drugs in the Aquatic Cycle that Result from Effluent Treatment and Abiotic/Biotic Reactions in the Environment: An Increasing Challenge Calling for Higher Emphasis on Measures at the Beginning of the Pipe

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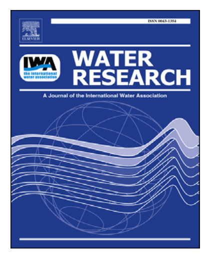

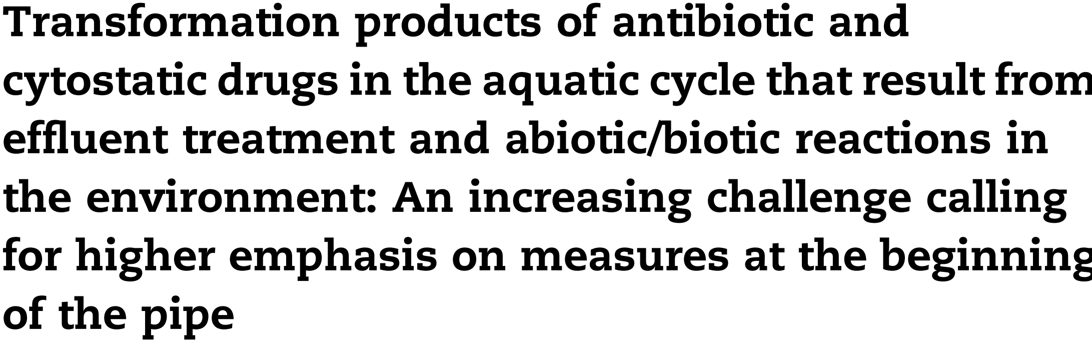

Transformation products of antibiotic and cytostatic drugs in the aquatic cycle that result from effluent treatment and abiotic/biotic reactions in the environment: An increasing challenge calling for higher emphasis on measures at the beginning of the pipe

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ABSTRACT

Pharmaceuticals may undergo transformation into new products during almost all possible processes along their life-cycle. This could either take place in the natural water environment and/or during water treatment processes. Numerous studies that address the issue of such transformation products (TPs) have been published, describing selected aspects of TPs in the environment and their formation within effluent and water treatment processes. In order to exemplify the number and quality of information published on TPs, we selected 21 active pharmaceutical ingredients from the groups of antibiotics and antineoplastics, and assessed the knowledge about their TPs that has been published until the end of May 2012. The goal of this work was to demonstrate, that the quality of data on pharmaceutical TPs greatly differs in terms of the availability of chemical structures for each TP, rather than to provide an exhaustive database of available TPs. The aim was to point out the challenge going along with so many TPs formed under different treatment and environmental conditions. An extensive review in the form of a table showing the existing data on 158 TPs for 15 compounds, out of 21 investigated, was presented. Numerous TPs are the result of different treatments and environmental processes. However, also numerous different TPs may be formed within only one type of treatment, applied under sometimes even very similar treatment conditions and treatments times. In general, the growing number of elucidated TPs is rationalized by ineffective removal treatments. Our results demonstrate a severe risk of drowning in much unrelated and non-assessable data, both from a scientific and from a technical treatment-related point of view. Therefore, limiting the input of pharmaceuticals into effluents as well as improving

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their (bio) degradability and elimination behavior, instead of only relying on advanced effluent treatments, is urgently needed. Solutions that focus on this "beginning of the pipe" approach should minimize the adverse effects of parent compounds by reducing and formation of TPs and their entrance into the natural environment.

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1. Introduction

The research on the presence of pharmaceuticals in the environment gained momentum in the 1990s; since then, it has been growing. Until today, a considerable number of review articles and several thousand original research articles on pharmaceuticals concerning their sources, their occurrence in different compartments of the environment, their fate and elimination by natural or human-driven processes, and possible effects on humans and organisms in the environment have been published (Halling-Sørensen et al., 1998; Ternes, 1998; Kümmerer, 2001; Heberer, 2002; Boreen et al., 2003; Bendz et al., 2005; Xia et al., 2005; Fent et al., 2006; Kümmerer, 2008; Onesios et al., 2009; Ding and He, 2010; Fatta-Kassinos et al., 2011a). In addition, the evaluation of analytical methods used for detection and quantification of pharmaceuticals in the environment has also developed further (Steger-Hartmann et al., 1996; Diaz-Cruz and Barceló, 2005; Kim and Carlson, 2005; Petrović et al., 2005; Fatta et al., 2007; Hao et al., 2007; Hernández et al., 2007; Hernando et al., 2007; Rodil et al., 2012; Masiá et al., 2014). As the knowledge on pharmaceuticals and their fate in the aquatic environment, together with more accurate and sensitive methods of their detection and analysis, increased, new questions and issues started to emerge. In the meantime, the focus in this area is not only on the parent compounds themselves, but also on the molecules resulting from incomplete mineralization in the body of treated organisms (metabolites) or abiotic reactions that can take place within advanced effluent treatment and once the parent compound is released into the environment (transformation products). However, research started to improve the knowledge on this new kind of micro-pollutants, i.e. transformation products (TPs), only recently. Knowledge on sources, properties, fate, and effects of TPs on human health and the natural environment is one such research focus with a tremendously increasing number of publications.

The sources of TPs are directly connected to the fate of parent compounds. Depending on their sources, TPs are described in the literature by different terms (Table 1). TPs are being formed in different processes in the environment as well as in waste water and potable water treatment (Zwiener, 2007; Schmidt and Brauch, 2008). After being administrated to humans or animals, pharmaceuticals may be metabolized by a variety of mechanisms and pathways within the body. The rate and extent of these processes are specific to each compound and may sometimes even be different between species. In the case of human pharmaceuticals, once these compounds and/or metabolites are excreted, they reach wastewater treatment plants (WWTPs), if such plants are in place, or directly reach surface water. In the case of veterinary pharmaceuticals, they are directly disposed in fields or used in biogas plants. In WWTPs, a further transformation of pharmaceuticals and metabolites may take place, for instance, by activated sludge during aerobic wastewater treatment or anaerobic digestion of sludge, which results in bacterial metabolites, also known as biotransformation products (bio-TPs) (Längin et al., 2009; Trautwein and Kümmerer, 2011). Hydrolysis and non-biotic oxidation reactions may also play a role in the transformation of pharmaceuticals in WWTPs (Kümmerer, 2009a). Additionally, so called advanced oxidation methods are under discussion for the treatment of wastewater and WWTP effluents in order to improve the removal rate of micro-pollutants such as pharmaceuticals. Processes used for these purposes are for example chlorination and advanced oxidation processes (AOPs), such as H_2O_2/

Table 1 – Examples of terms that have been used in the literature to describe transformation products according to their source.

Processes	Transformation products	Compartment
Biodegradation	Bio-transformation products, bacterial metabolites, biodegradation products, metabolites	Wastewater treatment plant, surface water bodies, anaerobic digesters, soils as far as related to bacteria or fungi
Photolysis, photocatalysis	Photo-transformation products, photoproducts	Surface water bodies, effluent treatment, drinking water treatment/disinfection
Chlorination, ozonation and advanced oxidation processes	Chlorination products, oxidation products, photo-oxidation products (if treatment by UV light is included), by products	Wastewater treatment plant, water treatment works, pre- and/or post- treatment of wastewater, drinking water treatment and disinfection
Other	Transformation products, intermediates, degradation products, stable transformation products	Used in general to all types of transformation products

UV, O_3/UV, TiO_2/UV, Fenton, and photo-Fenton. All of these processes do not often completely mineralize chemicals and can therefore frequently produce TPs (Zwiener, 2007; Kosjek and Heath, 2008; Radjenović et al., 2009b; Loos et al., 2013; Magdeburg et al., 2014; Richardson and Ternes, 2014; Trautwein et al., 2014; www.saicm.org). Together with WWTPs effluents; the mixture of pharmaceuticals, bio-TPs, and human metabolites enters surface water bodies. Once pharmaceuticals are introduced into the aquatic environment, they can undergo photolysis caused by exposure to the direct sunlight, in addition to already mentioned biodegradation and hydrolysis. As a result of this reaction, phototransformation products (photo-TPs) are formed. Furthermore, UV treatment, chlorination or treatment with ozone are also very common for water treatment in order to disinfect drinking water. Water treatment processes such as oxidation processes are applied, especially for the removal of micropollutants from drinking water, which cause odor or colour, as well as for disinfection. The most effective species generated in these processes, which reacts with the target compounds, is the hydroxyl radical (HO•). It is a very reactive molecule with low region-selectivity, i.e. in contrast to biological reaction mediated by enzymes, it reacts in a very unspecific way. This results in the formation of numerous TPs related to the treatment of pharmaceuticals.

In addition to publications on pharmaceuticals, numerous studies that address the issue of TPs have been published (Dewitte et al., 2008; Sirtori et al., 2010; Wang et al., 2010; Chen et al., 2012; Sturini et al., 2012; Lambropoulou and Nollet, 2014). Most of these works describe selected aspects of TPs in the environment and their formation within effluent and water treatment processes. Some reviews on TPs have been already published. For example, Fatta-Kassinos et al. (2011b) have summarized some general knowledge on how to identify TPs that are formed during the application of natural photolytic and AOPs as well as on potential biological effects that these products may cause. Additionally, Kosjek and Heath (2008) discussed the analytical challenges in the field of qualitative analysis of TPs generated during the AOPs and disinfection processes such as chlorination. Pérez and Barceló (2007) also provided an overview on strategies of analyzing unknown human and bacterial metabolites, using the mass spectrometry (MS) technique and focusing on the detection of metabolites in wastewater treatment plants.

Among research on pharmaceuticals, studies of antibiotics are very frequent (Kümmerer, 2009a, 2009b). Besides the natural background (some antibiotics are produced by bacteria in the environment), antibiotics are used in both human and veterinary medicine and in aquaculture (Kümmerer, 2009a). Therefore, sewage treatment plants as well as run-offs, e.g. from soils after the application of manure and heavy rain events, are sources of antibiotics in the environment (Daughton and Ternes, 1999; Kümmerer, 2009a). Researchers are interested in antibiotics not only because of their great usage, but also because of their direct influence on bacterial communities, which may impact geochemical cycles and soil fertility, and might possibly contribute to resistance development of potential human pathogens (Daughton and Ternes, 1999; Kümmerer, 2009a, 2009b).

In addition to the research on antibiotics, much recent attention has been given to cytostatic drugs and their mode of action. Cytostatic drugs are used to block the growth of cancer cells. They achieve this by influencing the cell cycle so that cell division and reproduction is inhibited (Eitel et al., 1999). Cytostatic drugs can exert carcinogenic, mutagenic, and/or teratogenic effects in animals and humans (Allwood et al., 2002). The measurement and significance of these properties under environmental conditions and possible associated risks are still unclear (Kümmerer and Al-Ahmad, 2010; Toolaram et al., 2014; Zhang et al., 2013; Kümmerer et al., in press). The use of such drugs in hospitals as well as increasingly in outpatient treatment for cancer therapy has considerably increased over the past decades due to the steady increase in the number of cancer patients around the world (Van Der Aa and Kommer, 2010; Yin et al., 2010; Stewart and Wild, 2014; Toolaram et al., 2014). After administration, most of these agents are incompletely metabolized in the body. Depending on the specific pharmaceutical, the rate of the excreted and unchanged pharmaceutical is different, ranging from a few percent up to nearly 100% (Eitel et al., 1999; Kümmerer and Al-Ahmad, 2010). The same holds true for antibiotics (Kümmerer and Henninger, 2003). Pharmaceuticals can; therefore, enter wastewater in their active forms via the urine and feces of patients undergoing chemotherapy. Therefore, sewage treatment plants are considered as the main source of introducing these agents to surface waters (Kümmerer and Al-Ahmad, 2010; Besse et al., 2012; Ferrando-Climent et al., 2013; Zhang et al., 2013; Kümmerer et al., in press).

Identification of products formed of incomplete degradation, e.g. in biodegradation experiments or photolysis studies, has become the most challenging step in environmental analysis (Krauss et al., 2010; Kosjek and Heath, 2011; Richardson and Ternes, 2014). Oxidation processes render molecules more polar. This often holds true for bio-TPs as well; however, polarity can sometimes also be reduced by, e.g. an alkylation step under anaerobic conditions. Due to the water-solubility of most pharmaceuticals and TPs, MS is often coupled with liquid chromatography (LC) and sometimes with gas chromatography (GC), which needs most often a prior derivatization step, thereby providing a more sensitive and selective tool for the separation/detection of the analytes in samples (Cardoza et al., 2005; An et al., 2010; Yuan et al., 2011). To separate unknown TPs from other compounds in the same sample, high performance liquid chromatography (HPLC) or ultrahigh performance liquid chromatography (UPLC) techniques are widely used (Jarman, 1973; Sanderson et al., 2005; Kwon, 2011; Shelver and Varel, 2012). Among the possible ionization techniques, atmospheric pressure ionization (API) methods such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are extensively employed, however, ESI is the most used (Kugelmann et al., 2011; Yuan et al., 2011; Luo et al., 2012).

In the absence of reference standards, which is most often the case for newly formed TPs, the interpretation of fragmentation patterns of tandem mass spectrometry (MS/MS) or ion trap multistage tandem mass spectrometry (MS^n) provides structural suggestions of TPs (Hernandez et al., 2008; Vasconcelos et al., 2009; Ge et al., 2010). In the past, low resolution mass spectrometry (LRMS) was used most often, but

Table 2 – List of selected pharmaceuticals.

Antibiotic agents			Cytostatic agents		
Compound name	Sub-class	CAS-RN[a]	Compound name	Sub-class	CAS-RN[a]
Cefuroxime	Cephalosporin	55268-75-2	Cyclophosphamide	Nitrogen mustard analogue	6055-19-2
Ceftriaxone	Cephalosporin	73384-59-5	Ifosfamide	Nitrogen mustard analogue	3778-73-2
Chlortetracycline	Tetracycline	57-62-5	Carboplatin	Platinium compound	41575-94-4
Doxycycline	Tetracycline	564-25-0	Cisplatin	Platinium compound	15663-27-1
Oxytetracycline	Tetracycline	79-57-2	5-fluorouracil	Pyrimidin analogue	51-21-8
Tetracycline	Tetracycline	60-54-8	Capecitabine	Pyrimidin analogue	154361-50-9
Ciprofloxacin	Fluoroquinolone	85721-33-1	Methotrexate	Folic acid analogue	59-05-2
Levofloxacin	Fluoroquinolone	100986-85-4	Hydroxycarbamide	Hydroxyurea	127-07-1
Ofloxacin	Fluoroquinolone	82419-36-1	Tamoxifen	Anti-estrogen	10540-29-1
Erythromycin	Macrolide	114-07-8	Imatinib	Protein Kinase Inhibitor	152459-95-5
Trimethoprim	Folic acid antagonist	738-70-5			

[a] Chemical abstracts service registry number.

provided only very limited information with respect to the reliability of the suggested structural formula, namely detected as peaks in chromatography. Degradation studies have often been performed at lab-scale, in which samples only contained the parent compound and a few TPs in relatively high concentration, but did not contain any additional water constituents such as nitrate or humic substances that can act as photo sensitizers. To assess the impact of environmental matrices on the degradation of compounds of interest, many studies were conducted by spiking a stock solution of a studied substance with raw water samples (Turiel et al., 2005). In such studies, a step of sample preparation is needed. Extraction by solid phase extraction (SPE) showed a successful separation and enrichment for the analysis of TPs (Cardoza et al., 2005). However, with unknown compounds, it is not fully clear whether all of them have been extracted and to what degree.

Recently, the advances in high-resolution mass spectrometry (HRMS) have enabled structure elucidation with a higher confidence because of high mass accuracy and resolution (Dewitte et al., 2008; Sirtori et al., 2010). Furthermore, HRMS can result in the detection and identification of TPs at lower concentrations, e.g. in the ng L^{-1} ranges in real environmental samples. More recently, hybrid instruments such as triple quadrupole/time-of-flight (QqQ/QToF) or linear ion trap/orbitrap (e.g. LTQ-Orbitrap) have been increasingly used to provide complementary information in order to elucidate and confirm unknown TPs (Calza et al., 2008; Radjenović et al., 2009a; Paul et al., 2010; López et al., 2014). An unambiguous confirmation can be typically obtained by the analysis of reference standards. Since standards of TPs often are not commercially available, a powerful structure elucidation technique such as nuclear magnetic resonance (NMR) or infrared (IR) spectroscopy can be used (Venta et al., 2005; DellaGreca et al., 2007; Kugelmann et al., 2011). However, because of the presence of several compounds in the sample, the interpretation of spectra is challenging. Furthermore, such expensive machines are not frequently available and low concentration of TP presents additional challenge.

In sum, much research has been published on the fate of parent compounds and related TPs that may be formed during different processes and under different conditions in the aquatic cycle. This paper uses the examples of selected pharmaceuticals and their TPs over a limited period to provide a critical review on what this knowledge might tell us. Two groups, antibiotics and anticancer drugs, were considered. Among the wide amount of such substances, a selection of 21 compounds was made, based on available knowledge in terms of potential effect, occurrence, persistence, and consumption. These compounds are representative of different families of compounds within each therapeutic group respectively (Table 2). Available information on these compounds was collected from various scientific publications. We assessed the knowledge about TPs that has been published until the end of May 2012 in order to exemplify the number and quality of information published on TPs. The aim of this study was to demonstrate, that the quality of data on pharmaceutical TPs greatly differs in terms of the availability of chemical structures for each TP, rather than to provide an exhaustive database of available TPs. To the best of our knowledge, this is the first work that addresses the challenge going along with so many TPs formed under different treatments and environmental conditions.

2. Materials and methods

Structures and availability of TPs' registry numbers in chemical abstracts service (CAS-RNs) was reviewed for the selected pharmaceuticals (Table 2) in the scientific literature via the following databases: Scopus (www.scopus.com), Web of Knowledge (www.apps.webofknowledge.com), PubMed (www.ncbi.nlm.nih.gov), and SciFinder (www.scifinder.cas.org). CAS-RNs are used worldwide to provide a nearly unique and unmistakable identifier for chemical substances that have been reported in the scientific literature (http://www.cas.org/content/chemical-substances/faqs). A literature research was conducted on data about TPs that has been published until the end of May 2012. Treatment processes (chlorination and AOPs, such as H_2O_2/UV, O_3/UV, TiO_2/UV, Fenton, and photo-Fenton) as well as naturally occurring transformations (biodegradation, photolysis) were taken into account when searching for TPs. Studies that investigated more complex treatment processes (e.g. a combination of above processes) were also included and short-written as "other" in Tables 1 and 3. Studies were considered that are related to lab-scale experiments (spiked synthetic/real water),

Table 3 – Transformation products (TPs) of investigated pharmaceuticals found with registry numbers in chemical abstracts service (CAS-RN).

Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
Antibiotic agents				
Cefuroxime 55268-75-2 $C_{16}H_{16}N_4O_8S$ 424.39	No data			No data
Ceftriaxone 73384-59-5 $C_{18}H_{18}N_8O_7S_3$ 554.58	No data			No data
Chlortetracycline 57-62-5 $C_{22}H_{23}ClN_2O_8$ 478.88	127-33-3 $C_{21}H_{21}ClN_2O_8$ 464.85	UPLC-ESI-QqQ-MS/MS	Biodegradation	Wu et al. (2011).
	1350552-48-5 $C_{21}H_{21}ClN_2O_8$ 464.85	HPLC-ESI-IT-MS/MS	Other	Chen et al. (2011).

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Table 3 – (continued)

Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	1350552-49-6 $C_{21}H_{21}ClN_2O_8$ 464.85	HPLC-ESI-IT-MS/MS	Other	Chen et al. (2011).
	1350552-50-9 $C_{20}H_{19}ClN_2O_8$ 450.03	HPLC-ESI-IT-MS/MS	Other	Chen et al. (2011).
	14297-93-9 $C_{22}H_{23}ClN_2O_8$ 478.88	HPLC-ESI-IT-MS/MS; standard control	Other	Chen et al. (2011).
		HPLC-ESI-QqQ-MS/MS	Other	Sanderson et al. (2005).
		UPLC-ESI-QqQ-MS/MS	Biodegradation	Shelver and Varel (2012).
		HPLC-ESI-QqQ-MS/MS	Biodegradation	Kwon (2011).
		UPLC-ESI-QqQ-MS/MS	Biodegradation	Wu et al. (2011).
	189624-83-7 $C_{22}H_{23}ClN_2O_8$ 478.88	HPLC-ESI-IT-MS/MS	Other	Chen et al. (2011).

	189624-84-8 $C_{22}H_{23}ClN_2O_8$ 478.88	HPLC-ESI-IT-MS/MS	Other	Chen et al. (2011).
	51262-22-7 $C_{20}H_{19}ClN_2O_8$ 450.83	HPLC-ESI-IT-MS/MS	Other	Chen et al. (2011).
	514-53-4 $C_{22}H_{23}ClN_2O_8$ 478.88	HPLC-ESI-IT-MS/MS; standard control UPLC-ESI-QqQ-MS/MS HPLC-ESI-QqQ-MS/MS HPLC-ESI-QqQ-MS/MS	Other Biodegradation Biodegradation Other	Chen et al. (2011). Shelver and Varel (2012). Arikan (2008). Sanderson et al. (2005).
	81163-11-3 $C_{22}H_{21}ClN_2O_7$ 460.86	HPLC-ESI-IT-MS/MS; standard control HPLC-ESI-QqQ-MS/MS	Other Biodegradation	Chen et al. (2011). Kwon (2011).

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Table 3 – (*continued*)

Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	4497-08-9 $C_{22}H_{21}ClN_2O_7$ 460.86	HPLC-ESI-IT-MS/MS; standard control	Other	Chen et al. (2011).
	60-54-8 $C_{22}H_{24}N_2O_8$ 444.43	HPLC-DAD-ESI-MS	Photodegradation	Chen et al. (2011).
Doxycycline 564-25-0 $C_{22}H_{24}N_2O_8$ 444.43	141-82-2 $C_3H_4O_4$ 104.06	GC–MS	AOPs	Yuan et al. (2011).
	100-21-0 $C_8H_6O_4$ 166.13	GC–MS	AOPs	Yuan et al. (2011).

	80-69-3 $C_3H_4O_5$ 120.06	GC–MS	AOPs	Yuan et al. (2011).
	56-81-5 $C_3H_8O_3$ 92.09	GC–MS	AOPs	Yuan et al. (2011).
	110-15-6 $C_4H_6O_4$ 118.09	GC–MS	AOPs	Yuan et al. (2011).
Oxytetracycline 79-57-2 $C_{22}H_{24}N_2O_9$ 460.43	100-21-0 $C_8H_6O_4$ 166.13	GC–MS	AOPs	Yuan et al. (2011).
	110-15-6 $C_4H_6O_4$ 118.09	GC–MS	AOPs	Yuan et al. (2011).

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Table 3 – (*continued*)

Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	123-76-2 $C_5H_8O_3$ 116.12	GC–MS	AOPs	Yuan et al. (2011).
	141-82-2 $C_3H_4O_4$ 104.06	GC–MS	AOPs	Yuan et al. (2011).
	14206-58-7 $C_{22}H_{24}N_2O_9$ 460.43	HPLC-ESI-MS; standard control	Other	Li et al. (2008).
	18695-01-7 $C_{22}H_{22}N_2O_8$ 442.42	HPLC-ESI-QqQ-MS/MS HPLC-ESI-MS; standard control	Other Other	Sanderson et al. (2005). Li et al. (2008).

	18751-99-0 $C_{22}H_{22}N_2O_8$ 442.42	HPLC-ESI-QqQ-MS/MS HPLC-ESI-MS; standard control	Other Other	Sanderson et al. (2005). Li et al. (2008).
	35259-39-3 $C_{22}H_{24}N_2O_9$ 460.43	HPLC-ESI-QqQ-MS/MS	Other	Sanderson et al. (2005).
	56-81-5 $C_3H_8O_3$ 92.09	HPLC-ESI-QToF-MS/MS	Photodegradation	Yuan et al. (2011).
	80-69-3 $C_3H_4O_5$ 120.06	HPLC-ESI-QToF-MS/MS	Photodegradation	Yuan et al. (2011).
	267244-10-0 $C_{21}H_{22}N_2O_9$ 446.41	HPLC-DAD-ESI-QMS	Other	Chen and Huang (2011).

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Table 3 – (*continued*)

Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
Tetracycline 60-54-8 $C_{22}H_{24}N_2O_8$ 444.43	1665-56-1 $C_{22}H_{22}N_2O_7$ 426.42	UPLC-ESI-QqQ-MS/MS HPLC-DAD-ESI-QMS	Biodegradation Other	Wu et al. (2011). Chen and Huang (2010).
	23313-80-6 $C_{22}H_{24}N_2O_8$ 444.43	HPLC-ESI-QqQ-MS/MS	Other	Sanderson et al. (2005).
	79-85-6 $C_{22}H_{24}N_2O_8$ 444.43	UPLC-ESI-QqQ-MS/MS HPLC-DAD-ESI-QMS	Biodegradation Other	Wu et al. (2011). Chen and Huang (2010).
	937181-90-3 $C_{22}H_{24}N_2O_9$ 460.43	HPLC-ESI-IT-MS^n; HPLC-APCI-MS HPLC-QqQ-MS/MS HPLC-APCI-MS/MS HPLC-UV-ESI-MS/MS HPLC-APCI-MS/MS	AOPs AOPs AOPs AOPs AOPs	Dalmázio et al. (2007). Khan et al. (2010). Wang et al. (2012). Wang et al. (2011a). Wang et al. (2011b).

	937181-91-4 $C_{22}H_{24}N_2O_{10}$ 476.43	HPLC-ESI-MS^n; HPLC-APCI-MS HPLC-QqQ-MS/MS	AOPs AOPs	Dalmázio et al. (2007). Khan et al. (2010).
	66241-98-3 $C_{20}H_{17}NO_9$ 415.35	HPLC-QqQ-MS/MS	AOPs	Khan et al. (2010).
	57-62-5 $C_{22}H_{23}ClN_2O_8$ 478.88	HPLC-APCI-MS/MS	AOPs	Wang et al. (2011b).
Ciprofloxacin 85721-33-1 $C_{17}H_{18}FN_3O_3$ 331.34	103222-12-4 (TP306)[a] $C_{15}H_{16}FN_3O_3$ 305.30	HPLC-ESI-MS/MS HPLC-ESI-QToF-MS/MS HPLC-ESI-LTQ-Orbitrap-MS/MS HPLC-UV-ESI-HRMS-MS/MS HPLC-MS HPLC-ESI-MS/MS HPLC-ESI-MS/MS HPLC-UV-MS HPLC-ESI-MS HPLC-QToF-MS/MS; HPLC-QqQ-MS/MS HPLC-ESI-MS/MS HPLC-ESI-IT-MS/MS HPLC-ESI-MS HPLC-EI-MS; GC–MS; H NMR HPLC-ESI-MS HPLC-UV-ESI-MS; HRMS; H NMR	AOPs AOPs AOPs AOPs AOPs AOPs AOPs Photodegradation Chlorination Biodegradation Photodegradation Photodegradation Chlorination Photodegradation Other Biodegradation	An et al. (2010). Paul et al. (2010). Calza et al. (2008). Dewitte et al. (2008). Huo et al. (2012). Kugelmann et al. (2011). Sturini et al. (2012). Cardoza et al. (2005). Dodd et al. (2005). Prieto et al. (2011). Turiel et al. (2005). Vasconcelos et al. (2009). Wang et al. (2010). Burhenne et al. (1997). Zhang and Huang (2005). Wetzstein et al. (1999).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	103237-52-1 $C_{17}H_{16}FN_3O_4$ 345.33	HPLC-ESI-MS	Photodegradation	Lester et al. (2011).
		HPLC-UV; MS; TLC; IR; NMR	Photodegradation	Torniainen et al. (1997a).
		HPLC-ESI-MS/MS	AOPs	Paul et al. (2007).
		HPLC-ESI-MS/MS	AOPs	Kugelmann et al. (2011).
		HPLC-EI-MS; GC–MS; H NMR	Photodegradation	Burhenne et al. (1997).
		HPLC-ESI-MS/MS	Photodegradation	Sturini et al. (2012).
	105394-83-0 $C_{16}H_{18}FN_3O$ 287.33	HPLC-UV-MS	Photodegradation	Cardoza et al. (2005).
		HPLC-ESI-MS/MS	Photodegradation	Vasconcelos et al. (2009).
		HPLC-ESI-MS	Photodegradation	Lester et al. (2011).
		HPLC-ESI-MS/MS	Photodegradation	Ge et al. (2010).
	105674-91-7 (TP263)[a] $C_{13}H_{11}FN_2O_3$ 262.24	HPLC-ESI-MS/MS	AOPs	An et al. (2010).
		HPLC-ESI-QToF-MS/MS	AOPs	Paul et al. (2010).
		HPLC-UV-ESI-HRMS-MS/MS	AOPs	Dewitte et al. (2008).
		HPLC-MS	AOPs	Huo et al. (2012).
		HPLC-ESI-MS/MS	AOPs	Kugelmann et al. (2011).
		HPLC-UV-MS	Photodegradation	Cardoza et al. (2005).
		HPLC-ESI-MS	Chlorination	Dodd et al. (2005).
		HPLC-QToF-MS/MS; HPLC-QqQ-MS/MS	Biodegradation	Prieto et al. (2011).
		HPLC-ESI-MS/MS	Photodegradation	Turiel et al. (2005).
		HPLC-ESI-MS	Chlorination	Wang et al. (2010).
		HPLC-EI-MS; GC–MS; H NMR	Photodegradation	Burhenne et al. (1997).
		HPLC-ESI-MS	Other	Zhang and Huang (2005).
		HPLC-UV-ESI-MS; HRMS; H NMR	Biodegradation	Wetzstein et al. (1999).
		HPLC-ESI-MS	Photodegradation	Lester et al. (2011).

		HPLC-ESI-MS/MS	AOPs	Paul et al. (2007).
		HPLC-UV; MS; NMR; TLC; IR	Photodegradation	Torniainen et al. (1997b).
		HPLC-ESI-LTQ-Orbitrap-MS/MS	Biodegradation	Girardi et al. (2011).
	109142-50-9 $C_{17}H_{18}FN_3O_4$ 347.34	HPLC-APCI-IT-MS/MS	Photodegradation	Ferdig et al. (2005).
	1219449-05-4 $C_{19}H_{21}FN_4O_3$ 372.39	HPLC-ESI-MS/MS	AOPs	An et al. (2010).
	1219449-06-5 $C_{17}H_{18}FN_3O_4$ 347.34	HPLC-ESI-MS/MS	AOPs	An et al. (2010).
	1219455-05-6 $C_{17}H_{19}N_3O_6$ 361.35	HPLC-ESI-MS/MS	AOPs	An et al. (2010).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	1219455-06-7 $C_{16}H_{19}N_3O_5$ 333.34	HPLC-ESI-MS/MS	AOPs	An et al. (2010).
	1219455-07-8 $C_{16}H_{19}N_3O_5$ 333.34	HPLC-ESI-MS/MS	AOPs	An et al. (2010).
	141673-55-4 $C_{16}H_{16}FN_3O_4$ 333.31	HPLC-ESI-QToF-MS/MS HPLC-ESI-LTQ-Orbitrap-MS/MS HPLC-ESI-MS HPLC-ESI-MS	AOPs AOPs Other Photodegradation	Paul et al. (2010). Calza et al. (2008). Zhang and Huang (2005). Lester et al. (2011).

Compound	Analytical method	Process	Reference
226903-07-7 (TP330)[a]	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
$C_{17}H_{19}N_3O_4$	HPLC-ESI-MS/MS	AOPs/Photodegradation	Sturini et al. (2012).
329.35	HPLC-UV-MS	Photodegradation	Cardoza et al. (2005).
	HPLC-ESI-MS/MS	Photodegradation	Turiel et al. (2005).
	HPLC-ESI-MS/MS	Photodegradation	Vasconcelos et al. (2009).
	HPLC-UV-ESI-MS; HRMS; H NMR	Biodegradation	Wetzstein et al. (1999).
	HPLC-ESI-MS	Photodegradation	Lester et al. (2011).
	HPLC-ESI-MS/MS	Photodegradation	Ge et al. (2010).
	HPLC-ESI-QToF-MS/MS	Photodegradation	Paul et al. (2010).
	HPLC-UV-MS/MS; HPLC-NMR	Photodegradation	Cardoza et al. (2003).
226903-12-4	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
$C_{17}H_{18}FN_3O_4$	HPLC-QToF-MS/MS; HPLC-QqQ-MS/MS	Biodegradation	Prieto et al. (2011).
347.34	HPLC-UV-ESI-MS; HRMS; H NMR	Biodegradation	Wetzstein et al. (1999).
	HPLC-ESI-LTQ-Orbitrap-MS/MS	Biodegradation	Girardi et al. (2011).
438571-49-4	HPLC-ESI-MS/MS	Photodegradation	Vasconcelos et al. (2009).
$C_{17}H_{19}N_3O_4$	HPLC-ESI-MS	Photodegradation	Lester et al. (2011).
329.35			
438571-50-7	HPLC-UV-MS	Photodegradation	Cardoza et al. (2005).
$C_{15}H_{17}N_3O_3$	HPLC-ESI-MS/MS	Photodegradation	Turiel et al. (2005).
287.31	HPLC-ESI-MS/MS	Photodegradation	Vasconcelos et al. (2009).
	HPLC-ESI-MS	Photodegradation	Lester et al. (2011).
	HPLC-ESI-MS/MS	Photodegradation	Sturini et al. (2012).

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Table 3 – (continued)

Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	918659-38-8 $C_{15}H_{13}FN_2O_5$ 320.27	UPLC-QToF-MS/MS	Biodegradation	Terzic et al. (2011).
	852546-79-3 $C_{13}H_{12}N_2O_3$ 244.25	HPLC-ESI-MS/MS	Photodegradation	Turiel et al. (2005).
	852546-80-6 $C_{15}H_{14}FN_3O_3$ 303.29	HPLC-ESI-MS/MS	Photodegradation	Turiel et al. (2005).
	852546-81-7 $C_{15}H_{17}N_3O_3$ 287.31	HPLC-ESI-MS/MS	Photodegradation	Turiel et al. (2005).

CAS / Formula / MW	Analytical method	Process	Reference
852546-82-8 $C_{15}H_{17}N_3O_3$ 287.31	HPLC-ESI-MS/MS	Photodegradation	Turiel et al. (2005).
860033-21-2 $C_{17}H_{16}FN_3O_5$ 361.32	HPLC-ESI-QToF-MS/MS HPLC-ESI-MS HPLC-ESI-MS	AOPs Chlorination Other	Paul et al. (2010). Wang et al. (2010). Zhang and Huang (2005).
860033-23-4 $C_{16}H_{16}FN_3O_4$ 333.31	HPLC-ESI-QToF-MS/MS HPLC-ESI-LTQ-Orbitrap-MS/MS HPLC-UV-ESI-HRMS-MS/MS HPLC-ESI-MS/MS HPLC-ESI-MS HPLC-ESI-MS HPLC-ESI-MS HPLC-ESI-MS/MS	AOPs AOPs AOPs AOPs Chlorination Other Photodegradation AOPs	Paul et al. (2010). Calza et al. (2008). Dewitte et al. (2008). Sturini et al. (2012). Wang et al. (2010). Zhang and Huang (2005). Lester et al. (2011). Paul et al. (2007).
918659-37-7 $C_{14}H_{11}FN_2O_4$ 290.25	HPLC-ESI-QToF-MS/MS	AOPs	Paul et al. (2010).

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Table 3 – (*continued*)

Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	93594-39-9 $C_{18}H_{18}FN_3O_4$ 359.35	HPLC-ESI-MS/MS HPLC-UV-MS	AOPs Photodegradation	Kugelmann et al. (2011). Cardoza et al. (2005).
	93594-40-2 $C_{21}H_{22}FN_3O_6$ 431.41	UPLC-QToF-MS/MS	Biodegradation	Terzic et al. (2011).
	93107-11-0 $C_{17}H_{19}N_3O_3$ 313.35	HPLC-ESI-MS/MS HPLC-MS/MS	Photodegradation Other	Sturini et al. (2012). Stieber et al. (2011).
	300665-06-9 $C_{13}H_{10}ClFN_2O_3$ 296.68	HPLC-ESI-MS	Chlorination	Dodd et al. (2005).

	1318139-22-8 $C_{15}H_{15}ClFN_3O_3$ 339.75	HPLC-ESI-MS	Chlorination	Dodd et al. (2005).
	99696-22-7 $C_{17}H_{17}ClFN_3O_3$ 365.79	HPLC-ESI-MS	Chlorination	Dodd et al. (2005).
	226903-13-5 $C_{17}H_{18}FN_3O_5$ 363.34	HPLC-UV-ESI-MS; HRMS; H NMR	Biodegradation	Wetzstein et al. (1999).
	1373395-26-6 $C_{17}H_{18}FN_3O_5$ 363.34	HPLC-MS	AOPs	Huo et al. (2012).

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Table 3 – (*continued*)

Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	1375414-96-2 $C_{16}H_{17}N_3O_4$ 315.32	HPLC-ESI-MS HPLC-ESI-QToF-MS/MS	Photodegradation Photodegradation	Lester et al. (2011). Paul et al. (2010).
	887407-22-9 $C_{17}H_{16}FN_3O_4$ 345.33	HPLC-ESI-HRMS	Biodegradation	Wetzstein et al. (2006).
	1375414-97-3 $C_{16}H_{17}N_3O_4$ 315.32	HPLC-ESI-MS HPLC-ESI-QToF-MS/MS	Photodegradation Photodegradation	Lester et al. (2011). Paul et al. (2010).
	1037621-00-3 $C_{17}H_{18}FN_3O_5$ 363.34	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).

	1037620-98-6 $C_{14}H_{18}FN_3O_4$ 311.31	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037620-97-5 $C_{13}H_{15}FN_2O_3$ 266.27	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	124487-37-2 $C_{17}H_{18}FN_3O_4$ 347.34	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037620-99-7 $C_{17}H_{16}FN_3O_5$ 361.32	HPLC-ESI-LTQ-Orbitrap-MS/MS HPLC-ESI-MS/MS	AOPs AOPs	Calza et al. (2008). Paul et al. (2007).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	226903-09-9 $C_{15}H_{16}FN_3O_2$ 289.3	HPLC-UV-ESI-MS; HRMS; H NMR HPLC-UV-ESI-HRMS-MS/MS	Biodegradation AOPs	Wetzstein et al. (1999). Dewitte et al. (2008).
	1031898-44-8 $C_{17}H_{18}FN_3O_4$ 347.35	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Dewitte et al. (2008).
	1031898-45-9 $C_{17}H_{16}FN_3O_5$ 361.34	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Dewitte et al. (2008).
	1031767-21-1 $C_{16}H_{16}FN_3O_5$ 349.31	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Dewitte et al. (2008).

	1031767-27-7 $C_{15}H_{18}FN_3O_3$ 307.32	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Dewitte et al. (2008).
	1031767-29-9 $C_{13}H_{14}FN_3O_2$ 263.27	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Dewitte et al. (2008).
	1031767-31-3 $C_{13}H_{16}FN_3O_3$ 281.28	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Dewitte et al. (2008).
	860033-20-1 $C_{17}H_{18}FN_3O_5$ 363.34	HPLC-ESI-MS	AOPs	Zhang and Huang (2005).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	141-82-2 $C_3H_4O_4$ 104.06	GC–MS	AOPs	Yuan et al. (2011).
	100-21-0 $C_8H_6O_4$ 166.13	GC–MS	AOPs	Yuan et al. (2011).
	57-13-6 CH_4N_2O 60.06	GC–MS	AOPs	Yuan et al. (2011).
	56-81-5 $C_3H_8O_3$ 92.09	GC–MS	AOPs	Yuan et al. (2011).

	56-40-6 $C_2H_5NO_2$ 75.07	GC–MS	AOPs	Yuan et al. (2011).
	110-85-0 $C_4H_{10}N_2$ 86.14	HPLC-UV-ESI-MS; HRMS; H NMR	Biodegradation	Wetzstein et al. (1999).
	226903-05-5 $C_{16}H_{18}FN_3O_2$ 303.33	HPLC-ESI-MS/MS HPLC-UV-ESI-MS; HRMS; H NMR	AOPs Biodegradation	Kugelmann et al. (2011). Wetzstein et al. (1999).
	1375414-98-4 $C_{17}H_{17}N_3O_5$ 343.33	HPLC-ESI-MS	Photodegradation	Lester et al. (2011).
	226903-11-3 $C_{17}H_{19}N_3O_5$ 345.35	HPLC-UV-ESI-MS; HRMS; H NMR	Biodegradation	Wetzstein et al. (1999).

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Table 3 – (continued)

Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	199742-51-3 $C_{13}H_{10}FNO_5$ 279.22	HPLC-UV-ESI-MS; HRMS; H NMR	Biodegradation	Wetzstein et al. (1999).
	226903-16-8 $C_{15}H_{17}N_3O_4$ 303.31	HPLC-UV-ESI-MS; HRMS; H NMR	Biodegradation	Wetzstein et al. (1999).
	1373395-27-7 $C_{17}H_{19}N_3O_6$ 361.35	HPLC-MS	AOPs	Huo et al. (2012).
	1275578-08-9 $C_{16}H_{18}FN_3O_3$ 319.33	HPLC-ESI-MS/MS	AOPs	Kugelmann et al. (2011).

	1275578-09-0 $C_{15}H_{16}FN_3O_4$ 321.3	HPLC-ESI-MS/MS	AOPs	Kugelmann et al. (2011).
	1275578-06-7 $C_{16}H_{19}N_3O_4$ 317.34	HPLC-ESI-MS/MS	AOPs	Kugelmann et al. (2011).
	1375414-99-5 $C_{17}H_{16}FN_3O_4$ 345.33	HPLC-ESI-MS	Photodegradation	Lester et al. (2011).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
Levofloxacin 100986-85-4 $C_{18}H_{20}FN_3O_4$ 361.37	115841-61-7 $C_{17}H_{16}FN_3O_4$ 345.33	HPLC-ESI-MS	Other	Cao et al. (2011).
	178964-53-9 $C_{17}H_{20}FN_3O_2$ 317.36	HPLC-ESI-MS	Other	Cao et al. (2011).
	117678-38-3 $C_{18}H_{20}FN_3O_5$ 377.37	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
	117707-40-1 $C_{17}H_{18}FN_3O_4$ 347.34	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).

	151250-74-7 $C_{13}H_{11}FN_2O_4$ 278.24	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
	151250-76-9 $C_{16}H_{18}FN_3O_4$ 335.33	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
	1263063-18-8 $C_{17}H_{20}FN_3O_5$ 365.36	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
	1263063-19-9 $C_{16}H_{20}FN_3O_4$ 337.35	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).

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Table 3 – (*continued*)

Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	1263063-20-2 $C_{16}H_{20}FN_3O_5$ 353.35	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
	1263063-21-3 $C_{16}H_{18}FN_3O_3$ 319.33	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
	1263063-22-4 $C_{16}H_{18}FN_3O_4$ 335.33	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).

	1263063-23-5 $C_{28}H_{32}F_2N_6O_6$ 586.59	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
	1263063-24-6 $C_{15}H_{18}FN_3O_5$ 339.32	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
Ofloxacin 82419-36-1 $C_{18}H_{20}FN_3O_4$ 361.37	82419-52-1 $C_{17}H_{18}FN_3O_4$ 347.34	HPLC-APCI-IT-MS/MS	Photodegradation	Ferdig et al. (2005).
	109-01-3 $C_5H_{12}N_2$ 100.16	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	69395-49-9 $C_4H_{10}N_2O$ 102.13	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037310-70-5 $C_{15}H_{20}FN_3O_3$ 309.34	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037310-71-6 $C_{17}H_{18}FN_3O_4$ 347.34	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037310-72-7 $C_{18}H_{21}N_3O_5$ 359.38	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).

	1037310-73-8 $C_{18}H_{20}FN_3O_5$ 377.37	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037310-74-9 $C_{18}H_{20}FN_3O_5$ 377.37	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037310-75-0 $C_{18}H_{20}FN_3O_5$ 377.37	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037310-76-1 $C_{18}H_{20}FN_3O_5$ 377.37	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	1037310-77-2 $C_{18}H_{18}FN_3O_5$ 375.35	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
Erythromycin 114-07-8 $C_{37}H_{67}NO_{13}$ 733.93	No data			No data
Trimethoprim 738-70-5 $C_{14}H_{18}N_4O_3$ 290.32	1182403-91-3 $C_{14}H_{18}N_4O_5$ 322.32	HPLC-ESI-QToF-MS/MS	AOPs	Radjenović et al. (2009a).

1182403-95-7 $C_{13}H_{18}N_4O_4$ 294.31	HPLC-ESI-QToF-MS/MS	AOPs	Radjenović et al. (2009a).
29606-06-2 $C_{14}H_{18}N_4O_4$ 306.32	HPLC-ESI-IT-MS; HPLC-QToF-MS/MS; H/D exchange HPLC-ESI-MS HPLC-ESI-ToF-MS	Biodegradation AOPs Photodegradation	Eichhorn et al. (2005). Luo et al. (2012). Sirtori et al. (2010).
860032-93-5 $C_{14}H_{20}N_4O_5$ 324.33	HPLC-ESI-IT-MS; HPLC-QToF-MS/MS; H/D exchange HPLC-ESI-QToF-MS/MS	Biodegradation AOPs	Eichhorn et al. (2005). Radjenović et al. (2009a).
1394121-65-3 $C_{14}H_{20}N_4O_4$ 308.33	NMR	Biodegradation	Yi et al. (2012).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	1394121-66-4 $C_{13}H_{20}N_4O_5$ 312.32	NMR	Biodegradation	Yi et al. (2012).
	86-81-7 $C_{10}H_{12}O_4$ 196.2	HPLC-ESI-MS HPLC-ESI-ToF-MS	AOPs Photodegradation	Luo et al. (2012). Sirtori et al. (2010).
	70035-83-5 $C_4H_6N_4O$ 126.12	HPLC-ESI-MS	AOPs	Luo et al. (2012).
	92440-76-1 $C_{14}H_{17}N_3O_4$ 291.3	HPLC-MS; NMR; TLC	Photodegradation	Bergh et al. (1989).

	93885-69-9 $C_{14}H_{16}N_2O_5$ 292.29	HPLC-MS; NMR; TLC	Photodegradation	Bergh et al. (1989).
	60729-91-1 $C_{14}H_{17}N_3O_4$ 291.3	HPLC-MS; NMR; TLC	Photodegradation	Bergh et al. (1989).
	72920-13-9 $C_{13}H_{15}N_3O_4$ 277.28	HPLC-MS; NMR; TLC	Photodegradation	Bergh et al. (1989).
	120749-06-6 $C_{14}H_{16}N_4O_3$ 288.3	HPLC-MS; NMR; TLC	Photodegradation	Bergh et al. (1989).
	21253-58-7 $C_{13}H_{16}N_4O_3$ 276.29	HPLC-MS; NMR; TLC	Photodegradation	Bergh et al. (1989).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	30806-86-1 $C_{14}H_{16}N_4O_4$ 304.3	HPLC-ESI-ToF-MS HPLC-ESI-MS	Photodegradation AOPs	Sirtori et al. (2010). Luo et al. (2012).
	55693-97-5 $C_{14}H_{18}N_4O_4$ 306.32	HPLC-ESI-ToF-MS	Photodegradation	Sirtori et al. (2010).
	1346601-32-8 $C_{14}H_{18}N_4O_4$ 306.32	HPLC-ESI-ToF-MS	Photodegradation	Sirtori et al. (2010).
	42310-45-2 $C_5H_8N_4O$ 140.14	HPLC-ESI-ToF-MS	Photodegradation	Sirtori et al. (2010).

	20781-06-0 $C_5H_6N_4O$ 138.13	HPLC-ESI-ToF-MS	Photodegradation	Sirtori et al. (2010).
		Cytostatic agents		
Cyclophosphamide 6055-19-2 $C_7H_{15}Cl_2N_2O_2P$ 261.09	27046-19-1 $C_7H_{13}Cl_2N_2O_3P$ 275.07	HPLC-ESI-QToF-MS; IR HPLC-ESI-MS/MS; IR HPLC-EI-MS; TLC	AOPs AOPs AOPs	Fernández et al. (2010). Venta et al. (2005). Jarman (1973).
	36761-83-8 $C_5H_{12}ClN_2O_2P$ 198.59	HPLC-EI-MS; TLC	AOPs	Jarman (1973).
	50650-73-2 $C_7H_{16}ClN_2O_2P$ 226.64	HPLC-EI-MS; TLC	AOPs	Jarman (1973).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
Ifosfamide 3778-73-2 $C_7H_{15}Cl_2N_2O_2P$ 261.09	No data			No data
Imatinib 152459-95-5 $C_{29}H_{31}N_7O$ 493.6	No data			No data
Carboplatin 41575-94-4 $C_6H_{14}N_2O_4Pt^{2+}$ 373.26	20115-64-4 $H_6N_2O_2Pt^{2+}$ 261.14	HPLC-UV	Photodegradation	Pujol et al. (1997).
	5445-51-2 $C_6H_8O_4$ 144.13	HPLC-UV	Photodegradation	Pujol et al. (1997).

	63700-88-9 $H_4N_2O_2Pt$ 259.12	HPLC-UV	Photodegradation	Pujol et al. (1997).
Cisplatin 15663-27-1 $Cl_2H_6N_2Pt^{+2}$ 300.05	31246-66-9 $Cl_2H_8N_2O_2Pt$ 334.06	HPLC-ESI-MS/MS	AOPs	Hernandez et al. (2008).
5-fluorouracil 51-21-8 $C_4H_3FN_2O_2$ 130.08	77413-15-1 $C_5H_7FN_2O_3$ 162.12	HPLC-ESI-MS	Photodegradation	Miolo et al. (2011).
	93713-25-8 $C_4H_5FN_2O_3$ 148.09	HPLC-ESI-MS	Photodegradation	Miolo et al. (2011).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	93713-26-9 $C_4H_5FN_2O_3$ 148.09	HPLC-ESI-MS	Photodegradation	Miolo et al. (2011).
	154121-06-9 $C_5H_7FN_2O_3$ 162.12	HPLC-ESI-MS	Photodegradation	Miolo et al. (2011).
Capecitabine 154361-50-9 $C_{15}H_{22}FN_3O_6$ 359.35	No data			No data

Hydroxycarbamide 127-07-1 $CH_4N_2O_2$ 76.05	No data			No data
Methotrexate 59-05-2 $C_{20}H_{22}N_8O_5$ 454.44	5939-37-7 $C_{20}H_{22}N_8O_6$ 470.44	HPLC-UV; IR	Biodegradation	Kiffmeyer et al. (1998).
Tamoxifen 10540-29-1 $C_{26}H_{29}NO$ 371.51	65-85-0 $C_7H_6O_2$ 122.12	HPLC-MS; NMR; TLC; IR	Photodegradation	DellaGreca et al. (2007).
	13002-65-8 $C_{26}H_{29}NO$ 371.51	HPLC-MS; NMR; TLC; IR	Photodegradation	DellaGreca et al. (2007).
	51777-15-2 $C_{17}H_{19}NO_2$ 269.34	HPLC-MS; NMR; TLC; IR	Photodegradation	DellaGreca et al. (2007).

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Parent compound CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Transformation products CAS-RN Molecular formula Molecular mass (g/mol) Chemical structure	Analytical technique	Treatment	Reference
	68732-11-6 $C_{26}H_{27}NO$ 369.5	HPLC-MS; NMR; TLC; IR	Photodegradation	DellaGreca et al. (2007).
	130605-17-3 $C_{26}H_{27}NO$ 369.5	HPLC-MS; NMR; TLC; IR	Photodegradation	DellaGreca et al. (2007).

[a] Here indicates the *m/z* ratio of a transformation product; UPLC: ultrahigh performance liquid chromatography; ESI: electrospray ionization; QqQ: triple quadrupole; MS: mass spectrometry; MS/MS: tandem mass spectrometry; HPLC: high performance liquid chromatography; DAD: diode array detector; UV: ultraviolet; QToF: Quadrupole time-of-flight; APCI: atmospheric pressure chemical ionization; MS^n: multistage tandem mass spectrometry; HRMS: high resolution mass spectrometry; GC: gas chromatography; NMR: nuclear magnetic resonance; IR: infrared spectroscopy; TLC: thin-layer chromatography; IT: ion trap; AOPs: advanced oxidation processes; Other: complex treatment processes or hydrolysis; QMS: a single-quadrupole mass spectrometry; H/D exchange: hydrogen/deuterium exchange.

and in which samples were collected from the aquatic environment or from full-scale WWTPs. However, human and mammal metabolites that were formed by metabolization of the compounds in target organisms were not considered in the data compilation. During the literature search, the following key words were used: transformation product; degradation product; photolysis; photocatalysis; photoproduct; photodegradation; chlorination; ozonation; oxidation; by-product; pharmaceutical; drug; and the names of the parent compounds. Available CAS-RNs of the reported TPs were obtained from the SciFinder database either by drawing the structure or finding articles with already assigned structures in the SciFinder database of TPs and their CAS-RNs.

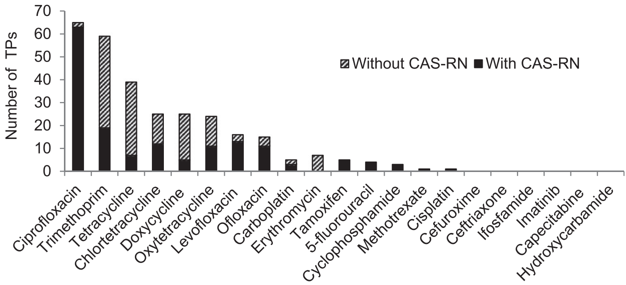

Fig. 1 – Summary of transformation products (TPs) owning chemical structures with/without registry number in chemical abstracts service (CAS-RN) for the selected antibiotic and anticancer drugs.

3. Results and discussion

In total, 294 TPs, identified with chemical structures in the literature, were found for 15 compounds out of the 21 selected as target compounds. 158 of these had an assigned CAS-RN, but 136 were not yet registered in the CAS system. However, for some of the selected pharmaceuticals, particularly cefuroxime, ceftriaxone, ifosfamide, imatinib, hydroxycarbamide and capecitabine, no research was found that had reported on TPs until the end of May 2012. This is likely due to the lack of the analytical methods, and the fact that these compounds have not yet been in the focus of research at that time. The most TPs were found for the two antibiotics ciprofloxacin and trimethoprim. In general, structures of TPs were more often reported for antibiotics (275 TPs) than for anticancer drugs (19 TPs). This could be related to i) the high occurrence of antibiotics in the environment due to their high consumption, and high interest in these compounds because of their contribution to resistance and ii) the fact that only a few of the studies have been done to assess the environmental impact of anticancer compounds, as these compounds are much less known and they are used at much lower amounts. All 158 TPs found with CAS-RNs (collected from 58 studies) are listed in Table 3. Numerous TPs are the result of treatment processes using AOPs (22 studies) and photo-degradation treatment (17 studies). Biodegradation was reported in 12 articles and 9 were assigned as “other”. Only two studies were found on chlorination (Table 3). However, some of the researchers investigated more than one type of treatment (e.g. Paul et al., 2010; Yuan et al., 2011; Sturini et al., 2012).

The majority of studies reported in Table 3 were done at lab-scale, i.e. by spiking a stock solution of the studied substance with synthetic water (43 studies) or with real water (6 studies). On the other hand, six investigations were based on samples collected from the aquatic environment, and in three others samples were collected from full-scale WWTPs.

Nevertheless, the differences in the number of TPs between different pharmaceuticals are not necessary correlated to the potential of possible transformations of the parent compound, but are usually directly correlated to the number of publications available on this topic (Fig. 1). For each of the two anticancer drugs, cisplatin and methotrexate, only one TP was described in the literature. In addition, only one investigation on TPs was found for the compounds doxycycline, 5-fluorouracil, carboplatin and tamoxifen in the studies by Yuan et al. (2011), Miolo et al. (2011), Pujol et al. (1997) and DellaGreca et al. (2007), respectively. Nevertheless, those studies identified many TPs (Table 3).

Among all selected pharmaceuticals, the most abundant group of TPs belongs to ciprofloxacin, one of the most widely used and studied antibiotics. Over 60 TPs for ciprofloxacin have been identified in different aquatic matrices. The majority of them have a CAS-RN (Fig. 1). It is important to realize that for ciprofloxacin, similar to other compounds, there is a list of TPs that have been reported just once, but other TPs were more frequently identified (Table 3). In many cases, the same TP was even formed from different treatments. Within this group, three transformation products with the CAS-RN 103222-12-4, which is also known as a human metabolite (Shah, 1991), 105674-91-7, and 226903-07-7 are the most common ones. These TPs were abbreviated as TP306, TP263, and TP330, indicating the *m/z* ratio of the product (Table 3). The abundance of these three TPs is interesting as well as the fact that they were found as a result in almost all types of reactions in the aquatic environment. They were also found in the water treatment processes. These TPs (TP306, TP263 and TP330) have also been identified in investigations on degradation of ciprofloxacin as photo-TPs by Lester et al. (2011) and as bio-TPs by Wetzstein et al. (1999). TP306 and TP263 have also been reported in studies on different types of oxidation and AOPs for ciprofloxacin, for example when treated by manganese dioxide (MnO_2) (Zhang and Huang, 2005), with TiO_2 irradiated by visible-light as well as UV-radiation (Paul et al., 2007), with Fenton's reagent (Kugelmann et al., 2011), during ozonation (Dewitte et al., 2008), and by the molecular imprinting technology onto TiO_2/fly-ash cenospheres (Huo et al., 2012). Furthermore, TP306 and TP263 were also identified as TPs of ciprofloxacin by chlorination (Dodd et al., 2005).

Besides typical lab-scale studies, a few researchers demonstrated the formation of these TPs in the aquatic environment by spiking a stock solution of ciprofloxacin with pond water (Cardoza et al., 2003), with river water (Turiel et al., 2005), and even in field systems (Cardoza et al., 2005). In the aforementioned studies, TP306, TP263, and TP330 were three among many other TPs that were detected as well (Table 3).

Different types of lamps and radiation wavelengths that are utilized for UV irradiation also make a difference in the formation of TPs, such as a high-pressure mercury lamp used

by Torniainen et al. (1997a) and a medium-pressure mercury lamp used by both Lester et al. (2011) and Vasconcelos et al. (2009). The same variety of conditions is observed in studies using a xenon lamp, resulting in different findings in relation to structural elucidation. For instance, ciprofloxacin was transformed to three TPs by the Suntest-CPS apparatus (Messrs, Heraeus) in a study by Burhenne et al. (1997); however, two other TPs were found by the merry-go-round photochemical reactor (Xujiang Electromechanical Plant) in a study by Ge et al. (2010) (Table 3). Additionally, it is important to note that in addition to different light sources, the entire experimental system of various studies is often very different, i.e. photoreactor and its geometry, initial concentration of parent compound, matrix of samples, pH and temperature of solution and many others. Fatta-Kassinos et al. (2011b) previously addressed this issue and gave an example of applying standardized tests for photolysis and surface water (i.e. OECD 316).

Chlorination is often applied as a disinfection process prior to the final discharge of WWTP effluent into streams, which can also affect the formation of TPs. In the context of ciprofloxacin, Dodd et al. (2005) investigated reactions between free available chlorine ($HOCl/OCl^-$) and ciprofloxacin, and described a formation of five TPs. Another study by Wang et al. (2010) identified four TPs formed by the reaction of ciprofloxacin with chlorine dioxide (ClO_2). A comparison of the identified TPs shows that only TP306 and TP263 are documented in both of these studies. Furthermore, monochlorinated analogues of TP306, TP263 and ciprofloxacin were identified as TPs by Dodd et al. (2005) while products with one and two aldehyde groups on the piperazine moiety were found by Wang et al. (2010) (Table 3). However, ClO_2 generates fewer chlorinated by-products and is less pH-dependent than free chlorine (Wang et al., 2010).

Generally, the same processes for one compound are often performed under different conditions by different research groups. This fact makes comparison and any discussion of treatment efficiencies difficult if not impossible.

Overall, many different approaches were applied in identifying unknown TPs. Identification is often performed by means of chromatographic and mass spectrometric instruments. Table 3 reveals an increasing use of LRMS, in 33 studies, whereas HRMS was reported in 14 studies. Cardoza et al. (2005) investigated photo-degradation of ciprofloxacin under different light sources, and the qualitative identification of TPs was only narrowed by using HPLC-UV-MS analysis of the treated samples. The authors compared the observed specific mass $[M+H]^+$ to the ones already published and confirmed in previous studies. However, an accurate confirmation of structures of TPs can only be done by comparison with a synthesized reference standard. In a study by Chen et al. (2011), HPLC-MS/MS was used to identify oxidation products of chlortetracycline by MnO_2. Among ten proposed structures, four were confirmed via comparison with authentic standards.

In case no standard is available, complementary techniques such as NMR and IR spectroscopy would be the best available tools in structural elucidation. A very early structure elucidation for TPs of ciprofloxacin was performed by utilizing mass spectrometry followed by final structure confirmation with NMR and IR techniques (Burhenne et al., 1997; Torniainen et al., 1997b). In a study by Cardoza et al. (2003), TP330 was found during the degradation of ciprofloxacin under fluorescent lights that were cycled diurnally at lab-scale. Initially, TP330 was identified using the HPLC-UV-MS technique. The relative molecular weight, MS/MS fragmentation patterns along with the spectral information obtained from NMR experiments were used to elucidate TPs structures. It is evident from Table 3 that NMR and IR spectroscopy have been employed in ten and six studies, respectively, while in two other studies, the identification of TPs was confirmed via comparison with standards. One has to be aware that NMR analysis needs enrichment or high testing concentrations which in turn may affect type and concentration of formed TPs, and in principle not all TPs that are formed within a certain process are detected within chromatographic analysis. This means also that highly polar products would not be retained on the chromatographic column, and less polar ones might be retained for too long during analysis. Furthermore they may possess a low ionization efficiency under the conditions selected and will therefore be below the limit of detection.

Studies that evaluated a specific treatment, in which the detected TPs were identified, did not always include a toxicity assessment which is not possible for unknown TPs or if TPs are not commercially available. Of the 58 studies reported in Table 3, only 14 studies included the evaluation of toxicity of the formed TPs. Toxicity was investigated by different methods, e.g. *Vibrio fischeri* luminescent bacterial assay (Calza et al., 2008; Vasconcelos et al., 2009; Sirtori et al., 2010; Yuan et al., 2011), residual antibacterial activity study (Wetzstein et al., 1999; Paul et al., 2010; Cao et al., 2011), mutagenicity test (Hernandez et al., 2008) or *Daphnia magna* toxicity assay (DellaGreca et al., 2007; Wang et al., 2011b; Yang and Chen, 2011; Wang et al., 2012). Most of the studies indicated either no difference between the parent compound and the TPs mixture, or even a decrease in toxicity during a transformation process. Only in two cases (Sirtori et al., 2010; Yuan et al., 2011) was the TPs mixture more toxic than the parent compound. However, in some studies there was evidence of a relation between time of the study and the toxicity of the reaction mixture, showing that toxicity increases at the beginning but then continuously decreases over the course of the treatment, e.g. ozonation, photodegradation (Calza et al., 2008; Yang and Chen, 2011; Wang et al., 2011b, 2012).

To summarize: Most of the investigations of TPs were done at lab-scale. The formed transformation products within the reactors were often identified by means of chromatographic and mass spectrometric instruments. However, the confirmation of structures of TPs can only be done in comparison to a synthesized reference standard or, in case no standard is available, by complementary techniques such as NMR and IR spectroscopy. For an accurate study of environmental samples where TPs are in trace as well as affected by the complexity of matrix, advanced sensitivity and accuracy of analytical methods is required to remove uncertainty in elucidation of structures. It is important to keep in mind that such a task is time-consuming and very expensive.

Moreover, as we have observed, not only one TP may be the result of different reactions (e.g. photodegradation,

biodegradation or AOP), but also numerous different TPs may be formed within one type of treatment, applied under different conditions. Only a minority of studies on the assessment of the ecotoxicological potency of TPs included long-term toxicity on non-target organisms. Furthermore, the question which endpoints are relevant for this kind of research is still unanswered. Investigations aiming at answering questions, such as how pharmaceuticals transform, what products result and what is the significance of these, are very costly and time-consuming if not impossible. Therefore, instead of only relying on advanced effluent treatments, solutions focusing on "beginning of the pipe" approach such as "benign by design" (Kümmerer, 2007; Rastogi et al., 2014a, 2014b) should minimize the adverse effects of parent compounds by reducing formation of TPs and their entrance into the natural environment.

4. Conclusions

Pharmaceuticals may undergo incomplete mineralization that leads to their transformation into new molecules by almost all possible processes, which could either take place in the natural aquatic environment and/or during water treatment processes. Today, the presence of TPs in the aquatic environment and the issue that they can actually pose a higher risk to environmental and human health than their parent is a fact. The growing number of elucidated TPs is rationalized by ineffective removal treatments.

Information available on TPs demonstrates that an already slight change in treatment conditions and processes results in the formation of different TPs. This in turn, on the one hand, makes it difficult to select the right conditions for effluent treatment, and on the other hand it presents a big challenge for the identification and assessment of TPs. The results presented in this paper demonstrate a severe risk of drowning in much unrelated and non-assessable data, both from a scientific and from a technical treatment-related point of view.

Therefore, from a practical and sustainability point of view, limiting the input of pharmaceuticals and their TPs as well as improving their (bio) degradability and elimination behavior, already during the optimization stages of drug design, i.e. "benign by design", is urgently needed. Solutions focusing on this "beginning of the pipe" approach should minimize the adverse effects of parent compounds and their TPs by ultimately reducing their entrance into the natural environment.

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ARTICLE

III

Characterization of Photo-Transformation Products of the Antibiotic Drug Ciprofloxacin with Liquid Chromatography–Tandem Mass Spectrometry in Combination with Accurate Mass Determination Using an LTQ-Orbitrap

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Characterization of photo-transformation products of the antibiotic drug Ciprofloxacin with liquid chromatography–tandem mass spectrometry in combination with accurate mass determination using an LTQ-Orbitrap

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ABSTRACT

The presence of pharmaceuticals, especially antibiotics, in the aquatic environment is of growing concern. Several studies have been carried out on the occurrence and environmental risk of these compounds. Ciprofloxacin (CIP), a broad-spectrum anti-microbial second-generation fluoroquinolone, is widely used in human and veterinary medicine. In this work, photo-degradation of CIP in aqueous solution using UV and xenon lamps was studied. The transformation products (TPs), created from CIP, were initially analyzed by an ion trap in the MS, MS/MS and MS^3 modes. These data were used to clarify the structures of the degradation products. Furthermore, the proposed products were confirmed by accurate mass measurement and empirical formula calculation for the molecular ions of TPs using LTQ-Orbitrap XL mass spectrometer. The degree of mineralization, the abundance of detected TPs and degradation pathways were determined. Eleven TPs were detected in the present study. TP1, which was never detected before, was structurally characterized in this work. All TPs still retained the core quinolone structure, which is responsible for the biological activity. As mineralization of CIP and its transformation products did not happen, the formation of stable TPs can be expected in waste water treatment and in surface water with further follow-up problems.

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1. Introduction

The presence of pharmaceuticals, especially antibiotics, in the aquatic environment is of growing concern. Several studies have been carried out on the occurrence and environmental risk of these compounds (Halling-Sørensen et al., 1998; Kümmerer, 2009). Photo induced reactions are known to play a key role among the abiotic transformation (Kümmerer, 2009). Photo-treatment is under discussion as a possible tool to remove micro-pollutants such as pharmaceutically active compounds through advanced sewage treatment and water treatment (Escher et al., 2009; Vasconcelos et al., 2009a). Ciprofloxacin (CIP), a broad-spectrum anti-microbial second-generation fluoroquinolone, is widely used in human and veterinary medicine. CIP was prescribed in 2010 in the order of 18.7 million defined daily doses (DDD) in Germany. Compared with 2009, this corresponded to an increase of 6.2% (Schwabe and Paffrath, 2011) reflecting a total amount of 9.5 tons (Gartiser et al., 2011). CIP has been found in hospital wastewater, sewage treatment plants and surface water; (Christian et al., 2003; Giger et al., 2003; Golet et al., 2002; Vasconcelos et al., 2009b; Watkinson et al., 2007). Initial studies from several groups have focused on photo-degradation of CIP under photolysis (Turiel et al., 2005; Vasconcelos et al., 2009a) and under advanced oxidation processes (An et al., 2010; Calza et al., 2008). Various transformation products (TPs) were detected (Burhenne et al., 1997; Paul et al., 2010; Sturini et al., 2012; Torniainen et al., 1996).

Mass spectrometry (MS) became the most selective instrument to identify unknown compounds and, using electrospray ionization (ESI), it is possible to obtain structurally significant fragment patterns (Fatta-Kassinos et al., 2011). As one of the latest LC/MS techniques, LTQ-Orbitrap enables high-resolution/high mass accuracy measurements on molecular ions (Makarov et al., 2006). Due to its very high resolution and mass accuracy, Orbitrap technology is routinely used in mixture analysis of peptides. There were also some literature reports on structural identification of drug metabolites using LTQ-Orbitrap (Peterman et al., 2006).

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In this work, photo-degradation of CIP in aqueous solution using UV and xenon lamps will be demonstrated. The major goals of this study are to identify and confirm detected degradation products by means of an instrument combination of liquid chromatography–tandem mass spectrometry (LC–MS/MS) and the accurate mass measurement of the molecular ions.

2. Materials and methods

2.1. Chemicals

Ciprofloxacin (CAS RN: 85721-33-1; purity >98%) was purchased from Sigma–Aldrich (Steinheim, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) and Sigma–Aldrich (Steinheim, Germany).

2.2. Photo-degradation and non-purgeable organic carbon (NPOC) monitoring

Photo-degradation experiments were carried out in a 1L immersion-type reactor (UV-Consulting Peschl, Mainz, Germany) filled with 750 mL of aqueous CIP solution in ultra-pure water. In order to meet the detection limits of non-purgeable organic carbon (NPOC) measurements, CIP concentration was 20 mg L^{-1}. Irradiation was performed using a medium-pressure mercury lamp with a power of 150 W (TQ 150, UV-Consulting Peschl, Mainz, Germany). The lamp emits polychromatic radiation with radiation flux Φ 47 W in the range 200–600 nm. The maximal intensities were at 254, 265, 302, 313, 366, 405/408, 436, 546, and 577/579 nm. The lamp was placed in a quartz tube with a cooling jacket. The temperature in all irradiation experiments was kept at 21.0 ± 1 °C and pH was neutral.

In order to simulate photolysis processes that occur in surface water, additional degradation experiments were conducted with a sunlight simulating xenon lamp (TXE 150, UV-Consulting Peschl, Mainz, Germany) that emits a spectrum similar to natural sunlight (300–800 nm). For analytical measurements aliquots of irradiated samples were collected at the following time points: 0, 2, 4, 8, 16, 32, 64 and 128 min. NPOC was determined using a Shimadzu TOC-5000 analyzer (Shimadzu, Duisburg, Germany) which is based on total oxidation on a platinum catalyst at a temperature of 680 °C. The instrument calibration was performed through the analysis of samples of potassium phthalate. NPOC was measured directly after samples were taken whereas samples for LC–MS analysis were stored at −20 °C until analysis.

2.3. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis

The primary elimination of CIP and TPs abundance are quantified using an Agilent Technologies HPLC 1100 series consisting of a G1322A degasser, two G1312A binary pumps, an auto sampler ALS G1329A + ALS Therm G1330B and a G1316A column oven. UV detection was performed at 278 nm using a G13658 detector (Agilent, Waldbronn, Germany). The chromatographic separations used a RP18 EC 125 mm × 4 mm, 5 μm Nucleodur reverse phase column (Macherey-Nagel, Düren, Germany). A gradient program was used for the mobile phase, combining solvents A (formic acid 0.1%) and B (acetonitrile) as follows: 5–15% B (up to 4 min), 15% B (up to 7 min), 15–40% B (up to 10 min), 40% B (up to 11 min), 40–5% B (up to 13 min), 5% B (up to 17 min). Flow rate was 0.7 mL min^{-1}, the injection volume was 5 μL and the column oven temperature was maintained at 40 °C. Mass spectra were obtained using an Esquire Mass spectrometer (6000^{Plus}, Bruker Daltonics, Bremen, Germany) equipped with atmospheric pressure electrospray ionization (AP-ESI) source. The mass spectrometer was operated in positive mode with −500 V end plate, +3300 V capillary voltage, 2068.43 hPa nebulizer pressure and 12 L min^{-1} dry gas flow at a temperature of 350 °C. The scan ranged from *m/z* 100 to 400. For further investigation of TPs, the samples were analyzed by the Auto MS^n mode, where product ions with highest peak intensity were isolated and fragmented up to MS^3 in order to gain more structural information. The injection volume was 20 μL. Structures and fragmentation patterns were drawn with ChemDraw Pro version 8.0 (CambridgeSoft Corporation, Cambridge, MA, USA). Data acquisition and evaluation were accomplished via ESI and software package Compass version 1.3 (Agilent Technologies, Bremen, Germany).

2.4. Accurate mass measurement

The accurate masses of CIP and its TPs were measured by the LTQ-Orbitrap XL mass spectrometer interfaced with a heated electrospray ionization (H-ESI) source (Thermo Scientific, Bremen, Germany). The chromatographic separations were performed by using a Dionex UHPLC system (Ultimate 3000) consisting of two HPG-3400-RS binary pumps, an auto sampler WPS-3000-TRS, a TCC-3000 column oven and a SRD-3600 degasser. UV detection was performed at 278 nm using a VWD-3400RS detector. The same chromatographic column and method as in Section 2.3 were used. The spectrometer was operated in positive mode under the following conditions: Solvent flow rate 0.5 mL min^{-1}, spray voltage 3.5 kV, capillary voltage 48 V and tube lens voltage 73 V. The heated capillary and desolvation temperatures were 350 °C and 400 °C, respectively. Nitrogen served as sheath, auxiliary and sweep gas with flow rate 74, 20 and 2 arb, respectively. MS data were recorded in the full scan mode (*m/z* 100–400). The acquisition method used was previously optimized in the tuning sections for the parent compound (capillary, magnetic lenses and collimating octapoles voltages) in order to achieve maximum sensitivity. All data were processed using Xcalibur/Qual Browser 2.1.0 SP1 build 1160 (Thermo Scientific, Bremen, Germany). The elemental composition tool was used to calculate molecular formulas, (ring and double-bond, RDB) values and the difference between theoretical and experimental *m/z* for product ions of CIP and its transformation products.

3. Results and discussion

3.1. Photo-degradation of CIP

The fate of CIP was monitored through combined evaluation of transformation products and assessment of mineralization during the process. As shown in Fig. 1a, the concentration of CIP rapidly decreased using a UV lamp. After an irradiation time of 4 min, 46% of the initial CIP concentration was already eliminated. After 128 min more than 98% of CIP was eliminated. However, mineralization of CIP and its transformation products did not happen. NPOC remained almost constant over the full irradiation time (Fig. 1a).

Similar degradation experiments were conducted with a xenon lamp. Results from photolysis using xenon lamp indicated that at the end of irradiation experiments only 61% of the initial CIP concentration was eliminated (Fig. 1b). This decrease again was not accompanied by a reduction in NPOC, which showed a similar course as obtained using UV lamp (Fig. 1).

3.2. Elucidation of transformation products

The degradation products created from CIP were initially analyzed by an ion trap in the MS, MS/MS and MS^3 modes. These data

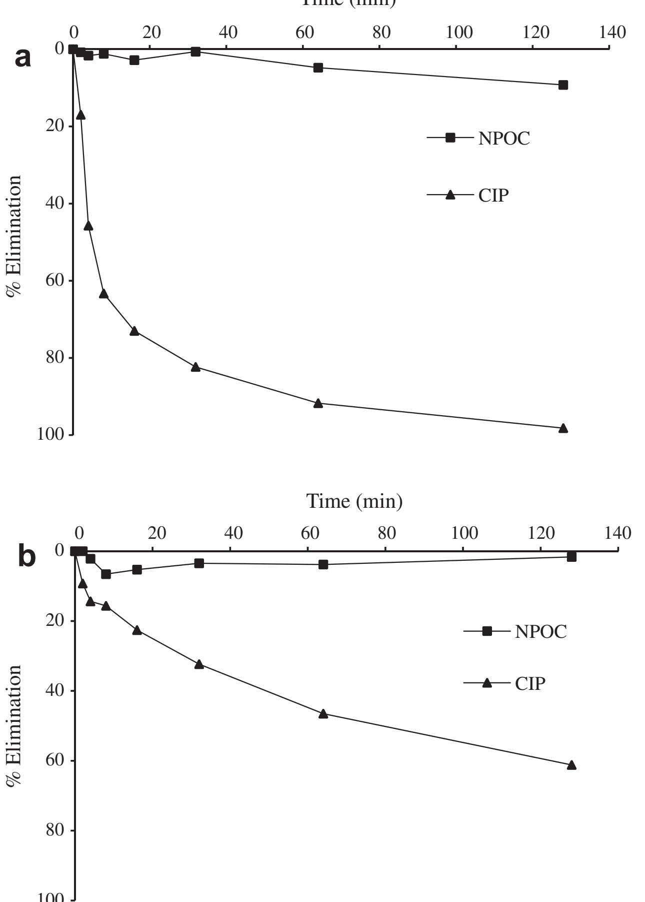

Fig. 1. Elimination of ciprofloxacin (CIP) and non-purgeable organic carbon (NPOC) during photo-degradation. (a) Using UV lamp, (b) using xenon lamp.

were used to clarify the structures of the degradation products. Furthermore, the proposed products were confirmed by accurate mass measurement and empirical formula calculation for the molecular ions of the degradation products.

3.2.1. Tandem mass spectrometry (MS^n) analysis

The structures of TPs were proposed on the basis of information derived from MS^n spectra compared with MS/MS, MS^3 spectra of CIP obtained under positive electrospray ionization (Table 1). Eleven TPs were identified under UV irradiation. However, xenon lamp irradiation did not result in any new TPs. The fragmentation pattern of Ciprofloxacin and the TPs, which were already identified in previous studies, were taken as a base for the structural elucidation of TP1. This product is structurally characterized for the first time in this work.

The fragments obtained of TP1, $[M+H]^+$ = 328.1 *m/z*, were used to clarify the structure of this photo-transformation product (Table 1). The MS^2 spectrum obtained of the precursor ion 328.1 *m/z* showed the formation of several product ions with *m/z* 300.1, 283.0, 272.0, 257.0, 229.0, 213.0, and 200.9 which formed through the elimination of C_2H_4, CHO_2, C_2H_2NO, C_3H_5NO, $C_4H_7N_2O$, $C_4H_7N_2O_2$ and $C_5H_6NO_3$, respectively (Fig. 2a). Fig. 2b gives a proposed fragmentation pattern for the structure of *m/z* = 328.1 according to the acquired mass spectral data. Additionally, the accurate mass measurement *m/z* 328.1286 was in agreement with the proposed elemental composition $C_{17}H_{18}N_3O_4$ of $[TP1+H]^+$ showing mass error of −0.61 mmu (Table 2). Furthermore, the calculated RDB value of 10.5 is increasing confidence in the proposed structure (Fig. 2 and Table 2).

For the other ten TPs, the obtained mass spectral information was compared to that previously reported in the literature and found to be compatible with the structures proposed herein (Fig. 4): TP6 (Wetzstein et al., 1999); TP7 (Lester et al., 2011); TP10 (An et al., 2010); TP2 and TP8 (Lester et al., 2011; Paul et al., 2010); TP9 (Vasconcelos et al., 2009b); TP3,TP4 and TP5 (Lester et al., 2011; Sturini et al., 2012; Turiel et al., 2005; Vasconcelos et al., 2009a); TP11 (Kugelmann et al., 2011). However, the oxidation product found by Kugelmann et al. (2011), with *m/z* 360 and described as C4b (Fig. 2 in Kugelmann et al. (2011)), was shown incorrectly possessing an additional hydroxyl group in position 8 which would mean specific mass of 376. However, in Fig. 4 the correct structure is shown.

3.2.2. Accurate mass analysis

The molecular formulas of the detected TPs, which were proposed by MS^n, were confirmed by accurate mass analysis, RDB values and mass deviation which matched the corresponding values of CIP. Using the elemental composition and ranges of the CIP parent structure, each TP was matched with a predicted chemical molecular and RDB value. The accurate mass measurement was in agreement with the proposed elemental composition of $[M+H]^+$ ions with a very low mass error of less than 1 mmu for all identified products. All calculated RDB values for TPs matched the predicted structures, increasing confidence in the proposed structures (Table 2). Regarding TP11, the only one transformation

Table 1
Chromatographic and mass spectral data of CIP and its identified TPs from LC–ESI–MS/MS; precursor in bold.

Compound	Retention time (min)	$[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)
TP1	4.0	**328.1**	300.1, 283.0, 272.0, 257.0, 229.0, 213.0, 200.9	**300.1** > 229.0, 213.0
TP2	6.1	**316.8**	299.1, 288.3, 255.3	**299.1** > 255.0, 227.2, 198.9
				255.3 > 226.9, 212.8, 142.8
TP3	6.3	**330.3**	312.1, 286.4, 269.0, 243.0, 217.0, 199.9	**286.4** > 268.0, 243.0, 217.0, 176.0
				217.0 > 175.7
TP4	6.5	**288.4**	270.1, 271.1, 245.0	**270.1** > 116.9, 227.0
TP5	7.0	**306.9**	289.0, 263.0, 286.1, 245.0, 290.0, 264.0	**289.0** > 268.0, 203.0, 268.1
				263.0 > 232.9
TP6	7.5	**346.2**	328.4, 300.1, 269.8	**328.4** > 298.1, 241.0
				300.1 > 317.7, 223.8, 255.1, 265.9
CIP	8.0	**332.2**	314.1, 288.1, 268.1, 245.0, 231.0, 204.9	**288.1** > 268.1, 245.0, 230.9, 198.0
				245.0 > 223.0, 204.0, 189.0
TP7	11.3	**344.7**	326.1, 227.0, 241.0	**326.1** > 270.0, 229.9
TP8	11.7	**316.3**	298.1	**298.1** > 227.0, 185.1
TP9	12.3	**330.3**	312.4, 231.0, 259.0	**312.4** > 305.5, 213.0
TP10	12.9	**348.8**	330.1, 350.1, 231.0	**231.0** > 213.0
TP11	13.3	**360.0**	342.2, 318.1	**342.2** > 231.0
				318.1 > 246.0

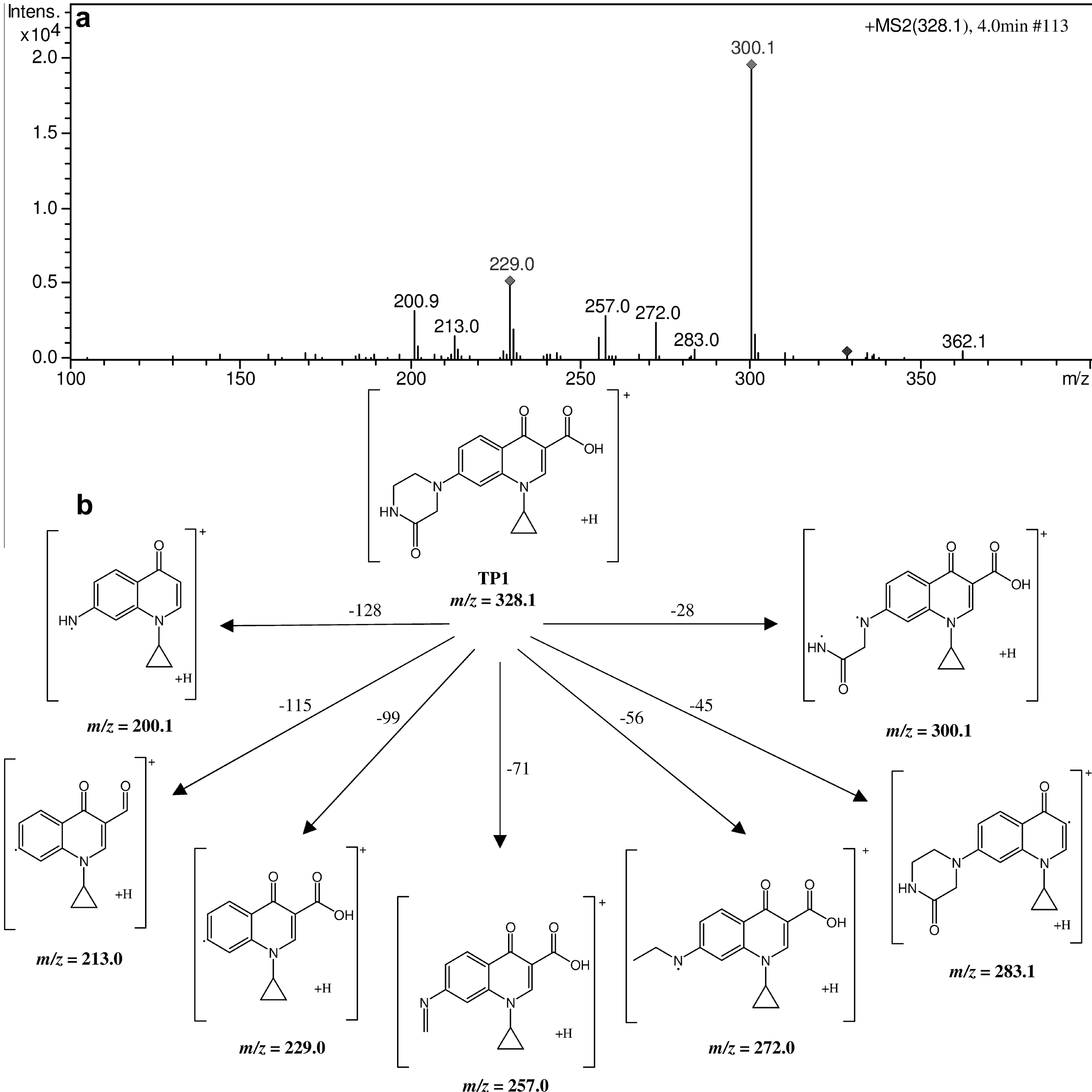

Fig. 2. (a) MS^2 spectrum of detected transformation product TP1 (m/z 328.1) at retention time = 4 min during photo-degradation (time point 8 min) of CIP using UV lamp, (b) proposed fragmentation patterns for TP1.

Table 2
Accurate mass measurements found by LTQ-Orbitrap XL for CIP and its identified transformation products during photolysis using UV and xenon lamps. Proposed chemical formulas, ring and double bond (RDB) values and the difference between the theoretical and experimental m/z for product ions were obtained from software Xcalibur (Thermo Scientific, Bremen, Germany) based on CIP elemental composition and ranges.

Compound	Molecular formula	Retention time (min)	Accurate mass $[M+H]^+$ (m/z)		Mass error (mmu)	RDB
			Experimental	Theoretical		
TP1	C17 H18 O4 N3	3.97	328.1286	328.1292	−0.61	10.5
TP2	C16 H18 O4 N3	5.37	316.1285	316.1292	−0.70	9.5
TP3	C17 H20 O4 N3	5.49	330.1440	330.1448	−0.79	9.5
TP4	C15 H18 O3 N3	5.74	288.1337	288.1343	−0.57	8.5
TP5	C15 H17 O3 N3 F	6.28	306.1240	306.1248	−0.89	8.5
TP6	C17 H20 O5 N3	6.50	346.1393	346.1397	−0.44	9.5
CIP	C17 H19 O3 N3 F	6.96	332.1399	332.1405	−0.61	9.5
TP7	C17 H18 O5 N3	10.78	344.1234	344.1241	−0.69	10.5
TP8	C16 H18 O4 N3	11.20	316.1289	316.1292	−0.27	9.5
TP9	C17 H20 O4 N3	11.76	330.1447	330.1448	−0.12	9.5
TP10	C17 H19 O4 N3 F	12.25	348.1349	348.1354	−0.49	9.5
TP11	C18 H19 O4 N3 F	12.82	360.1346	360.1354	−0.77	10.5

product of CIP, with *m/z* 360 as well possessing no carbon more than CIP, has been reported and described as compound 14 by Guo et al. (2013). However, This TP, which has the elemental composition $C_{17}H_{15}N_3O_5F$, does not match with the accurate mass measurement *m/z* 360.1346 of TP11 in our study. It showed a big mass error of 35.56 mmu and thus that structure cannot be assigned to the TP11. Therefore, based on the results of high-resolution mass spectrometry we assume the structure of TP11 (see Fig. 4) which is in accordance with the findings of Kugelmann et al. (2011), as described in Section 3.2.1, as the most probable.

3.3. Pathways of transformation

TPs generated during photo-degradation of CIP were analyzed by LC–ESI–MS and their abundance was plotted as a function of irradiation time using UV and xenon lamps (Fig. 3). In case of UV irradiation, the relative peak areas of all TPs exhibit similar trends (Fig. 3a). All peaks increase dramatically, and then they decrease rapidly with increasing irradiation time. After 128 min of irradiation, all identified TPs as well as CIP are completely disappeared. Unlike using UV, irradiation using a xenon lamp shows slower product formation and at the end of the experiment all detected products are still present (Fig. 3b). However, no new TPs have been formed.

Data obtained from structure elucidation, along with abundance profile of TPs during photo-experiments were used to construct the transformation pathways of CIP and its TPs under both UV and xenon irradiation. Transformation pathways are proposed in Fig. 4 indicating that TP3, TP11, TP5, TP7, TP1 are primary products regarded as precursors of TP6, TP10, TP4, TP2 and TP8, respectively. However, TP11 was possibly formed by the addition of carbonyl group to the parent molecule of CIP. TP9 could be formed via TP7. The transformations of CIP were to be found to occur mainly through substitution of fluorine, defluorination, hydroxylation of the quinolone core and breakdown of the piperazine ring (Fig. 4). Those are well known transformation modes of CIP (Albini and Monti, 2003; An et al., 2010; Mella et al., 2001; Sunderland et al., 2001). As all TPs still retain the core quinolone structure, which is responsible for the biological activity, further follow-up problems can be expected.

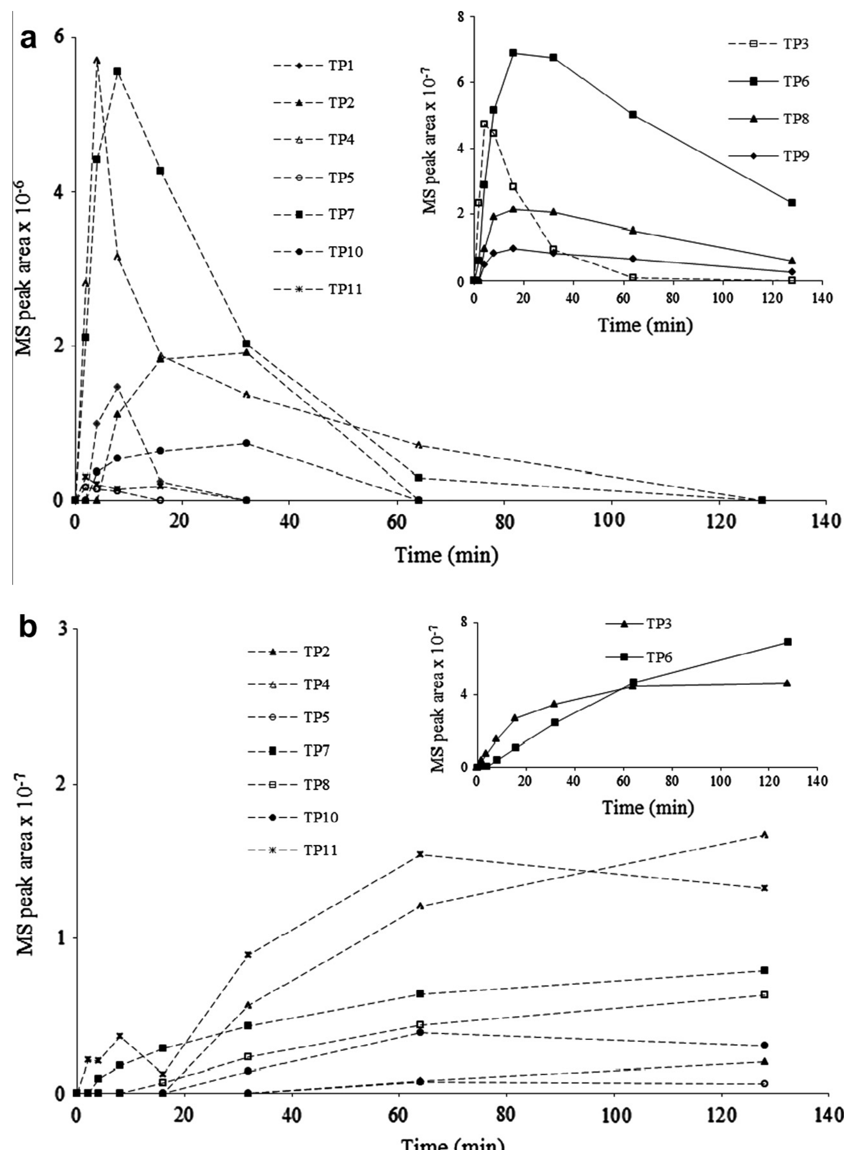

Fig. 3. Course of detected photo-transformation products of CIP plotted as a function of the irradiation time monitored by LC–ESI–MS. (a) Using UV lamp, (b) using xenon lamp.

Fig. 4. Proposed structures and transformation pathways followed by CIP during photo-degradation using UV and xenon lamps.

4. Conclusion

The results presented in this paper demonstrate that photo-oxidation treatment of sewage and drinking water contaminated with Ciprofloxacin can successfully remove the parent compound. However, our findings from NPOC measurements clearly demonstrate that mineralization does not take place. The formation of stable TPs can be expected in waste water treatment and in surface water. Certainly additional TPs formed that are not detected by the applied analytical methods. Such products could be non-ionisable under the condition of ESI selected herein, or they may be lost during the chromatographic analysis. The formation of TPs maintaining the core quinolone structure, has to be expected with resulting in follow-up problems. As little is still known about environmental fate and toxic properties of TPs, further studies on this drug and its TPs, including toxicity tests *in vitro* as well as *in silico*, are in progress.

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ARTICLE

IV

Genotoxic Effect of Ciprofloxacin During Photolytic Decomposition Monitored by the *In Vitro* Micronucleus Test (MNvit) in HepG2 Cells

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RESEARCH ARTICLE

Genotoxic effect of ciprofloxacin during photolytic decomposition monitored by the in vitro micronucleus test (MNvit) in HepG2 cells

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Abstract
Purpose Ciprofloxacin (CIP), a broad-spectrum, second-generation fluoroquinolone, has frequently been found in hospital wastewaters and effluents of sewage treatment plants. CIP is scarcely biodegradable, has toxic effects on microorganisms and is photosensitive. The aim of this study was to assess the genotoxic potential of CIP in human HepG2 liver cells during photolysis.
Methods Photolysis of CIP was performed in aqueous solution by irradiation with an Hg lamp, and transformation products were monitored by HPLC-MS/MS and by the determination of dissolved organic carbon (DOC). The cytotoxicity and genotoxicity of CIP and of the irradiated samples were determined after 24 h of exposure using the WST-1 assay and the in vitro micronucleus (MN) test in HepG2 cells.
Results The concentration of CIP decreased during photolysis, whereas the content of DOC remained unchanged. CIP and its transformation products were not cytotoxic towards HepG2 cells. A concentration-dependent increase of MN frequencies was observed for the parent compound CIP (lowest observed effect level, 1.2 μmol L^{-1}). Furthermore, CIP and the irradiated samples were found to be genotoxic with a significant increase relative to the parent compound after 32 min ($P<0.05$). A significant reduction of genotoxicity was found after 2 h of irradiation ($P<0.05$).
Conclusions Photolytic decomposition of aqueous CIP leads to genotoxic transformation products. This proves that irradiated samples of CIP are able to exert heritable genotoxic effects on human liver cells in vitro. Therefore, photolysis as a technique for wastewater treatment needs to be evaluated in detail in further studies, not only for CIP but in general.

Keywords Ciprofloxacin · Fluoroquinolones · Wastewater treatment · Photo-oxidation · Photolysis · Transformation products · Hepatoma cell line (HepG2) · Genotoxicity · Cytotoxicity · Micronucleus test (MNvit)

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1 Introduction

The presence of pharmaceuticals, especially antibiotics, in the environment has attracted attention in the scientific community around the world (Halling-Sørensen et al. 2000; Heberer 2002; Kümmerer 2009a, b, c). Antibiotics are discharged into the environment via domestic waste, municipal wastewater and after veterinary use. Pharmaceuticals

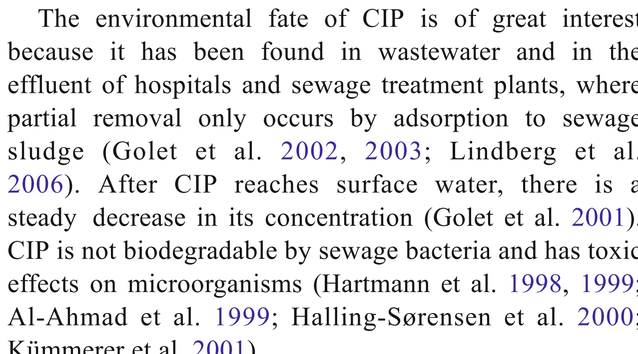

Fig. 1 Structure of ciprofloxacin

have been detected in wastewater effluents, river waters and groundwaters (Blackwell et al. 2004; Miao et al. 2004; Kim and Carlson 2007). Introduction of antibiotics into the environment may exert selection pressure on microorganisms (Kümmerer et al. 2004; Kümmerer 2009b) and subsequently lead to changes in the antibiotic susceptibility of the microbes and/or modify the predominant microbial species (Hu et al. 2007). This is of utmost importance for the treatment of infections in humans.

Antibiotics are a diverse group of chemicals that can be divided into subgroups such as β-lactams, quinolones, tetracyclines, macrolides, sulfonamides and others. The active compounds of antibiotics are often complex molecules. Ciprofloxacin (CIP), a broad-spectrum, second-generation fluoroquinolone antibiotic, possesses both basic and acidic functionalities (Fig. 1). The acid constants are 6.16 and 8.63 (Kümmerer 2009a). It inhibits the prokaryotic-specific enzyme gyrase. Ciprofloxacin also shows residual inhibitory activity against the functionally and structurally related mammalian topoisomerase II. Consequently, CIP has been correlated with genotoxic effects in both prokaryotic and eukaryotic test systems (Herbold et al. 2001). It has been reported that quinolones have some toxic effects on the central nervous, cardiovascular, and gastrointestinal systems and that they also lead to ondrotoxicity, reproductive and developmental toxicity, genotoxicity, carcinogenicity and phototoxicity (Christ et al. 1988; Stahlmann and Lode 1998). The in vitro genotoxicity of CIP has been demonstrated with sister chromatid exchange, unscheduled DNA synthesis (Takayama et al. 1995) and chromosomal aberrations in human lymphocytes (Gorla et al. 1999). The in vivo genotoxicity of CIP has been demonstrated with the micronucleus test (Pino 1995) as well as its ability to induce chromosomal aberrations in human lymphocytes (Gorla et al. 1999), mice (Mukherjee et al. 1993) and rats (Basaran et al. 1993). In contrast, several other in vivo studies on CIP revealed no genotoxic effects, and the compound was not found to be carcinogenic in rodent long-term bioassays (Herbold et al. 2001).

Nevertheless, in vivo evidence for photocarcinogenic activity mediated by CIP and other fluoroquinolones is a well-known fact and has been tested in mice exposed to UVA after systemic administration of CIP (Klecak et al. 1997).

The environmental fate of CIP is of great interest because it has been found in wastewater and in the effluent of hospitals and sewage treatment plants, where partial removal only occurs by adsorption to sewage sludge (Golet et al. 2002, 2003; Lindberg et al. 2006). After CIP reaches surface water, there is a steady decrease in its concentration (Golet et al. 2001). CIP is not biodegradable by sewage bacteria and has toxic effects on microorganisms (Hartmann et al. 1998, 1999; Al-Ahmad et al. 1999; Halling-Sørensen et al. 2000; Kümmerer et al. 2001).

As an advanced sewage treatment technique, photolytic or photocatalytic decomposition is under discussion as a possible tool for the removal of active compounds (Vasconcelos et al. 2009a, b; Escher et al. 2009; Fatta-Kassinos et al. 2011). It is already known for quinolones that photolytic decomposition probably occurs via a free radical mechanism, with loss of the carboxylic group at the *ortho* position (Sanchez et al. 2005; Vasconcelos et al. 2009a, b). However, data on toxic properties, especially the genotoxicity of the photolytic transformation products, have not been studied.

To obtain more information on the potential risks of CIP to environmental and human health, the genotoxic and cytotoxic potential of CIP and of its transformation products after photolytic decomposition in aqueous solutions was studied in the human-derived hepatoma cell line (HepG2) by means of the in vitro micronucleus (MNvit) assay.

To our knowledge, there has been no previous study dealing with the evaluation of the cytotoxicity and genotoxicity of the photolytic transformation products of aqueous CIP by means of the MNvit assay in HepG2.

2 Materials and methods

2.1 Chemicals

Ciprofloxacin (purity >98%, CAS 85721-33-1) and benzo(*a*)pyrene (B(*a*)P, CAS 50-32-8) were purchased from Sigma-Aldrich (Steinheim, Germany). Cytochalasin B (Sigma-Aldrich, Steinheim, Germany, CAS 14930-96-2) was diluted in dimethyl sulphoxide (DMSO; Carl Roth, Karlsruhe, Germany, CAS 67-68-5) in order to obtain a stock solution of 3.33 mg L^{-1}, which was kept in the dark at 4° C until use. Trypsine/EDTA and phosphate-buffered saline (PBS) were purchased from PAA Laboratories (Parsching, Austria). Giemsa stain (Merck, Darmstadt, Germany) for microscopy and Triton X as well as general laboratory chemicals were obtained from Sigma-Aldrich (München, Germany). WST-1 reagent was purchased from Roche (Mannheim, Germany).

2.2 Generation of transformation products of aqueous ciprofloxacin solutions

2.2.1 Photolysis and DOC monitoring

Photolysis experiments were performed in an 800-mL Pyrex glass, cylindrical immersion-type photoreactor containing 650 mL of aqueous CIP solution (20 mg L^{-1}) dissolved in aq. bidest. (Millipore GmbH Schwalbach, Germany). Irradiation was performed using an apparatus from Heraeus (Hanau, Germany) equipped with a TQ 150 medium-pressure mercury lamp (150 W). The lamp emits polychromatic radiation in the range from 200 to 436 nm. The peak intensities were at 254, 265, 302, 313 and 366 nm. The lamp was placed in a quartz tube encased in a cooling jacket. Chamber temperatures were regulated by a pressurized water-cooling circuit. The temperature in all the irradiation experiments was kept at 21.0±1°C, pH 7.0. Aqua bidest. was used to prepare all solutions. For HPLC analysis, samples of the irradiated aqueous solutions were taken during the irradiation process at the following time points: 0, 2, 4, 8, 16, 32, 64 and 128 min. The concentration of dissolved organic carbon (DOC) was determined with a TOC analyzer (TOC 5000, Shimadzu GmbH, Duisburg, Germany).

2.2.2 HPLC analysis

In order to monitor the transformation products of CIP in aqueous solution during the photolytic decomposition process, HPLC analyses were performed using an LC10 HPLC system (Shimadzu) equipped with two pumps (LC10AT), an automatic sampler, a column oven and a fluorescence detector (exc., 278 nm; em., 445 nm). Data acquisition and evaluation were accomplished with the Shimadzu Class LC10 software package. A reverse phase column (RP18 CC 125×4 mm Nucleodur 100-5) and a pre-column (RP18 CC 8×4 mm Nucleosil 100-5) were used. A gradient programme was used for the mobile phase, combining solvent A (formic acid 0.1% in water, *v*/*v*) and solvent B (acetonitrile) as follows: 5–15% B (up to 10 min), 15–40% B (up to 12 min), 40–5% B (up to 13 min) and 5% B (up to 15 min). The flow rate was set at 1 mL/min, the injection volume was 5 μL, and the column oven temperature was maintained at 35°C. The retention time (RT) of CIP was 6.4 min. Quality control was previously performed by preparing solutions of eight known concentrations, ranging from 0.001 to 1 mg/L of CIP in water, with an R^2 value above 0.998 that were analysed by the previously mentioned fluorescence detector. The RT of CIP was 11.2 min. All samples of photolysed aqueous CIP solutions were diluted 1:20 before measurement. The limit of detection was 0.001 mg/L and the limit of quantification was 0.005 mg/L. Water was used as blank.

2.2.3 LC-MS/MS analysis

Transformation products were identified in fractions obtained after 0, 2, 4, 8, 16, 32, 64 and 128 min by liquid chromatography with tandem mass spectroscopy (LC-MS/MS). The apparatus and the method used are described elsewhere (Haddad and Kümmerer, in preparation). The organic products forming during photolysis were expressed as *m*/*z* values obtained from LC-MS/MS analysis. The calculated peak areas represent the ion peak area divided by the maximum peak area observed for the ion during the reaction time course×100%.

2.3 HepG2 cell line culture

This cell line was originally established from human liver tumour biopsy (Aden et al. 1976) and was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were cultivated in DMEM supplemented with 15% foetal calf serum (FCS Gold, PAA Parsching, Austria), 1% penicillin/streptomycin (PAA) and 2 mmol L^{-1} L-glutamine (PAA) in a humidified incubator at 37°C in a CO_2 atmosphere. Cells were grown as monolayer cultures in flasks with a 75-cm^2 surface (Greiner Bio-One, Frickenhausen, Germany) with 15 mL medium and subcultured every 5 days. In order to transfer the cells into new flasks, they were washed twice with PBS and trypsinised for 5 min at 37°C using 2 mL trypsin–EDTA solution. After incubation, 8 mL medium was added and the cell suspension was centrifuged at 300×*g* for 5 min. As HepG2 cells tend to stick together, they were separated with a 10-mL syringe and needle (0.4×19) and subsequently transferred into new flasks for subcultivation or directly utilized in the assays. Before use, HepG2 cells were taken from liquid nitrogen and washed twice with fresh culture medium. The cells used in all the experiments were restricted to passages between 5 and 10.

2.4 Chemical treatment

For the bioassays, all irradiated aqueous solutions (0 to 128 min, three independent photolysis experiments) were sterile-filtered (0.2 μm) and stored at −20°C. For the WST-1 assay, sample aliquots of 4 μL were added to the medium, resulting in a final volume of 200 μL (0.4 μg L^{-1} CIP at time point 0 min). In a first MNvit experiment, cells were exposed to various concentrations of CIP ranging from 0.1 to 100 μmol L^{-1} to assess the concentration–response relationship for CIP. Furthermore, in a second MNvit experiment, treatment of cells was performed by the addition of 0.1 mL of each photolysed sample to a final culture volume of 5 mL (0.4 μg mL^{-1} CIP at sampling time point 0 min). Triton X (0.01%, *v*/*v*) and B(*a*)P (1 μmol L^{-1}) dissolved in DMSO were used as positive controls in the WST-1 assay and the MNvit test, respectively. The final DMSO concentration in the

medium did not exceed 1% (*v*/*v*). Solvent control for the experiments with CIP and its irradiated solution was prepared by the addition of respective volumes of aq. bidest. to the culture medium. The positive controls and solvent controls were included concurrently in each experiment.

2.5 WST-1 assay

Cell viability was determined by the reductive cleavage of a tetrazolium dye to formazan (WST-1 assay). The WST-1 assay was conducted according to the manufacturer's protocol. Ten thousand HepG2 cells were seeded in 96-well microtitre plates and cultured for 24 h. After a 24-h treatment period, the culture medium was removed and 10 μL WST-1 reagent+190 μL phenol red-free culture medium were added and incubated for 1 h. The absorbance of the formed water-soluble formazan in the culture medium was measured at 435 vs. 620 nm (reference). The mean values of five wells per concentration were evaluated. Every sample was tested in three independent replicates (three 96-well microtitre plates). For evaluation, the mean absorbance of each time point was compared with the solvent as control. A reduction of the absorbance below 70% of the control level (Millipore) indicates cytotoxicity. Triton X at 0.01% (*v*/*v*) was used as the positive control.

2.6 The CB-MNvit assay

The assay was carried out according to OECD guideline 487 (OECD 2010) and Fenech (2007). The cytokinesis-block in vitro micronucleus (CB-MNvit) assay was performed using a quadriperm chamber technique (Yamamoto et al. 2005). Confluent cells were detached by trypsinisation from culture flasks (Greiner Bio-One) and seeded into chambers of QuadriPERM dishes (Falcon, Heidelberg, Germany). The cell suspensions, adjusted to a cell density of 80,000 cells per culture (5 mL), were spread on microscope slides kept in the chambers of QuadriPERM dishes, i.e. each single chamber represents a separate cultivation compartment. Cells were allowed to adhere for 24 h and subsequently exposed to the chemicals. After an incubation period of 24 h, cells were washed twice with PBS to remove and wash out chemicals from each slide and subcultivated for as long as 26 h in the presence of cytochalasin B (final concentration, 3 μg mL^{-1}) to block cell division. Next, cultures were washed twice with PBS and hypotonically treated with 5 mL trisodium citrate solution (1.5%, *v*/*v*) at 37°C for 3–5 min. Cells were then fixed two times with freshly prepared fixative solution (methanol, acetic acid and formaldehyde 60:20:1, *v*/*v*/*v*) and stained with 5% Giemsa solution until the nuclei became clearly distinguishable from the cytoplasm (8–10 min). All slides were coded and scored using a light microscope (Leitz DMR). Evaluation of cells was done at 400-fold magnification, and micronuclei in binucleated cells (BNC) were selected according to the criteria described by Umegaki and Fenech (2000) and Fenech et al. (2003). Round or oval non-refractile bodies detached from the nucleus, with smooth outlines and with a diameter not greater than one third the large diameter of the main nuclei, were considered as MN. For each sample, the number of MN in 1,000 BNC was examined. In order to estimate cytotoxic effects, the cytokinesis-block proliferation index (CBPI) of each culture was also calculated according to the formula CBPI=(M1+2(M2)+3(M3)+4(M4)/*N*), where M1 to M4 represent the number of cells with one to four nuclei, respectively, and *N* is the total number of cells scored according to Surralles et al. (1995) by assessing 500 cells. In all cultures used for the evaluation of MN formation, cytostasis was ≤40% compared with the corresponding controls. Experiments were repeated three times independently (*N*=3).

2.7 Statistical analysis

Values shown are the means±SD for the indicated number of independent experiments. Statistical significance was assessed by one-way ANOVA followed by LSD post hoc pairwise comparisons. Values of *P* lower than 0.05 were considered statistically significant.

3 Results

3.1 Phototransformation products of CIP

As shown in Fig. 2, the concentration of CIP gradually decreased during photolytic decomposition (bars). However, DOC did not decrease markedly over the course of the irradiation experiment (line). Table 1 shows the temporal evolution of CIP transformation products obtained during photolysis as preliminary results from LC-MS/MS analysis on the basis of

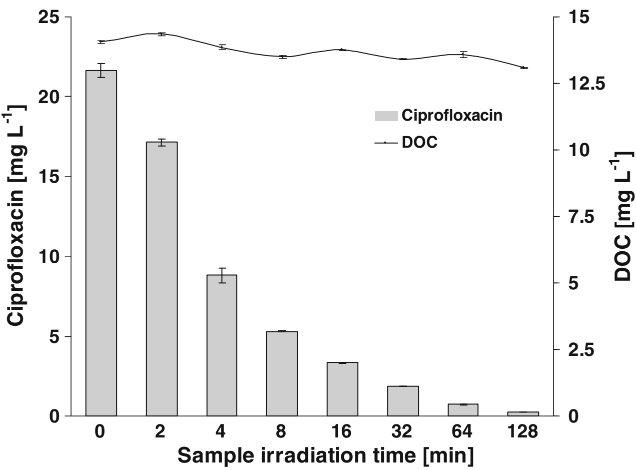

Fig. 2 Decay of ciprofloxacin (*bars*) and of dissolved organic carbon concentrations (*DOC*, *line*) during the photolysis experiments. Values are the means±SD from two independent experiments

Table 1 Temporal evolution of transformation products during photolysis

Sample irradiation time (min)	Relative MS peak area (%)					
	CIP (*m/z* 332)	I (*m/z* 362.5)	II (*m/z* 317.7)	III (*m/z* 330.1)	IV (*m/z* 346.2)	V (*m/z* 317.7)
0	100	0	0	0	0	0
2	87	0	0	34	6	0
4	57	0	0	94	43	0
8	40	94	53	100	71	75
16	27	100	100	56	93	100
32	16	13	82	14	100	100
64	5	0	0	0	61	80
128	1	0	0	0	21	0

The initial solution contained 20 mg L^{-1} CIP at pH 7 and 21±1°C. The formed organic transformation products were expressed as *m/z* values obtained from MS/MS analysis. The calculated peak areas represent the ion peak area divided by the maximum peak area observed for the ion during the reaction time course×100%

accurate mass determination. Data represent relative MS peak areas of the ciprofloxacin ion and the transformation products formed. Transformation was found to occur through the substitution of fluorine (defluorination), hydroxylation of the quinolone core and the breakdown of the piperazine ring (details are described in Haddad and Kümmerer, in preparation). No prominent reaction products were detected which might have been responsible for the increased genotoxic activity after 32 min of photolytic treatment.

3.2 Cytotoxicity of CIP and irradiated aqueous ciprofloxacin solutions

In the WST-1 assay, the viability of cells treated with the phototransformation products were found to be in the range of 90–110% compared with the untreated control. Therefore, they were considered as non-cytotoxic to human liver cells (Fig. 3). Nevertheless, a significant decrease of viability compared with the initial CIP solution at time point 0 min was found for the fraction sampled after 32 min of irradiation ($P<0.05$). The positive control (Triton X, 0.1%, *v*/*v*) shows a decline of cell viability to 16% of the control.

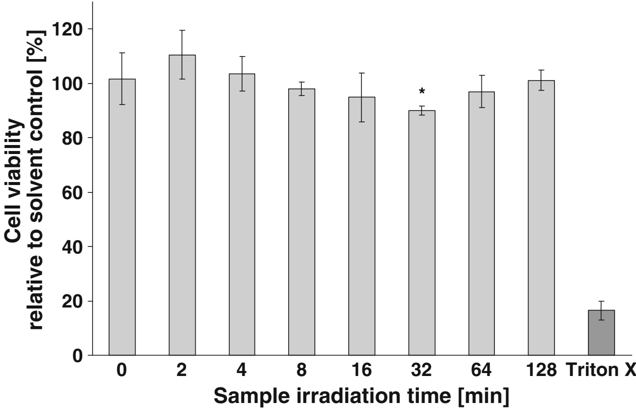

Fig. 3 Cell viability of human liver cells (HepG2) after exposure to solutions of ciprofloxacin and to samples of photolysed ciprofloxacin. Initial concentration of CIP at time point 0 min was 0.4 mg L^{-1}≙1.2 μmol L^{-1}. Cell viability was measured by tetrazolium salt cleavage (WST-1 assay) after 24 h of exposure. Positive control was Triton X (0.1%, *v*/*v*). Values are the means±SD from three independent experiments. *$P<0.05$ vs. samples treated at time point 0 min

3.3 Micronucleus induction by CIP

Figure 4 shows the level of induced MN in HepG2 cells by CIP at concentrations of 0.1 μmol L^{-1} up to 100 μmol L^{-1}. CIP induced a significant formation of micronucleated cells (lowest observed effect concentration) at 1 μmol L^{-1} (*P*<

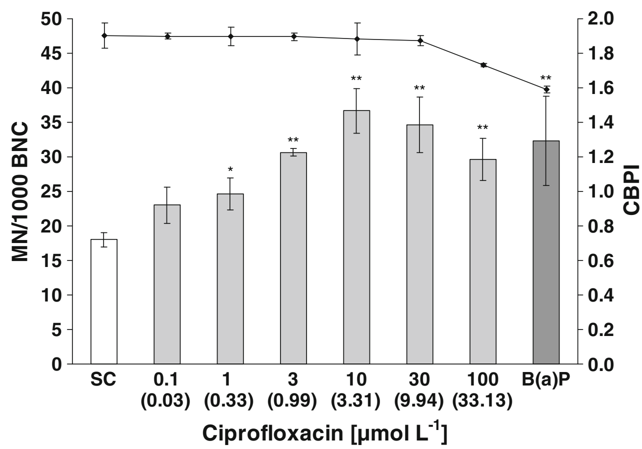

Fig. 4 Induction of micronuclei (*bars*) by ciprofloxacin and cytokinesis-block proliferation index (*squares*) in HepG2 cells after 24 h of exposure. The cells were exposed for 24 h to ciprofloxacin followed by subcultivation for 26 h in the presence of cytochalasin B (3 μg mL^{-1}). *Values* represent the means±SD of three independent experiments. One thousand BNC were evaluated per experimental point. *Values in parenthesis* are concentrations expressed as milligrams per litre. *MN* micronuclei, *BNC* binucleated cells, *CBPI* cytokinesis-block proliferation index. *$P<0.05$, **$P<0.01$ (vs. solvent control)

0.05) with the highest induction at 10 μmol L^{-1} (36.6±3.2 MN/1,000 BNC, $P<0.05$; Fig. 4). At higher concentrations (above 10 μmol L^{-1}), the level of micronuclei steadily declined. MN formation induced by CIP was accompanied by cytotoxic effects as measured by the CBPI at the highest concentration (100 μmol L^{-1}, Fig. 4).

3.4 Micronucleus induction by photolysed aqueous ciprofloxacin solutions

The genotoxic effects of CIP solution (at 0 min) and of photolysed aqueous ciprofloxacin solutions (collected at time points 2, 4, 8, 16, 32, 64 and 128 min) in HepG2 cells are shown in Fig. 5. The frequency of MN in solvent-treated cells was found to be 16.33±1.15 MN/1,000 BNC, whereas the frequency of induced micronuclei by photolysed ciprofloxacin solutions showed a tendency to increase after treatment with samples obtained between 16 and 64 min of irradiation compared with the non-irradiated sample (0 min); however, significant effects were found only after 32 min of irradiation ($P<0.05$). MN frequencies declined for samples that were irradiated longer than 32 min. Cells treated with the photolysed solutions collected at 128 min induced a mean frequency of 20.3±0.72 MN/1,000 BNC, and this effect was significantly lower than in the non-photolysed CIP (24.33±1.42, $P<0.05$). Nevertheless, MN frequency of this sample remained significantly higher than the frequency found in the solvent control ($P<0.05$). No inhibition of cell proliferation (CBPI>1.8) was found for all samples of the photolysis experiment. In the positive control B(*a*)P, the CBPI decreased to 1.66±0.01 (Fig. 5).

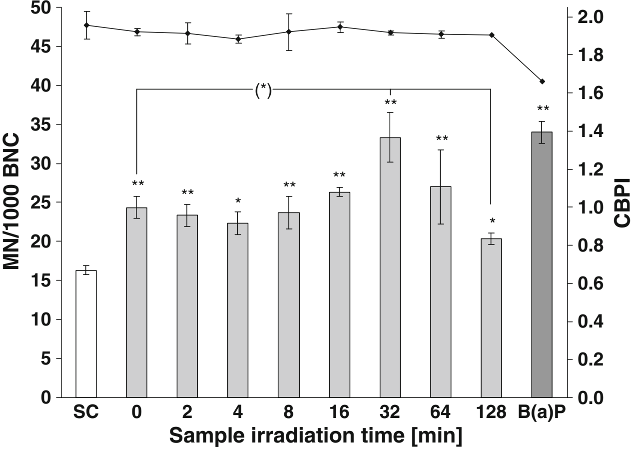

Fig. 5 Induction of micronuclei (*bars*) and cytokinesis-block proliferation index (*squares*) in HepG2 cells by ciprofloxacin and by photolysed samples after 24 h of exposure. Initial CIP concentration at time point 0 min was 0.4 mg L^{-1}≙1.2 μmol L^{-1}. *Values* represent the means±SD of three independent experiments. *SC* solvent control (positive control—B(*a*)P, 1 μmol L^{-1}), *MN* micronuclei, *BNC* binucleated cells, *CBPI* cytokinesis-block proliferation index. *$P<0.05$, **$P<0.01$ (vs. samples treated at time point 0 min)

4 Discussion

CIP is known as an antibiotic that is frequently prescribed for the treatment of bacterial infections. In the literature, the antibacterial feature of the drug is attributed to DNA binding, resulting in a given inhibition of bacterial DNA topoisomerases (Suh and Lorber 1995). CIP has shown to be present in the aquatic environment. In the study presented here, we evaluated the cytotoxic and genotoxic potential of CIP and of photolysed ciprofloxacin samples in the HepG2 cell line in vitro.

The viability and the proliferation of cells after treatment with CIP and its photolysed transformation products were assessed by the WST-1 assay and by the determination of the CBPI in the MNvit assay. The results show that the viability of the cells exposed to photolysed samples of CIP is almost in the same range as the untreated control, thereby confirming that the photolytically decomposed products are not cytotoxic to HepG2 cells. Also, the cell viability of HepG2 cells treated with the parent compound was not reduced at concentrations up to 30 μg mL^{-1}. Nevertheless, our results show evidence of cell proliferation inhibition at concentrations of around 100 μg mL^{-1}, which is in accordance with the data given by Koziel et al. (2010). However, the inhibitory concentrations of the drug vary in the literature, which may result from the different experimental conditions and cell types. For example, Gürbay et al. (2002) showed that CIP was not cytotoxic to human fibroblasts at any of the concentrations tested when the cells were incubated with CIP for 24 h. In contrast, Jun et. al. (2003) identified apoptotic effects in Jurkat T cells at concentration levels as low as 2.5 μg mL^{-1}.

The in vitro micronucleus test is a genotoxicity test for the detection of micronuclei in the cytoplasm of interphase cells. MN may originate from acentric chromosome fragments (i.e. lacking a centromere) or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division (OECD 2010). Thus, genetic damage becomes manifested in the newly formed daughter cell (Reifferscheid et al. 2008). The HepG2 cell line retains the activities and many of the morphological characteristics of liver parenchymal cells. They show metabolic competence responsible for the activation of various xenobiotics, namely phase I and II enzymes that play key roles in the activation and detoxification of DNA-reactive carcinogens (Darroudi et al. 1996; Majer et al. 2004).

Accordingly, our results showed that CIP and samples of photolysed aqueous CIP exert heritable genotoxic effects in the HepG2 cell line. An aqueous CIP solution (0 min) at a concentration of 0.4 mg L^{-1} (1.2 μmol L^{-1}) was found to meet the lowest observed effect level, as was assessed previously by dose–response experiments. The genotoxic potency of the photolysed samples obtained over the course

of the irradiation experiments showed a characteristic pattern, which supports the following conclusion: (1) the genotoxic potency of CIP (0 min) was significantly enhanced after photolytic decomposition for the fraction sampled at the 32-min time point and (2) 128 min of photolysis was in turn sufficient to induce a significant decrease of genotoxicity relative to the parent compound. These results are in accordance with Sanchez et al. (2005) who showed that the photolytic decomposed products of CIP and other fluoroquinolones are more genotoxic than the drug itself. Similar results were obtained by Pereira et al. (2007). The reason for the increased peak of activity after 32 min of photolysis remains unclear and certainly needs to be investigated further. Preliminary results from LC-MS/MS analysis did not reveal any relevant findings at this time point (32 min) in comparison to samples taken after 16 min of photolytic decomposition (Table 1). But it seems likely that there are still additional transformation products with a smaller mass or products that are not detectable by electrospray ionisation. Furthermore, previous studies conducted by Vasconcelos et al. (2009a, b) and Paul et al. (2010) actually contributed to the identification of an intermediary product formed during the photolytic decomposition of ciprofloxacin. Our results show that phototreatment of CIP enhances genotoxic potency, leading to higher levels of induced micronuclei. However, phototreatment as a target-aimed procedure represents a valuable option to eliminate or at least reduce the hazard of CIP in aqueous solution if the treatment time is long enough. These results are in accordance with Burhenne et al. (1997) and Vasconcelos et al. (2009a, b) who proved that variations in pH and exposure time in the photolysis of CIP can result in the formation of different by-products via different pathways. Photoinduced transformation of fluoroquinolones, and especially of CIP, is a process that is, to a great extent, dependent on the photogeneration of the $\pi\ \pi^*$ triplet form (Mella et al. 2001). These transient species are more electrophilic than the ground state, and if they are not formed or its quenching product is in the original ground state, the photoreactivity of fluoroquinolones such as CIP decays. The result is a more gradual decrease of the CIP concentration. In comparison, for some other processes, degradation is more dependent on the presence or formation of oxidants in solution, which accelerates the decrease of the CIP concentration (Vasconcelos et al. 2009a, b).

Fluorescence was not detected in the final transformation products of CIP, indicating that the aromatic core π system has been modified by photoreactions and/or subsequent reactions. However, the products formed could not be further identified and are probably numerous as the DOC level was nearly constant throughout the photolytic treatment. Even though the core quinolone was modified but remained intact, there is a decrease in antimicrobial potency during photolytic treatment, as found by Paul et al. (2010). Together with our findings, this could be seen as the rationale in an attempt to explain the characteristic course of associated changes in biological reactivity during the photolytic treatment of CIP. We propose an overlying effect between a residual telomerase-disturbing activity and the formation of non-detectable transient species, presumably ROS, capable of inducing oxidative damage in eukaryotic cells.

However, it is important to keep in mind that first and foremost, the aim of this study was to compare the cytotoxicity and genotoxicity of the parent compound and its photoproducts; to this purpose, we chose the experimental strategy and endpoints used. This explains our focus on biological outcomes using a metabolically competent human cell line as a very first step in understanding. In order to evaluate the relevance of our findings for the induction of heritable mutations or for the induction of cancer in humans involved with exposure to ciprofloxacin or its transformation products, more detailed studies, especially those applying in vivo toxicity tests, will be necessary. The pathway triggering such an increased genome-damaging effect—regardless of how weak it appears—must be explored in greater detail. A compilation of biotests within the eukaryotic system is required to obtain comprehensive health and ecotoxicological risk assessment data. Such a suitable combination could be composed by the utilization of overlapping endpoints comprising parameters for cytotoxicity and especially to verify heritable genome-damaging effects. For instance, the comet assay (for measuring DNA damage and repair capacity) and the micronucleus test (with fluorescence in situ hybridization technique using chromosome-specific probes) could be performed, complementing other effect-directed approaches with different human cell lines or primary cells like peripheral blood mononuclear cells.

Additional analytical work is recommended as the main transient species involved in DNA damage by exerting enhanced strand breaking activity and generating photoexcitation of CIP seem to be short-lived intermediates.

5 Conclusions

Photolytic decomposition is under discussion as a possible tool to remove micro-pollutants such as pharmaceutically active compounds through advanced sewage treatment. The results presented in this paper not only raise questions about the suitability and sustainability of photolytic decomposition as an advanced treatment technique with regard to CIP but also reveal the need to emphasize the requirement to control the treatment time parameters. Therefore, before the application of such a technology can be recommended, further, as well as site- and case-specific, investigations are necessary to be able to judge whether or not such

approaches produce new problems, e.g. concerning drinking water safety. In countries lacking effluent treatment and/or in the case of insufficient removal of CIP and related compounds due to direct emission into water bodies, or wherever high sunlight intensity predominates, there is also the possibility of an incomplete photolytic decomposition of CIP in surface water bodies. This is of special importance for regions where surface water or effluents are used for irrigation or where water reuse is commonplace, e.g. in arid countries. In this case, the genotoxic transformation products could be introduced directly into the food chain. Therefore, additional restrictions to prevent further input into the water cycle have to be taken into account. Thus, to simulate environmental conditions, our strategy was not geared to optimizing irradiation efficiency by the implementation of sophisticated photolytic decomposition technologies; rather, the principal aim of our study was to select standard conditions as described elsewhere to allow comparability between results and to consider the outcome.

The results presented here demonstrate that the application of light for the removal of CIP from water can result in an increase of genotoxicity if it is not performed under controlled conditions. Photolytic and photocatalytic decomposition methods for the removal of micro-pollutants in sewage and drinking water treatment can be successful for the removal of the parent compound. However, as found in this study, the situation can deteriorate after treatment because even more genotoxic by-products may be formed. Altogether, it can be assumed that the optimal strategy to decrease microbial toxicity or genotoxicity, as shown by the example of CIP, can arise from minor structural modifications in molecular architecture and do not necessarily require energy-intensive advanced photolytic processes, which may mineralize compounds. Therefore, such treatments need to be evaluated in detail in further studies, not only for CIP but in general.

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ARTICLE

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Initial hazard screening for genotoxicity of photo-transformation products of ciprofloxacin by applying a combination of experimental and *in-silico* testing

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Initial hazard screening for genotoxicity of photo-transformation products of ciprofloxacin by applying a combination of experimental and *in-silico* testing☆

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ABSTRACT

Ciprofloxacin (CIP) is a broad-spectrum antibiotic found within μg/L concentration range in the aquatic environment. It is a known contributor of *umuC* induction in hospital wastewater samples. CIP can undergo photolysis to result in many transformation products (TPs) of mostly unknown toxicity. The aims of this study were to determine the genotoxicity of the UV mixtures and to understand the possible genotoxic role of the stable TPs. As such, CIP and its UV-irradiated mixtures were investigated in a battery of genotoxicity and cytotoxicity *in vitro* assays. The combination index (CI) analysis of residual CIP in the irradiated mixtures was performed for the umu assay. Further, Quantitative Structure–Activity Relationships (QSARs) predicted selected genotoxicity endpoints of the identified TPs. CIP achieved primary elimination after 128 min of irradiation but was not completely mineralized. Nine photo-TPs were identified. The irradiated mixtures were neither mutagenic in the Ames test nor genotoxic in the *in vitro* micronucleus (MN) test. Like CIP, the irradiated mixtures were *umuC* inducing. The CI analysis revealed that the irradiated mixtures and the corresponding CIP concentration in the mixtures shared similar *umuC* potentials. QSAR predictions suggested that the TPs may be capable of inducing chromosome aberration, MN *in vivo*, bacterial mutation and mammalian mutation. However, the experimental testing for a few genotoxic endpoints did not show significant genotoxic activity for the TPs present as a component of the whole mixture analysis and therefore, further genotoxic endpoints may need to be investigated to fully confirm this.

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1. Introduction

The risk to human and ecosystem health from the presence of pharmaceuticals as micropollutants has remained a debatable issue in the scientific community (Richardson and Ternes, 2011). This concern is partially due to the sparsity in chronic toxicity data from environmental exposures (Taylor and Senac, 2014). Of particular interest are the antineoplastics and antibiotics drugs since by design they are intended to be toxic to cells (Taylor and Senac, 2014; Toolaram et al., 2014; Bergheim et al., 2015). In this regard, monitoring of not only the inherent cytotoxicity but also genotoxicity nature would be paramount to a risk assessment scheme as many of these drugs can directly or indirectly interact with deoxyribonucleic acid (DNA) (Toolaram et al., 2014). Amongst the antibiotic pharmaceuticals is ciprofloxacin (CIP), a broad-spectrum second-generation fluoroquinolone (FQ) that was identified as the main source of *umuC* genotoxicity in the hospital wastewater investigated by Hartmann et al. (1998). Over the years, it has been detected frequently within the μg/L concentration range in the aquatic environment (Hartmann et al., 1998; Martins et al., 2008).

The mode of action (MOA) of CIP involves the binding of the quinolone moiety to the bacterial DNA gyrase which leads to the stabilization of the cleavable complex, preventing the enzyme turnover and thereby inhibiting the resealing of DNA strand breaks (Clerch et al., 1992; Albertini et al., 1995). The formation of intra- and inter-strand adducts could arrest DNA replication, inducing the

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SOS system and producing lesions that would prompt excision repair (Clerch et al., 1996). Quinolones binds differently to eukaryotic topoisomerase II mainly because of the difference in structural DNA and therefore the genotoxic potential is lower in eukaryotic organisms than prokaryotic organisms (Albertini et al., 1995; Clerch et al., 1996). The MOA of CIP in eukaryotic organisms is believed to be the same as in bacteria resulting in DNA strand breaks that if not repaired can lead to clastogenicity and/or cytotoxicity (Lynch et al., 2003). DNA damage using *in vitro* assays includes micronucleus (MN) formation, chromosome aberration (CA), unscheduled DNA synthesis, induction of HPRT mutation cells and thymidine kinase (TK) mutation (Bredberg et al., 1989; Albertini et al., 1995; Chételat et al., 1996; Curry et al., 1996; Gibson et al., 1998; Lynch et al., 2003; Garcia-Käufer et al., 2012).

Environmental monitoring of FQs including CIP showed that their lower concentrations in sewage effluent compared to raw sewage could be attributed to the sorption process in the wastewater treatment plant (WWTP) (Giger et al., 2003). Therefore, an improvement in FQ removal from wastewater would require WWTP to have longer hydraulic retention time and this could enhance the conditions to promote horizontal gene transfer processes enabling the passage of plasmids and transpose encoding antibiotic resistance (Manaia et al., 2010). Manaia et al. (2010) estimated 1–5% of the total enterobacteria species were CIP resistant in the treated wastewater from domestic WWTPs. For FQ, the induction of the SOS repair response system may enhance bacterial survival and could eventually lead to antimicrobial resistance (Cirz et al., 2005; Dörr et al., 2009). Bacteria only needs to be exposed at sub-inhibitory concentrations antibiotics to foster resistance (Kümmerer, 2004).

In the aquatic environment, the fate of CIP is governed by several mechanisms such as photodegradation, adsorption and biotransformation (Cardoza et al., 2005). CIP was reported as not readily biodegradable (Al-Ahmad et al., 1999; Kümmerer et al., 2000). Investigations of lab-scaled photolysis as a treatment method (UV lamp) or as simulated sunlight (Xenon lamp) to degrade CIP has shown the formation of transformation products (TPs) with structures that retained the core quinolone molecule but with alterations, substitutions and/or deletion of its substituents (Chételat et al., 1996; Sánchez et al., 2005; Vasconcelos et al., 2009; Paul et al., 2010; Garcia-Käufer et al., 2012; Haddad and Kümmerer, 2014). Moreover, Haddad and Kümmerer (2014) identified the same photo-TPs in both UV and Xe lamp photolysis. Several *in vitro* genotoxicity assays have shown that the irradiated mixtures containing TPs and CIP may be mutagenic in the Ames test and genotoxic to several cell lines including mouse lymphoma, human hepatic carcinoma cells (HepG2) and human T lymphocyte cells (Jurkat cells) (Chételat et al., 1996; Sánchez et al., 2005; Garcia-Käufer et al., 2012). Even though CIP is a known *umuC* inducer, none of these studies have monitored the changes in genotoxicity of treated CIP using the umu test. However, quantitative structure activity relationships (QSAR) predicted that some TPs may be capable of inducing the *umuC* gene at lower concentrations than CIP (Li et al., 2014).

In this study we monitored the genotoxicity of CIP and its mixture of TPs after UV irradiation as both a whole mixture analysis using a battery of genotoxicity assays (including the umu assay for SOS repair response) and an individual TP analysis with *in silico* predictions using QSAR models. UV light was selected for the photolysis treatment method since it resulted in higher relative abundance of the individual photo-TPs (Haddad and Kümmerer, 2014). This was important since the aims of the study were to determine the genotoxicity of the UV mixtures and to understand the possible genotoxic role of the stable TPs. The design of the study largely followed an effect driven approach for TP assessment as proposed by Escher and Fenner (2011) with recommendations for genotoxicity characterization of TPs from Toolaram et al. (2014).

2. Materials and methods

2.1. Photodegradation and mineralisation monitoring

Photodegradation of CIP (CAS RN: 85721-33-1; from Sigma–Aldrich) was performed in a 1L immersion-type reactor (UV-Consulting Peschl) using a 150 W medium-pressure mercury lamp (TQ 150, UV-Consulting Peschl, Text S1). The reactor was filled with CIP solution in Millipore water (20 mg/L) and irradiated for 128 min. The initial CIP concentration was selected based on the detection limits for non-purgeable organic carbon (NPOC) measurements using a Shimadzu TOC-5000 analyser. Further information on experimental-setup can be found in Haddad and Kümmerer (2014).

2.2. Liquid chromatography analysis

Detection, identification and quantification, of CIP and its TPs were performed on Agilent Technologies 1100 HPLC series connected to a mass spectrometer Bruker Daltonics Esquire 6000 ^{Plus} equipped with an atmospheric pressure electrospray ionization (AP-ESI) source. Chromatographic Separation was performed on a RP18 EC 125 mm $\times$ 4 mm, 5 μm Nucleodur reverse phase column (Macherey–Nagel). Additionally, the accurate masses of CIP and its TPs were measured by LTQ-Orbitrap XL mass spectrometer interfaced with a heated electrospray ionization (H-ESI) source (Thermo Scientific). All LC instruments, chromatographic parameters and mass spectrometer settings have been detailed in Haddad and Kümmerer (2014).

2.3. QSAR predictions

Structure illustrations were performed with MarvinSketch 5.8.0. using simplified molecular input line entry specification (SMILES) codes. These SMILES codes were introduced into various computer based QSAR models for predicting the effects on a number of genotoxicity endpoints.

In silico toxicity predictions of CIP and its TPs were performed using a set of QSAR software each with different algorithms and training sets. The software included CASE Ultra V.1.4.6.6 (MultiCASE Inc.) (Saiakhov et al., 2013), and Leadscope software V.3.0.11–1 with training sets from 2012 SAR Genetox Database (Leadscope) (Roberts et al., 2000). Also Oasis Catalogic software from Laboratory of Mathematical Chemistry (University Bourgas, Bulgaria) predicted mutagenicity based on bacterial mutagenicity (module mutagenicity v.04) in *Salmonella typhimurium* (*Salmonella* Catalogic model, SC).

All *in silico* models had validated database and training sets (Roberts et al., 2000; Chakravarti et al., 2012; Saiakhov et al., 2013). Further information on each model can be seen in Supplementary (Table S1). These models also have been applied in other works (Mahmoud et al., 2014; Rastogi et al., 2014).

2.4. Genotoxicity testing

Prior to testing, samples were kept at 4 °C for 24 h to reduce the presence of short lived reactive oxygen species that can affect the bioassays (Vasquez et al., 2013) and to ensure that mostly stable transformation products were considered in the mixtures. Then the samples were sterile filtered (0.2 μm) and frozen in aliquots at −150 °C. All tests were performed at least twice with 3 replicates per bacterial test and 2 replicates for the *in vitro* MN test. Sample pH

was measured and adjusted if necessary to pH7.0 ± 0.2 with 1 M NaOH or 1 M HCl prior to performing bioassays.

2.4.1. Bacterial mutagenicity – Ames-test

The Ames test with *S. typhimurium* TA100 and TA98 was performed based on the Ames MPF 98/100 Aqua test manual (Xenometrix AG) and used a microplate format that was adapted from the fluctuation assay. This test was done also with metabolic activation using Aroclor 1254-induced rat liver homogenate (S9). A detailed description on this test procedure is provided in the supplementary (Text S2).

Classification as positive for mutagenicity followed a >2 fold increase in the number of revertants over that of the baseline number of revertants.

2.4.2. Bacterial genotoxicity: umu test

The umu test was performed according to ISO 13829 (ISO, 2000) with *S. typhimurium* TA1535 psk 1002 (Leibniz Institute DSMZ – German Collection of Microorganisms and Cell cultures). This test was done also with Aroclor 1254-induced rat liver homogenate (S9) for metabolic activation. Further details of the test procedure is given in Supplementary (Text S2).

The calculation of growth (G) and induction ratio (IR) were according to ISO 13829. However, classification as positive for *umuC* induction was taken when IR ≥ 2 and G ≥ 0.5.

2.4.3. Mammalian genotoxicity: in vitro micronucleus assay using flow cytometry

The *in vitro* MN test was performed with Chinese hamster ovary cells (CHO-K1, American Type Culture Collection). These cells had a doubling rate of 16–18 h. The test was designed and executed using the guidelines of the *In vitro* MicroFlow Kit (Litron Laboratories) and Bryce et al. (2010). The details of the *in vitro* MN test and the cell staining procedure can be found in the Supplementary (Text S2).

Flow cytometry analysis was performed with BD Biosciences FACSCalibur using the gating and settings recommended by the *In vitro* MicroFlow Kit protocol. 20,000 nucleated cells per samples were analysed for MN formation, and cytotoxicity (EMA+ and relative survival). Samples were classified as MN inducing when MN frequency increased ≥3 fold over the mean negative control value and cytotoxic if there was 50% reduction in relative survival.

2.5. Comparison of the umuC induction between CIP and its irradiated mixtures

Under the assumption that the TPs may exhibit similar activity as CIP since the quinolone core was retained in the identified TPs, the relationship between CIP and its irradiated mixtures was investigated in the umu assay using both non-linear regression curves and combination index (CI) computation. A dose–response curve was constructed for IR of CIP from the non-cytotoxic to the marginally cytotoxic range using Four Parameter Logistic Curve function (Sigmaplot 12) with equation:

$$y = \min + (max - min)/(1 + (x/EC50)^{-slope} \quad (1)$$

Where min = bottom of the curve; max = top of the curve; slope = slope of the curve at its midpoint; EC50 (mg/L) = x value for the curve point that is midway between the max and min parameters, x (mg/L) = CIP concentration to cause effect (y). This curve was used to predict the CIP concentrations that would cause similar effect as those observed in the irradiated mixtures. The residual CIP concentrations found in the irradiated mixtures after treatment for 2 min, 4 min, 8 min and 16 min and under 1:400, 1:2000 and 1:4000 dilutions were plotted also to show their relationship to CIP dose–response curve.

The combination index has been well defined and used to quantify drug–drug interactions in mixtures (Chou and Talalay, 1984; Kortenkamp and Altenburger, 1999; Zhao et al., 2010). Based on our results (see Section 3.2.2.), we assumed that CIP may be the main component in the irradiated mixtures that was responsible for the induction of the *umuC* gene. Therefore, we adopted the following equation from the Cassee et al. (1998):

$$CI = \frac{d_1}{D_1} = \frac{CIPmix}{CIPpred} \quad (2)$$

In this case, d_1 was the concentration of the residual CIP (mg/L) in the photolysis mixture (CIP mix) and D_1 was the concentration of CIP (mg/L) that would produce the same effect (CIP predicted). Genotoxicity is limited by cytotoxicity and thus, instead of a median toxicity parameter such as EC_{50}, the CI was calculated for each effect observed by the mixture that has an IR ≥ 2 and G ≥ 0.5 to provide a better comparison within the non-cytotoxic but genotoxic range. A graph was then plotted of the computed CI versus the irradiation time. According to LC-MS and NPOC data, mixtures obtained from 2 min to 16 min of photolysis contained most of the TPs (Fig. 1). Therefore, these mixtures were selected for the CI analysis.

2.6. Statistical analysis and software

The statistical significance was determined by ANOVA (Holm-Sidak method, overall significance level p ≤ 0.05) and was used to assist in the determination of significant differences between controls and samples. All statistical analysis and graphs were processed using Microsoft Excel 2010 (Microsoft Corporation) and Sigmaplot 12.0 (Systat Software, Inc).

3. Results and discussion

3.1. CIP and its photolytic products

CIP under UV photolysis gradually underwent primary elimination and transformation with time. After an irradiation time of 46 min >99% of parent compound was eliminated (Fig. 1). The relative peak areas of all TPs exhibited a similar trend with peaks increasing significantly, and then decreasing rapidly with irradiation time (Fig. 1). After 64 min of irradiation, all identified TPs as well as CIP were not detectable. However, complete mineralization of CIP did not occur as only 70% of total NPOC was eliminated at the end of the photolysis (Fig. 1). This may indicate that there were several other TPs formed that could not be identified using our analytical protocol.

Nine known stable TPs of CIP were found (Table 1). The details of their formation and elucidation are detailed in Haddad and Kümmerer (2014). As was similarly reported by several researchers, all of the proposed TP structures retained the quinolone molecule and the cyclopropyl ligand of CIP but the transformation occurred mostly to the piperazine moiety (Chételat et al., 1996; Sánchez et al., 2005; Vasconcelos et al., 2009; Paul et al., 2010; Garcia-Käufer et al., 2012; Haddad and Kümmerer, 2014).The proposed structure of TP5 was the human metabolite desethylenciprofloxacin, that is known to have a lesser microbial activity than CIP (Shah, 1991). Using our analytical settings, TP 5 occurred at <1% of the area mass of CIP. Only three TPs, namely TP 3, 6 and 8, occurred at ≥10% of the initial CIP peak area in the mixture and thus fulfilled the criterion of the U.S. Food and Drug Administration (FDA) to identify relevant TPs for further assessment (FDA, 1998). Currently relative peak area quantification were used to assist in

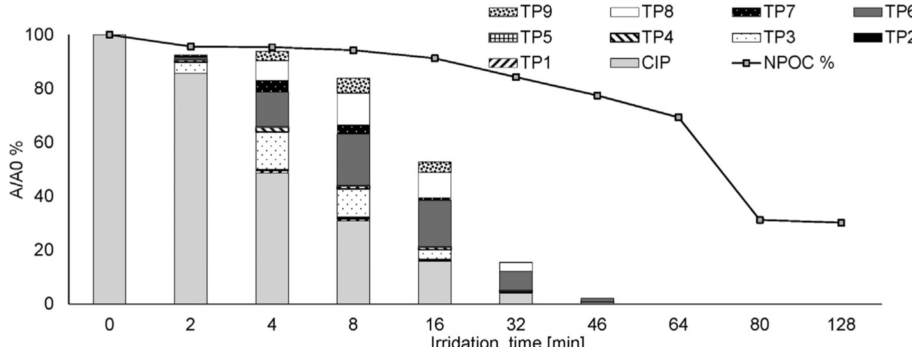

Fig. 1. Time course of the relative peak area of ciprofloxacin (CIP), its transformation products (TPs) (bars), and the non-purgeable organic carbon (NPOC) (line) during the photodegradation experiment. A/A0; A is the area of TP at specified irradiation time point and A0 is the area of CIP at 0 min monitored by LC-MS.

identifying possible relevant TPs from analytical data. However, this may not be the best way for determining the relevant TPs in the mixture as the measured concentration may be small due to low analytical sensitivity as a result of low ionisation rates in mass spectrometry or low molar extinction coefficient in case of UV–vis detection. Therefore, this method only provided an idea of a relative change in concentration of the identified TPs as pure standards of the TPs were not available for absolute quantification of actual TP concentration. As such, an initial hazard screening should at least include whole mixture toxicity in conjunction with QSAR for individual TPs.

3.2. Genotoxicity characteristics of CIP and its mixtures of photo-TPs

3.2.1. Bacterial mutagenicity

The Ames test using the frameshift strain TA98 and the base substitution strain TA100 were negative for mutagenicity in CIP and its photolytic mixtures (Table 2). Chételat et al. (1996) showed that even with concomitant irradiation and mutagenicity testing, there was a slight but insignificant increase in strain TA100 revertants. They also have determined that the photoproducts did not enhance the gyrase-mediated genotoxicity but the notable increase in revertants of another strain tested namely, TA104, was most probably from short lived reactive oxygen species (Chételat et al., 1996).

QSAR modelling predicted a few TPs were mutagenic to Salmonella (Table 1). The inconclusive prediction by CASE Ultra for TP5 believed to be desethylenciprofloxacin shared the same positive structure alert as in the CIP structure (Table S2). Using classification rule 4 of the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use M7 guideline (ICH, 2014), TP 5 may not be mutagenic in strain TA100 or TA98 since experimentally CIP was not mutagenic in these strains. In the rule based model of Oasis, three TPs namely TP 7, 8 and 9 were predicted positive in the Ames test (Table 1). However, in the Leadscope model based on both statistical and rule based criteria, none of the TPs were predicted to cause bacterial mutagenicity (Table 1). Further, the Leadscope model predicted that like CIP, all TPs may cause *in vitro* mammalian mutation (Table 1) and this was not tested for here.

QSAR estimations by different models would have their respective weaknesses and strengths and as such predictions may be different. Regardless, experimentally, these photolytic mixtures containing the photo-TPs and CIP were not mutagenic to strains TA100 and TA98. This may be as a result of the low concentration of the relevant TPs or mixture interactions or simply that the strains tested here were not suitable to demonstrate the mutagenicity predicted by QSAR analyses.

3.2.2. Bacterial genotoxicity

Relative growth inhibition testing with *S. typhimurium* TA1535 psk 1002 in the umu test revealed that bacterial growth was less affected when exposed to the photolysis mixtures. Even though CIP was present at lower concentrations in these mixtures and therefore cytotoxicity was expected to be lower, it cannot be excluded that the presence of the various TPs did not enhance the toxicity of the mixtures. Similarly, Paul et al. (2010) deduced that the TPs in their photolytic and photocatalytic treated CIP solution did not significantly influence the overall cytotoxicity to *E.coli* K12. A similar conclusion was made by Ge et al. (2015) and Sturini et al. (2015) in their studies. Paul et al. (2010) postulated that transformation of the piperazine moiety and the fluoride ion would diminish the antimicrobial potency of CIP. In our case, the TPs were mostly altered at these two positions often resulting in a deflourination and/or breakage of the piperazine moiety (Table 1). Sukul and Spiteller (2007) further suggested that dealkylated TPs have a much lower antimicrobial potency than deflourinated TPs.

The irradiated mixtures were positive for *umuC* induction without any metabolic activation following a similar diminishing pattern as in the primary elimination of CIP (Figs. 1 and 2). As such, it was hypothesized that CIP may be the main contributor to the levels of *umuC* induction in the photolytic samples. CIP was also tested with metabolic activation which led to a detoxification of the samples (Fig. 2). The observed minimum genotoxic concentration of CIP was 0.004 mg/L (−S9) and 0.025 mg/L (+S9). This was similar to the 0.005 mg/L reported by Hartmann et al. (1998) for the umu test.

The CI of the measured CIP concentration in the treated mixtures (residual CIP) relative to the predicted CIP concentration based on the single-substance toxicodynamics was computed and illustrated in Fig. 3a and b. In such a graph, the CI of 1 indicated that the predicted CIP concentration and its residual concentration in the treated mixture produced the same level of *umuC* induction.

The CI analysis revealed that the genotoxicity of the mixtures were not greater than the parent compound (Fig. 3). The 1:2000 dilution of mixtures for 4 min and 8 min had CIs relatively >1. However, this could be attributed to errors in prediction of CIP concentration from the curve fitting model. In fact, Fig. 3a showed that the corresponding concentration and effect for these time points were within the confidence band of the measured effects of CIP. As such, the CI analysis revealed that the SOS repair response induction potential of the irradiated mixtures containing the TPs and CIP was not greater than CIP itself.

From the QSAR modelling of Li et al. (2014), the genotoxicity potential of quinolones was stronger when there was the addition

Table 1
Proposed structures of photo TPs and their corresponding QSAR predictions for selected genotoxicity and mutagenicity endpoints.

TP ID	Retention time (min)	Structure	Molar mass ($gmol^{-1}$)	Case ultra					Leadscope				Oasis
				Genotoxicity				Mutagenicity	Genotoxicity		Mutagenicity		Mutagenicity
				A		B	C	D	A	B	D	E	D
				A7U	A7V	A7S	A64	A2H					
CIP	8.5		331.35	+	§	−	+	−	+	−	−	+	−
TP1	4.6		327.34	+	§	§*	−	−	+	−	−	+	−
TP2	6.5		315.33	+	§	−	−	−	+	−	−	+	−
TP3	***6.7***		***329.36***	+	***§***	**-**	+	**-**	+	**-**	**-**	+	**-**
TP4	7.0		287.32	+	§	§*	−	−	+	−	−	+	−
TP5	7.5		305.31	+	§	−	+	§*	+	−	−	+	−
TP6	***7.8***		***345.36***	+	***§***	***§****	+	**-**	+	**-**	**-**	+	**-**
TP7	11.5		343.34	+	§	−	OD	−	+	−	−	+	+
TP8	***12.0***		***315.33***	+	***§***	***§****	***OD***	**-**	+	**-**	**-**	+	+
TP9	12.5		329.36	+	§	−	+	−	+	−	−	+	+

A = In vitro Chromosome Aberration; B = In vivo Micronucleus Composite; C = Other Genotoxicity Tests; D = Salmonella Mutagenicity; E = Mammalian Mutagenicity.
A7S: MN *in vivo*; A7U: Chromosome aberration composite *in vitro*; A7V: Chromosome aberration in CHO cells; A64: Unscheduled DNA synthesis induction; A2H: Mutagenicity Ames *Salmonella typhimurium*.
− = Negative alert for activity; + = Positive alert for activity; § = Inconclusive because of the presence of unknown structural fragments from the training set; §* = Inconclusive because of the presence of both positive and deactivating alerts in the molecule; OD = molecule fragments are out of domain.
TPx in bold and italics represents the TPs that are ≥ 10% relative abundance to CIP.

Table 2
Bacterial mutagenicity of CIP and its mixtures of photo-TPs.

Time points	Ames bacterial mutagenicity[1]			
	Number of revertants			
	TA 98		TA100	
	−S9	+S9	−S9	+S9
NC	2 ± 2	2 ± 2	3 ± 1	1 ± 1
0	2 ± 1	1 ± 1	4 ± 2	1 ± 0
2	n.t	n.t	n.t	n.t
4	n.t	n.t	n.t	n.t
8	4 ± 1	3 ± 3	5 ± 2	2 ± 1
16	3 ± 1	4 ± 2	4 ± 2	2 ± 1
32	3 ± 1	3 ± 1	6 ± 2	1 ± 2
64	4 ± 3	2 ± 2	5 ± 1	1 ± 2
128	1 ± 1	2 ± 2	4 ± 1	1 ± 0
PC	45 ± 2	48 ± 0	48 ± 0	48 ± 0

Data presented are the mean values ± the standard deviation. n.t: not tested; PC: positive control 0.1 μg/ml 4-NQO + 2 μg/ml 2-NF (−S9) and 5 μg/ml 2-AA (+S9). NC: negative control of Millipore water; 1: samples tested at 1:2000 dilution; T0 = CIP at 0.01 mg/L.

of bulky groups in position 1 (cyclopropyl ligand of CIP), a negative charge or bulkier group at position 7 (piperazine moiety of CIP) as well as small electronegative species in position 6 (fluoride ion of CIP), position 3 (carboxylic group of CIP) and position 8 of the molecule. Li et al. (2014) did QSAR predictions for umu test with several known TPs including TP3 (as P5) and TP5 (as P1) identified here and concluded that some TPs were predicted to show stronger *umuC* induction than CIP. Since the predicted LOECs for these two TPs by Li et al. (2014) suggested that they have greater genotoxic potential than CIP, it would be expected that they contribute to an increased genotoxicity in the umu test. However, our irradiated samples containing these TPs did not demonstrate this. In actuality, the presence of any of the TPs did not enhance the genotoxicity after photolysis probably because of their interaction whether antagonistic or synergistic in the mixtures and/or they did not occur at concentrations that can cause an observable effect. The retention of the quinolone moiety would suggest that the TPs have an intrinsic ability to bind to DNA and possibly induce the SOS repair response system. However, the lipophilicity, cell penetration and DNA binding affinity may be altered because of the changes in

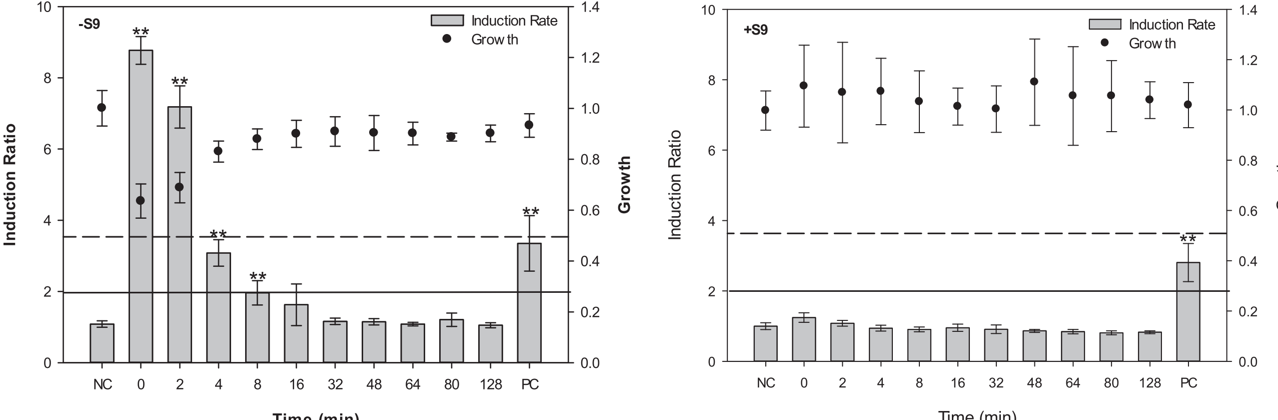

Fig. 2. *Umu C* induction of CIP and its irradiated mixtures without metabolic activation (left) and with metabolic activation (right). Results represented are from a 1:2000 dilution of each sample. Dotted lines represent growth threshold of G ≥ 0.5, solid line represents the induction ratio threshold of IR ≥ 2. ** represents p ≤ 0.01 of samples compared to the negative control (NC). NC = Millipore water as sample, T0 = CIP at 0.01 mg/L. PCs are the positive controls of 4-nitroquinoline-1-oxide (4-NQO) without S9 and 2-aminoanthracene (2-AA) in the presence of S9.

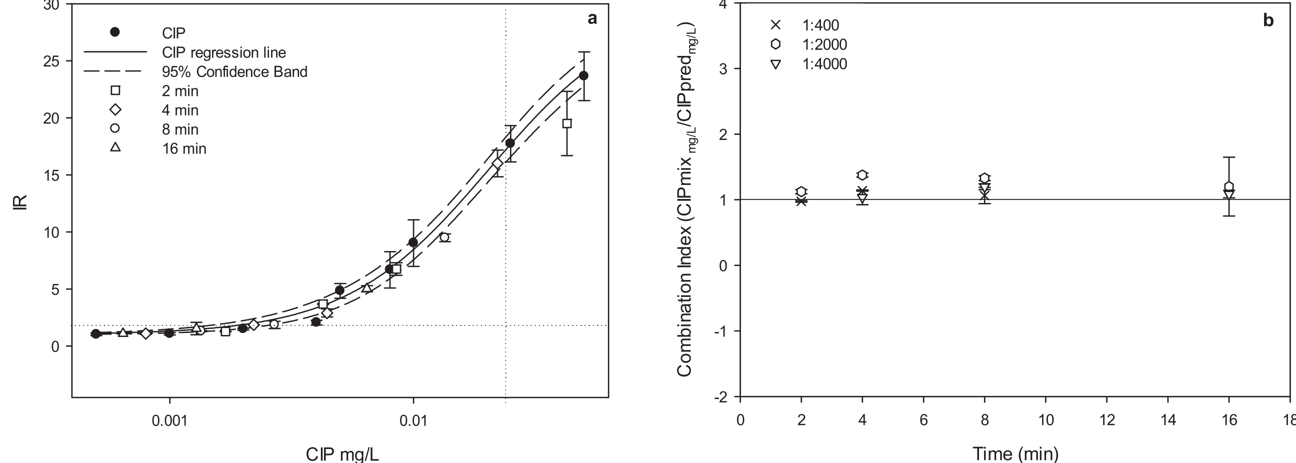

Fig. 3. (a) Dose–response relationship of CIP and effect of residual CIP in its irradiated mixtures in the umu test. Dotted lines represent the threshold of IR ≥ 2 and G ≥ 0.5. (b) Combination index of residual CIP in irradiated mixtures and predicted CIP to cause similar effects of IR ≥ 2 and G ≥ 0.5 versus irradiation time.

the substituents. Since the TPs were neither synthesized nor commercially available, confirming the structural alteration that would influence the bacterial cytotoxicity and genotoxicity was not possible in this work.

Several authors have noted that there was a correlation between the genotoxicity of SOS chromotest and the mutagenicity observed in strain TA 102 of Ames test possibility because the SOS response system was induced in both tests (Power and Phillips, 1993; Albertini et al., 1995). Since CIP induced the SOS repair response system that would compensate DNA damage, it is possible that bacteria can become resistant to CIP (Mamber et al., 1993; Power and Phillips, 1993; Clerch et al., 1992; Yim et al., 2011). Nonetheless, in our case, the photolytic mixtures may not pose a threat towards enhancing bacterial resistance stemming from induction of the SOS repair response system since they did not increase the induction of *umuC* gene. Further, the mixtures of TPs were not more cytotoxic than CIP and therefore would not cause added selection pressure to microbial communities that would favour resistance mutants. The *umuC* inducing effect of CIP was not observed below the minimum genotoxic concentration of CIP which is at environmentally relevant concentrations (μg/L), even within the photolytic mixtures tested. Therefore, photolysis would be an effective method to reduce the *umuC* potential of CIP as an environmental contaminant, as long as CIP is removed from the mixture. Haddad and Kümmerer (2014) have identified similar TPs after simulated sunlight photolysis. Thus, it may be possible that the mixtures generated from natural irradiation could also result in compounds with less potency than CIP. However, bearing in mind that kinetics of formation and concentration of the TPs may be different for direct sunlight photolysis; it is still possible that other mixture interactions could occur that may influence bacterial genotoxicity and cytotoxicity. Further, natural photolysis could lead to the formation of reactive oxygen species that may play a greater role in environmental genotoxicity.

3.2.3. Mammalian genotoxicity

Our experiments demonstrated that CHO-K1 cells treated with CIP at concentrations 1 mg/L (5% CIP v/v) to 4 mg/L (20% CIP v/v) had relative survival rates of ≤60% and induced apoptosis/necrosis (EMA+ ≥5%) while producing no significant MN induction. Cytotoxicity testing of our diluted photolytic mixtures with the CHO-K1 cells resulted in slight but insignificant reductions on their relative survival and no significant effect on apoptosis/necrosis (EMA+) after exposure (Table 3).

No significant effect on MN formation was found in CHO-K1 cells exposed to CIP and its irradiated mixtures (Table 3). Gibson et al. (1998) found a statistically significant increase in MN formation in CHO-K1 cells exposed to CIP but reasoned that this was of no biological significance since the MN counts were within the historical negative control. No studies of CHO-K1 cells exposed to CIP photolysis mixtures were found in our literature research. However, many photogenotoxicity *in vitro* studies performed with other cell lines revealed CIP and irradiated mixtures induced MN, CA and/or other DNA damage (Chételat et al., 1996; Sánchez et al., 2005; Garcia-Käufer et al., 2012). These authors have postulated that the increase in photogenotoxicity observed may be due to the presence of short lived reactive species rather than the stable TPs (Chételat et al., 1996; Sánchez et al., 2005; Garcia-Käufer et al., 2012). Nevertheless, the photolysis mixtures generated here should be investigated in other cell lines such as CHO-K5 cells, V79 cells, Hep G2 cells and mouse lymphoma L5178Y $TK^{+/-}$ in which CIP is known to induce MN before it can be excluded that the mixture of TPs were not inherently genotoxic to mammalian cells.

QSAR prediction suggested that MN formation was not likely *in vivo* from exposure to CIP. In fact, CIP was not reported to induce MN *in vivo* especially at the tissue concentrations achieved from the therapeutic dose (Albertini et al., 1995; Herbold et al., 2001). However, CASE Ultra MN *in vivo* (A7S) model identified some positive structural alerts for TPs 1, 4, 6 and 8 which were not found in CIP and this risk should not be excluded from further evaluations of the TPs (Table S2). QSAR modelling predicted that like CIP, the TPs would be positive for mammalian mutation involving mouse lymphoma mutation assay and for *in vitro* chromosome aberration in cell lines such as Chinese hamster lung cells and human peripheral blood lymphocytes but with uncertainty in CHO cell lines (model A7V) due to the presence of unknown fragments (Table 1). Further, unscheduled DNA synthesis was predicted for some TPs. These TPs had either a retention or substitution in the fluoride position of the CIP molecule (TP 3, 5 6) or like TP 9, the presence of a tertiary amide group (Table S2).

4. Conclusion and outlook

The retention of the quinolone moiety in the photo-TPs would suggest they could bind to the DNA similarly as CIP but the alterations of the substituents may affect their affinity to DNA binding and subsequently their potency and toxic nature. QSAR predictions suggested that there were a few TPs that may be genotoxic to bacteria and mammals. The battery of genotoxicity assays employed covered a few endpoints with one cell line and a few bacterial strains. It was able to demonstrate that the photolytic samples were *umuC* inducing and this may most likely be because

Table 3
Mammalian genotoxicity of CIP and its mixtures of photo-TPs.

Time points	*In Vitro* micronucleus[1]		
	Cytotoxicity		Genotoxicity
	Relative survival (%)	EMA+ (%)	MN (%)
NC	100 ± 2.54	0.26 ± 0.10	1.49 ± 0.37
0	78.88 ± 4.43	0.22 ± 0.06	1.16 ± 0.05
2	94.61 ± 5,72	0.24 ± 0.01	1.12 ± 0.12
4	94.51 ± 7.21	0.32 ± 0.06	1.26 ± 0.04
8	102.44 ± 2.23	0.28 ± 0.06	1.04 ± 0.04
16	102.18 ± 18.30	0.24 ± 0.08	1.10 ± 0.00
32	96.69 ± 13.34	0.53 ± 0.00	0.99 ± 0.06
64	99.53 ± 11.11	0.39 ± 0.19	1.46 ± 0.37
128	94.46 ± 27.77	0.27 ± 0.18	0.90 ± 0.05
MMC	78.85 ± 31.31	0.22 ± 0.03	3.97 ± 0.13
VB	57.12 ± 12.93	0.82 ± 0.15	4.62 ± 0.11

Data presented are the mean values ± the standard deviation. MMC: 0.1 μg/ml mitomycin C; VB: 0.01 μg/ml vinblastine sulphate; NC: negative control of Millipore water; 1: samples tested at 2.5% v/v; T0 min = CIP at 0.5 mg/L.

of the presence of CIP rather than the TPs. It was possible that the effect of the TPs was masked by antagonistic mixture interactions or that the concentrations of the TPs in the mixture were not sufficient to cause any observable effect in the bioassays.

Therefore, we have observed that while photolysis provided a mean for the removal of CIP, the genotoxicity and cytotoxicity potential of the resultant mixtures could be dependent primarily on the concentration of residual CIP. The genotoxic risk of the TPs in the environment was not particularly defined in this study as CIP was determined to be the main genotoxin in the bioassays used. As such, this study provided only an initial hazard screening of the particular mixtures generated here. Further work is especially recommended with photolytic mixtures for mammalian genotoxicity using other cell lines and concentration ranges. The TPs that were predicted as genotoxic would require a more comprehensive assessment that would include chemical analytic characterization, exposure analyses (including biodegradation studies) and effect driven analyses for TP threshold identification to determine their environmental risks.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.12.040.

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Supplementary Data

for

Initial hazard screening for genotoxicity of photo-transformation products of Ciprofloxacin by applying a combination of experimental and *in-silico* testing

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Text S1: Description of UV lamp

The medium pressure mercury lamp TQ 150 total radiation flux Φ from 200 nm to 600 nm is 47 W m^{-2} and the maximal intensities for whole spectral distribution were at following wavelengths (UV-Consulting Peschl)

Wavelength (nm)	Intensities (W m^{-2})
254	4.0
265	1.4
302	1.8
313	4.3
366	6.4
405/408	3.2
436	4.2
546	5.1
577/579	4.7

Text S2: Genotoxicity testing procedure

Genotoxicity testing

Genotoxicity testing was done in a battery of three assays, namely, the Ames bacterial mutagenicity test, the umu test and the *in vitro* micronucleus (MN) test.

Bacterial mutagenicity: Ames MPF 98/100 Aqua

Materials: Ames MPF 98/100 Aqua test kit containing Exposure medium, Reversion indicator medium, Growth medium, Aroclor 1254-induced rat liver homogenate (S9), and positive controls: 4-nitroquinoline-N-oxide (4-NQO) and 2-nitrofluorene (2-NF) and 2-aminoanthracene (2-AA) was supplied by Xenometrix AG.

Test organism: Salmonella typhimurium TA98 and TA100 was bought from Xenometrix AG.

Method: An overnight culture was prepared and grown until the OD600 nm reached ≥ 2.0. In a 24-well plate, bacteria were added to the exposure medium and the samples (1:2000 diluted) in the presence or absence of metabolic activation (+/- S9). The plates were then exposed for 90 min at 37°C (MaxQ600, Thermo Scientific) while shaking (250 rpm). After which, the exposed mixture was diluted with reversion indicator medium and transferred into 384-well plates for 48 h incubation at 37°C. During this time, the pH dependent reversion indicator dye would change from purple to yellow in the presence of bacterial growth. The result was colorimetrically scored by eye to give the number of revertants (yellow coloured wells) out of the 48 wells for each irradiation time. Positive controls used for the MPF assay without metabolic activation were a mixture of 4-NQO and 2-NF at a final concentration of 0.1 µg/ml and 2 µg/ml respectively. 2-AA at a final concentration of 5 µg/ml was used for the test performed with S9 mix. Millipore water was used as the negative control.

Before the testing of mutagenicity, the cytotoxicity of samples was assessed to dismiss the possibility of false 'negative' mutagenicity results. This was done by assessing the growth of the TA98 strain through the measurement of absorbance 600 nm after 90 minutes exposure with the test samples using an exposure plate similarly prepared as that for the mutagenicity test.

Analysis: Classification as positive for mutagenicity occurred when the response was ≥ 2 fold increase in the number of revertants over that of the baseline number of revertants (the mean revertants of the negative control plus standard deviation (SD)). The statistical significance determined by ANOVA (Holm-Sidak method, overall significance level $p \leq 0.01$) was also used to assist in the determination of positive results.

Bacterial genotoxicity: Umu Test

Materials: TGA- culture medium comprised of tryptone from Sigma-Aldrich Chemie GmbH and sodium chloride (NaCl), 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid (HEPES), and d(+)-glucose (anhydrous), ampicillin sodium salt, magnesium chloride hexahydrate ($MgCl_2.6H_2O$) and potassium chloride (KCl) from Carl Roth GmbH. B-Buffer and phosphate buffer were prepared from disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), , sodium dihydrogenphosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) and sodiumdodecylsulphate (SDS) from Sigma-Aldrich Chemie GmbH and magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) and potassium chloride (KCl) from Carl Roth GmbH. The stop reagent contained sodium carbonate (Na_2CO_3) from Carl Roth GmbH. Ortho-Nitrophenol-ß-d-galactopyranoside (ONPG) was also obtained from Carl Roth GmbH. Positive controls included 4-nitroquinoline-1-oxide (4-NQO) and 2-aminoanthracene (2-AA) (Sigma-Aldrich Chemie GmbH) dissolved in dimethyl sulfoxide (DMSO). Aroclor 1254-induced rat liver homogenate (S9) was brought from Xenometrix, AG while the co-factor salt NADP sodium salt and D-glucose-6-phsophate di sodium salt were obtained from Carl Roth GmbH and Applichem, respectively.

Test organism: Salmonella typhimurium TA1535 psk 1002 was bought from Leibniz Institute DSMZ- German Collection of Microorganisms and Cell cultures.

Method: The umu test for genotoxicity testing was performed according to ISO 13829{International Organization for Standardization, 2000}. An overnight culture of *S. typhimurium* TA1535 psk 1002 was grown for 15 h at 37°C shaking at 250 rpm (MaxQ600, Thermo Scientific). After which the OD 600 nm was measured and a 1:10 dilution of the overnight culture was re-incubated until the bacteria were in log phase (OD 600 nm = 0.4-0.6). Then plate A was prepared containing the samples, 10x concentrated TGA medium, and bacteria with and without S9 mix. Plate A was incubated for 2 h at 37 °C while shaking at 250 rpm after which plate B was prepared by a 1:10 dilution of the contents of plate A in TGA media. Plate B was incubated further for 2 h at 37 °C and 250 rpm. Thereafter, the optical density OD 600 nm of the contents of plate B was measured using BioTek synergy HT. Then the β-galactosidase activity was determined by placing 30 µl of the contents of plate B to a new plate (plate C) containing the B-Buffer and following with the addition of the ONPG solution. Plate C was incubated for 30 min at 28°C, 250 rpm, after which the reaction was stopped using the stop reagent. Then the OD420 nm of plate C was measured to calculate the induction ratio.

Calculation and Analysis: The calculation of growth (G) and induction ratio (IR) were performed according to ISO 13829. However, classification as positive for *umuC* induction was assessed when the $IR \geq 2$ and $G \geq 0.5$. The statistical significance determined by ANOVA (Holm-Sidak method, overall significance level $p \leq 0.01$) was also used to assist in the determination of positive results.

Mammalian genotoxicity: *In vitro* micronucleus assay using flow cytometry

Materials: Reagents for the staining and lysis of cells for flow cytometry analysis were purchased from Litron Laboratories, Rochester, NY (*In Vitro* MicroFlow kit). The content of

the *In Vitro* MicroFlow kit included Buffer Solution, Nuclei Acid Dye A Solution (EMA dye), RNase Solution, Nucleic Acid Dye B Solution (SYTOX Green dye) and Incomplete Lysis Solutions 1 and 2. 6 µm PeakFlow™ Green flow cytometry reference beads were bought from Invitrogen. Positive controls used were Mitomycin C (MMC) and Vinblastin sulphate (VB) dissolved in Dimethyl sulfoxide (DMSO) all obtained from Sigma Aldrich Chemie GmbH.

The cell culture solutions included HAM's F12 culture medium with stable L-glutamine combined with 10% fetal bovine serum (FBS superior) and 1% Penicillin/Streptomycin from Biochrom. Trypsin/EDTA-Solution (0.05%/0.02%) and phosphate buffer salt (PBS) solutions were also obtained from Biochrom.

Cell line: Chinese hamster ovary cells (CHO-K1) were bought from American Type Culture Collection (ATCC). These cells had a doubling rate of 16-18 hours.

Method: CHO-K1 Cells were maintained for at least two weeks prior to the test in the combined HAM's F12 medium at 37°C, 5% CO_2 in a humid atmosphere (Thermo Scientific MIDI 40 CO_2 incubator). Then the cells were trypsinized and plated at 12000 cells/ml/well into a 24 well pate. The cells were allowed to attach for 44 h at 37°C, 5% CO_2 in a humid atmosphere. After that the media was aspirated and replaced with 1 ml solutions containing the samples (2.5% v/v) in media. The positive controls were MMC at 0.1 µg/ml and VB at 0.01 µg/ml. The 24 well plate was then placed for 30 h at 37°C, 5% CO_2 in a humid atmosphere. Then the cell staining and lysis protocol of the *InVitro* MicroFlow Kit was followed.

Briefly, the cell staining and lysis protocol includes placing the 24 well plate on ice for 20 minutes after the 30 h exposure time. After that the solution was aspirated and 300 µl of EMA solution was added to each well. The plate was exposed to fluorescence light for 30 min to undergo photoactivation of the dye. Then the EMA dye was aspirated and the cells were washed with 1ml of cold buffer solution. 500 µl of complete lysis solution 1 was added to each well and

incubated for 1 h at 37°C, 5% CO_2 in a humid atmosphere. Cytometry reference beads were added to complete lysis solution 2 which was later added to each well after the incubation period. The plate was then kept at room temperature in dark for at least 30 min prior to flow cytometry analysis.

Analysis: Flow cytometry analysis was performed using BD Biosciences FACSCalibur according to the gatings and settings recommended by the *In vitro* MicroFlow Kit protocol. 20,000 nucleated cells per samples were analysed for MN formation, and cytotoxicity (EMA+ and relative survival). The validity criteria for the test were defined as suggested by Bryce et al (2010). Samples were classified as positive when MN frequency increased ≥ 3 of the mean negative control value. Samples were determined to be cytotoxic if there was 50% reduction in relative survival. The statistical significance was determined by ANOVA (Holm-Sidak method, overall significance level $p \leq 0.05$).

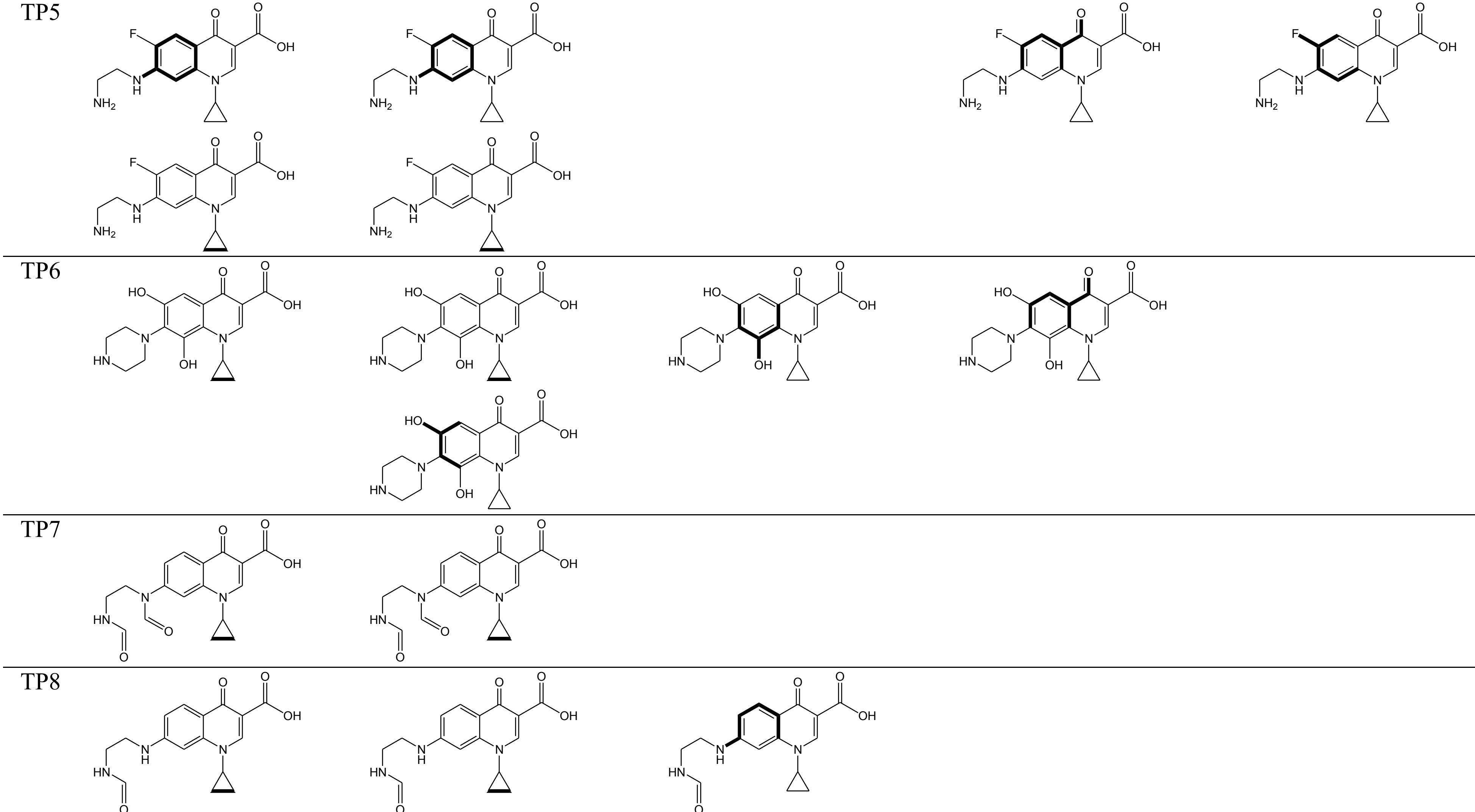
Table S1: Description of the applied QSAR models

Toxicity	QSAR software	Model	Description	Endpoint	References
Genotoxicity	CASE Ultra v.1.4.6.6 (MultiCASE Inc.)	A7U Chromosome Aberration *In vitro* composite	model developed for more than one cell line	*In vitro* Chromosome abberation	Chakravarti et al., 2012; Saiakhov et al., 2013
		A7V Chromosome Aberration *In vitro* CHO cells	model developed for Chinese Hamster Ovary (CHO) cells		
		A7S Micronucleus *In vivo* composite	model develop from rat and mice data	*In vitro* MN formation	
		A64 UDS Induction	model developed for unscheduled DNA induction with primary rat liver, human peripheral blood lymphocytes and fibroblast	Unschedule DNA synthesis	
Mutagenicity		A2H Salmonella Ames mutagenicity	model developed *S. typhimurium* strains (+/- S9) as a composite from NTP, GENETOX, USEPA and FDA database	Bacterial mutagenicity	
Genotoxicity	Leadscope V.3.0.11-1	*In vitro* Chromosome Aberration average model	model developed with CHO, CHL, HPBL and other mammalian cell culture from 2012 Genetox Database from Leadscope	*In vitro* Chromosome abberation	Roberts et al., 2000
		Micronucleus *in vivo* composite model	model developed with rat and mice data from 2012 Genetox Database from Leadscope	*In vitro* MN formation	
Mutagenicity		Salmonella composite	model developed with data from *S. typhimurium* TA 97, TA 98, TA 100, TA1535, TA 1536, TA 1537, TA 1538 from 2012 Genetox Database from Leadscope	Bacterial mutagenicity	

		Mammalian mutation *in vitro*	model developed for mammalian mutation including mouse lymphoma mutation gene mutation assays at the thymidine kinase (tk) locus using L5178Y cells in culture from 2012 Genetox Database from Leadscope	Mammalian mutagenicity	
Mutagenicity	Oasis	Mutagenicity v .04	model developed with data from *S. typhimurium*+/-S9 using NTP database	Bacterial mutagenicity	Laboratory of Mathematical Chemistry, 2014

Table S2: Positive structural alerts in CIP and its photo-TPs for selected genotoxicity and mutagenicity endpoints from QSAR modelling using Case Ultra software.

TP ID	Case Ultra				
	Genotoxicity				Mutagenicity
	A		B	C	D
	A7U	A7V	A7S	A64	A2H
CIP					
TP1					
TP2					
TP3					
TP4					



TP5
TP6
TP7
TP8

TP9

Bold lines represent positive structural alerts

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