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Selected Aspects of Biodegradation Testing of Pharmaceuticals Including Development of a New Biodegradation Test

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II. Lutterbeck, C. A., **Baginska, E.**, Machado. Ê. L., Kümmerer, K., Removal of the anti-cancer drug methotrexate from water by advanced oxidation processes: Aerobic biodegradation and toxicity studies after treatment. Chemosphere 2015; 141:290–6. doi: 10.1016/j.chemosphere.2015.07.069

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V. **Baginska, E.**, Haiß, A., Kümmerer, K., Biodegradation screening of chemicals in an artificial matrix simulating the water–sediment interface. Chemosphere 2015;119: 1240–6. doi: 10.1016/j.chemosphere.2014.09.103

VI. Gutowski, L., **Baginska, E.**, Olsson, O., Leder, C., Kümmerer, K., Assessing the environmental fate of S-metolachlor, its commercial product Mercantor Gold® and their photoproducts using a water–sediment test and *in silico* methods. Chemosphere 2015; 138:847–855. doi:10.1016/j.chemosphere.2015.08.013.

Selected Aspects of Biodegradation Testing of Pharmaceuticals Including Development of a New Biodegradation Test

This cumulative thesis and the publications listed on the following page are submitted to the Faculty of Sustainability of Leuphana University Lüneburg to earn the academic degree of

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Dedication

I would like to dedicate this Thesis to my Brother

Wojtek

Mój Duduś, moje serce

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List of symbols and abbreviations

5-FU	5-fluorouracil
ACN	acetonitril
AOP	Advanced oxidation process
BOD	Biological oxygen demand
CAS-RN	Chemical abstracts registry number
CBT	Closed bottle test
CFU	Colony forming unit
CIP	Ciprofloxacin
CYC	Cyclophosphamide
DEG	Diethyleneglycol
FDA	Fluorescence detector
HPLC	High performance liquid chromatography
IR	Infrared spectroscopy
ISO	International Standard Organization
LC-MS	Liquid chromatography mass spectrometry
MTX	Methotrexate
NMR	Nuclear Magnetic Resonance
NPOC	Non-purchable organic carbon
OECD	Organization of Economic Cooperation and Development
QSAR	Quantitative Structure Activity Relationship
STP	Sewage treatment plant
ThOD	Theoretical oxygen demand
TP	Transformation product
UV	Ultraviolet
WST	Water sediment test
WWTP	Wastewater treatment plant

Summary

Among all attenuation processes, biodegradation plays one of the most important role and is one of the most desirable processes in the environment especially since products released during this bio-reactions, can be once again reintroduced into the natural element cycles. To assess biodegradation, a variety of biodegradation test procedures have been developed by several international organizations. OECD guidelines for ready biodegradability testing represent one of the most prominent group of internationally used screening biodegradation tests (series 301A-F). These tests are usually very simple in their designs and allow for the fast and cheap screening of biodegradability. However, because of their stringency, the test conditions are not close to simulating environmental conditions and may lead to unrealistic results. To overcome these limitations, OECD introduced simulation tests which are designed to investigate the behavior of chemicals in specified environmentally relevant compartments. Despite the fact that simulation tests give more insight into the fate of chemicals in the environment, they are not applied frequently as they are often tedious, time consuming and expensive. Consequently, there is a need to provide a new biodegradation testing method that would combine complex testing environment as in simulation tests, easiness in handling and good data repeatability as in screening biodegradation tests. Another challenge is an adaption of the existing biodegradation testing methods to new types of samples, i.e. mixtures of transformation products (TPs).

The research on the presence of pharmaceuticals in the environment gained momentum in the 1990s; since then, it has been growing. Their presence in the environment is a well-established fact. A wide range of pharmaceuticals is continuously detected in many environmental compartments such as surface waters, soils, sediments, or ground waters. After pharmaceuticals reach the natural aquatic environment they may undergo a number of processes such as: photolysis (under direct sunlight), hydrolysis, oxidation and reduction reactions, sorption, biodegradation (by bacteria of fungi), and bioaccumulation. These processes, may cause their elimination from aquatic environment, if reaction is complete, or creation of new compounds i.e., transformation products (TPs). What is more, processes, like chlorination and advanced oxidation processes (AOPs), such as H_2O_2/UV, O_3/UV, TiO_2/UV, Fenton, and photo-Fenton, or UV treatment which might be applied in water or wastewater treatment, may also lead to the TPs introduction into aquatic environment. The research on the TPs brings many new challenges. From one side, there is a constant need for the development of a sensitive and reliable analytical separation, detection, and structure

elucidation methods. Furthermore, there is a need for the preparation of appropriate assays for the investigation of properties of new compounds, especially those answering the question if TPs pose a higher risk to the aquatic ecosystems than their parent compounds. Among numerous groups of pharmaceuticals, two are of great importance: antibiotics since they might promote emergence and maintenance of antimicrobial resistance in the aquatic environment; and cytostatic drugs. Cytostatic drugs can exert carcinogenic, mutagenic and/or teratogenic effects in animals and humans.

The challenges of biodegradation testing presented in this thesis, encompasses these different areas of interest and was divided into three objectives: 1) Identification of the knowledge gaps and data distribution of the two groups of pharmaceuticals antibiotics and cytostatic drugs (article I); 2) Increasing the knowledge on biodegradation of cytostatic drugs and their TPs (articles II, III, and IV) and 3) Establishment of a biodegradation test with closer to simulation tests conditions, that could be affordable and to support better understanding on processes in water sediment interface construction – screening water-sediment test. Further validation of the test with an insight into sorption and desorption processes (articles V and VI).

Article I focuses on the identification of the knowledge gaps and data distribution of the two groups of pharmaceuticals antibiotics and cytostatic drugs. A literature research was conducted on data about parent compounds and TPs that has been published. The 21 selected antibiotics and cytostatic drugs were investigated. Information available on TPs demonstrates that already a slight change in treatment conditions and processes results in the formation of different TPs. This in turn makes it, on the one hand, 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. What is more, there is 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 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

Articles II, **III** and **IV** address the issue of increasing the knowledge on biodegradation of cytostatic drugs and their TPs. Biodegradation testing was conducted according to OECD 301D guideline (Closed Bottle Test) and toxicity evaluation assays using the luminescent bacteria *Vibrio fischeri*. Samples with TPs were tested for biodegradation and toxicity when during the photodegradation studies (UV-Lamp, Xenon Lamp, UV/H_2O_2, UV/Fe^{2+}/H_2O_2, and UV/TiO_2), observed mineralization was <70%. The experimental data from the presented study confirms that not all of photodegradation treatments are able to completely remove cytostatic drugs. What is more, it is difficult to predict, what properties formed TPs would have, and what would be the influence on environment once released.

Articles V and **VI** are about establishment of a biodegradation test with closer simulation tests conditions, that could be affordable and to support better understanding on processes in water-sediment interface construction – screening water-sediment test (WST). The WST provides testing conditions (presence of water-sediment interface) that are more complex than those in standardized screening tests, where only biodegradation performed by bacteria suspended in the water phase is taken into account. An indirect method for the evaluation of compound mineralization, adopted from biodegradation screening tests, makes WST less laborious than standardized simulation OECD tests where analytical methods validated for each compound and radiolabelling are required. The limitation for application of the WST test is the toxicity against inoculum of the tested compound. However, the inclusion of a 'toxicity control' allows excluding false negative results. The WST is also not suited for volatile and low water soluble compounds. Further advantages of WST (**article VI**) were achieved by integration of a 'sterile control' into the test system. This, together with screening the water phase for the test substances e.g., by HPLC, gives a good indication of possible sorption and other non-biotic processes of the tested compounds. Consequently, the WST can be a good starting point for further investigations of sorption and desorption in different sediment compartments. WST can be applied for the first screening of the substance behavior in water-sediment interface and is a good starting point providing the information that allows planning the direction of further research especially in combination with *in silico* tools.

Zussamenfassung

Der biologische Abbau von organischen Substanzen spielt eine bedeutende Rolle und ist einer der erstrebenswertesten Prozesse in der Umwelt, vor allem, da die bei diesen Bio-Reaktionen freigesetzten Produkte insbesondere bei vollständiger Mineralisierung wieder in die natürlichen Elementzyklen reassimiliert werden können. Um die biologische Abbaubarkeit zu prüfen, wurde eine Vielzahl von biologischen Testverfahren von mehreren internationalen Organisationen entwickelt. OECD-Prüfrichtlinien für die biologische Abbaubarkeitsprüfung stellen eine der bekanntesten Gruppe von international eingesetzten biologischen Abbautests dar (301A-F). Diese Tests sind in der Regel sehr einfach in ihren Designs und ermöglichen das schnelle und kostengünstige Screening der biologischen Abbaubarkeit. Aufgrund ihrer Stringenz entsprechen die Testbedingungen nicht der Realität von Umgebungsbedingungen und können zu unvollkommenen, fehlerhaften (unrealistischen) Ergebnissen führen. Um diese Einschränkungen zu überwinden, führte die OECD Simulationstests ein, die das Verhalten von Chemikalien in bestimmten umweltrelevanten Kompartimenten untersuchen sollen. Trotz der Tatsache, dass Simulationstests mehr Einblicke in das Verhalten von Chemikalien in der Umwelt geben, werden sie nicht häufig angewendet, da sie oft langwierig, zeitaufwändig und teuer sind. Folglich besteht die Notwendigkeit, ein neues biologisches Testverfahren bereitzustellen, das komplexe Testumgebungen, wie bei Simulationstests, mit der Einfachheit in der Handhabung und der guten Datenwiederholbarkeit kombiniert, wie es bei Screeningtests für biologische Abbauarbeit gegeben ist. Eine weitere Herausforderung istdie Anpassung der bestehenden biologischen Abbauprüfmethoden an neue Probenarten, d.h. Mischungen von Transformationsprodukten (TPs) und die Bereitstellung neuer effizienterer Methoden.

Die Forschung über das Vorkommen von Arzneimitteln in der Umwelt hat seit den 1990er Jahren eine zunehmende Dynamik. Ihre Präsenz in der Umwelt ist eine allgemein anerkannte Tatsache. In vielen Umweltkompartimenten wie Oberflächengewässern, Böden, Sedimenten oder Grundwässern wird kontinuierlich eine breite Palette von Arzneimitteln nachgewiesen. Nachdem Arzneimittel die natürliche aquatische Umwelt erreichen, können sie eine Reihe von Prozessen wie Photolyse (unter direktem Sonnenlicht), Hydrolyse, Oxidations- und Reduktionsreaktionen, Sorption, biologischer Abbau (durch Mikroorganismen) und Bioakkumulation durchlaufen. Diese Prozesse können die vollständige Eliminierung von Chemikalien und Arzneimitteln aus der aquatischen Umgebung bewerkstelligen, aber auch zu neuen Verbindungen wie TPsführen. Darüber hinaus können durch Chlorierung und

fortgeschrittene Oxidationsprozesse wie Behandlung mit H_2O_2/UV, O_3/UV, TiO_2/UV, die Fenton- und Photo-Fentonreaktion oder UV-Bestrahlung, die in der Wasser- oder Abwasserbehandlung angewendet werden, TPs entstehen, die wiederrum in die aquatische Umwelt gelangen. Die Herausforderungen in der Forschung bezüglich dieser TPs sind nicht nur die Entwicklung empfindlicher und zuverlässiger analytischer Nachweis- und Strukturaufklärungsmethoden, sondern auch die Etablierung geeigneter Assays für die Untersuchung der Eigenschaften dieser neuen Verbindungen. Es stellt sichvor allem die Frage, ob die TPs ein höheres Risiko darstellen als die Muttersubstanzen (Ausgangsprodukte). Unter zahlreichen Gruppen von Arzneimitteln scheinen zwei von großer Bedeutung zu sein: Antibiotika, da sie die Entstehung und Erhaltung der antimikrobiellen Resistenz in der aquatischen Umwelt fördern können sowie Zytostatika, welche karzinogene, mutagene und / oder teratogene Effekte bei Versuchstieren und Menschen haben.

Die Anforderungen der in dieser Arbeit vorgestellten biologischen Abbauprüfungen umfassen verschiedene Bereiche. Dementsprechend hatte die vorgelegte Arbeit drei Ziele: 1) Ermittlung der Wissenslücken zu Antibiotika und Zytostatika (Artikel I), 2) Erforschung des biologischen Abbaus von Zytostatika und deren TPs (Artikel II, III und IV) und 3) Etablierung eines biologischen Abbautestverfahrens mit Annäherung an reale Bedingungen, das eine kostengünstige Alternative darstellt und ein besseres Verständnis für die Wechselwirkungen von Substanzen zwischen Wasser- und Sedimentphasen erlaubt und Einblicke in das Sorptions- und Desorptionsverhalten von Prüfsubstanzen bietet (Artikel V und VI).

Der Artikel I konzentriert sich auf die Identifizierung von Wissenslücken und Daten von zwei Gruppen von Arzneimitteln: Antibiotika und Zytostatika. Es wurde eine Literaturrecherche über Muttersubstanzen und TPs durchgeführt. 21 ausgewählte Antibiotika und Zytostatika wurden betrachtet. Informationen, die über TPs verfügbar sind, zeigen, dass eine bereits geringfügige Änderung der Behandlungsbedingungen und Prozesse zur Bildung von verschiedenen TPs führt. Dies wiederum macht es einerseits schwierig, die richtigen Bedingungen für die Abwasserbehandlung auszuwählen und setzt gleichzeitig voraus, TPs vor der Behandlung zu indentifizieren und zu bewerten, was Sowohl aus wissenschaftlicher als auch aus technischer, behandlungsbezogener Sicht insbesondere bei schwer interpretierbaren Daten und nicht beurteilbaren Stoffen ein nahezu unlösbares Unterfangen darstellt. Daher ist aus praktischer und nachhaltiger Sicht die Begrenzung des Eintrags von Arzneimitteln und deren TPs in die Umwelt genauso erstrebenswert, wie die Zielsetzung während des

Arzneimitteldesigns, die Moleküle bezüglich ihrer (Bio-) Abbaubarkeit und ihres Eliminierungsverhaltens zu optimieren. Dies entspricht dem Gedanken von "benign by design".. Lösungen, die sich auf diesen "beginning of the pipe"-Ansatz konzentrieren, sollten die nachteiligen Wirkungen der Muttersubstanzen und ihrer TPs minimieren, indem sie letztendlich ihren Eintritt und Verbleib in die natürliche Umgebung reduzieren.

Die Artikel II, III und IV befassen sich mit der Erforschung des biologischen Abbaus von Zytostatika und deren TPs. Die Bioabbautests wurden gemäß der OECD 301D-Richtlinie (CBT) und die Toxizitätassays unter Verwendung des Lumineszenzbakteriums Vibrio fischeri durchgeführt. TPs wurden getestet, wenn während der Photoabbaustudien (UV-Lampe, Xenonlampe, UV/H_2O_2, UV/Fe^{2+}/H_2O_2 und UV/TiO_2) die beobachtete Mineralisierung weniger als 70% betrug. Die experimentellen Daten aus der vorgestellten Studie bestätigen, dass nicht alle Photodegradationsbehandlungen in der Lage sind, Zytostatika vollständig zu entfernen. Darüber hinaus konnte gezeigt werden, dass es schwierig ist zu prognostizieren, welche TPs entstehen und welche Eigenschaften und Wirkungen sie in in der Umwelt haben werden.

Die Artikel V und VI beschreiben die Entwicklung und Anwendung eines neuen biologischen Abbautestverfahrens, den Screening-Wasser-Sediment-Test (WST), der näher an Umweltbedingungen angelehnt ist als einfache genormte Tests, aber eine preiswertere Alternative mit geringerem Arbeitsaufwand im Vergleich zu herkömmlichen Simulationstests darstellt und gleichzeitig ein besseres Verständnis für Prozesse an der Wasser-Sediment-Schnittstelle ermöglicht. Der WST liefert Testbedingungen (Vorhandensein von Wasser-Sediment-Grenzflächen), die komplexer sind als bei standardisierten Screening-Tests, bei denen nur der biologische Abbau der gelösten Substanz durch die in der Wasserphase suspendierten Bakterien berücksichtigt wird. Eine indirekte Methode zur Bewertung der Mineralisierung, die aus Screening-Tests zur biologischen Abbaubarkeit übernommen wurde, macht den WST weniger aufwendig als Simulations-OECD-Tests, bei denen analytische Methoden und radioaktiv markierte Verbindungen benötigt werden. Über den Endpunkt Sauerstoffverbrauch sind Aussagen zur Mineralisierung der Testsubstanzen mit Hilfe eines Summenparameters möglich. Es kann aber auch eine stoffspezifische Analytik wie bei den OECD Simulationstests durchgeführt werden. Wie bei allen Tests, die die biologische Abbaubarkeit prüfen, ist es auch beim WST-Test nicht möglich, Substanzen zu prüfen, die gegenüber dem Inokulum hemmend oder toxisch wirken. Die Einbeziehung einer "Toxizitätskontrolle" erlaubt jedoch, falsch negative Ergebnisse auszuschließen. Der WST eignet sich für flüchtige, wasserlösliche und schwer wasserlösliche Verbindungen. Ein weiterer Vorteil ist die Einführung einer „Sterilkontrolle“ (Artikel VI). Diese ermöglicht in Kombination mit dem Screening von Testsubstanzen bzw. deren TPs in der Wasserphase und ggf. im Sediment, z. B. durch HPLC, Hinweise auf eine mögliche Sorption und andere nicht-biotische Prozesse zur Elimination der getesteten Verbindungen. Folglich kann der WST ein guter Ausgangspunkt für weitere Untersuchungen von Sorption und Desorption in verschiedenen Sedimentkomponenten sein. Insgesamt kann der WST für das erste Screening des Stoffverhaltens in der Wasser-Sediment-Grenzfläche gut angewendet werden und ist ein guter Ausgangspunkt für die Planung in welche Richtung die weiteren Forschungen (auch in Kombination mit in silico Methoden) gehen sollen.

1. Introduction and motivation

1.1. Biodegradability Testing

Among all of attenuation processes, biodegradation plays one of the most important role and is one of the most desirable processes in the environment especially since products released during this bio-reactions, can often be once again reintroduced into the natural element cycles especially in the case of complete mineralization (Cowan et al., 1996). Chemicals that are characterized as being biodegradable are broken down into smaller non-toxic compounds by bacteria or fungi during their growth; in the best case they are fully mineralized (i.e., transformed to inorganic compound and/or used for biomass production). The biodegradability and rate of biodegradation of chemicals are important for the estimation of parameters such as exposure to aquatic organisms, persistency, toxicity, bioaccumulation, etc. (Blok and Booy, 1984; Raymond et al., 2001; OECD, 2006). The rate and to the what extent a substance is biodegraded is also an important information used in Quantitative Structure Activity Relationships (QSAR) models, for predicting the properties of chemical compounds (Cowan et al., 1996; Rücker and Kümmerer, 2012).

To assess biodegradation, a variety of biodegradation test procedures have been developed by several international organizations, for instance the Organization of Economic Co-operation and Development (OECD), or International Organization for Standardizations (ISO) standards. Among them, OECD guidelines for ready biodegradability testing represent the most prominent group of internationally used screening biodegradation tests (301A-F) (OECD, 2006; Guhl and Steber, 2006; Strotmann et al., 1995). All these ready biodegradability screening tests are very simple in their designs and they use indirect measurement of the elimination of test compounds e.g., decrease of oxygen, carbon dioxide evolution or pressure change. That allows for the fast and cheap screening of biodegradability (OECD, 2006). However, because of their stringency, the test conditions are not close to simulating environmental conditions and may lead to unrealistic results. Therefore the results of these tests should be considered as guiding only (i.e., they allow only for the classification of the compound as ready/ not ready biodegradable, but not to fully predict its behavior in natural environment) (Cowan et al., 1996; Alexander, 1985).

The simulation tests described in the OECD guidelines are designed to overcome these limitations since they allow to investigate the behavior of chemicals in specified environmentally relevant compartments e.g., soil (OECD 307), sediment (OECD 308), aerobic sewage system (OECD 303A). Furthermore, the concentrations used in these tests should be in the same range as found in the environment. Despite the fact that simulation tests give more insight into fate of chemicals in the environment, they are not applied frequently as they are often tedious, time consuming and expensive. One of the reasons for that might be that it involves working with radiolabeled substances, which requires specific apparatus, equipment, knowledge, and safety measures as well as the safe disposal of radioactive waste. One of such a simulation tests, described in the OECD 308 guideline (OECD, 2002), is designed to investigate aerobic and anaerobic transformation of chemicals in aquatic sediment systems.

In most of the standard biodegradation tests, the source of inoculum is the bacterial mixture taken usually directly from surface waters or STPs effluents. So complex biological communities, in combination with the indirect measurement of biodegradation processes, makes proper interpretation of biodegradation tests results challenging. Especially, when we interpret negative results as compounds possible influence on metabolism, community structure or reproduction of microorganisms, or even all the above. This interpretation is even more complicated, when a sample consists not only of parent compound, but additionally comprises a mixture of compounds' transformation products (TPs).

1.2. Pharmaceuticals in the Environment

The research on the presence of pharmaceuticals in the environment gained momentum in the 1990s; since then, it has been growing. Their presence in the environment is a well-established fact. A wide range of pharmaceuticals is continuously detected in many environmental compartments such as surface waters (Ashton et al., 2004; Bendz et al., 2005; Hilton and Thomas, 2003; Hirsch, 1999; Mompelat et al., 2009; Ternes, 1998), soils (Ho et al., 2014; Aznar et al., 2014; Song et al., 2010), sediments (Kim and Carlson, 2007; Chen et al., 2013; Lahti and Oikari, 2012), or ground waters (Barnes et al., 2008; Hirsch, 1999). They may enter these environmental compartments via different pathways. Main sources of pharmaceuticals in the aquatic environment are STPs' effluent, manufacturing plants, household waste (inappropriate disposal), landfill effluent, aquaculture, or the use of contaminated sludge or manure in agriculture (Kümmerer, 2008).

Many pharmaceuticals related to the human medicine, may undergo only incomplete resorption and metabolism within the body and therefore enter the raw sewage in their active forms or as metabolites (Andreozzi et al., 2003). Additionally, the inappropriate disposal of out-of-date or unwanted drugs is also source of pharmaceuticals in raw sewage (Kümmerer, 2008). After reaching the STP, some of the pharmaceuticals might be biodegraded by activated sludge or eliminated by sorption. However, since STPs are not designed to eliminate this class of contaminants, part of them remains unchanged and reaches aquatic environment through effluent (Fatta-Kassinos et al., 2011a; Roberts and Thomas, 2006; Heberer, 2002; Yu et al., 2006). Therefore, STPs are the main point sources of human pharmaceuticals in the aquatic environment. Because of limited biodegradability and a constant release into the environment, many pharmaceuticals can be regarded as persistent or at least pseudopersistent (Kümmerer, 2008).

After pharmaceuticals reach the natural aquatic environment they may undergo number of processes such as: photolysis (under direct sunlight), hydrolysis, oxidation and reduction reactions, sorption, biodegradation (by bacteria of fungi), and bioaccumulation. These processes may cause their elimination from aquatic environment, if reaction is complete, or creation of new compounds i.e., TPs (Kümmerer, 2008).

Aforementioned reactions are not the only sources of pharmaceuticals' TPs in the aquatic environment. Technical processes, like chlorination and advanced oxidation processes (AOPs), such as treatment with H_2O_2/UV, O_3/UV, TiO_2/UV, Fenton, and photo-Fenton, are under discussion for the treatment of STP effluents in order to improve the removal rate of micro-pollutants such as pharmaceuticals. Since all of these processes often do not completely mineralize chemicals they can consequently produce TPs (Zwiener, 2007; Kosjek and Heath, 2008; Radjenović et al., 2009; Loos et al., 2013; Magdeburg et al., 2014; Richardson and Ternes, 2014; Trautwein et al., 2014). Another treatment, which may lead to the TPs introduction into aquatic environment, is UV treatment. It is applied in water treatment in order to disinfect effluents or drinking water. Water treatment processes such as oxidation processes are applied, especially for the removal of micro-pollutants from drinking water, which cause odor or color, as well as for disinfection.

Numerous studies, that address the issue of pharmaceuticals' TPs, have been published (e.g. Dodd and Huang, 2004; Calza et al., 2008; DellaGreca et al., 2007; Dewitte et al., 2008; Fernández et al., 2010; Khan et al., 2010; Prieto et al., 2011; Terzic et al., 2011; Vasconcelos et al., 2009). The research on TPs brings many new challenges. From one side, there is a constant need for the development of a sensitive and reliable analytical separation, detection, and structure elucidation methods. Additionally, there is a need for the preparation of appropriate assays for the investigation of properties of new compounds, especially those answering the question if TPs pose a higher risk to the aquatic ecosystems than their parent compounds (Kosjek and Heath, 2008; Pérez and Barceló, 2007; Fatta-Kassinos et al., 2011b).

Two groups of the pharmaceuticals are of special importance: antibiotics and cytostatic drugs. Researchers are interested in antibiotics not only because of their extensive 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. These drugs have a specific mode of action (blocking the growth of cancer cells by influencing the cell cycle), and can exert carcinogenic, mutagenic and/or teratogenic effects in experimental animals and humans (Allwood et al., 2002; Eitel et al., 2000; Kümmerer et al., 2016).

Biodegradation testing of chemicals including pharmaceuticals and their TPs is a new challenge. First of all, there is a need to provide a new biodegradation testing method that would combine complex testing environment as in simulation tests, easiness in handling and good data repeatability as in screening biodegradation tests. Another challenge is the need for an adaption of the existing biodegradation testing methods to new types of samples and compounds including mixtures of TPs and providing new, more efficient testing methods of above mixtures.

2. Aims and objectives

The challenge of biodegradation testing presented in this thesis, encompasses different areas and was divided into three objectives:

1) Identification of the knowledge gaps and data distribution of the two groups of pharmaceuticals i.e., antibiotics and cytostatic drugs (article I).

2) Increasing the knowledge on biodegradation of cytostatic drugs and their TPs (articles II, III, and IV).

3) Development, validation and establishment of a biodegradation test with closer to simulation tests conditions that would be affordable and would allow a better understanding of processes in water sediment interfaces – screening water-sediment test. Extended validation of the test with an insight into sorption and desorption processes (article V and VI).

3. Research approach

Objective 1: Identification of the knowledge gaps and data distribution of the two groups of pharmaceuticals antibiotics and cytostatic drugs (article I).

The scientific literature was researched 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). A literature research was conducted on data about parent compounds and TPs. In total 21 pharmaceuticals from two groups namely antibiotics and cytostatic drugs were selected based on available knowledge in terms of potential effects, occurrence, persistence, and consumption. 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. Studies were considered that are related to lab-scale experiments (spiked synthetic/real water), and in which samples were collected from the aquatic environment or from full-scale waste water treatment plants (WWTPs). However, human and mammal metabolites that were formed by metabolization of the compounds in target organisms were not considered in the data compilation.

Objective 2: Increasing the knowledge on biodegradation of cytostatic drugs and their TPs (articles II, III, and IV).

To fill the research gaps on cytostatic drugs and their TPs, the following testing design was applied. First the photodegradation studies were conducted. The applied processes were: UV-Lamp, Xenon Lamp, UV/H_2O_2, UV/Fe/H_2O_2, and UV/TiO_2 photodegradations. Compounds tested were: Cyclophosphamide (CYC), 5-Fluorouracil (5-FU), and Methotrexate (MTX). Toxicity and biodegradation of the parent compound were tested. Additionally, if during the photodegradation studies, observed mineralization was <70%, these samples were tested further for their biodegradation and toxicity (Figure 1). Biodegradation testing was conducted according to OECD 301D guideline (Closed Bottle Test (CBT)) and toxicity evaluation with assays using the luminescent bacteria *Vibrio fischeri*. Additionally, in the samples the NPOC (non-purchable organic carbon) was measured and analytical evaluation by HPLC or LC-MS was performed.

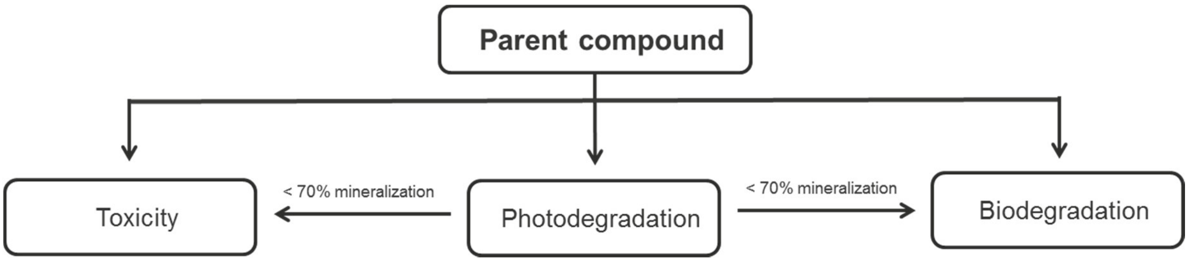

Figure 1: Experimental design of TPs analysis (article II, III, IV).

Objective 3: Establishment of a new biodegradation test – screening water-sediment test (WST). Further validation of the test with an insight into sorption and desorption processes (articles V and VI).

The aim of this research was to establish an aerobic screening water sediment test (WST) that will be characterized by good reproducibility. The increased reproducibility was achieved by creating an artificial and standardized medium based on the existing OECD guidelines. Inoculum was prepared from mixed sources of surface water and sediment as well as effluent from sewage treatment plant (OECD guideline 302C (OECD, 1981)). Since the low bacterial density was suspected in WST the mineral medium composition, which contained of mineral salts, trace elements, and growth factors, was based on the OECD guideline 301D (OECD, 1992) where low bacterial density is used. Artificial sediment (OECD guideline 218 (OECD, 2004)) was composed of kaolin clay (5% dry weight), quartz sand (grain size 0.8 - 0.2 mm; 92% dry weight), peat (2% dry weight), and calcium carbonate (<0.1% dry weight). What is more, an additional series of test vessels (toxicity control and sterile control) were introduced to improve the interpretation of the data. Moreover, in this test no radiolabeled test compounds were used and the degradation measurement was focused on an indirect method (pressure change in the closed vessels) which is cheap and simple, i.e. the measurement of a pressure difference with the OxiTop® system (WTW, 2006).

Two pre-tests were conducted to optimize the test design. Because the indirect measurement method of compound mineralization (i.e., change in pressure of the head space) was used in the WST, the first pre-test was conducted to determine the optimum theoretical oxygen demand (ThOD) with minimum background influence, for the given test conditions. Secondly for the choice of the optimum quality control, biodegradation of three well biodegradable compounds was investigated, namely: Aniline, diethylene glycol (DEG), and sodium acetate.

To further validate the WST and its possible applications biodegradation of two pharmaceuticals, namely acetaminophen and ciprofloxacin, in the WST was investigated. Ciprofloxacin was expected to be not biodegradable but influenced by sorption, therefore removal from water phase was investigated. For that, water samples were collected with a syringe through sample port, at days 0, 1 and 28. The samples were stored at -20 °C until they were measured by the HPLC-fluorescence detection (FDA) (Shimadzu Prominence HPLC system; Duisburg, Germany).

Further evaluation of WST with an insight into sorption and desorption processes.

In order to gain more insight in processes that take place during WST and expand the possible knowledge gain (beside biodegradation processes), an accompanying battery of tests was applied to WST in order to investigate the sorption and desorption possibilities of micro-pollutants. Because of the test design, processes such as biodegradation, sorption, elimination from water phase, and abiotic degradation can be investigated in this test simultaneously.

Furthermore, a pesticide S-metolachlor and its commercial product Mercantor Gold® (86.5% w/w) was used in this test in order to extend the possible application of WST. Pesticides and their TPs are next to the pharmaceuticals an important group of micro-pollutants that may cause unwanted effects on non-target organisms such as humans, animals, other plants, and the environment due to their toxicity and their bioaccumulation potential or just their unwanted presence in the environment. The direct application on fields and possible entering surface waters by wash-off from plants or during surface runoffs makes these compounds suitable for WST.

This research focused on investigation of the impact of Mercantor Gold®'s adjuvants on the active substance fate in the simulated water-sediment system. For this purpose such a test as the WST is most suitable compared to simple biodegradation tests. Additionally, the biodegradability of photo-TPs of both S-metolachlor and Mercantor Gold® in the water sediment system using WST was investigated to evaluate the elimination of the parent compound, its photo-TPs and the possible generation of bio-TPs resulting from photo-TPs. Monitoring by HPLC-UV was applied to screen for accessible TPs and primary elimination of parent compounds and TPs. The structure elucidation of generated bio-TPs was accomplished using LC-UV-MS/MS.

In addition the dissipation of the S-metolachlor from water phase was monitored in each vessel of the test series (test, toxicity control, and sterile control) at days 0, 1, and 28. Samples were collected through sample port and analyzed by HPLC. Furthermore, extraction of S-metolachlor from sediment was performed by the use of extraction solution containing a mixture of acetonitrile (ACN) and water (9:1). In parallel, the extraction from sediment with 0.01 M $CaCl_2$ aqueous solution was performed to evaluate the strength of sorption of S-metolachlor onto sediment (OECD, 2000). Analysis of S-metolachlor and TPs was performed by HPLC-UV and LC-MS/MS Bruker Daltonic Esquire 6000 plus ion-trap mass spectrometer (IT-MS) equipped with the Bruker data analysis system (Bruker Daltonic GmbH, Bremen, Germany).

4. Results and discussion

Objective 1: Identification of the knowledge gaps and data distribution of the two groups of pharmaceuticals antibiotics and cytostatic drugs (article I).

In total, 294 TPs, identified with chemical structures in the literature published until 2012, 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. In general, structures of TPs were by far more often reported for antibiotics (275 TPs) than for anticancer drugs (19 TPs). 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 i.e. how “fashionable” the parent compound is in research and how easy to measure. 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 after isolation and enrichment of the target compound. 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, 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, sometimes even depending on the duration or other specific conditions of the treatment. 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; Rastogi et al., 2014b) should minimize the adverse effects of parent compounds by reducing formation of TPs and their entrance into the natural environment. The research also demonstrated that more data e.g. on biodegradation of the selected pharmaceuticals and additional tests for pharmaceuticals and chemicals are needed.

Objective 2: Increasing the knowledge on biodegradation of cytostatic drugs and their TPs (articles II, III, and IV).

Among all the applied treatment processes, UV/TiO_2 and UV/Fe/H_2O_2 were most efficient ones for the elimination of 5-FU and MTX. Since the threshold of <70% of mineralization was adopted, the biodegradation screening was conducted only for the samples after treatments: UV-Lamp and Xe-Lamp for CYC; UV-Lamp, Xe-Lamp, and UV/H_2O_2 for 5-FU and UV/H_2O_2 for MTX.

None of the parent compounds (CYC, MTX and 5-FU) could be classified as ready biodegradable. The classification of CYC as a non-readily biodegradable compound is in line with the results of previous CBT experiments (Kümmerer et al., 1996). Moreover, even in biodegradation experiments that simulate sewage treatments plants (which contain a much higher density and diversity of bacteria) and at concentrations ranging from low ng L^{-1} to a few hundred mg L^{-1}, CYC was not eliminated (Steger-Hartmann et al., 1997; Kiffmeyer et al., 1998; Buerge et al., 2006). MTX showed the highest level of biodegradation (44%) among tested compounds. Kiffmeyer et al. (1998), reported a 95% of MTX removal in conditions simulating sewage treatment plant. However MTX was classified as not readily biodegradable in a study performed with the 'manometric respiration test' OECD 301 F (Henschel et al., 1997). The biodegradation of MTX appears to be strongly dependent on the bacterial density and elimination could also be due to sorption to sludge.

The classification of 5-FU as not ready biodegradable is in accordance with the results of CBT by Kümmerer and Al-Ahmad (1997). On the other hand, in an OECD confirmatory test, Kiffmeyer et al., 1998 verified a full elimination of 5-FU within a few days. According to the authors, the biodegradation rate is dependent on the initial concentration, and the best results were obtained at the lowest 5-FU concentrations (i.e., 5 mg L^{-1}), which is still very high, compared to its concentration in sewage or the environment. The outcome of research carried out with activated sludge indicated a complete removal of 5-FU from the wastewater of an oncological ward (Mahnik et al., 2007). More recently, Kosjek et al., 2013 investigated the degradation of 5-FU in batch biodegradation experiments at a scale of 0.5 L (inoculum of activated sludge with initial concentration of 5.4 g L^{-1}). The authors found that 5-FU was completely eliminated at lower concentrations (1 and 10 mg L^{-1}), whereas a toxic effect occurred at higher concentrations (20 and 100 mg L^{-1}), retarding or even inhibiting the elimination. Yu et al., 2006 observed incomplete removal of 5-FU (<60%), even at lower concentrations (1 and 50 µg L^{-1}). Despite the results of some studies presented above, showing a fast elimination of 5-FU, it is noteworthy that all these tests were performed with higher bacterial densities (2.5 g L^{-1} dry mass in the OECD 303A and 5.4 g L^{-1} in the study performed by Kosjek et al. (2013)), whereas the CBT test is characterized by a low bacterial density (500 CFU mL^{-1}), which may explain the different outcomes. Moreover, the results of the Zahn-Wellens Test (Kümmerer and Al-Ahmad, 1997), which is also performed with a higher bacterial density (1 g L^{-1} dry mass), showed no biodegradation after 28 days. Although performing this test with a high initial concentration of 5-FU (854 mg L^{-1}), Kümmerer and Al-Ahmad (1997) observed no toxicity in the toxicity control, which excludes the hypothesis of a toxic effect that could occur at higher concentrations resulting in retarding or inhibiting, as suggested by some authors (Kosjek et al., 2013; Kiffmeyer et al., 1998). Likewise, no toxic effect was observed by the authors in the toxicity control of the CBT. Therefore, considering the above information, the low biodegradability of 5-FU in the CBT is likely associated with the low bacterial density.

Comparison between the biodegradation of parent compound and TPs mixture after the treatment processes showed that UV/H_2O_2 increased the biodegradability of formed TPs compared to the parent compound for 5-FU, however, slightly decreased biodegradation of TPs of MTX. Also UV-photo treatment improved the biodegradation of TPs’ mixture from

5-FU, whereas treatment with Xe-Lamp had no influence on improving the biodegradability of the above. This may be because of the different lamps (i.e. wavelengths/spectra and radiation energy) Since there was no elimination observed for CYC under UV and Xe-Lamp treatments, consequently, there was no difference in biodegradation level between samples before and after these treatments.

The increase in biodegradation of TPs usually followed the reduction in toxicity for all of the compounds. A significant reduction was observed for chronic luminescence inhibition and growth inhibition endpoints in luminescent bacteria *Vibrio fischeri* assays.

The results demonstrated that more knowledge on fate of parent compounds and TPs is desirable.

Objective 3: Establishment of a new biodegradation test – Screening water-sediment test (WST). Further validation of the test with an insight into sorption and desorption mechanisms (articles V and VI).

First of all, some of the important parameters were optimized for the best performance of the test. In the WST, the concentration of tested substance was adjusted so that by the use of indirect measurement it would be able to differentiate the mineralization of the compound from the high background degradation of the organic fraction (peat) in the artificial sediment. Aniline in all four concentrations achieved similar rates and levels of biodegradation; 64 ± 17%; 80 ± 4%; 62 ± 10%; 68 ± 84% for 40, 20, 10, and 4 mg L^{-1} respectively. Best values in regards to standard deviation were obtained at a test concentration of 20 mg L^{-1} aniline which corresponds to a ThOD of 48.2 mg L^{-1}. Optimum test substance concentration was therefore decided to be 40 mg L^{-1} in terms of ThOD. The reference compound was selected from the three proposed namely aniline, sodium acetate, and DEG. The ready biodegradability of aniline along with the clearly separated and structured biodegradation curve makes this compound the optimum choice for a reference compound in the WST. Artificial sediment content had been adjusted to improve the oxygen penetration into sediment. For that purpose the content of clay was reduced from 20 to 5%. This improved the water exchange between water and sediment phases and therefore exchange of oxygen. Moreover, to reduce the biological oxygen demand (BOD) of the sediment, and therefore high background readings, the organic fraction was decreased to 1% (2% of peat consisting of 50% of carbon). The cultivation time was decreased from one month to 10 days. The shortened cultivation period of inoculum used in the test, was applied in order to limit the decrease in bacterial diversity, reduce change of the inoculum, while still increasing the bacterial density and its adaptation to WST conditions.

To investigate possible discrepancy of inoculum performance, two WSTs with aniline, sodium acetate and DEG, were conducted one and half years apart and in two different seasons (summer and winter). As expected, there was no significant difference of biodegradation rate of sodium acetate since it is ready biodegradable compound. DEG and aniline also reached similar levels of biodegradation in both tests, with only slight difference in the adaptation time of inoculum expressed as lag phase. These results, gave a proof of a good reproducibility of conditions (especially inoculum) in WST regardless the season when experiments are performed.

Additionally, biodegradation of two pharmaceuticals, namely acetaminophen and ciprofloxacin was investigated for the possible application of the test.

Acetaminophen

The inoculum in the WST was in an optimum condition, as could be seen from ‘quality control’ which was biodegraded in 85 ± 14%. Acetaminophen was degraded to 60 ± 28%. Acetaminophen in tested at a concentration that was not toxic to inoculum with biodegradation level in ‘toxicity control’ of 67 ± 15%. Since the ‘Toxicity control calculated’ (sum of oxygen demand in the test and quality assay) and ‘toxicity control’ measured corresponded well to each other, it was concluded that degradation of both compounds occurred in parallel and was not impacted because bacterial toxicity. Therefore, the ‘Toxicity control’ proved to be a good tool to investigate bacterial inhibition by the tested substances. The potential of acetaminophen to be biodegraded is in agreement with literature data (Gusseme et al., 2011; Lin et al., 2010; Wu et al., 2012b). Since the biodegradation achieved in the WST was similar to those in simulation test systems it can be suggest that the WST is a good indicator system for screening under simulating environment biodegradation conditions.

Ciprofloxacin (CIP)

Similarly in this test series, the inoculum was in good condition since ‘quality control’ reached 66 ± 18% of degradation. CIP showed no biodegradability with a negative biodegradation curve that reached -46 ± 12% at day 28. The negative development of the biodegradation curve for CIP is a result of normalization of the test results by the ‘blank’ series, therefore this result should be considered as 0% degradation. Furthermore, low biodegradability in ‘toxicity control’ 5 ± 11% and correlation of toxicity controls between calculated and tested indicated that the test substance has inhibited the bacterial activity during the WST. These results are in agreement with literature data (Al-Ahmad et al., 1999; Girardi et al., 2011).

The comparison of the substance removal from water phase between test and sterile controls performed by analytical screening of compound concentration in water phase, gave more information on the influence of the abiotic processes e.g., sorption or hydrolysis of CIP. CIP concentration in the water phase during the test demonstrated that CIP was partially removed from water phase in all three test series after 8 h and almost completely removed from the water phase at the end of the test. That indicates that sorption will be the main removal process of CIP from aquatic environment. A more rapid CIP elimination was obtained in the sterile control which could be explained by the zwitterionic nature of CIP. Decreasing the pH value by sodium azide promoted the share of cationic form of CIP and increased sorption effects. Numerous studies showed CIP having a strong potential to be adsorbed on clay (Li et al., 2011; Wu et al., 2012a; Wang et al., 2011) and peat (humic substances) (Aristilde and Sposito, 2010).

Further evaluation of WST with an insight into sorption and desorption mechanisms was investigated using a pesticide S-metolachlor and its commercial formulation Mercantor Gold® that contains adjuvants which could impact biodegradation and sorption.

Behavior of pure S-metolachlor, Mercantor Gold®, and their photo-TPs in the WST.

The inoculum was in very good condition since the biodegradation of sodium acetate in the quality control reached 75 ± 9%. The results demonstrate for S-metolachlor no biodegradability (6 ± 14%) likewise the photolysis mixture of S-metolachlor after 8h of irradiation (-10 ± 8%). The negative development of the S-metolachlor biodegradation was a result of normalization of the test results by blank series and should therefore be considered as 0%. No toxic effects on bacteria (biodegradation in toxicity control > 25%) were observed by any tested substance in the toxicity control (biodegradation level in 'toxicity control' of 37 ± 9% for S-metolachlor and 37 ± 7% for the photolysis mixture). Moreover, in both cases, calculated toxicity controls (sum of oxygen demand in the test and quality assay) and toxicity controls measured corresponded well to each other, concluding that degradation of sodium acetate was not inhibited by the test compound.

For Mercantor Gold® in WST the inoculum was of very good activity (quality control was biodegraded in 79 ± 9%, n = 2). No difference was observed between biodegradation of

Mercantor Gold® and its photolysis mixture. The biodegradability of Mercantor Gold® reached 11 ± 16% and photolysis mixture 11 ± 6%. Both, Mercantor Gold® and its photo-TPs were not toxic to the test bacteria as biodegradation in toxicity control reached 44 ± 7% and 48 ± 10%, respectively. Additionally, calculated toxicity control corresponded well to toxicity control obtained, concluding that degradation of both (sodium acetate and Mercantor Gold®) occurred in parallel and was not inhibited. The slightly higher degradation rates of Mercantor Gold® compared with the pure S-metolachlor and their photolysis mixtures could be within measurement error and therefore were not significant, especially when biodegradation was evaluated based on indirect measurements such as monitoring pressure. Gutowski et al. (2015) compared biodegradability of S-metolachlor and S-metolachlor in Mercantor Gold® in Closed Bottle test and Manometric Respiratory test and neither biodegradability nor a difference between commercial formulation and the pesticide alone was found. The difference between above mentioned and current study was the presence of a sediment layer and inoculum used. The inoculum in WST was a mixture of microbial communities from natural water bodies and secondary effluent from sewage treatment plant whereas for the other tests only inoculum from secondary effluent of sewage treatment plant was used. Both factors can impact the result.

The photo-TPs were also resistant to the biodegradation in the WST. Although, both substances and their photolysis mixtures were not degraded it is worthy to mention that there was always a small difference in biodegradation on favor of the commercial product. This finding might be interpreted as the influence of the adjuvants on the biodegradation process or their biodegradability. These adjuvants, which were in fact a mixture of hydrocarbons and the surfactant dodecylbenzenesulfonic acid calcium salt, could have contributed to biodegradation in a low extent (Khleifat, 2006; Scott and Jones, 2000). Dodecylbenzenesulfonic acid is known to be biodegradable in the environment by anaerobic and aerobic bacteria (Denger and Cook, 1999; Manousaki et al., 2004). This is in agreement with the results of Katagi (2006, 2008). In another study by Mersie et al. (2004), metolachlor was found to be partially degraded in a water-sediment test and formation of oxanilic acid and ethane sulfonic acid transformation products was reported. Moreover, these authors reported a decline in concentration of the above-mentioned TPs after 56 days of incubation, suggesting further transformation to new products. However, these metabolites were not found in our WST. The main reason might be due to different sediment used or another test design applied in our study.

Dissipation of the S-metolachlor from water phase.

HPLC-UV measurements revealed that S-metolachlor was similarly removed from the water phase in all three test series of the WST (test, toxicity and sterile controls). The compound was gradually dissipating, starting from the 1st day to reach 33.6% removal on 28th day. Likewise the pure compound, the S-metolachlor in Mercantor Gold® was continuously dissipating to reach higher removal of 52.8% on the 28th in all three test series. This result could be explained by one of the Mercantor Gold® adjuvants (dodecylbenzenesulfonic acid calcium salt), which is an anionic surfactant that will directly interact with the sediment. Katagi (2008) indicated that due to their structures, surfactants can determine the distribution properties of pesticides in soil and sediment systems (i.e. mobility, leaching, sorption etc.). Generally, this was confirmed by a study of Gonzalez et al. (2010), who reported increased soil mobility of organochlorine pesticides due to the presence of anionic surfactants (sodium dodecyl sulfate). Therefore S-metolachlor in Mercantor Gold® could easier permeate to sediment than pure S-metolachlor.

The WST allowed for tracking the elimination of the parent substance from the water phase which was probably due to its sorption onto sediment particles. The extraction of S-metolachlor was conducted in order to investigate whether S-metolachlor could be re-extracted from sediment taken from WST. This information is especially important, since desorption processes also play an important role in the fate and distribution of chemicals in the environment. For this purpose two different solvents were selected. One highly polar consisting of ACN/water (9:1) and as a second one a solution of 0.01 M $CaCl_2$ as suggested by OECD Test Guideline 106 (OECD, 2000). Recovery rate for S-metolachlor as pure compound and as constituent of and Mercantor Gold® with $CaCl_2$ was 55.4% and 50.0%, respectively. Extraction with ACN/water turned out to be more effective with recovery rates of 96.9% (S-metolachlor) and 93.1% (Mercantor Gold®). This might suggest that a part of S-metolachlor in both formulations was immobilized by the sediment under natural conditions. The nature of S-metolachlor sorption onto artificial sediment might differ depending on the individual fraction that it consists of: mineral, clay and organic. Thus, it might be assumed that S-metolachlor was sorbed to one of the three sediment components under natural conditions. Although the detailed sorption mechanisms of pesticides are not yet fully understood some authors proved that sorption of metolachlor increases with increased soil organic matter content (Gannon et al., 2013; Westra et al., 2015). Therefore only extraction with highly polar ACN allowed obtaining high recovery compared with $CaCl_2$ solution. One can say that in natural aquatic environment S-metolachlor might display similar behavior and could be partially immobilized in bottom sediments.

Determination of transformation products in WST.

The results from the WST after 28 days show that the photolysis product with the specific mass m/z 266.2 was eliminated and it reveals that two products with the m/z 264.2 (Rt 9.7 and 12.0 min) were formed. The formation of bio-TPs from photolysis mixtures of S-metolachlor in water was also demonstrated by Gutowski et al. (2015) using a Manometric Respiratory test, OECD 301F. Since, this study shows an identical degradation of photo-TPs with the m/z 266.2 and the formation of two new products with the m/z 264.2 (Rt 9.7 and 12.0 min), it confirms that WST delivers the similar conditions for biotic transformation as a Manometric Respiratory test (Gutowski et al., 2015). These observed follow up bio-TPs were found only in test and toxicity control series and not in the sterile control confirming their biological generation. Abiotic processes like hydrolysis or other non-biotic chemical transformations can be excluded to play a significant role in the parent compound and its photo-TPs fate in water.

The WST proved to be an appropriate tool for the first screening of a various substances behavior (pesticides, pharmaceuticals) in water-sediment interface and a good starting point providing the information that allows planning direction of further research. What is more, obtained results on biodegradation and sorption were in agreement with literature data for all of the tested compounds namely acetaminophen, ciprofloxacin and S-metolachlor. Additionally, false negative results, that are a result of the toxicity against inoculum of the tested compound, can be eliminated by the inclusion of a ‘toxicity control’. Integration of a ‘sterile control’ into the test system combined with screening the water phase for the test substances, gives a good indication of possible sorption and other non-biotic processes of the tested compounds. This all makes the WST a reliable tool for biodegradation and sorption investigation.

5. Concluding remarks and outlook

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 makes it, on the one hand, 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. There is 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 (article I).

Furthermore, the experimental data from the study on cytostatic drugs confirms that not all the treatments are suitable for each compound. What is more, it is difficult to predict, what properties TPs would have, and what would be the influence on environment once released (articles II, III and IV).

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, 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.

The WST (article V) provides testing conditions (presence of water-sediment interface) that are more complex than those in standardized screening tests, where only biodegradation performed by bacteria suspended in water phase is taken into account. An indirect method for the evaluation of compound mineralization, adopted from biodegradation screening tests, makes WST less laborious than standardized simulation OECD tests where analytical methods validated for each compound and radiolabelling are required. The limitation for application of the WST test is the toxicity against inoculum of the tested compound. However, the inclusion of a ‘toxicity control’ allows excluding false negative results. The WST is also not suited for volatile and low water soluble compounds.

Further advantages of WST (article VI) were achieved by integration of a ‘sterile control’ into the test system. This, together with screening the water phase for the test substances e.g., by HPLC, gives a good indication of possible sorption and other non-biotic processes of the tested compounds. Consequently, WST can be a good starting point for further investigations of sorption and desorption in different sediment compartments. WST can be applied for the first screening of the substance behavior in water-sediment interface and a good starting point providing the information that allows planning the direction of further research especially in combination with *in silico* tools.

Summarizing, the WST is more efficient than other screening tests, since it gives a better understanding of the environmental fate of organic compounds, which is essential to estimate its effects and risks for an environmental risk assessment, and at the same time affordable workload level. Future development of biodegradation in water-sediment interface should focus on investigation of chemicals behavior in anoxic conditions which are common to deep water bodies. Another direction of further research would be adjustment of WST conditions in order to test chemicals in lower, closer to those detected in the environment, concentrations.

Biodegradation and other tests are needed to get further insights in the fate of micro-pollutants into the aquatic environment. The work and tests performed and developed in this thesis do contribute to this. In the long run, however, even if we provide new and efficient testing methods it is still impossible to test every single compound and its TPs. Therefore there is a need to focus on the introduction of chemicals into the natural environment. Design of more environment friendly chemicals, limitation of the introduction of chemicals into environment e.g. by efficient elimination of these compounds via wastewater treatment plants; or peoples' education on proper usage is also important.

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List of publications

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Michael, I., Ines Vasquez, M., Hapeshi, E., Haddad, T., **Baginska, E.**, Kümmerer, K., Fatta-Kassinos, D., Metabolites and transformation products of pharmaceuticals in the environment as contaminants of emerging concern. Book chapter in: Transformation products of emerging contaminants in the environment: Analysis, processes, occurrence, effects and risks, Vol. 1 edited by Dimitra A. Lambropoulou, Leo M. L. Nollet, 01/2014: chapter 14: pages 413-457; John Wiley & Sons Ltd., ISBN: 978-1-118-33959-6

Contributions to international conferences

Poster presentations

Lamoree, M., Weiss, J., **Baginska, E**., Haddad, T., Leder, C., Vasconcelos, T., Roche, P., Boireau, V., Hebert, A., Roig, B., Mompelat, S., Kümmerer, K., Formation and structure elucidation of stable transformation products of pharmaceuticals in the water. Poster, SETAC North America 32nd Annual Meeting, Boston 2011

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Toolaram, A.P., **Baginska, E.**, Lutterbeck, C.A., Huynh, K.D., Schneider, M., Kümmerer, K., Environmental fate of Methotrexate: photodegradation, biodegradability and mutagenicity assessment. Pharmaceuticals in the environment: is there a problem? PHARMAS Conference, Nimes, 2013, June 3rd-4th France

Baginska, E., Lutterbeck, C.A., Kümmerer, K., Biodegradability of cytostatic drugs: Cyclophosphamide, 5-Fluorouracil and Methotrexate and their photo -transformation products resulting from treatment with UV and simulated sunlight radiation in Closed Bottle Test, Pharmaceuticals in the environment: is there a problem? PHARMAS Conference, Nimes, 2013, June 3rd-4th, France

Baginska, E., Gutowski, L., Haiss, A., Olsson, O., Kümmerer, K., Environmental fate of S-metolachlor in its pure form and as a part of commercial product - Mercantor Gold®: biodegradation and sorption onto sediment, SETAC North America 34th Annual Meeting, Nashville 2013

Publication list and scientific conference contribution

Article	Title	Authors	Author status	Weighting factor	Publication status	Conference contributions
1	Biodegradation screening of chemicals in an artificial matrix simulating the water-sediment interface	Ewelina Baginska; Annette Haiß; Klaus Kümmerer	Co-author with predominant contribution [Überwiegen der Anteil]	1.0	Chemosphere 2015; 119:1240–6 DOI: 10.1016/j.chemosphere.2014.09.103 (IF= 4.068 (5-year), Q1)	Pharmaceutical products in the Environment: Is there a problem? PHARMAS Conference, Nimes, 2013, June 3rd-4th, France
2	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	Tarek Haddad, Ewelina Baginska, Klaus Kümmerer	Co-author with equal contribution [Gleicher Anteil]	1.0	Water Research 2015; 72:75–126 DOI: 10.1016/j.watres.2014.12.042 (IF= 6.769 (5-year), Q1)	
3	Assessing the environmental fate of S-metolachlor, its commercial product Mercantor Gold® and their photoproducts using a water–sediment test and *in silico* methods	Lukasz Gutowski, Ewelina Baginska, Oliver Olsson, Christoph Leder, Klaus Kümmerer	Co-author with equal contribution [Gleicher Anteil]	1.0	Chemosphere 2015; 138:847–855 DOI: 10.1016/j.chemosphere.2015.08.013 (IF= 4.068 (5-year), Q1)	SETAC North America 34th Annual Meeting, Nashville; 17th-23rd November 2013
4	Removal of the anti-cancer drug methotrexate from water by advanced oxidation processes:	Carlos Alexandre Lutterbeck, Ewelina Baginska, Ênio Leandro Machado,	Co-author with important	0.5	Chemosphere 2015a; 141:290–6 DOI:	

	Aerobic biodegradation and toxicity studies after treatment	Klaus Kümmerer	contribution [Wichtiger Anteil]		10.1016/j.chemosphere.2015.07.069 (IF= 4.068 (5-year), Q1)	
5	Degradation of cyclophosphamide and 5-fluorouracil by UV and simulated sunlight treatments: Assessment of the enhancement of the biodegradability and toxicity	Carlos Alexandre Lutterbeck, Marcelo Luís Wilde, Ewelina Baginska, Christoph Leder, Ênio Leandro Machado, Klaus Kümmerer	Co-author with important contribution [Wichtiger Anteil]	0.5	Environmental Pollution 2016; 208:467–76 DOI: 10.1016/j.envpol.2015.10.016 (IF= 5.008 (5-year), Q1)	Pharmaceutical products in the Environment: Is there a problem? PHARMAS Conference, Nimes, 2013, June 3rd-4th, France
6	Degradation of 5-FU by means of advanced (photo)oxidation processes: UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2 – Comparison of transformation products, ready biodegradability and toxicity	Carlos Alexandre Lutterbeck, Marcelo Luís Wilde, Ewelina Baginska, Christoph Leder, Ênio Leandro Machado, Klaus Kümmerer Machado, Klaus Kümmerer	Co-author with important contribution [Wichtiger Anteil]	0.5	Science of The Total Environment 2015b;527-528:232–45 DOI: 10.1016/j.scitotenv.2015.04.111 (IF= 4.317 (5-year), Q1)	
			Total	4.5		

Declaration

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

Ewelina Baginska
Lüneburg, 7th June 2017

Appendices

Article I

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.

Haddad, T., **Baginska, E.**, Kümmerer, K., Water Research 2015;72:75–126. doi: 10.1016/j.watres.2014.12.042

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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.

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.

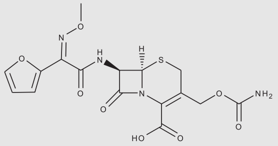
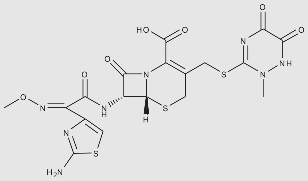
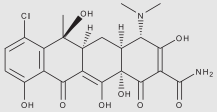
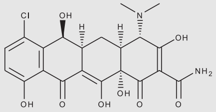
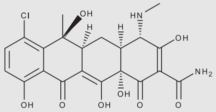
Recently, the advances in high-resolution mass spectrometry (HRMS) have enabled structure elucidation with a higher confidence because of high mass accuracy and resolution (Dewitte et al., 2008; Sirtori et al., 2010). Furthermore, HRMS can result in the detection and identification of TPs at lower concentrations, 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

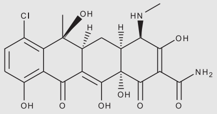
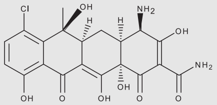
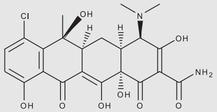
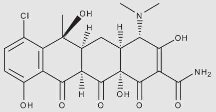
Structures and availability of TPs' registry numbers in chemical abstracts service (CAS-RNs) was reviewed for the selected pharmaceuticals (Table 2) in the scientific literature via the following databases: Scopus (www.scopus.com), Web of Knowledge (www.apps.webofknowledge.com), PubMed (www.ncbi.nlm.nih.gov), 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).

(continued on next page)

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 HPLC-ESI-QqQ-MS/MS UPLC-ESI-QqQ-MS/MS HPLC-ESI-QqQ-MS/MS UPLC-ESI-QqQ-MS/MS	Other Other Biodegradation Biodegradation Biodegradation	Chen et al. (2011). Sanderson et al. (2005). Shelver and Varel (2012). Kwon (2011). 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).

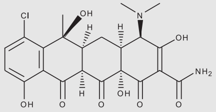
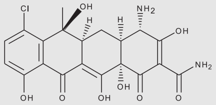
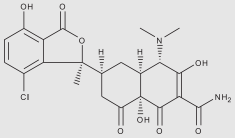
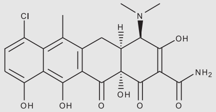
	189624-84-8 $C_{22}H_{23}ClN_2O_8$ 478.88 	HPLC-ESI-IT-MS/MS	Other	Chen et al. (2011).
	51262-22-7 $C_{20}H_{19}ClN_2O_8$ 450.83 	HPLC-ESI-IT-MS/MS	Other	Chen et al. (2011).
	514-53-4 $C_{22}H_{23}ClN_2O_8$ 478.88 	HPLC-ESI-IT-MS/MS; standard control UPLC-ESI-QqQ-MS/MS HPLC-ESI-QqQ-MS/MS HPLC-ESI-QqQ-MS/MS	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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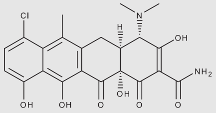
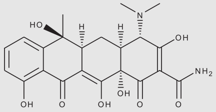
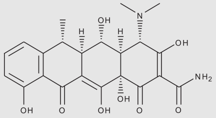
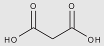
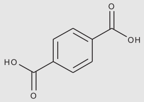
Table 3 – (*continued*)

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).

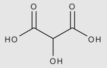
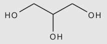
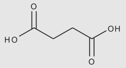
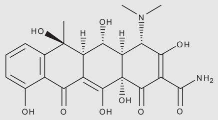
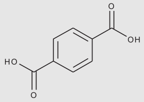
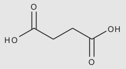
	80-69-3 $C_3H_4O_5$ 120.06 	GC–MS	AOPs	Yuan et al. (2011).
	56-81-5 $C_3H_8O_3$ 92.09 	GC–MS	AOPs	Yuan et al. (2011).
	110-15-6 $C_4H_6O_4$ 118.09 	GC–MS	AOPs	Yuan et al. (2011).
Oxytetracycline 79-57-2 $C_{22}H_{24}N_2O_9$ 460.43 	100-21-0 $C_8H_6O_4$ 166.13 	GC–MS	AOPs	Yuan et al. (2011).
	110-15-6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

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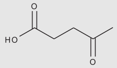
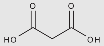
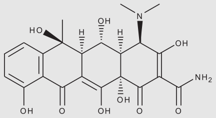
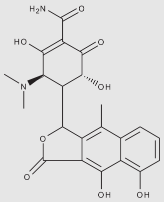
Table 3 – (*continued*)

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).

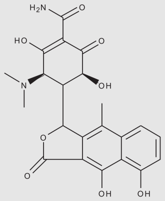
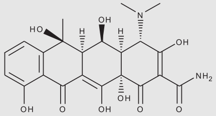
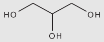
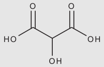
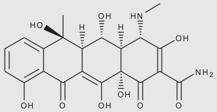
	18751-99-0 $C_{22}H_{22}N_2O_8$ 442.42 	HPLC-ESI-QqQ-MS/MS HPLC-ESI-MS; standard control	Other Other	Sanderson et al. (2005). Li et al. (2008).
	35259-39-3 $C_{22}H_{24}N_2O_9$ 460.43 	HPLC-ESI-QqQ-MS/MS	Other	Sanderson et al. (2005).
	56-81-5 $C_3H_8O_3$ 92.09 	HPLC-ESI-QToF-MS/MS	Photodegradation	Yuan 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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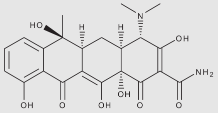
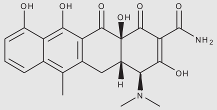
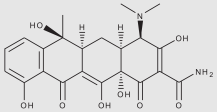
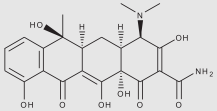
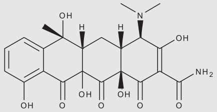
Table 3 – (*continued*)

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).

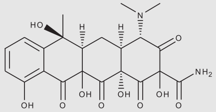
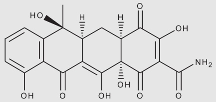
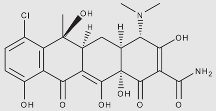
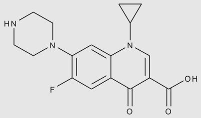
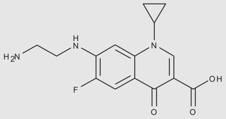
	937181-91-4 $C_{22}H_{24}N_2O_{10}$ 476.43 	HPLC-ESI-MS^n; HPLC-APCI-MS	AOPs	Dalmázio et al. (2007).
		HPLC-QqQ-MS/MS	AOPs	Khan et al. (2010).
	66241-98-3 $C_{20}H_{17}NO_9$ 415.35 	HPLC-QqQ-MS/MS	AOPs	Khan et al. (2010).
	57-62-5 $C_{22}H_{23}ClN_2O_8$ 478.88 	HPLC-APCI-MS/MS	AOPs	Wang et al. (2011b).
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	AOPs	An et al. (2010).
		HPLC-ESI-QToF-MS/MS	AOPs	Paul et al. (2010).
		HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
		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-ESI-MS/MS	AOPs	Sturini et al. (2012).
		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-IT-MS/MS	Photodegradation	Vasconcelos et al. (2009).
		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).

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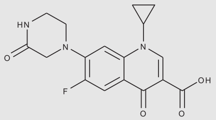
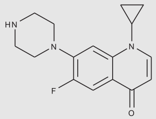
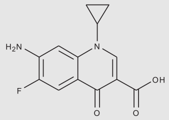
Table 3 – (*continued*)

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
		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).
	103237-52-1	HPLC-ESI-MS/MS	AOPs	Kugelmann et al. (2011).
	$C_{17}H_{16}FN_3O_4$	HPLC-EI-MS; GC–MS; H NMR	Photodegradation	Burhenne et al. (1997).
	345.33 	HPLC-ESI-MS/MS	Photodegradation	Sturini et al. (2012).
	105394-83-0	HPLC-UV-MS	Photodegradation	Cardoza et al. (2005).
	$C_{16}H_{18}FN_3O$	HPLC-ESI-MS/MS	Photodegradation	Vasconcelos et al. (2009).
	287.33	HPLC-ESI-MS	Photodegradation	Lester et al. (2011).
		HPLC-ESI-MS/MS	Photodegradation	Ge et al. (2010).
	105674-91-7 (TP263)[a]	HPLC-ESI-MS/MS	AOPs	An et al. (2010).
	$C_{13}H_{11}FN_2O_3$	HPLC-ESI-QToF-MS/MS	AOPs	Paul et al. (2010).
	262.24	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).

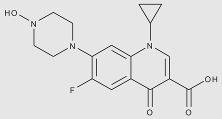
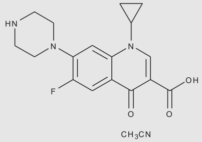
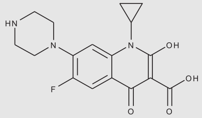
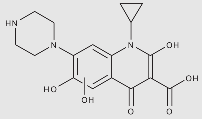
		HPLC-ESI-MS/MS	AOPs	Paul et al. (2007).
		HPLC-UV; MS; NMR; TLC; IR	Photodegradation	Torniainen et al. (1997b).
		HPLC-ESI-LTQ-Orbitrap-MS/MS	Biodegradation	Girardi et al. (2011).
	109142-50-9 $C_{17}H_{18}FN_3O_4$ 347.34 	HPLC-APCI-IT-MS/MS	Photodegradation	Ferdig et al. (2005).
	1219449-05-4 $C_{19}H_{21}FN_4O_3$ 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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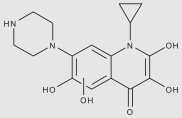
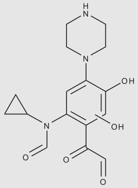
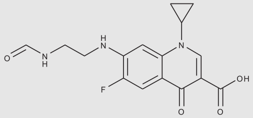
Table 3 – (*continued*)

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
	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).

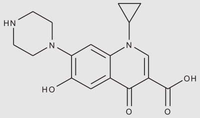
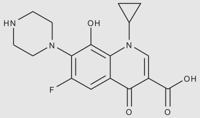
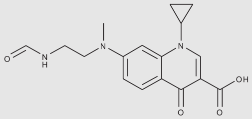
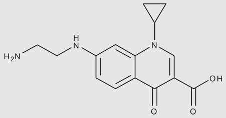
226903-07-7 (TP330)[a] $C_{17}H_{19}N_3O_4$ 329.35 	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	HPLC-ESI-MS/MS	AOPs/Photodegradation	Sturini et al. (2012).
	HPLC-UV-MS	Photodegradation	Cardoza et al. (2005).
	HPLC-ESI-MS/MS	Photodegradation	Turiel et al. (2005).
	HPLC-ESI-MS/MS	Photodegradation	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 $C_{17}H_{18}FN_3O_4$ 347.34 	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	HPLC-QToF-MS/MS; HPLC-QqQ-MS/MS	Biodegradation	Prieto et al. (2011).
	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 $C_{17}H_{19}N_3O_4$ 329.35 	HPLC-ESI-MS/MS	Photodegradation	Vasconcelos et al. (2009).
	HPLC-ESI-MS	Photodegradation	Lester et al. (2011).
438571-50-7 $C_{15}H_{17}N_3O_3$ 287.31 	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-ESI-MS	Photodegradation	Lester et al. (2011).
	HPLC-ESI-MS/MS	Photodegradation	Sturini et al. (2012).

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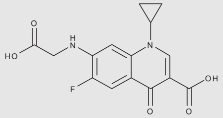
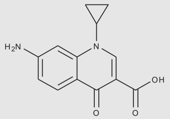
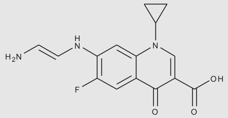
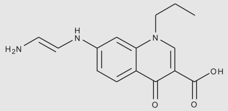
Table 3 – (*continued*)

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).

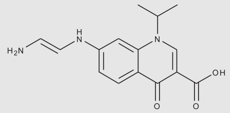
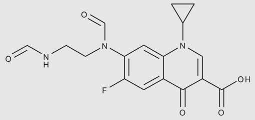
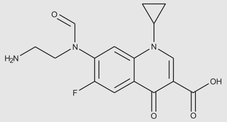
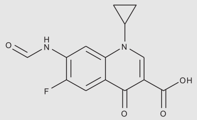
852546-82-8 $C_{15}H_{17}N_3O_3$ 287.31 	HPLC-ESI-MS/MS	Photodegradation	Turiel et al. (2005).
860033-21-2 $C_{17}H_{16}FN_3O_5$ 361.32 	HPLC-ESI-QToF-MS/MS HPLC-ESI-MS HPLC-ESI-MS	AOPs Chlorination Other	Paul et al. (2010). Wang et al. (2010). Zhang and Huang (2005).
860033-23-4 $C_{16}H_{16}FN_3O_4$ 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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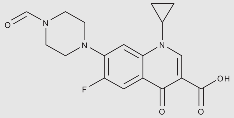
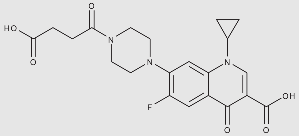
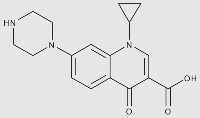
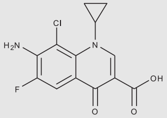
Table 3 – (*continued*)

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).

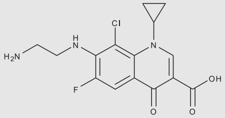
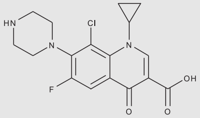
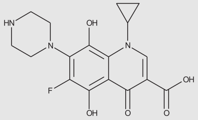
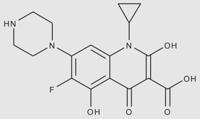
	1318139-22-8 $C_{15}H_{15}ClFN_3O_3$ 339.75 	HPLC-ESI-MS	Chlorination	Dodd et al. (2005).
	99696-22-7 $C_{17}H_{17}ClFN_3O_3$ 365.79 	HPLC-ESI-MS	Chlorination	Dodd et al. (2005).
	226903-13-5 $C_{17}H_{18}FN_3O_5$ 363.34 	HPLC-UV-ESI-MS; HRMS; H NMR	Biodegradation	Wetzstein et al. (1999).
	1373395-26-6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

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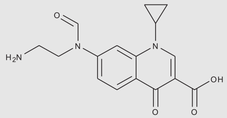
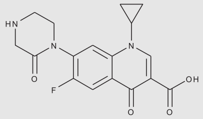
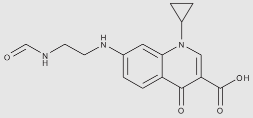
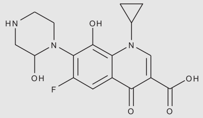
Table 3 – (*continued*)

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).

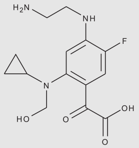
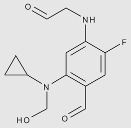
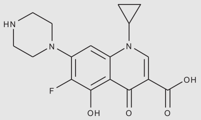
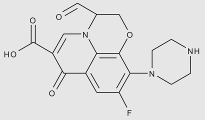
	1037620-98-6 $C_{14}H_{18}FN_3O_4$ 311.31 	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037620-97-5 $C_{13}H_{15}FN_2O_3$ 266.27 	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	124487-37-2 $C_{17}H_{18}FN_3O_4$ 347.34 	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037620-99-7 $C_{17}H_{16}FN_3O_5$ 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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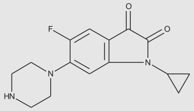
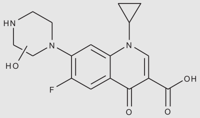
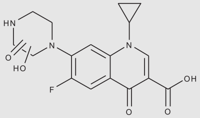
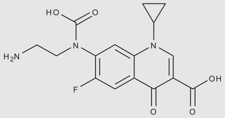
Table 3 – (*continued*)

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
	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).

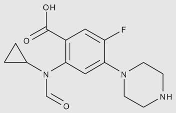
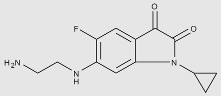
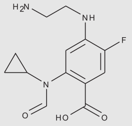
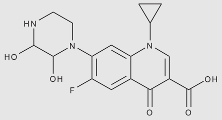
	1031767-27-7 $C_{15}H_{18}FN_3O_3$ 307.32 	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Dewitte et al. (2008).
	1031767-29-9 $C_{13}H_{14}FN_3O_2$ 263.27 	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Dewitte et al. (2008).
	1031767-31-3 $C_{13}H_{16}FN_3O_3$ 281.28 	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Dewitte et al. (2008).
	860033-20-1 $C_{17}H_{18}FN_3O_5$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

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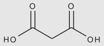
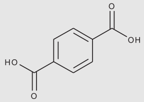
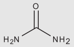
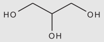
Table 3 – (*continued*)

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
	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).

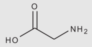
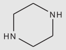
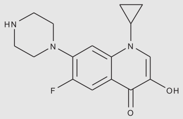
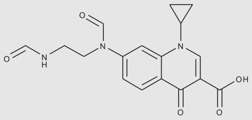
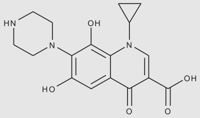
	56-40-6 $C_2H_5NO_2$ 75.07 	GC–MS	AOPs	Yuan et al. (2011).
	110-85-0 $C_4H_{10}N_2$ 86.14 	HPLC-UV-ESI-MS; HRMS; H NMR	Biodegradation	Wetzstein et al. (1999).
	226903-05-5 $C_{16}H_{18}FN_3O_2$ 303.33 	HPLC-ESI-MS/MS HPLC-UV-ESI-MS; HRMS; H NMR	AOPs Biodegradation	Kugelmann et al. (2011). Wetzstein et 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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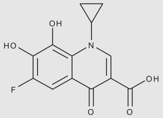
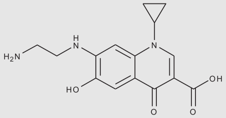
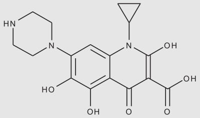
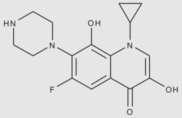
Table 3 – (*continued*)

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).

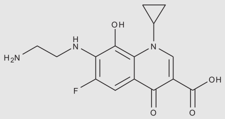
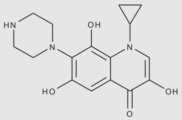
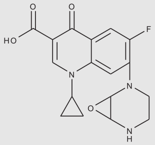
	1275578-09-0 $C_{15}H_{16}FN_3O_4$ 321.3 	HPLC-ESI-MS/MS	AOPs	Kugelmann et al. (2011).
	1275578-06-7 $C_{16}H_{19}N_3O_4$ 317.34 	HPLC-ESI-MS/MS	AOPs	Kugelmann et al. (2011).
	1375414-99-5 $C_{17}H_{16}FN_3O_4$ 345.33 	HPLC-ESI-MS	Photodegradation	Lester et al. (2011).

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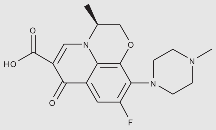
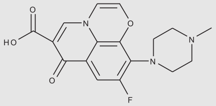
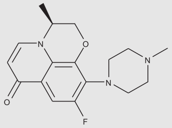
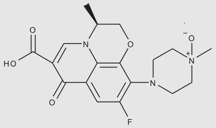
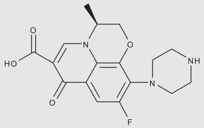
Table 3 – (*continued*)

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
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).

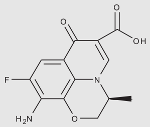
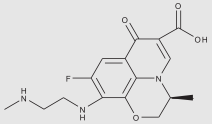
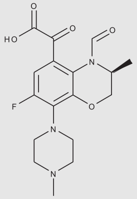
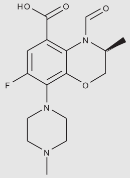
	151250-74-7 $C_{13}H_{11}FN_2O_4$ 278.24 	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
	151250-76-9 $C_{16}H_{18}FN_3O_4$ 335.33 	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
	1263063-18-8 $C_{17}H_{20}FN_3O_5$ 365.36 	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
	1263063-19-9 $C_{16}H_{20}FN_3O_4$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

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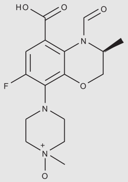
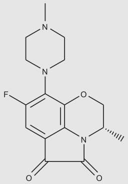
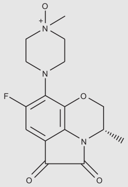
Table 3 – (*continued*)

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).

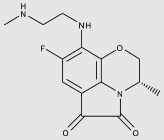
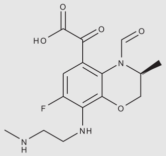
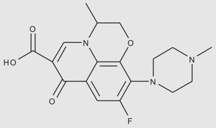
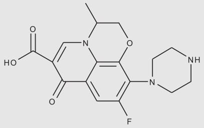
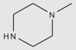
	1263063-23-5 $C_{28}H_{32}F_2N_6O_6$ 586.59 	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
	1263063-24-6 $C_{15}H_{18}FN_3O_5$ 339.32 	HPLC-UV-ESI-HRMS-MS/MS	AOPs	Witte et al. (2009).
Ofloxacin 82419-36-1 $C_{18}H_{20}FN_3O_4$ 361.37 	82419-52-1 $C_{17}H_{18}FN_3O_4$ 347.34 	HPLC-APCI-IT-MS/MS	Photodegradation	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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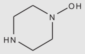
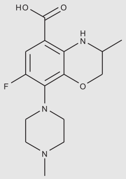
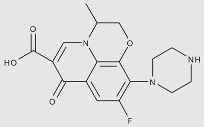
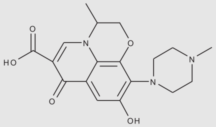
Table 3 – (continued)

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
	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).

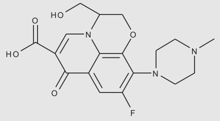
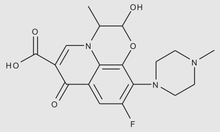
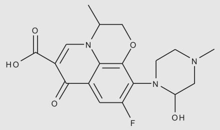
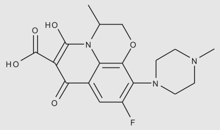
	1037310-73-8 $C_{18}H_{20}FN_3O_5$ 377.37 	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037310-74-9 $C_{18}H_{20}FN_3O_5$ 377.37 	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037310-75-0 $C_{18}H_{20}FN_3O_5$ 377.37 	HPLC-ESI-LTQ-Orbitrap-MS/MS	AOPs	Calza et al. (2008).
	1037310-76-1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

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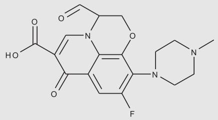
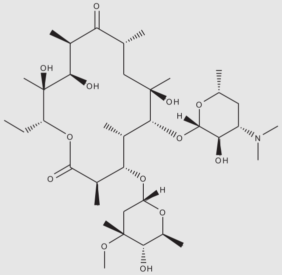
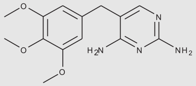
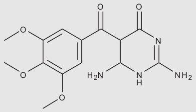
Table 3 – (*continued*)

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
	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).

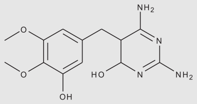
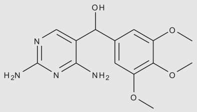
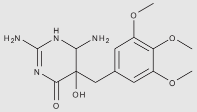
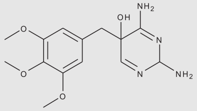
1182403-95-7 $C_{13}H_{18}N_4O_4$ 294.31 	HPLC-ESI-QToF-MS/MS	AOPs	Radjenović et al. (2009a).
29606-06-2 $C_{14}H_{18}N_4O_4$ 306.32 	HPLC-ESI-IT-MS; HPLC-QToF-MS/MS; H/D exchange HPLC-ESI-MS HPLC-ESI-ToF-MS	Biodegradation AOPs Photodegradation	Eichhorn et al. (2005). Luo et al. (2012). Sirtori et al. (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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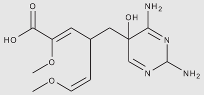
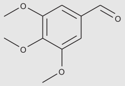
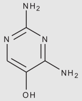
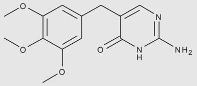
Table 3 – (*continued*)

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
	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).

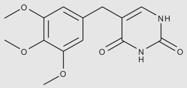
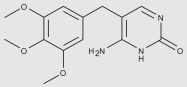
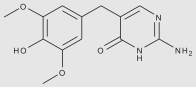
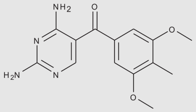
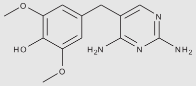
	93885-69-9 $C_{14}H_{16}N_2O_5$ 292.29 	HPLC-MS; NMR; TLC	Photodegradation	Bergh et al. (1989).
	60729-91-1 $C_{14}H_{17}N_3O_4$ 291.3 	HPLC-MS; NMR; TLC	Photodegradation	Bergh et al. (1989).
	72920-13-9 $C_{13}H_{15}N_3O_4$ 277.28 	HPLC-MS; NMR; TLC	Photodegradation	Bergh et al. (1989).
	120749-06-6 $C_{14}H_{16}N_4O_3$ 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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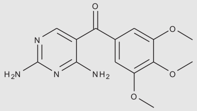
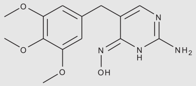
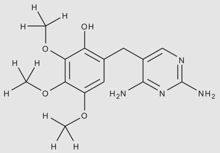
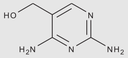
Table 3 – (*continued*)

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
	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).

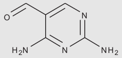
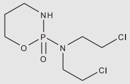
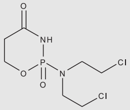
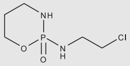
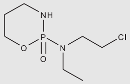
	20781-06-0 $C_5H_6N_4O$ 138.13 	HPLC-ESI-ToF-MS	Photodegradation	Sirtori et al. (2010).
		Cytostatic agents		
Cyclophosphamide 6055-19-2 $C_7H_{15}Cl_2N_2O_2P$ 261.09 	27046-19-1 $C_7H_{13}C_{l2}N_2O_3P$ 275.07 	HPLC-ESI-QToF-MS; IR HPLC-ESI-MS/MS; IR HPLC-EI-MS; TLC	AOPs AOPs AOPs	Fernández et al. (2010). 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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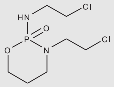
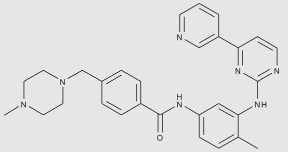
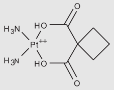
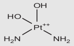
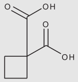
Table 3 – (*continued*)

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
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).

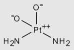
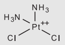
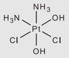
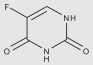
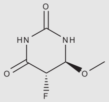
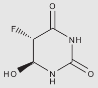
	63700-88-9 $H_4N_2O_2Pt$ 259.12 	HPLC-UV	Photodegradation	Pujol et al. (1997).
Cisplatin 15663-27-1 $C_{l2}H_6N_2Pt^{+2}$ 300.05 	31246-66-9 $Cl_2H_8N_2O_2Pt$ 334.06 	HPLC-ESI-MS/MS	AOPs	Hernandez et al. (2008).
5-fluorouracil 51-21-8 $C_4H_3FN_2O_2$ 130.08 	77413-15-1 $C_5H_7FN_2O_3$ 162.12 	HPLC-ESI-MS	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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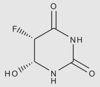
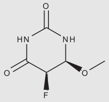
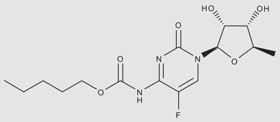
Table 3 – (*continued*)

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
	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

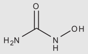
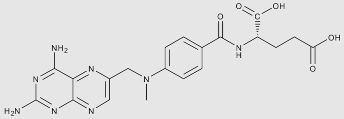
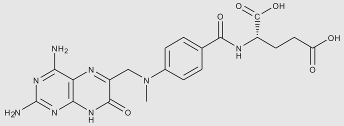
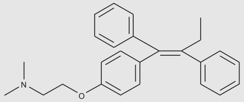
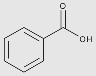
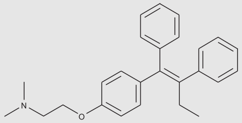
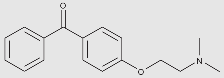
Hydroxycarbamide 127-07-1 $CH_4N_2O_2$ 76.05 	No data			No data
Methotrexate 59-05-2 $C_{20}H_{22}N_8O_5$ 454.44 	5939-37-7 $C_{20}H_{22}N_8O_6$ 470.44 	HPLC-UV; IR	Biodegradation	Kiffmeyer et al. (1998).
Tamoxifen 10540-29-1 $C_{26}H_{29}NO$ 371.51 	65-85-0 $C_7H_6O_2$ 122.12 	HPLC-MS; NMR; TLC; IR	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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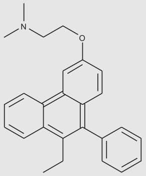
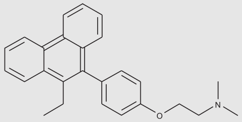
Table 3 – (*continued*)

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
	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.

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).

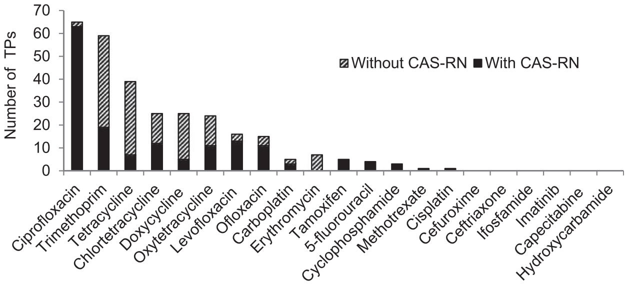


Fig. 1 – Summary of transformation products (TPs) owning chemical structures with/without registry number in chemical abstracts service (CAS-RN) for the selected antibiotic and anticancer drugs.

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 II

Removal of the anti-cancer drug methotrexate from water by advanced oxidation processes: Aerobic biodegradation and toxicity studies after treatment.

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Removal of the anti-cancer drug methotrexate from water by advanced oxidation processes: Aerobic biodegradation and toxicity studies after treatment

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HIGHLIGHTS

- AOP's were compared with respect to degree and kinetics of elimination and mineralization of MTX.
- The parent compound was easily eliminated in all the treatments.
- None of the treatments fully mineralized MTX and formation of stable intermediates was observed.
- MTX and its photolytic mixture were not biodegradable.
- Photolytic mixture was less toxic than MTX itself.

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ABSTRACT

Anti-cancer drugs are discussed as high risk substances in regard to human health and considered as problematic for the environment. They are of potential environmental relevance due to their poor biodegradability and toxicological properties. Methotrexate (MTX) is an antimetabolite that was introduced in the pharmaceutical market in the 40's and still today is one of the most consumed cytotoxic compounds around the world. In the present study MTX was only partially biodegraded in the closed bottle test (CBT). Therefore, it was submitted to three different advanced oxidation processes (AOPs): UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2. The irradiation was carried out with a Hg medium-pressure lamp during 256 min whereas the analytical monitoring was done through LC-UV–MS/MS and DOC analysis. MTX was easily removed in all the irradiation experiments, while the highest mineralization values and rates were achieved by the UV/Fe^{2+}/H_2O_2 treatment. The lowest resulted from the UV/H_2O_2 reactions. The UV/H_2O_2 treatment resulted in little biodegradable transformation products (TPs). However, the same treatment resulted in a reduction of the toxicity of MTX by forming less toxic TPs. Analysis by LC-UV–MS/MS revealed the existence of nine TPs formed during the photo-catalytic treatments. The pH of the solutions decreased from 6.4 (t 0 min) to 5.15 in the UV/H_2O_2 and from 6.4 (t 0 min) to 5.9 in the UV/TiO_2 at the end of the experiments. The initial pH of the UV/Fe^{2+}/H_2O_2 experiments was adjusted to 5 and after the addition of H_2O_2 the pH decreased to around 3 and remained in this range until the end of the treatments.

1. Introduction

Pharmaceuticals are ubiquitous substances and several studies have detected their presence in wastewaters (Kümmerer, 2001; Escher et al., 2011), surface waters (Buerge et al., 2006; Fatta-Kassinos et al., 2011), drinking waters (Heberer, 2002; Mompelat et al., 2009) and ground waters (Heberer, 2002). Among the various classes of pharmaceuticals, the anti-cancer drugs require a special attention because of an increasing demand for the chemotherapy treatment (Hoppe-Tichy, 2010). Anti-cancer drugs are discussed

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as high risk substances in regard of human health and considered as problematic for the environment because of their special properties in combination with poor biodegradability (Toolaram et al., 2014). Methotrexate (MTX) is a mutagen and a teratogen anticancer drug which belongs to the subgroup of the antimetabolites and acts by blocking enzyme activity and disrupting the DNA synthesis.

It is widely used at high dose for the chemotherapy of various forms of cancer (bronchial, breast and ovarian cancer, lymphomas, leukemia) and has been sold since the 1940s (Rubino, 2001). MTX is a highly polar compound with negligible bioaccumulation and sorption to organic matter (Table S1). Up to 90% of the unchanged drug can be excreted by urine and feces. Therefore, it can be expected in the water cycle. Some studies reveal the presence of MTX in hospital and sewage treatment plant (STP) effluents, and even in surface waters at a concentration range of ng/L (Halling-Sørensen et al., 1998; Castiglioni et al., 2005; Yin et al., 2010; Besse et al., 2012).

These findings demonstrate that the traditional biological, physical and chemical water and wastewater treatments are ineffective in removing or mineralizing MTX. In those cases, advanced oxidation processes (AOP's) may be suitable methods, which may yield good elimination. Besides, the AOP's represent an interesting alternative, since they can be employed in association with biological treatments for wastewater remediation, as a pre-treatment, increasing the biodegradability by a partial oxidation, or as a post-treatment for the degradation of persistent compounds (De la Cruz et al., 2012).

Over the last years many studies have reported the application of UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2 systems to degrade pharmaceutical compounds (Arslan-Alaton and Dogruel, 2004; Elmolla and Chaudhuri, 2010; De la Cruz et al., 2012; Sleman et al., 2012). However, often incomplete mineralization can result in the formation of unwanted reaction products (so called transformation products, TPs) of unknown properties.

The objectives of the present work were: (i) to evaluate the biodegradability of MTX, (ii) to compare the efficiency of the elimination of MTX as well as its degree of mineralization by UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2 (iii) to evaluate the biodegradability of its photo-TPs, and (iv) to assess the toxicity of MTX and its photo TPs.

2. Materials and methods

As methotrexate exhibits mutagenic and teratogenic properties, the work with this compound requires strict safety precautions (Allwood et al., 2002).

2.1. Chemicals

All solvents used in our studies were of HPLC grade and all chemicals were of analytical reagent grade or higher (Text S1).

2.2. Advanced photo treatments

The assays were performed in an 800 mL batch photo-reactor containing 600 mL of MTX with an initial concentration of 20 mg/L. The high concentration was used in order to allow for performing subsequent biodegradation testing. A medium-pressure mercury lamp (TQ150, UV Consulting Peschl, Mainz) with an ilmasil quartz immersion tube was used to irradiate the samples. A measured lamp output of 2057 mJ/cm^2 was obtained using an UVpad Spectral Radiometer (Opsytec Dr. Gröbel GmbH, Ettlingen, Germany). The emission of the used UV lamp was measured with UV-pad Spectral Radiometer (Opsytec Dr. Gröbel GmbH, Ettlingen, Germany) at a distance of 4 cm from the emission source in an aluminum box in the range of 200–440 nm (Fig. S1).

The solutions were constantly stirred in order to ensure homogeneity and a circulating cooling system (WKL230, LAUDA, Berlin) was used to keep the temperature between 20 ± 2 °C. The pH values were also monitored during all the treatments. The experiments were performed during 256 min with samplings at regular time points in a geometrical row (2, 4, 8, 16, 32, 64, 128 and 256 min) to check the degradation and mineralization rates of the compounds.

Nonlinear regression analyses were performed using an exponential decay model with the statistical software Prism 5 (Graphpad Inc., CA, USA). The functions "one phase decay" and "two phase decay" were used to calculate the rate constants and the half-lifes of the three processes. A more detailed description of data analysis is available in the Supplementary Material (Text S2).

2.2.1. UV/H_2O_2

Three different concentrations of hydrogen peroxide (9.8 mM, 14.7 mM and 19.6 mM) were tested in order to determine the optimum concentration range in our experiments. The mineralization degrees were used to evaluate the performance of the processes. Aiming to avoid any direct oxidation of MTX by hydrogen peroxide, the lamp was firstly turned on and afterwards the hydrogen peroxide was added. The pH of the samples collected during the assays was adjusted to 7 (±1) with NaOH, whereas the residual, unreacted H_2O_2 was destroyed by the addition of catalase made from bovine liver (1 unit will decompose 1.0 μmole of H_2O_2 per min at pH 7.0 at 25 °C).

2.2.2. UV/Fe^{2+}/H_2O_2

The experiments were performed based on the mineralization results of the UV/H_2O_2 experiments. The initial concentrations of H_2O_2 and $FeSO_4 \cdot 7H_2O$ were 9.8 mM and 1.56 mM respectively. The pH was adjusted to 5 with H_2SO_4 (2 M, 98%) (although the literature defines the optimal pH for the Fenton reactions at around 3, preliminary experiments showed no better results than the ones performed in pH 5). Furthermore two additional experiments varying the concentrations of hydrogen peroxide (15.5 mM and 19.6 mM) and of the catalyst (2.14 mM) were also performed. In order to precipitate the ferric ions after the irradiation was finished the pH of the aliquots was adjusted to 7 ± 1 with NaOH. Afterwards catalase was used to destroy the residual, unreacted H_2O_2 and the samples were then centrifuged at 4000 rpm for 5 min and subsequently filtered through 0.2-μm filter membranes (CHROMAFIL® Xtra.Typ: PES 20/25, Macherey–Nagel, Germany).

A simple and practical spectrophotometric method based on the reaction of H_2O_2 with ammonium metavanadate in acidic medium was used for monitoring the H_2O_2 consumption during the tests (Nogueira et al., 2005). The concentrations of ferric and ferrous ions were determined using a colorimetric method with 1–10 phenantroline (APHA/AWWA/WPCF, 2012).

2.2.3. UV/TiO_2

Preliminary experiments with different concentrations (100, 500 and 1000 mg/L) were performed in order set the best titanium dioxide concentration. Before starting the photo-catalytic assays, solutions containing MTX and TiO_2 were constantly stirred for 30 min in the dark to reach adsorption equilibrium on the TiO_2 surface. The samples collected during the treatments were centrifuged at 4000 rpm for 5 min and then filtered through 0.2-μm membrane filters (CHROMAFIL® Xtra.Typ: PES 20/25, Macherey–Nagel, Germany).

2.3. Chemical analysis

The primary elimination of MTX and the identification of TPs were monitored by means of liquid chromatography tandem mass spectrometry (LC-MS/MS). The analysis in positive ion mode was performed on an Agilent 1100 module (Agilent Technology, Waldbronn, Germany) LC coupled to a low resolution ion trap mass spectrometer (MS) Bruker Daltonic Esquire 6000^{plus} ion trap (Bruker Daltonik GmbH, Bremen, Germany) in order to conduct a first screening. The ionization was done by electrospray ionization (ESI). A more detailed description of the chromatographic conditions can be found in the Supplementary material (Text S3).

Dissolved organic carbon (DOC) measurements in three replicates were done to determine the degree of mineralization using a TOC (total organic carbon) analyzer (TOC VCPN 5050, Shimadzu GmbH, Duisburg, Germany) equipped with an ASI-V auto sampler.

2.4. Biodegradation

The closed bottle test (CBT) was performed in order to screen the biodegradability of MTX as well as the mixture of photodegradation products. The CBT is recommended as a first, simple test for the assessment of the biodegradability of organic compounds (Nyholm, 1991; OECD, 1992b). It was carried out in accordance with the OECD standardized procedure (OECD, 1992b) in a dark room at constant temperature (20 ± 1 °C) for 28 days. The test principles, procedures and composition of the CBT are described in detail in Text S4.

A threshold of less than 70% mineralization of MTX (measured based on DOC removal) at the end of each treatment processes (256 min) in the photodegradation experiments was adopted as trigger value to perform biodegradation tests on the photo treated solutions. This value was chosen for practical reasons, i.e., because the CBT is run in duplicate and demands relatively high volumes to be performed, the more mineralization is attained in the photodegradation tests, the more volume would be necessary to reach the required concentration of the compound to run the CBT. So, the threshold was set in order to avoid the need to generate high volumes of the photodegraded samples (the photoreactor has a useful volume of 600 mL).

2.5. Toxicity

The marine bacterium *Vibrio fischeri* was used to determine the toxicity of MTX and the oxidation by-products formed during the treatment. The samples were analyzed before (0 min) and after (256 min) advanced treatments. A newly developed automated method, that combines the conventional short-term luminescence inhibition test according to EN ISO 11348 and the *Photobacterium phosphoreum* growth inhibition test (DIN 38412-37) was used (Menz et al., 2013). A more detailed description of the method can be found elsewhere (Menz et al., 2013).

The test allows for the determination of three different endpoints: (i) the acute luminescence inhibition (acute LI) after 30 min, (ii) chronic luminescence inhibition (chronic LI) after 24 h and (iii) growth inhibition (GI) after 14 h. The samples were tested in triplicate at different concentrations (i.e. dilutions) in every experiment and repeated independently in different tests at least in duplicate.

Significant inhibition differences between the samples before and after the treatments were identified by One Way ANOVA, following Tukey's Multiple Comparison Test (overall significance level = 0.05) with the statistical software Prism 5 (Graphpad Inc., CA, USA).

3. Results and discussion

Control tests to evaluate the contribution of photolysis, dark Fenton and adsorption mechanisms in the removal of MTX were also carried out. The UV-only control experiment showed that the parent compound underwent direct UV-photolysis and was completely degraded after 128 min but no significant DOC removal (<5%) was observed after 256 min. On the other side, the parent compound of MTX was easily degraded in the dark Fenton tests (98% in 2 min) and a DOC removal of ≈50% was observed after 256 min (Fig. S2)

In addition, the experiments under dark conditions (adsorption on TiO_2 in the absence of UV light) showed a quite low removal at 500 mg/L TiO_2 (<20% as DOC) after 256 min.

3.1. UV/H_2O_2

No significant difference on the DOC removal of MTX was observed using the highest H_2O_2 concentrations (Fig. S3) when compared to the lowest. From these results it became evident that the process was optimal at a H_2O_2 concentration of 9.8 mM above which an inhibitory effect took place (Litter, 2005). The scavenging effect of H_2O_2 when added in excess is a well-known phenomenon (Augugliaro et al., 2006). In this process the formation of perhydroxyl radicals ($HO_2^{\bullet}$) which are much less reactive (redox potential 1.0 V) than the hydroxyl radicals (2.8 V) can retard the reaction, as shown on the Eq. (1):

$$H_2O_2 + {}^{\bullet}OH \rightarrow H_2O + HO_2^{\bullet} \qquad (1)$$

Therefore, the subsequent experiments were performed with the lowest concentrations, i.e., 9.8 mM of H_2O_2. As can be seen in the Fig. 1, almost 50% of the DOC removal occurs during the first 64 min. So, after a faster initial mineralization, the DOC removal reaches a plateau and suggests the formation of hardly mineralized TPs. The application of a modified pseudo-first order kinetic model fits well to the reaction and supports this assumption. This model considers both the presence of oxidizable and non-oxizidable matter and attributes the remaining DOC of the mixture to the presence of refractory compounds (Martins et al., 2010). The parent compound was almost completely degraded (>99%) in 960 s (16 min) and a decrease of the pH from 6.4 (*t* 0 min) to 5.15 (*t* 256 min) was observed at the end of the reactions.

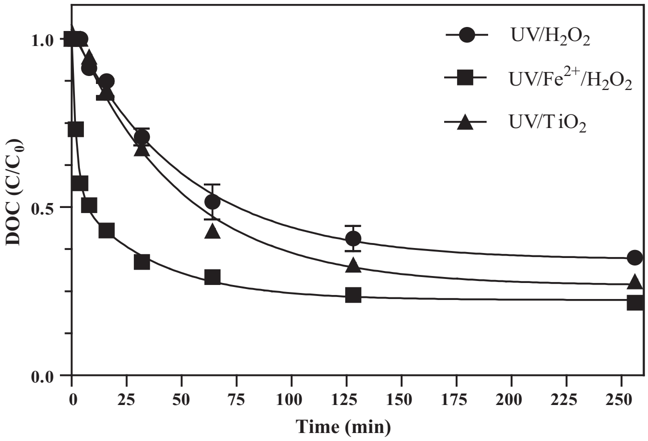

Fig. 1. Kinetics of the mineralization of MTX under the three different treatment reactions. Values are the means ± SD from two independent experiments. Initial pH values of the UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2 were 6.4, 5 and 6.4, respectively.

3.2. UV/Fe^{2+}/H_2O_2

Considering the results obtained in the UV/H_2O_2 reactions, three preliminary baseline experiments (varying the H_2O_2 and Fe^{2+} concentrations) were performed in order to verify the most appropriate reaction conditions. The results showed similar DOC removals after 256 min in the three experiments (Fig. S4). So, the tests were conducted with the lowest concentrations i.e., 9.8 mM and 1.56 mM of H_2O_2 and Fe^{2+} respectively.

The results showed that the reactions fitted well to the two phase decay kinetic model. Based on the Fig. 1 again one can clearly distinguish the presence of two phases in the mineralization process: one very fast until the first 32 min, achieving a mineralization of 66% and the other much slower achieving a final DOC removal of 78% at the end of 256 min (i.e. 12% in the last 224 min). The very fast thermal reaction between Fe(II) and H_2O_2 is completed in a few minutes probably due to the quick consumption of hydrogen peroxide during the first 32 min. The monitoring of the H_2O_2 concentration supports this. Almost 90% of the initial concentration of H_2O_2 was consumed within 2 min (Fig. S5), and after 32 min it was not possible to detect the presence of H_2O_2 in the solution anymore. Another possible explanation for this slowdown can be the formation of new recalcitrant TP's. The monitoring of the parent compound showed an almost complete elimination of MTX (>99%) after 8 min (480 s). After the addition of H_2O_2 the pH decreased to around 3 and remained in this range until the end of the treatment.

Prior research performed with 2,4-dichlorophenol also observed the presence of two phases during the photo-Fenton treatment (Karci et al., 2012). In this study a faster TOC removal occurred during the first 20 min and, after this treatment time, the reaction almost stopped, probably due to the entire consumption of hydrogen peroxide. These observations clearly indicate that the concentration of H_2O_2 is decisive for the change in kinetics.

After the complete consumption of the hydrogen peroxide, the DOC removal continued through the iron recycling caused by the photochemical reduction of ferric ions (Eq. (2)) leading to the production of $^{\cdot}OH$ and Fe^{2+} (Augugliaro et al., 2006). The fast oxidation is typical for Fenton reactions being indicative of a direct and fast $^{\cdot}OH$ oxidation step that slows down as soon as Fe^{3+} starts to accumulate (Arslan-Alaton and Dogruel, 2004). This observation is in agreement with our findings. It can be noted that at 32 min Fe^{2+} reached its lowest value (6 mg/L) and in the absence of H_2O_2 the reaction rates strongly decreased (Fig. S6). After this time point an iron recycling happens, with a measured Fe^{2+} concentration of 13.5 mg/L at 256 min, resulting in a slower reaction rate.

$$Fe(OH)^{2+} + h\nu \rightarrow Fe^{2+} + HO^{\cdot} \quad (2)$$

3.3. UV/TiO_2

During the UV/TiO_2 treatment noticeable enhancement of the DOC removal could be observed when the TiO_2 amount increased from 100 mg to 500 mg (50.4–72%) (Fig. S7). This improvement is associated with an increased number of catalyst active sites that are available for photo-catalytic reactions leading to mineralization of the parent compound and of intermediately formed transformation products. Nevertheless, a further increase up to 1000 mg/L caused a significant reduction in mineralization (54.5%). Although higher catalyst loadings may produce an increased availability of active sites, an excess of TiO_2 reduces the light penetration, and hence, the photo activated volume of the suspension shrinks (Konstantinou and Albanis, 2004). This "loss" of photons results in a plateau or even a decrease in the mineralization rate (Hapeshi et al., 2010).

Catalysts loadings up to 400–500 mg/L have been reported as optimum concentrations in most of the studies, while only slight enhancements or even decreases are observed when TiO_2 concentration is further increased up to 2000 mg/L (Konstantinou and Albanis, 2004). Similar observations were reported in other studies involving pharmaceuticals compounds (Elmolla and Chaudhuri, 2010; Hapeshi et al., 2010). So, all the following experiments were performed with a catalyst loading of 500 mg/L.

The results presented in the Fig. 1 show that almost 60% of the mineralization happened during the first 64 min. Afterwards a decrease of the reaction rate occurred until 256 min where a mineralization of 72% was achieved. In the same way as for the UV/H_2O_2 treatment, the modified pseudo-first order kinetic model also fitted well to the UV/TiO_2 reactions indicating the formation of new refractory TP's. A slight pH decrease from 6.4 to 5.9 was observed after 256 min of the reaction. The parent compound was almost completely eliminated (≈82%) after 4 min (240 s).

Calza et al. (2014) investigated the photo-catalytic degradation of MTX (15 mg/L) in ultrapure water using a catalyst load of 200 mg/L and a 40 Watt lamp with maximum emission at 360 nm. The authors verified a half-life of 3 min and a complete removal of MTX after 30 min irradiation.

3.4. Biodegradation

According to the results of the photo treatments only samples from the UV/H_2O_2 reactions reached less than 70% mineralization and were therefore collected and tested in the CBT experiments.

The CBT results were classified as valid according to the OECD guideline, since biodegradation in quality control exceeded 60% ThOD within 14 days from the start of the experiment (Fig. S8). The biodegradation of the parent compound reached 44 ± 3% for $n = 2$, and so MTX cannot be classified as a ready biodegradable substance (biodegradation level was below 60% ThOD). No toxic effects were observed as toxicity control exceeded 25% ThOD and correlated well with calculated toxicity control. Kiffmeyer et al. (1997), reported a biodegradation of 95% of MTX after seven days in an OECD confirmatory test. However MTX was classified as not readily biodegradable in a study performed with the 'manometric respiration test' OECD 301 F (Henschel et al., 1997)

A decrease of the biochemical oxygen demand (BOD) was observed for the samples collected after the UV/H_2O_2 treatment (photo-TPs generated after 256 min) after 28 days of the CBT (Fig. 2), indicating a less biodegradability of the photolytic mixture

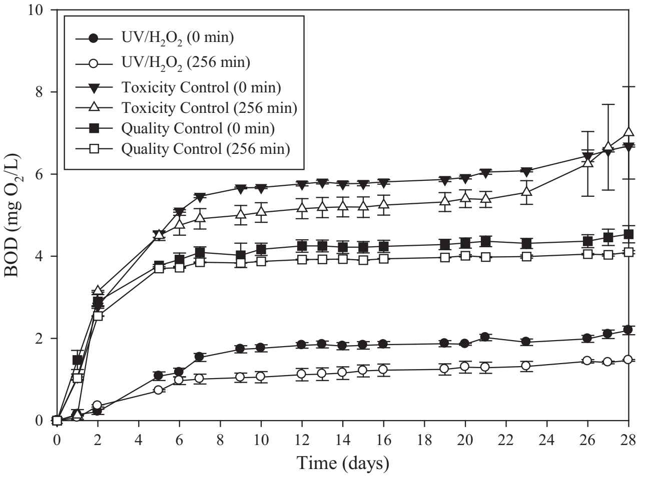

Fig. 2. Degradation in Closed Bottle Test of methotrexate (0 min) and of the photodegradation mixture (256 min of irradiation).

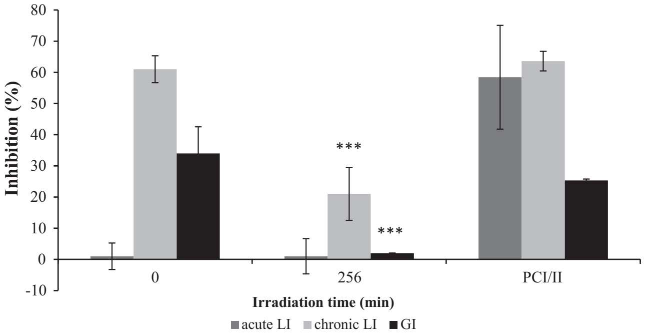

Fig. 3. Toxicity tests with *V. fischeri* before (0 min) and after (256 min) the UV/H_2O_2 treatment ($n = 2$) considering three endpoints: acute Luminescence inhibition after 30 min (acute LI), chronic Luminescence inhibition after 24 h (chronic LI) and Growth inhibition after 14 h (GI) Positive control I (PCI): 4.5 mg/L 3,5-Dichlorophenol (acute LI), Positive control II (PCII): 0.05 mg/L Chloramphenicol (chronic LI, Growth Inh.). The untreated and treated samples were submitted to final dilutions of 1:4. Statistically significant differences (***) compared to the untreated samples were identified by one way ANOVA following Tukey's Multiple Comparison Test ($P < 0.0001$).

in comparison to MTX. The mixture of photo-TPs was not toxic to test bacteria.

3.5. Toxicity

As well as for the biodegradation tests, the same threshold of less than 70% mineralization was adopted to perform the tests. Therefore, the toxicity assays were performed only with samples that were submitted to the UV/H_2O_2 treatment. The concentration range was defined based on the EC_{50} of the chronic inhibition (4.07 mg/L), obtained in previous studies carried out in our laboratory (Lutterbeck et al., 2014).

As can be seen in the Fig. 3 no significant difference regarding the acute luminescence inhibition was measured in the samples before and after the UV/H_2O_2 treatment. However, statistically significant toxicity reductions were observed in the chronic luminescence inhibition (from 61% to 21%) and in the growth inhibition (from 34% to 2%). So it can be concluded that the intermediates formed during the reaction, although not biodegradable, are less toxic than the parent compound.

3.6. Process comparison

The UV/Fe^{2+}/H_2O_2 reactions presented the best results for the mineralization of MTX and its by-products (78.4%) followed by UV/TiO_2 and UV/H_2O_2, with DOC removals of 72.1% and 65.1%, respectively (Fig 1). The kinetics of the UV/H_2O_2 and UV/TiO_2 showed that both reactions fitted well to the modified pseudo-first order reaction model and exhibited similar rate constants and half-lifes (Martins et al., 2010) (Table S3). On the other hand, the application of the "two phase decay model" equation allowed to identify two distinct stages in the mineralization of MTX by the UV/Fe^{2+}/H_2O_2 treatment. The first was very fast with a rate constant of 0.4277 ± 0.067 min^{-1} and a half-life of 1.62 min whereas in the second the calculated rate constant was more than 15 times lower than in the first stage.

Taking into account only the elimination of the parent compound, which was monitored by LC-MS, UV/TiO_2 was the fastest reaction, followed by UV/Fe^{2+}/H_2O_2 and UV/H_2O_2. In the photocatalysis process, MTX was almost completely eliminated after

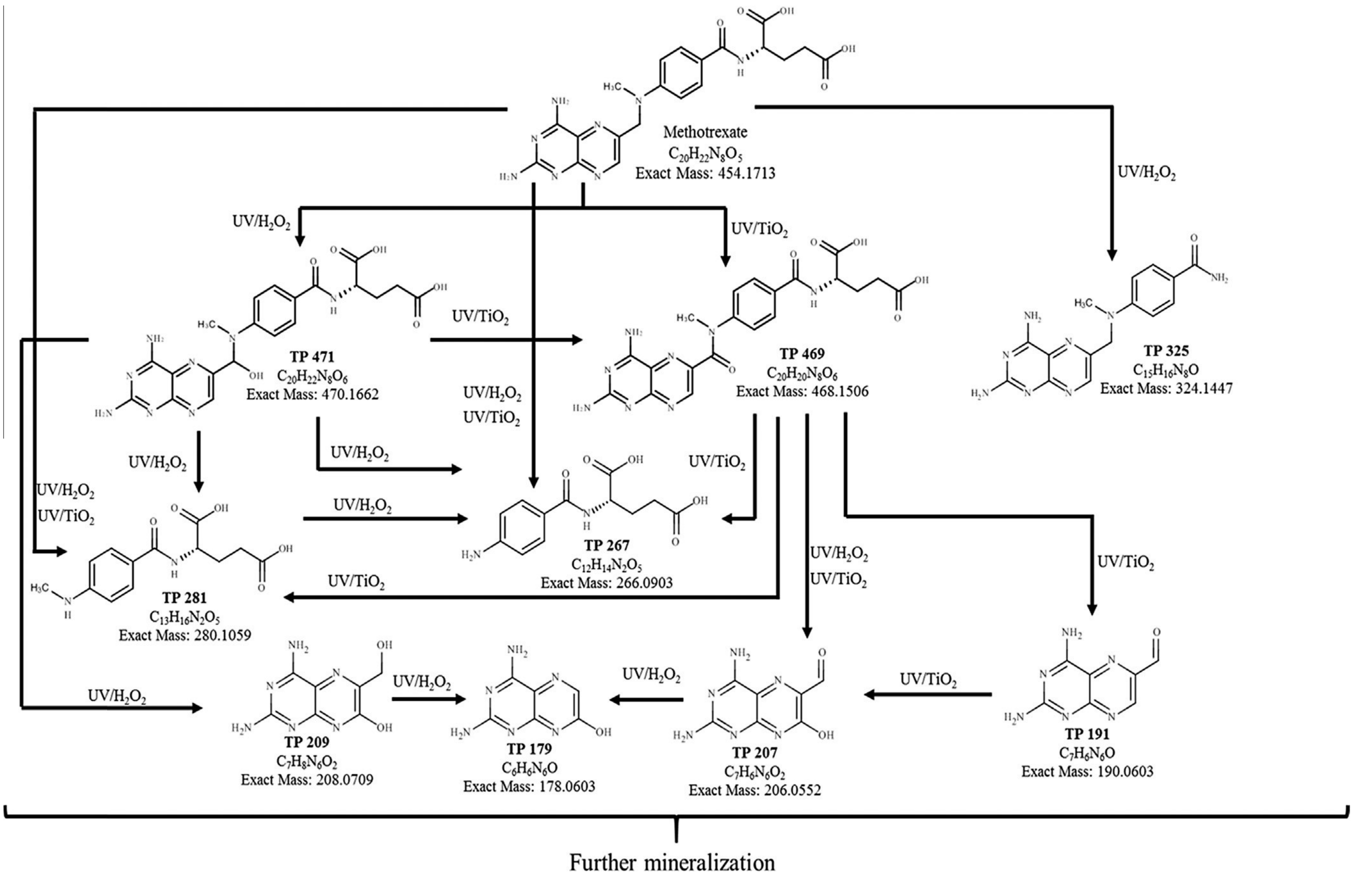

Fig. 4. Proposed degradation pathway of MTX by means of the UV/H_2O_2 and UV/TiO_2 processes.

4 min (240 s), whereas in the photo-Fenton and UV/H_2O_2 reactions these times were of 8 min (480 s) and 16 min (960 s) (Fig. S9), respectively. Table S4 presents the rate constants and half-lifes of the three processes.

3.7. Identification and kinetics of transformation products

The LC-MS/MS analysis allowed the identification and the proposition of the structures of nine transformation products (TPs) that were formed during UV/H_2O_2 and UV/TiO_2 processes (Fig. 4, Table S5 and Text S4). It was not possible to identify TPs formed during the UV/Fe^{2+}/H_2O_2 process, probably due to the high elimination and mineralization rates of the process.

The protonated form $[M^{+}H]^{+}$ of TP 469 has an elemental composition of $C_{20}H_{21}N_8O_6$ and was found in the UV/TiO_2 process. TP 469 is proposed to be formed through an intermediary TP 470 (found previously in the UV/H_2O_2 but not in the UV/TiO_2 process) formed through an N-Alpha-hidroxylation step followed by Hemiaminal oxidation (hydrogen abstraction) forming TP 469.

TPs 281 and 267 were formed in both photocatalytic processes (UV/H_2O_2 and UV/TiO_2). The formation of TP 281 is likely due a C–N Hemiaminal cleavage on the methylene moiety from parent compound, TP 469 and 470. An N-Dealkylation from TP 281 might lead to the formation of TP 267, as a secondary TP. However, as TP 267 was found in the same amount as for TP 281 after 2 min of process, it can also be proposed to be formed as a primary TP (i.e. directly from MTX). The fragmentation pattern of both TPs originated several product ions allowing their identification and elucidation. Calza et al. (2014) also observed the formation of TPs 281 and 267 during the UV/TiO_2 processes.

TP 209 is proposed to have molecular formula $[M^{+}H]^{+}$ of $C_7H_9N_6O_2$ and was formed likely due N-Dealkylation on the methylene moiety and a double hydroxylation on the originated fragment. Other possibility is that TP 209 can be formed from TP 470 through an N-Dealkylation followed by a hydroxylation step. The product ions formed by its fragmentation pattern support this assumption. Previous studies have also reported the formation of TP 209 in the photocatalytic processes (Calza et al., 2014).

TP 207 was formed during the UV/H_2O_2 and UV/TiO_2 processes and has 237 Da less than MTX. As well as for TP 209, TP 207 might be formed from TP 468 through an N-Dealkylation on the methylene moiety and further hydroxylation on the 2,4 diamino pteridine moiety. Another proposed pathway for the formation of TP 207 is the cleavage of the C–N bond and further hydroxylation on the methylene moiety. TP 207 was identified as 2,4-diaminopter idine-6-carboxylic acid, a biotransformation product already described in previous studies (Kosjek et al., 2015). TP 191 (formed only in the UV/TiO_2 processes) might be a secondary TP formed through an N-Dealkylation step from TP 468.

TP 471, TP 325 and TP 179 were formed in very low ratio levels being not possible to generate a fragmentation pattern up to MS^2. Nevertheless, TP 471 has 16 Da more than MTX and it is proposed to be formed through a hydroxylation step. However, because of the absence of a MS/MS fragmentation pattern, it is difficult to predict the exact position where the hydroxylation could took place. TP 471 or MTX-OH was already described as biotransformation product of MTX and reported as non-biodegradable (Kiffmeyer et al., 1997). Therefore, in this study it is proposed a N-Alpha hydroxylation from the MTX.

TP 325 was found only in the UV/H_2O_2 process. It has less 130 Da than the parent compound and was probably formed through an Amide Dealkylation from the side chain of MTX. Lastly, the ($[M^{+}H]^{+}$) of TP 179 has an elemental composition of $C_6H_7N_6O$ which corresponds to one oxygen atom and one carbon atom less than TP 207.

The profile of the peak area of the identified TPs shows that all of them were transient during the treatment once they were formed and further fully degraded (Fig. S10).

4. Conclusions

The presence of pharmaceuticals in the aquatic environments has motivated the search for effective effluent and drinking water treatments that may remove such compounds from water. Nevertheless, comparative studies of treatment methods are rarely performed. In the present work, three different AOP's under the same operational conditions were tested. Albeit MTX was easily eliminated in all the reactions, none of the treatments attained a full mineralization, which resulted in the generation of stable transformation products. So, the results demonstrate that at least for practical application of AOP knowledge on primary elimination is insufficient to select the appropriate treatment period. The combination of several analytical methods is necessary to get a more complete understanding of the dynamics of the processes. Additionally, such knowledge is urgently needed to conduct treatment in a way that formation of TPs is minimized. On the one hand, however, the long treatment times needed for the highest degree of mineralization of the parent compound and TPs suggest that a practical application is rather limited if higher volumes of effluent or raw water have to be treated as it would be nearly impossible to work with irradiation times longer than a few minutes for practical reasons. On the other hand, the results clearly demonstrate that even at very long treatment times the further improvement is not sufficient, since only a little increase in the degree of mineralization is reached after a certain time point (e.g. 64 min in photo catalytic treatment). The UV/H_2O_2 process showed not to be a good pre-treatment option, since the photolytic mixture was even more refractory than the parent compound. However, the same process significantly reduced the toxicity of MTX after 256 min as far as the toxicity endpoints monitored in our study are concerned. Whilst this could be seen as an advantage the since the persistence of the formed intermediates is a clear disadvantage.

Acknowledgments

This research was financially supported by EU under the 7th Framework, grant agreement n° 265346. Carlos Alexandre Lutterbeck thanks the Brazilian Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarship (Grant Nr. 290136/2011-3). The authors also wish to acknowledge the Pharmacy of the Hospital Lüneburg for kindly providing methotrexate. Special thanks to Dr. Marcelo Luís Wilde for the help with the analytical work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.07.069.

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Supplementary Material

Removal of the anti-cancer drug Methotrexate from water by advanced oxidation processes: aerobic biodegradation and toxicity studies after treatment

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Nachhaltige Chemie und Stoffliche Ressourcen, Institut für Nachhaltige Chemie und Umweltchemie, Fakultät für Nachhaltigkeit, Leuphana Universität Lüneburg, Scharnhorststraße 1/C13, D-21335 Lüneburg, Germany. Tel.: +49 4131 677 2893.

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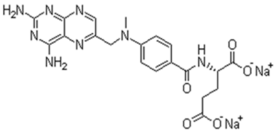
Table S1 – Structure and main physico-chemical properties of methotrexate.

Structure	**Chemical Formula**	**Molecular Weight**	**Solubility in water**	**Log K_{ow}**	**Unchanged Excretion**
	$C_{20}H_{20}N_8Na_2O_5$	498,4 g/mol $^{-1}$	0.9 mM at pH5 to 20 mM at pH7$^{(1)}$	-1.28 $^{(2)}$	90% $^{(3)}$

MW: molecular weight; WS: water solubility; log Kow: partition coefficient (octanol/water);
References: (1)(Rubino, 2001) (2)(Sanderson and Thomsen, 2009) (3)(Besse et al., 2012)

Text Supplementary Material S1 - Chemicals:

Acetonitrile (HiPerSolv CHROMANORM, LC–MS grade, BDH Prolabo) and formic acid (analytical grade) were purchased from VWR International GmbH (Darmstadt, Germany). Sodium hydroxide (NaOH 1 M) and sulfuric acid (H_2SO_4 2 M, 98 %) were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and titanium dioxide (TiO_2 P25) was purchased from Evonik Degussa GmbH (Frankfurt, Germany). Hydrogen peroxide (H_2O_2 30 % w/w) was acquired from Merck-Group (Darmstadt, Germany) whereas iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), catalase from bovine liver (2,000-5,000 units/mg protein), ammonium metavanadate (NH_4VO_3) and 1,10-phenantroline ($C_{12}H_8N_2$) from Sigma-Aldrich Biochemie GmbH (Hamburg, Germany). Methotrexate disodium (TEVA GmbH, Ulm, Germany) was kindly provided and handled by the pharmacy of the Hospital Lüneburg, Germany (therapeutic infusions bags were prepared on-demand). In order to avoid any scavenging effect of other species and so evaluate only the degradation of MTX, all the solutions were prepared using ultrapure water (Q_1:16.6 mΩ and Q_2: 18.2 mΩ).

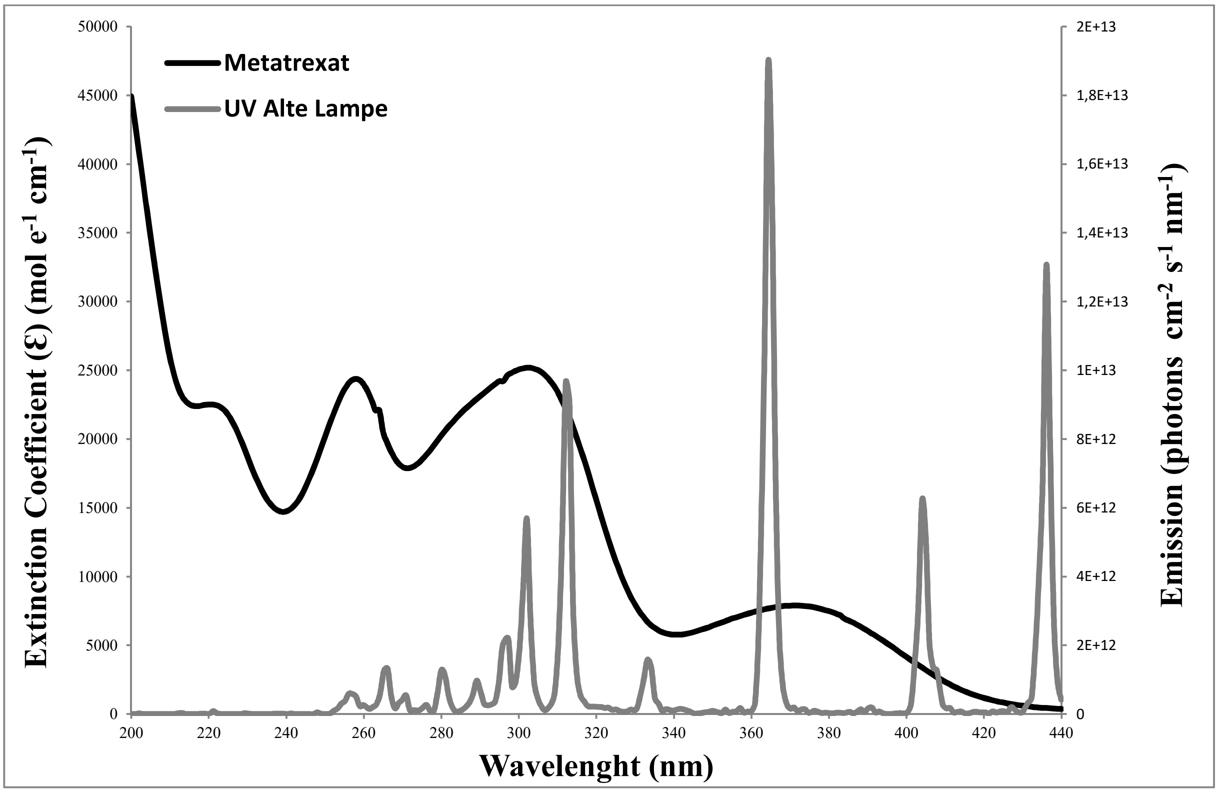


Fig. S1 – Intensity of light emitted by the used UV lamp and molar extinction coefficient of MTX for the range of 200 nm to 440 nm. (Concentration of 10 mg/L)

Text Supplementary Material S2 – Kinetic analysis:

A one phase decay model was used to calculate the rate constants of the mineralization of MTX by the UV/H_2O_2 and UV/TiO_2 treatments. The following equation was used:

$y = (y_0 - \text{plateau})*\exp(^{-K*X}) + \text{plateau}$

Where: y_0 = y value when x (time) is zero and is expressed in the same units as y; plateau= y value at infinite times, expressed in the same units as y; K = is the rate constant, expressed in reciprocal of the X axis time units. If X is in minutes, then K is expressed in inverse minutes; Tau is the time constant, expressed in the same units as the X axis; It is computed as the reciprocal of K; Half-life is in the time units of the X axis and it is computed as ln(2)/K; Span is the difference between y_0 and Plateau, expressed in the same units as your Y values.

A two phase decay model was adopted to calculate the rate constants of the mineralization of MTX by the UV/Fe^{2+}/H_2O_2 treatment. The following equation was used:

$\text{Spanfast} = (y_0 - \text{plateau})*\text{percentfast}*.01$

$\text{spanslow} = (y_0 - \text{plateau})*(100-\text{percentfast})*.01$

$y = \text{plateau} + \text{spanfast}*\exp^{(-\text{Kfast}*X)} + \text{spanslow}*^{\exp\ (-\text{Kslow}*X)}$

Where: y_0 = y value when x (time) is zero and is expressed in the same units as y; plateau= y value at infinite times, expressed in the same units as y; Kfast and Kslow = the two rate constants expressed in inverse minutes; percent fast is the fraction of the span (from Y_0 to plateau) accounted for by the faster of the two components; half-life (fast) and half-life (slow) are in the time units of the x axis and are computed as ln(2)/K

Text S3 LC-MS/MS analysis

The chromatographic separation of the sample was performed on a Nucleodur RP-18 endcapped 100-3 column, 2µm (Macherey-Nagel, Düren, Germany) coupled to a precolumn, (Nucleodur C18 ec 4-2, 3µm; Macherey-Nagel, Düren, Germany). For elution, 0.1 % formic acid in deionized water (solution A) and acetonitrile LC-MS grade (solution B) were used by applying the following gradient: isocratic from 0 min. until 5 min 5 % B, linear gradient 20 min 70 % B, isocratic until 25 min 70 % B, 27 min 5 % B, 33 min 5 % B. The sample injection volume was 10 µL and flow rate was 0.3 mL/min.

Mass spectra were obtained using a Bruker Daltonic Esquire 6000 ion trap mass spectrometer (MS) equipped with a Bruker data analysis system (Bruker Daltonik GmbH, Bremen, Germany). The MS was connected to the Agilent Technologies HPLC 1100 series. Ionization was done by an electrospray (ESI: electrospray ionization) module. The positive mode was used. Setting of scan mode was full-MS without fragmentation of molecular ions. The operation conditions of the source were: -500 V end plate and -3580 V capillary voltages relative to the needle, 30 psi nebulizer pressure, and 12.0 mL min^{-1} nitrogen dry gas flow at dry temperature of 350°C. For quantification, the molecule ion for MTX (m/z 455.1) at a retention time of 12.2 min was used.

Text Supplementary Material S4 – Aerobic Biodegradability:

The aerobic biodegradability of MTX and its TP's was investigated in the Closed Bottle Test (CBT) according to the OECD 301D guideline(OECD, 1992).

All the vessels used in the test contained the same mineral salt solution prepared in accordance to the test guideline. The effluent collected from the municipal STP **AGL GmbH**, Lüneburg, Nord, Germany (73 000 inhabitant equivalents) was used as inoculum source. Two drops of inoculum were added to 1 L of the mineral medium solution, which resulted in bacterial density of approx. 500 CFU/mL.

Each test consisted of four different series running in parallel and in duplicate, respectively. The first series corresponded to the "blank" series, which contained only mineral medium and inoculum. The vessels used in the "quality control" series were prepared using readily biodegradable sodium acetate in a concentration corresponding to 5 mg/L theoretical oxygen demand ($ThODNH_3$, calculated without considering a possible nitrification) as the only organic carbon source besides the inoculum. The "test" vessel series contained inoculum and the test compound. The "toxicity control" was the fourth series and contained in addition to the inoculum the test compound and sodium acetate in a concentration corresponding to 5 mg/L ThOD, respectively. A tested compound is considered to be inhibitory for the bacteria if the biodegradation does not reach more than 25% of ThOD within 14 days. Additionally, to determine the toxic effects, the oxygen consumption measured in the toxicity controls was compared with the predicted level calculated from the oxygen consumption in the quality control and in the test vessel, respectively.

During the whole test the consumption of oxygen in the bottles was measured daily with an optical oxygen sensor system (Fibox 3 PreSens, Regensburg, Germany) using sensor spots in the bottles. Besides, the temperature and the pH (day 0 and 28) were also controlled. A more detailed description of the test can be found elsewhere (Kummerer et al., 1996; Trautwein et al., 2008; Mahmoud and Kummerer, 2012; Friedrich et al., 2013).

According to the OECD guideline, a compound is classified as ‘‘readily biodegradable” if biodegradability, expressed as a percentage of oxygen consumed in the test vessel compared to maximum consumption (ThOD), exceeds 60 % within a period of ten days starting from the day where oxygen consumption reaches 10 % ThOD. The ThOD of MTX was calculated based on the molecular formula of the compound.

Table S1 summarize the composition of the aerobic biodegradation test series of the CBT

Table S2 – Composition of the aerobic biodegradation test series in the CBT.

	1	**2**	**3**	**4**
Test Series	**Blank**	**Quality Control**	**Test Compound**	**Toxicity Control**
Mineral medium	+	+	+	+
Inoculum	+	+	+	+
Test substance			+	+
Sodium acetate		+		+

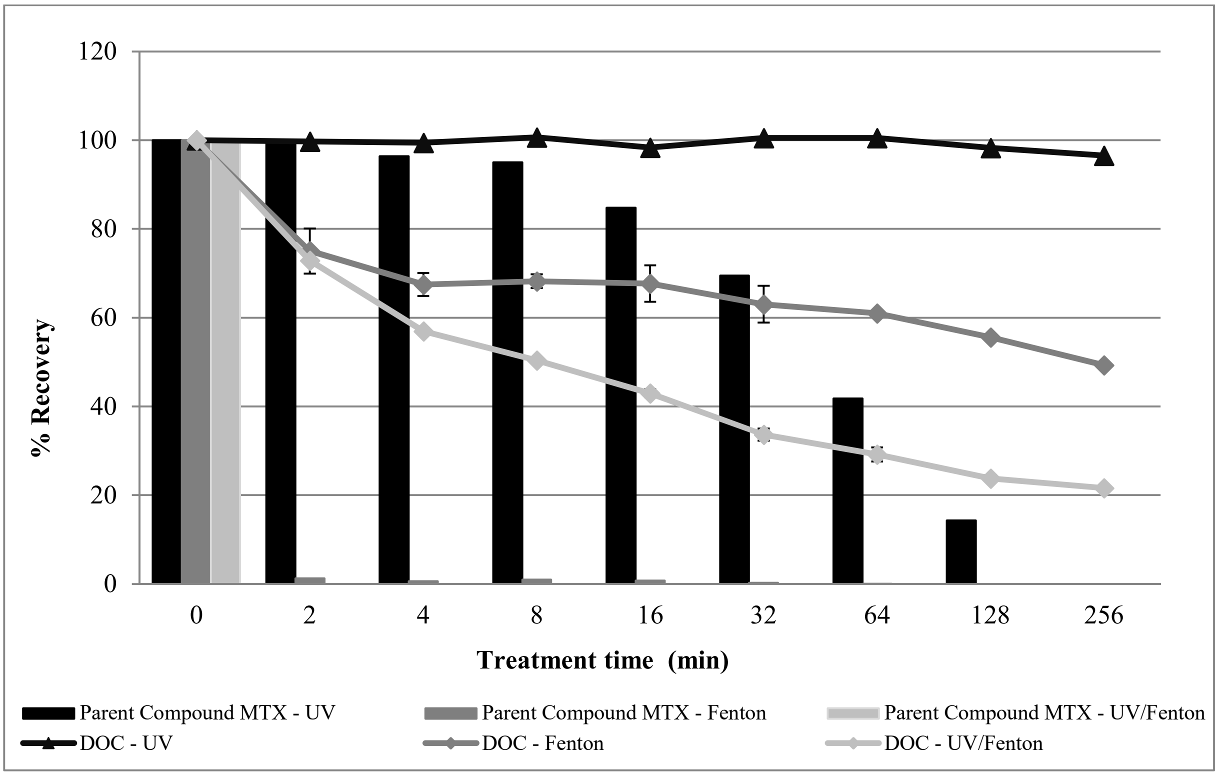


Fig. S2 – Degradation and mineralization rates of MTX and its photo-transformations products through UV, Fenton and UV/Fenton treatments.

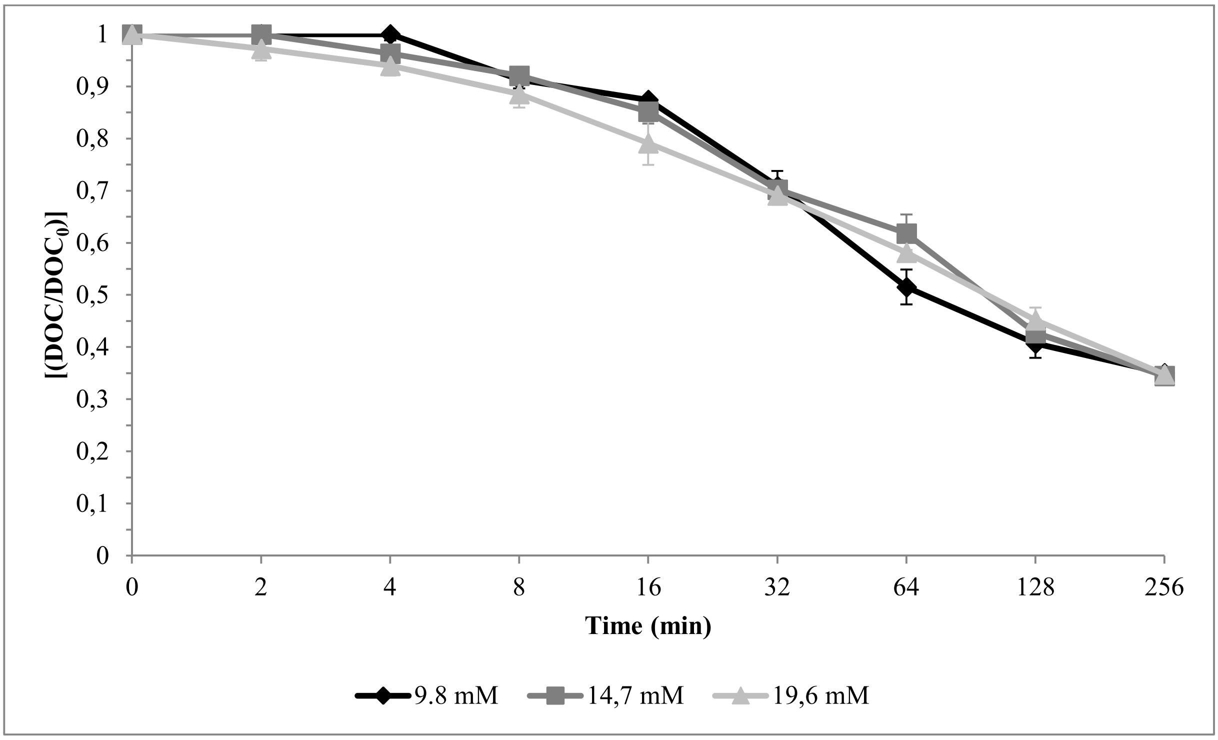


Fig. S3 - Effect of the H_2O_2 concentration on the mineralization degrees of MTX

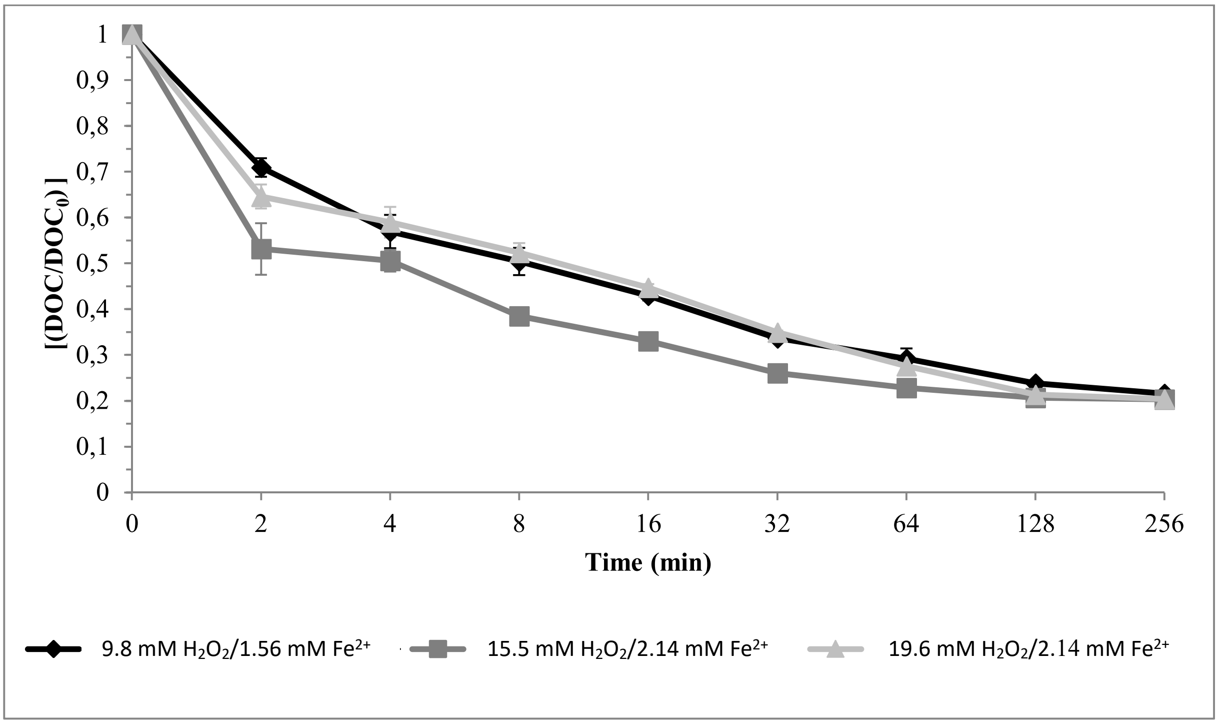


Fig S4 - Mineralization of MTX under UV/Fe^{2+}/H$_2$O$_2$, with different concentrations of H$_2$O$_2$ and Fe^{2+}

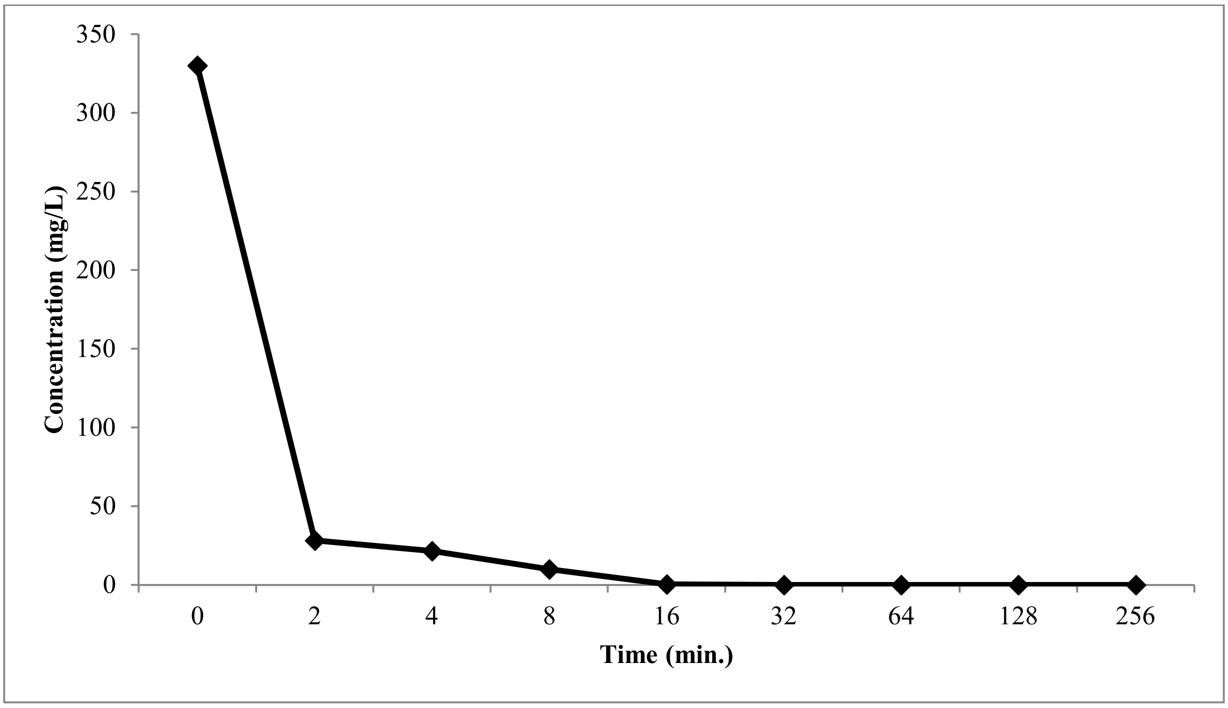


Fig. S5 – Consumption of H$_2$O$_2$ during the photo-Fenton treatment.

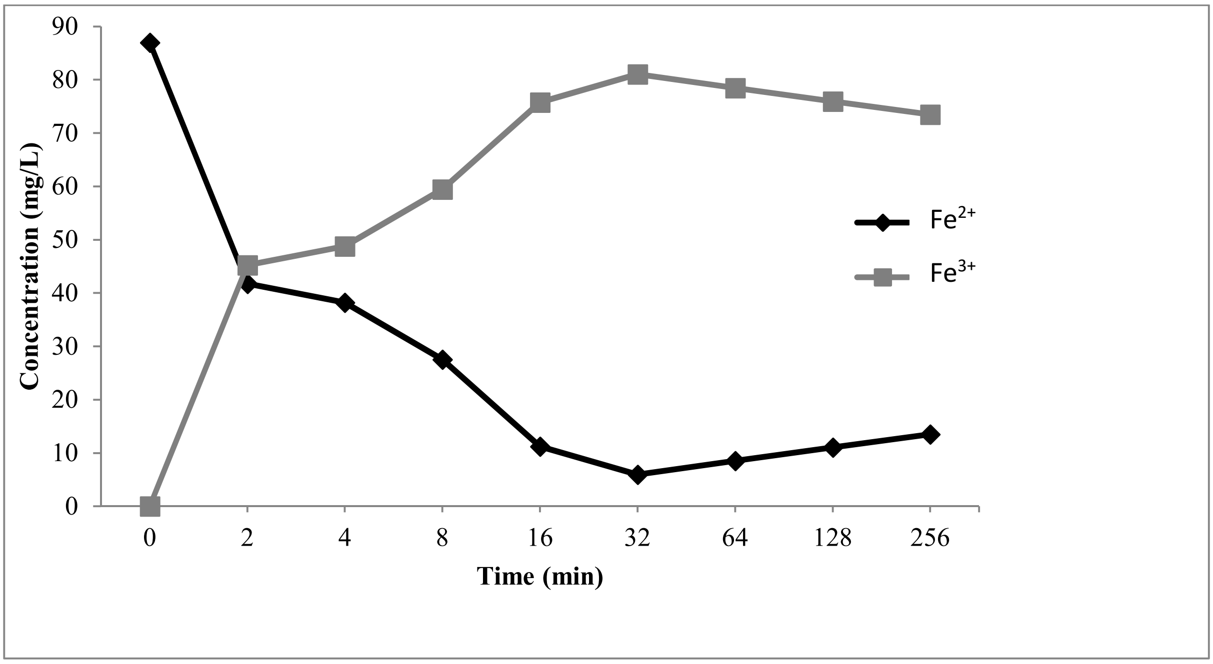


Fig. S6 – Consumption of ferric and ferrous ions during the UV/Fe^{2+}/H_2O_2

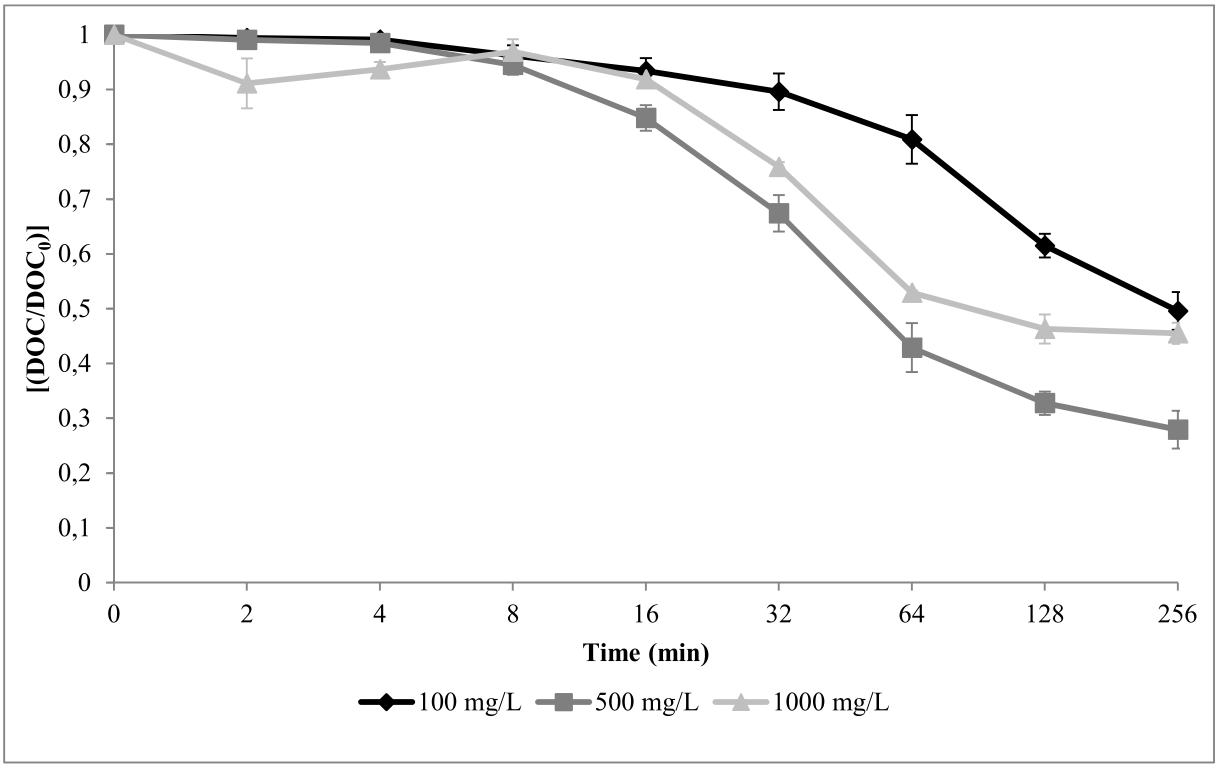


Fig. S7- Effect of catalyst loading on the mineralization degrees of MTX

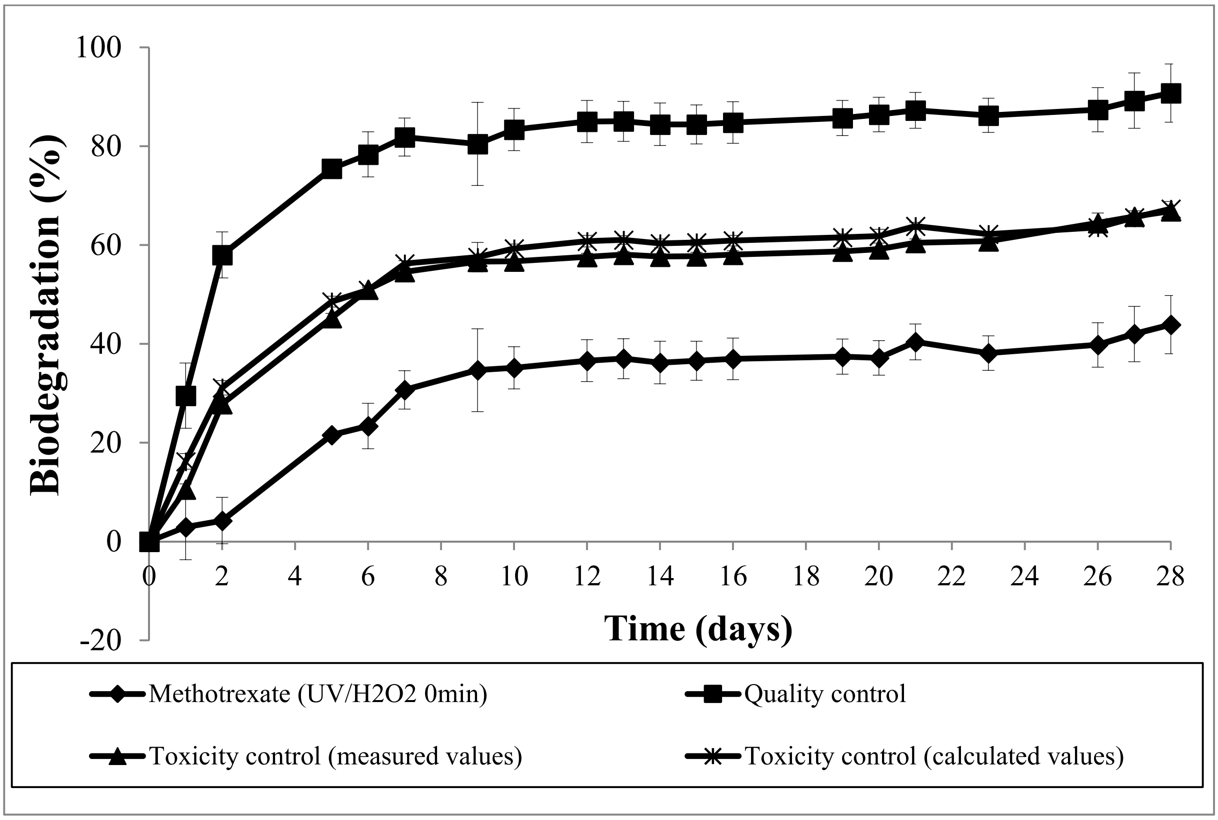


Fig S8– Closed Bottle Test (CBT) of MTX (n=2)

Table S3 – Apparent mineralization rate constants of methotrexate under different AOP's and the corresponding $t_{0.5}$.

Process	**K'$_{fast}$ (min $^{-1}$)**	**$t_{1/2\ fast}$ (min)**	**K'$_{slow}$ (min $^{-1}$)**	**$t_{1/2\ slow}$ (min)**	**R^2**	**SS**
UV/H_2O_2	0.0196 ± 0.002	35.43	—	—	0,995	0,0031
UV/Fe^{2+}/H_2O_2	0.4277 ± 0.067	1.62	0,0274 ± 0,006	25.34	0.998	0,0013
UV/TiO_2	0.0207 ± 0.002	33.51	—	—	0.993	0,0055

Table S4 – Apparent degradation rate constants of methotrexate under UV/H_2O_2 and UV/TiO_2 and corresponding $t_{0.5}$.

Process	**Time point (s)**	**k (s $^{-1}$)**	**R^2**	**$t_{1/2}$ (s)**
UV/H_2O_2	480	0,0016	0,983	433
UV/Fe^{2+}/H_2O_2	240	0,0035	0,987	198
UV/TiO_2	240	0,0039	1	177.7

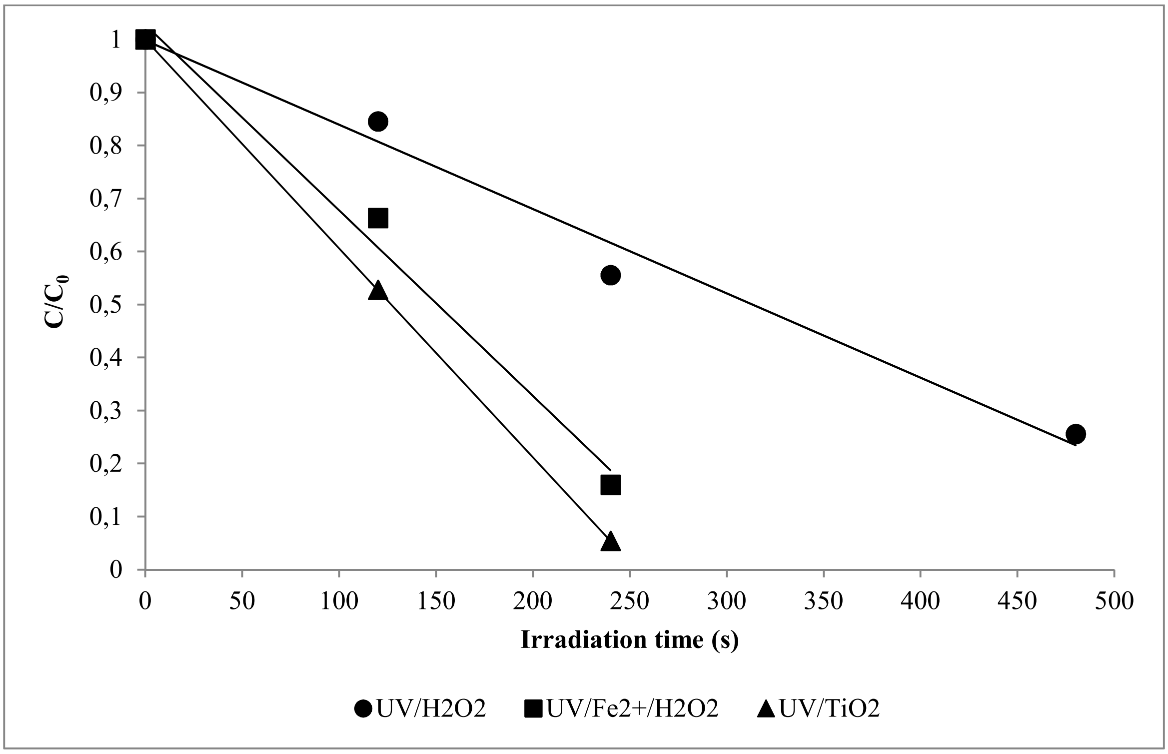


Fig. S9 – Kinetics of the primary elimination of MTX by UV/H_2O_2, UV/ Fe^{2+}/H_2O_2 and UV/ TiO_2.

Table S5. MS information of the TPs of MTX identified by means of LC-IT-MS (positive ion mode) in different degradation processes UV/H_2O_2 and UV/TiO_2

TP (LC-IT-MS)	**Rt (min)**[a]	**Proposed Molecular Formula**	**ESI(+) MS $[M+H]^+$ Precursor Ions m/z**[c]	**ESI(+) MS^2 Product Ions m/z (Relative abundance, %)**
MTX	12.1	$C_{20}H_{23}N_8O_5$	455.1	308 (100), 175 (10.2), 134 (6.5)
TP 179[b]	1.6	$C_{13}H_{17}N_2O_5$	179.0	
TP 191[c]	1.4	$C_7H_7ON_6$	191.0	
TP 207[b,c]	1.5	$C_7H_7O_2N_6$	207.0	
TP 209c	1.6	$C_7H_9N_6O_2$	208.9	163 (20.68), 191 (63.04), 192 (43.37)
TP 267[b,c]	2.3	$C_{12}H_{15}N_2O_5$	266.7	84.2 (16.28), 120 (100), 130.1(43.5), 174.8 (9.5), 191.8 (34.64), 249 (71.11)
TP 281[b,c]	7.5	$C_{13}H_{17}N_2O_5$	281.1	106.1 (0.76), 108.3 (6.43), 130.1 (4.18), 134.1 (100), 149.1 (0.99), 186.8 (2.94), 205 (0.96) 267 (0.93)
TP 325[b]	3.7	$C_{15}H_{17}ON_8$	325.1	
TP 469[c]	3.7	$C_{20}H_{21}O_6N_8$	469.0	
TP 471[b]	13.5	$C_{20}H_{23}O_6N_8$	470.9	

[a] Chromatography retention time

[b] UV/H_2O_2,

[c] UV/TiO_2

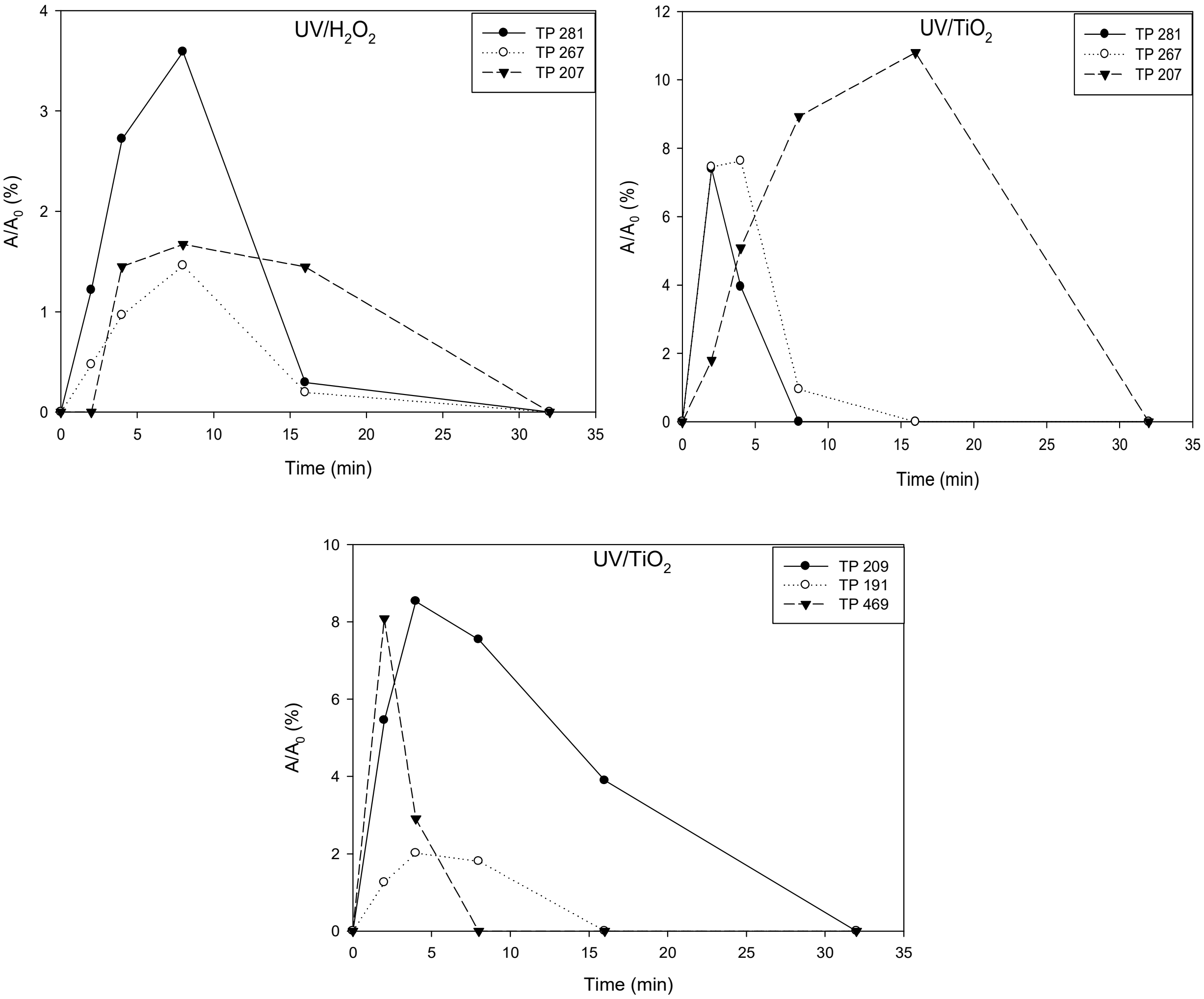
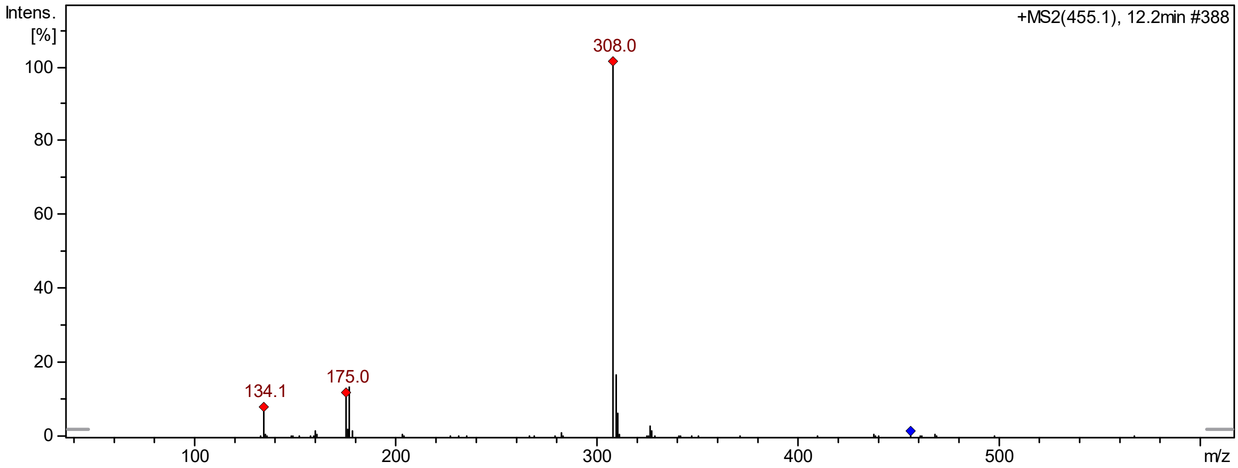
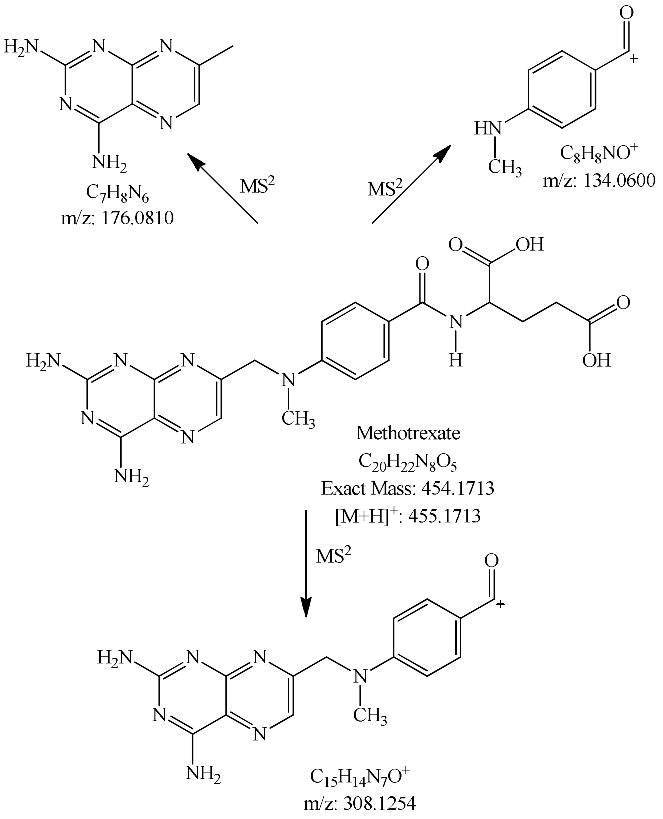


Fig. S10 - Profile of the peak area ratio (A/A_0) of the identified TPs formed during the UV/H_2O_2 and UV/TiO_2 processes.

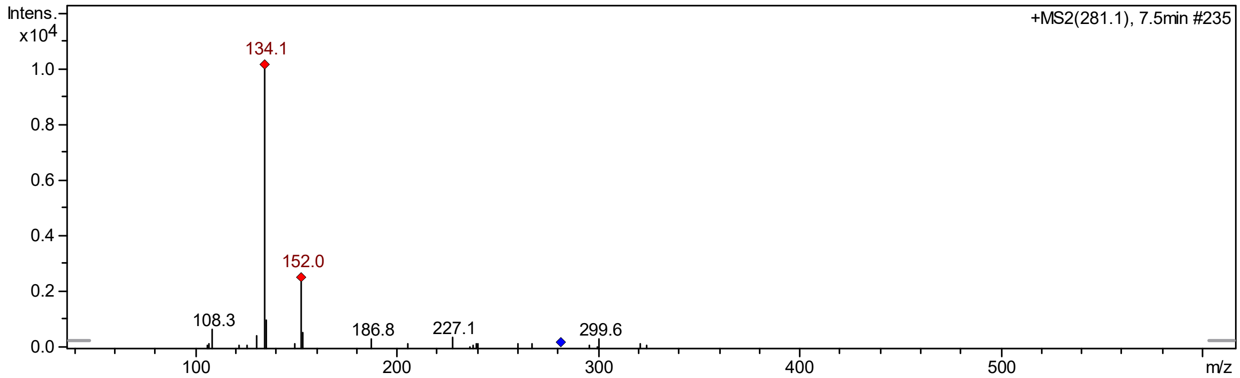
Text S4 – Identification and elucidation of the TPs from MTX by means of LC-MS/MS.

MTX





TP 281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x10^4
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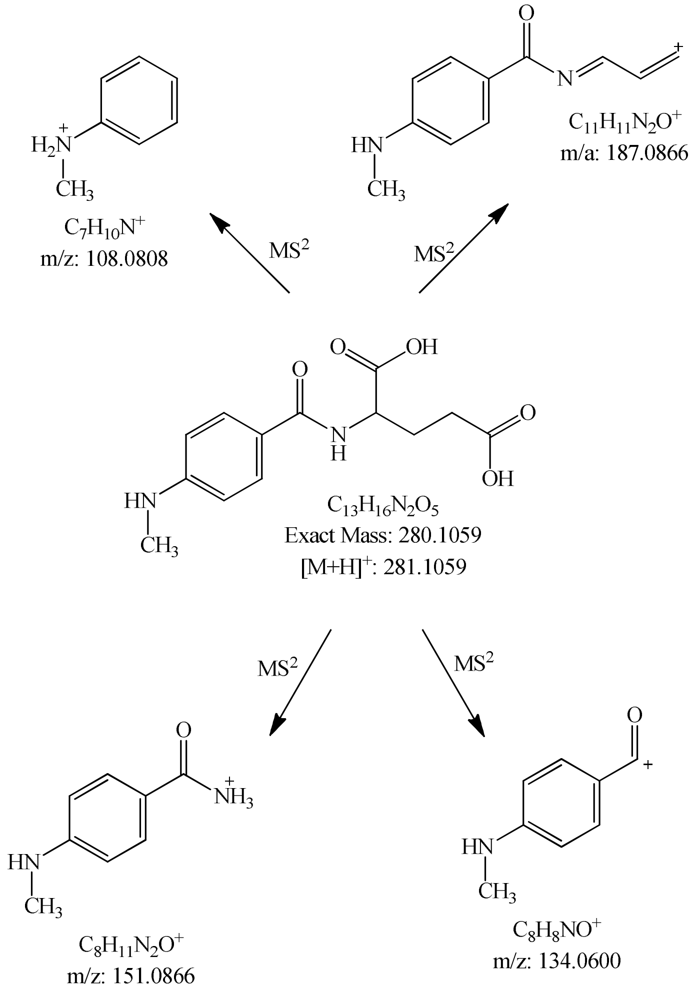
0.8
0.6
0.4
0.2
0.0
+MS2(281.1), 7.5min #235
134.1
152.0
108.3
186.8
227.1
299.6
100
200
300
400
500
m/z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CH3
C7H10N+
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MS2
MS2
O
N
+
HN
CH3
C11H11N2O+
m/a: 187.0866
O
OH
O
N
H
O
HN
CH3
OH
C13H16N2O5
Exact Mass: 280.1059
[M+H]+: 281.1059
MS2
MS2
O
NH3+
HN
CH3
C8H11N2O+
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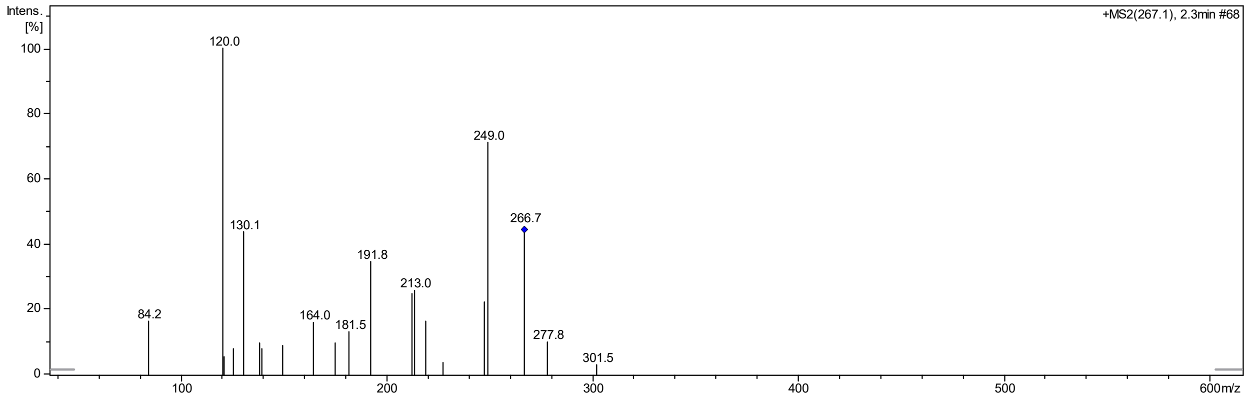
O
+
HN
CH3
C8H8NO+
m/z: 134.0600

TP 267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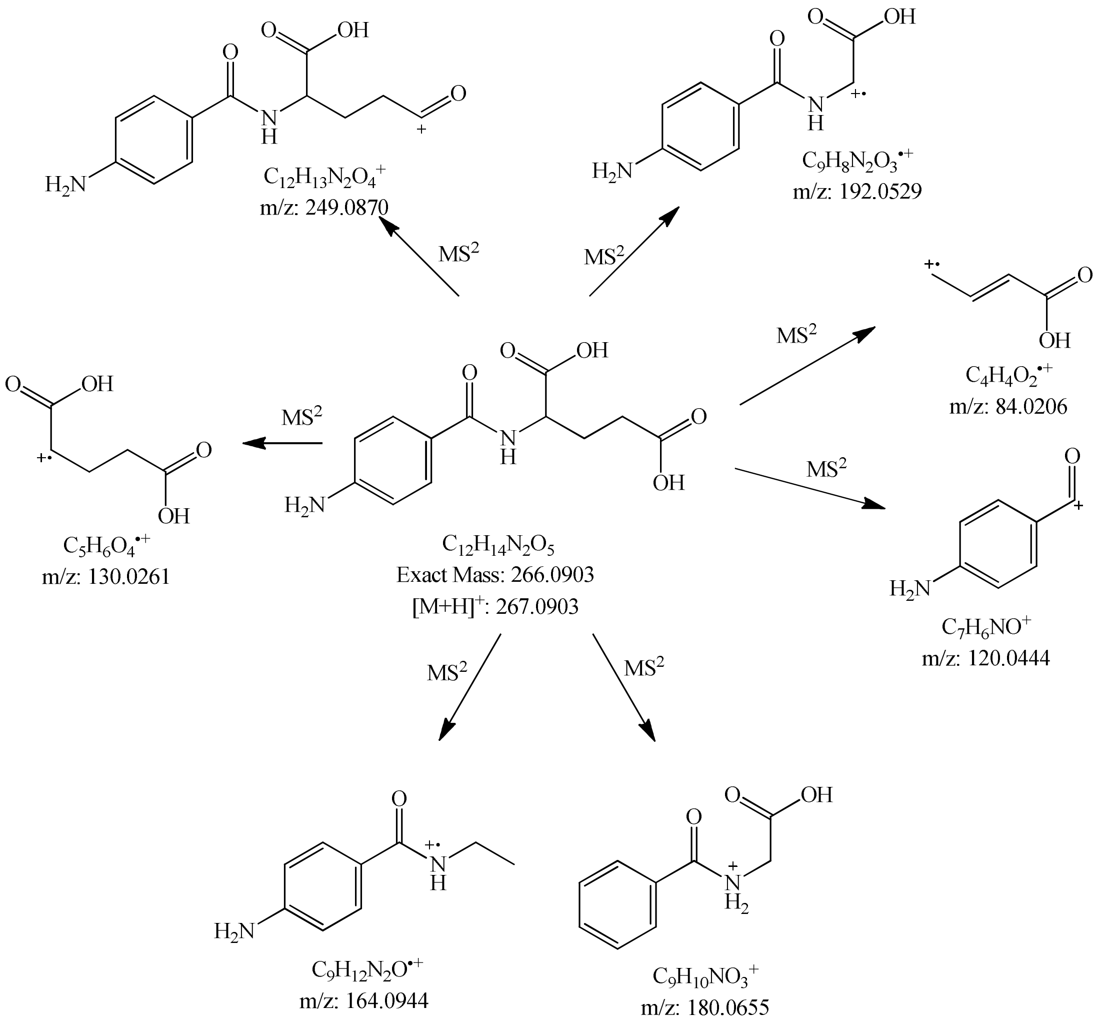
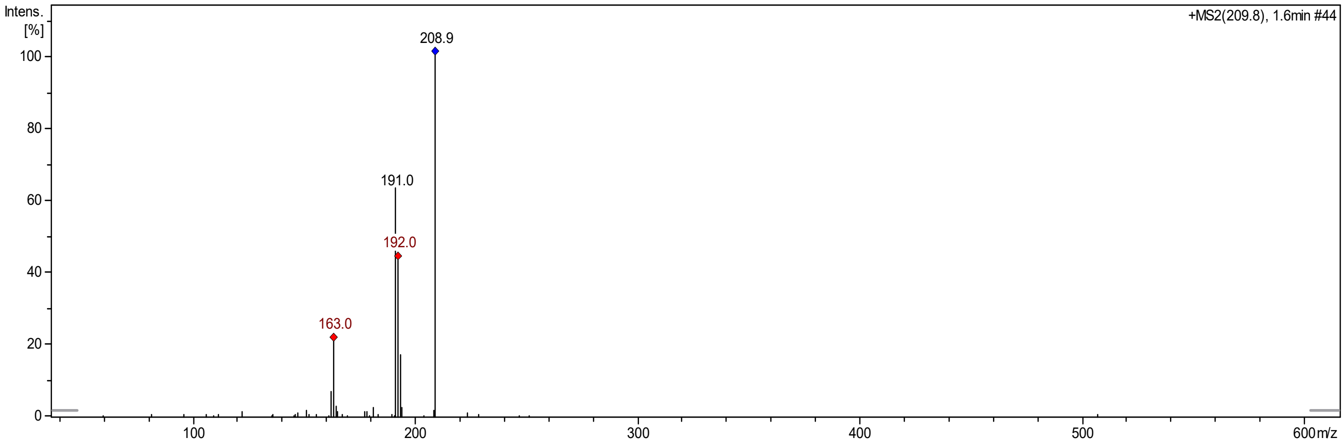
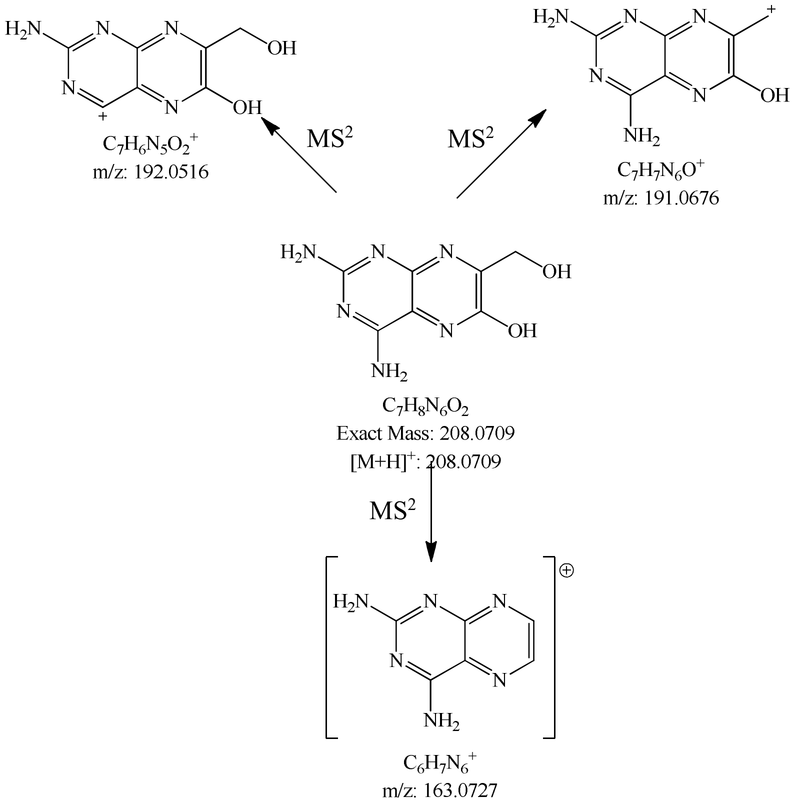
0
20
40
60
80
100
120.0
249.0
266.7
130.1
191.8
213.0
84.2
164.0
181.5
277.8
301.5
100
200
300
400
500
600m/z


$C_{12}H_{13}N_2O_4^+$
m/z: 249.0870
$C_9H_8N_2O_3^{\bullet+}$
m/z: 192.0529
$C_4H_4O_2^{\bullet+}$
m/z: 84.0206
$C_5H_6O_4^{\bullet+}$
m/z: 130.0261
$C_{12}H_{14}N_2O_5$
Exact Mass: 266.0903
$[M+H]^+$: 267.0903
$C_7H_6NO^+$
m/z: 120.0444
$C_9H_{12}N_2O^{\bullet+}$
m/z: 164.0944
$C_9H_{10}NO_3^+$
m/z: 180.0655
MS^2

TP 209





$C_7H_6N_5O_2^+$
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$C_7H_7N_6O^+$
m/z: 191.0676

$C_7H_8N_6O_2$
Exact Mass: 208.0709
$[M+H]^+$: 208.0709

$C_6H_7N_6^+$
m/z: 163.0727

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Sanderson, H., Thomsen, M., 2009. Comparative analysis of pharmaceuticals versus industrial chemicals acute aquatic toxicity classification according to the United Nations classification system for chemicals. Assessment of the (Q)SAR predictability of pharmaceuticals acute aquatic toxicity and their predominant acute toxic mode-of-action. Toxicology Letters 187, 84-93.

Trautwein, C., Kümmerer, K., Metzger, J.W., 2008. Aerobic biodegradability of the calcium channel antagonist verapamil and identification of a microbial dead-end transformation product studied by LC–MS/MS. Chemosphere 72, 442-450.

Article III

Degradation of cyclophosphamide and 5-fluorouracil by UV and simulated sunlight treatments: Assessment of the enhancement of the biodegradability and toxicity.

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journal homepage: www.elsevier.com/locate/envpol

Degradation of cyclophosphamide and 5-fluorouracil by UV and simulated sunlight treatments: Assessment of the enhancement of the biodegradability and toxicity

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ABSTRACT

The presence of pharmaceuticals in the environment has triggered concern among the general population and received considerable attention from the scientific community in recent years. However, only a few publications have focused on anticancer drugs, a class of pharmaceuticals that can exhibit cytotoxic, genotoxic, mutagenic, carcinogenic and teratogenic effects. The present study investigated the photodegradation, biodegradation, bacterial toxicity, mutagenicity and genotoxicity of cyclophosphamide (CP) and 5-fluorouracil (5-FU). The photodegradation experiments were performed at a neutral to slight pH range (7–7.8) using two different lamps (medium-pressure mercury lamp and a xenon lamp). The primary elimination of the parent compounds was monitored by means of liquid chromatography tandem mass spectrometry (LC-IT-MS/MS). NPOC (non-purgeable organic carbon) analyses were carried out in order to assess mineralization rates. The Closed Bottle Test (CBT) was used to assess ready biodegradability. A new method using *Vibrio fischeri* was adopted to evaluate toxicity. CP was not degraded by any lamp, whereas 5-FU was completely eliminated by irradiation with the mercury lamp but only partially by the Xe lamp. No mineralization was observed for the experiments performed with the Xe lamp, and a NPOC removal of only 18% was registered for 5-FU after 256 min using the UV lamp. Not one of the parent compounds was readily biodegradable in the CBT. Photo transformation products (PTPs) resulting from photolysis were neither better biodegradable nor less toxic than the parent compound 5-FU. In contrast, the results of the tests carried out with the UV lamp indicated that more biodegradable and non-toxic PTPs of 5-FU were generated. Three PTPs were formed during the photodegradation experiments and were identified. The results of the *in silico* QSAR predictions showed positive mutagenic and genotoxic alerts for 5-FU, whereas only one of the formed PTPs presented positive alerts for the genotoxicity endpoint.

Abbreviations: 5-FU, 5-fluorouracil; AI, acute luminescence inhibition; CBT, closed bottle test; CFU, colony forming units; CI, chronic luminescence inhibition; CP, cyclophosphamide; DNA, deoxyribonucleic acid; DOC, dissolved organic carbon; EC, effective concentration; GI, growth inhibition; HPLC, high performance liquid chromatography; LC, liquid chromatography; LOEC, lowest observed effect concentration; LSSTP, laboratory-scale sewage treatment plant; MS, mass spectrometry; NPOC, non purgeable organic carbon; OECD, organization for economic co-operation and development; PTPs, photo-transformation products; QSAR, quantitative structure–activity relationship; TOC, total organic carbon; ThOD, theoretical oxygen demand; UV, ultraviolet.

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1. Introduction

Pharmaceuticals were already identified as environmental contaminants in the 70s (Hignite and Azarnoff, 1977). Because of the stability of many compounds, the removal of these substances through conventional water and wastewater treatments is often incomplete and inefficient (Wang and Lin, 2014; Zhang et al., 2013). Pharmaceuticals and their metabolites can undergo several physical–chemical and biological processes (e.g., dilution, hydrolysis, non-biotic oxidation, biodegradation, photolysis and sorption to sediments). These natural and technical processes can lead also to the formation of stable transformation products (TPs), which can be more harmful and persistent than the parent compounds (Illés et al., 2014; Gros et al., 2006).

Since many times biodegradation plays only a minor role when it comes to the elimination of the pharmaceuticals, photolysis in technical systems (e.g. potable water treatment, waste water treatment) and natural waters by irradiation with sunlight has been suggested as a significant degradation pathway of these compounds (Wang and Lin, 2014; Mahmoud and Kümmerer, 2012).

Although growing attention has been paid to the presence of pharmaceuticals in the environment, data about important classes such as anticancer drugs is still scarce. However, even at low concentrations, these compounds are likely to present a hazard, as they can have cytotoxic, genotoxic, mutagenic, carcinogenic and teratogenic effects on non-target organisms (Allwood et al., 2002; Kümmerer et al., 2014). Furthermore, most of the anticancer drugs are characterized by low biodegradability, which suggests that conventional sewage treatment plants will be unable to remove these kinds of compounds from water (Toolaram et al., 2014).

Cyclophosphamide (CP) and 5-Fluorouracil (5-FU) are among to most commonly consumed cytotoxic drugs worldwide, and several studies have found evidence for their presence in different water compartments in concentrations ranging from ng/L up to µg/L (Buerge et al., 2006; Gómez-Canela et al., 2013; Catastini et al., 2008; Mahnik et al., 2007; Weissbrodt et al., 2009; Kosjek et al., 2013; Besse et al., 2012). Table 1 lists the chemical structures, Chemical Abstract Services numbers, molecular formulas, excretion rates and consumption in several countries of CP and 5-FU.

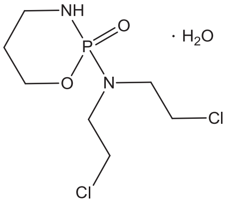
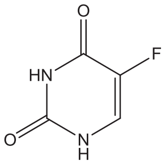
Recently, some researchers have tried to understand the environmental fate of CP and 5-FU with the help of photodegradation, biodegradation and toxicity studies (e.g. Kosjek et al., 2013; Lin et al., 2013; Lin and Lin, 2014). However, to the best of our knowledge, there have been no studies comparing the elimination and mineralization of both compounds by sunlight and UV irradiations. This kind of study may contribute to an assessment of risk resulting from the environmental and human exposure to these compounds and potential TPs from its release in natural aquatic environments until its re-uptake in wastewater and drinking water treatment plants equipped with UV radiation systems.

Many of the studies on these compounds are insufficient because they focus only on primary elimination; however, TPs can, as indicated above, be even more recalcitrant and toxic than the parent compounds (Magdeburg et al., 2014; Lutterbeck et al., 2015a). In light of the difficulties of obtaining a complete mineralization (time, costs and formation of recalcitrant TPs), it makes sense to reduce the impact of these compounds by improving their biodegradability and by reducing their toxicity. Some studies have assessed the acute toxicity of anticancer drugs and of the TPs formed during treatments (Lin and Lin, 2014; Calza et al., 2014). Unfortunately, short-term assays can underestimate the real toxic potential of these compounds (Backhaus and Grimme, 1999). Therefore, a toxic evaluation should encompass different toxicity endpoints (acute, chronic and growth).

Finally, it is important to note here that the TPs identified in the processes are, in general, formed in low concentrations in complex compartments, and for this reason, it is exceedingly difficult to isolate and assess a given compound. In addition, many of these compounds are not commercially available, and individual experimental analyses of their toxicity are, therefore, almost impossible. Nevertheless, it is possible to gain valuable insights regarding the toxicological properties of anticancer drugs and related TPs by using *in silico* tools based on quantitative structure–activity relationships (QSARs).

Considering the hazard that anticancer drugs may pose to different living organisms, even at low concentrations, the present study aimed to: (i) evaluate the photochemical degradation and mineralization of CP and 5-FU under UV (Hg medium pressure lamp) and simulated sunlight irradiation (i.e. Xe lamp) treatments; (ii) assess the biodegradability before and after the photochemical experiments; (iii) evaluate the toxicity of the compounds before and after the treatments using different endpoints; (iv) identify possible transformation products formed during photodegradation experiments and (v) use the new insight of *in silico* QSAR predictions to assess the mutagenicity and genotoxicity of 5-FU and PTPs.

Table 1
Chemical structures, Chemical Abstract Services numbers, molecular formulas, consumption and excretion rates of cyclophosphamide and 5-fluorouracil.

Substance name, chemical formula and CAS number	Chemical structure	Annual consumption (in kg, per country)	Unchanged drug excretion (%)	Reference
Cyclophosphamide monohydrate		27–272 (Taiwan, 2005)		Lin et al. (2013)
$C_7H_{15}Cl_2N_2O_2P \cdot H_2O$		55 (UK, 2001)	20	Rowney et al. (2009)
6055-19-2		305.73 (France, 2008)	>25	Besse et al. (2012)
		260–288 (Germany, 2010)	11–20	Mompelat et al. (2009)
			20	Kümmerer and Al-Ahmad (2010)
			15–25	Kovalova et al. (2009)
			10–40	Zhang et al. (2013)
			5–25	Gómez-Canela et al. (2013)
5-Fluorouracil		119 (Austria, 1997)	2–35	Mahnik et al. (2007)
$C_4H_3FN_2O_2$		74–297 (Taiwan, 2005)		Lin et al. (2013)
51-21-8		5593.37 (UK, 2001)	7–11	Rowney et al. (2009)
		1733.2 (France, 2008)	20	Besse et al. (2012)
			5–15	Kovalova et al. (2009)
			15	Zhang et al. (2013)

2. Materials and methods

Anticancer drugs are recognized as dangerous substances and must be handled carefully. Strict safety precautions were taken for all the experiments performed in our laboratories (Allwood et al., 2002; Eitel et al., 1999). The waste generated during these experiments was disposed and treated as hazardous, and the instruments used were carefully cleaned after usage.

2.1. Chemicals

Acetonitrile (HiPerSolv CHROMANORM, LC–MS grade, BDH Prolabo, 99.9%) and formic acid (98–100%) were purchased from VWR International GmbH (Darmstadt, Germany). CP (95%, Endoxan, Baxter Oncology, Round Lake, IL, USA) and 5-FU (99%, Medac Gesellschaft für Klinische Spezialpräparate Gmbh Hamburg, Germany) were prepared by the pharmacy of the Hospital Lüneburg, Germany (Therapeutic infusions bags were prepared on demand). In order to minimize any scavenging effect of other species and to evaluate only the degradation of the two compounds, all the solutions were prepared using ultrapure water (Q_1:16.6 mΩ and Q_2: 18.2 MΩ Ultra Clear Water, Evoqua Water Technologies GmbH, Barsbüttel, Germany).

2.2. Photodegradation

The experiments were carried out in an 800 mL batch photoreactor containing 600 mL of the solutions with an initial concentration of 20 mg/L. The higher initial concentrations were used to allow for subsequent biodegradation experiments, to enable the identification of transformation products (TPs) formed during the photodegradation experiments and to evaluate the possible effects on *Vibrio fischeri*. The samples were irradiated using a xenon lamp (TXE 150 W, UVConsulting Peschl, Mainz, Germany) and a medium-pressure mercury lamp (TQ150, UV Consulting Peschl, Mainz, Germany) with an ilmasil quartz immersion tube. Both lamps exhibit a polychromatic spectrum. A lamp output of 398.4 W/m^2 (200–440 nm) was measured for the Hg lamp using an UVpad Spectral Radiometer (Opsytec Dr. Gröbel GmbH, Ettlingen, Germany), whereas for the Xe lamp, a total lamp output of 260.2 W/m^2 was measured using the function spectroradiometer of a BLACK-Comet UV–VIS Spectrometer (model C) (StellarNet Inc., Florida, USA). Fig. S1 shows the intensity of light emitted by the UV and Xe lamps and the molar extinction coefficient of 5-FU and CP for the range of 200 nm–800 nm.

Magnetic stirring was used throughout the experiments. A circulating cooling system (WKL230, LAUDA, Berlin) was used to maintain the temperature between 20 ± 2 °C. Each test was performed in neutral to slightly basic pH range (7–7.8) for 256 min, and samples were taken at regular time points (2, 4, 8, 16, 32, 64, 128 and 256 min) to check the elimination, transformation and mineralization rates of the compounds.

2.3. Instrumental analysis

The monitoring of the primary eliminations of CP and 5-FU and the identification of TPs generated during the photolysis experiments were carried out by means of liquid chromatography tandem mass spectrometry (LC-IT-MS/MS). The analysis in positive ion mode was performed in an Agilent 1100 module (Agilent Technology, Waldbronn, Germany) HPLC-tandem mass spectrometer (MS) Bruker Daltonic Esquire 6000^{plus} ion trap (Bruker Daltonik GmbH, Bremen, Germany). The ionization was performed by electrospray ionization (ESI).

A Dionex Ultimate 3000 UHPLC system (Dionex, Idstein, Germany) tandem LTQ Orbitrap-XL with H-ESI source (Thermo Scientific, Bremen, Germany) (LC-HRMS) was used to analyse the primary elimination of 5-FU and to identify TPs in negative ion mode.

A more detailed description of the operational conditions and the LOQ and LOD of both compounds can be found in the supplementary material (Text S1 and Tables S1 and S2).

Mineralization was measured using non-purgeable organic carbon (NPOC) with a TOC (total organic carbon) analyzer (TOC VCPN 5050, Shimadzu GmbH, Duisburg, Germany).

2.4. Determination of the kinetic of degradation

In order to estimate the kinetics of the elimination for the substances, the following pseudo-first order relationship was adopted according to Equation (1):

$$ln\frac{C}{C_0} = -kt \tag{1}$$

where C is the concentration of the compound; C_0 is the initial concentration of the compound; k is the rate constant; t is the reaction time. Half-lives were calculated with the help of the following equation (Equation (2)):

$$t_{1/2} = \ln 2/K \tag{2}$$

2.5. Biodegradation

The Closed Bottle Test (CBT) was used to evaluate the biodegradation of CP, 5-FU and PTPs formed during photodegradation experiments. CBT is an aerobic biodegradation test that is recommended as a very first step when assessing the biodegradability of organic compounds (Nyholm, 1991; OECD, 1992). The test was performed, following the OECD guidelines, with low nutrient content and under low bacterial density conditions (similar to natural surface water environments), at 20 ± 1 °C, in the dark, for 28 days (OECD, 1992). Each CBT was run in duplicate.

The inoculum was collected from the final effluent of a municipal sewage treatment plant (STP) serving 73,000 inhabitant equivalents (AGL GmbH, Lüneburg Nord, Germany). A more detailed explanation of the principles, procedures and composition of the CBT is given in Text S2.

2.6. Toxicity assays

The assays were performed according to Menz et al. (2013), using the luminescent bacteria strain *V. fischeri* NRRL-B-11177 (Hach-Lange GmbH) for the combined assessment of short-term luminescence inhibition after 30 min (LI30min), long-term luminescence inhibition after 24 h (LI24h) and growth inhibition after 14 h (GI14h). A more detailed description of the method can be found elsewhere (Menz et al., 2013). Samples were analysed before (0 min) and after (256 min) the photochemical treatments.

Previous toxicity tests performed in our laboratories (Lutterbeck et al., 2014) showed that the lowest observed effect concentration (LOEC) of CP is 120 mg/L (much higher than the concentration used in our assays, namely 20 mg/L). Therefore, in this study, the toxicity assays were performed only with 5-FU. These assays were carried out based on prescreening tests performed in our laboratories. We considered the results of these tests to establish concentration ranges for performing the tests and, in this way, compare the efficiency of both lamps after the treatments. The obtained EC_{50} values of chronic tests (0.45 mg/L) were considered in order to establish the concentration ranges to be tested.

Significant inhibition differences between the samples before and after the treatments were identified by One Way ANOVA, following Tukey's Multiple Comparison Test (overall significance level = 0.05) with the statistical software Prism 5 (Graphpad Inc., CA, USA).

2.7. *In silico predictions for toxicity of 5-FU and the generated photo-TPs*

In order to assess the toxic effects of the parent compound of 5-FU and of the PTPs formed during the photodegradation experiments, *in silico* predictions using the software CASE Ultra 1.5.2.0 were performed. A set of different models, which enable the prediction of the mutagenic and genotoxic activities, was selected. More specifically, a combination of statistical and rule-based systems in line with the recently implemented ICH guideline M7 (International Conference on Harmonization (ICH), 2014) was applied (GT1_A7B, GT1_AT_ECOLI, GT_EXPERT, PHARM_ECOLI, PHARM_SALM). Chemical structure illustrations were performed by using the plugin MarvinSketch (5.8.0, 2012) ChemAxon (http://www.chemaxon.com). Simplified molecular input line entry specification (SMILES) codes from the molecular TPs structures in their neutral form were used for input of molecular structures.

3. Results and discussion

3.1. *Photodegradation*

As can be seen in Fig. S1 (Supplementary Material), the absorbance of CP above 200 nm is almost negligible, whereas 5-FU has the maximal absorbance at 265 nm, with a low absorption at wavelengths higher than 290 nm.

As shown in Fig. 1A and B, CP was neither eliminated nor mineralized in the experiments with both lamps. These results can be attributed to the very low absorbance of CP in the emissions wavelength range of the Hg lamp (200–600 nm) and the Xe lamp (200–800 nm).

These findings confirm the results of previous studies. In a study performed with a sunlight simulator (Suntest CPS, Atlas, equipped with a 1.5 kW Xe lamp), Lin et al. (2013) also observed no degradation of CP through direct photolysis. Furthermore, in experiments carried out with an UV low pressure lamp emitting light at 254 nm (most intensive emitted wavelength), CP turned out to be the most resistant substance among a group of 30 pharmaceuticals and personal care products (Kim and Tanaka, 2009). According to Kim and Tanaka (2009), the low degradation rates that are achieved only with UV irradiation can be attributed to the presence of the amide bonds in the chemical structure of CP (UV max. absorption at 193 nm).

Table 2
Degradation rate constants of 5-fluorouracil, using Hg and Xe lamps and the corresponding $t_{1/2}$.

Lamp	Time point (min)	K (min^{-1})	R^2	$t_{1/2}$ (min)
Hg	16	0.055	0.92	12.5
Xe	256	0.0015	0.99	462.1

In contrast to CP, 5-FU underwent direct photolysis when exposed to irradiation from both lamps, albeit much faster in the case of the Hg lamp (Fig. 1A). As suggested by the results of a previous study (Kosjek et al., 2013), it was assumed that the degradation of 5-FU by the mercury lamp obeys pseudo-first order kinetics. A linear relationship of ln C/C_0 versus t was established from 0 min to 16 min. The results showed a rate constant of 0.055 min^{-1} and a half-life of 12.5 min (Table 2).

After 32 min, a complete primary elimination of 5-FU was observed. However, NPOC measurements showed a degree of mineralization of only 18% (±5.1%) after 256 min of the treatment, indicating the formation of PTPs that are resistant to direct photolysis (Fig. 1B). As can be seen in Fig. 1B, after the elimination of the parent compound the mineralization rate slows down. This behavior shows presence of 2 steps in the mineralization process: the elimination 5-FU (faster reaction) and the formation of stable intermediates which in turn react on a second phase into the final products (slower reaction). In a study performed with a low pressure monochromatic UV mercury lamp with peak emission at 254 nm, Kosjek et al. (2013) observed a degradation rate constant of 0.045 min^{-1} and a half-life of 15 min for 5-FU, which is similar to the results of our experiments (Table 2).

Since the absorbance of 5-FU at wavelengths >290 nm is very limited, the parent compound was only partially degraded (32.2%) when the Xe lamp was used, and no significant NPOC variation was observed after 256 min of exposition, indicating negligible mineralization. This finding also suggested that possible PTPs of 5-FU are likely to be resistant to photolysis under these conditions. These results also demonstrate that 5-FU photolysis under simulated sunlight irradiation obeyed pseudo first-order kinetics (Table 2).

Lin et al. (2013) studied the photodegradation of 5-FU in real surface waters by using a sunlight simulator Suntest CPS equipped with a 1.5 kW Xe arc lamp and emitting radiation in the range of

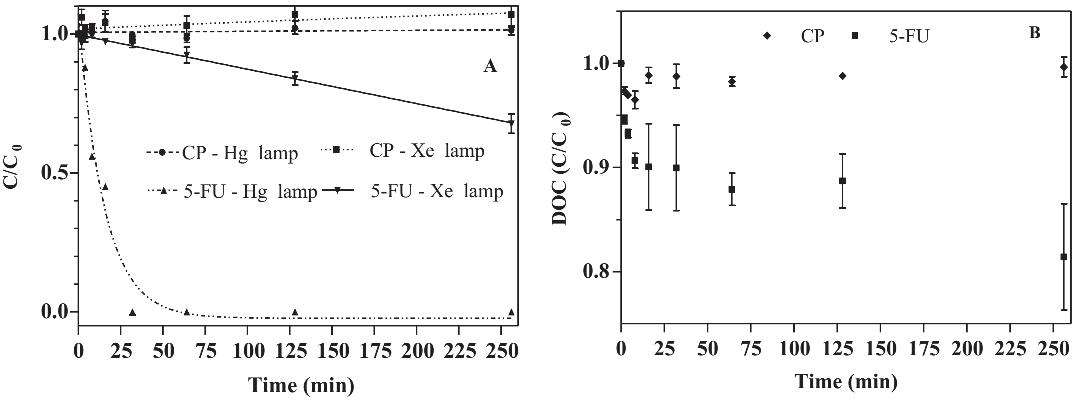


Fig. 1. **A** – Primary elimination of the parent compounds (20 mg/L) using the Hg and Xe Lamps during 256 min (n = 2). **B** – Mineralization degrees of the target compounds during 256 min using Hg Lamp. (n = 2). In the case of 5-FU there seem to be a change in kinetics between 128 und 256 min.

290–800 nm. The authors confirmed the appearance of PTPs that were not susceptible to photodegradation. Furthermore, a primary elimination of 50% from the initial concentration of 5-FU occurred after 9.8 h of irradiation, while a mineralization had not even occurred after 42 h. Lin et al. (2013) attributed this fact to the lack of chromophore moiety, whose absence precludes direct photolysis of the generated by-products. The half-life of 5-FU obtained in our experiments (7.7 h) was similar to the one observed in the study by Lin et al. (2013) (Table 2).

Herrmann et al. (2015) monitored the concentration of dissolved oxygen during photodegradation of gabapentin using an Hg medium pressure lamp with a higher intensity (560.7 W/m^2) and verified the consumption of dissolved oxygen. The H_2O_2 concentration was qualitatively measured, proving that direct photolysis might lead to the formation of HO• radicals. Accordingly, it might be proposed that indirect photolysis can take place through reactive oxygen species (ROS) such as $^1O_2$ and $•O_2^-$, HO•, $HO_2•$ and H_2O_2. Besides, studies have proposed the formation of ROS through photosensitizers and even through self-sensitization (Ge et al., 2009).

However, as CP does not present chromophores moieties absorbing photons in the emission range of the lamps used in the present study (see Fig. S1), no transformation under these kinds of conditions was observed. Therefore, it might be proposed that HO• radicals are not involved in the photodegradation of CP, which is in line with the literature (Lin et al., 2013). Thus, CP does not undergo photodegradation (Fig. 1A, B).

In contrast to CP, 5-FU absorbs UV radiation, and HO• radicals might be formed through dissolved oxygen or self-sensitization (Du et al., 2014; Sun et al., 2014). Thus, the proposed degradation of 5-FU might follow different pathways based on ROS and through direct photolysis.

It might be inferred that 5-FU underwent direct photolysis leading to a singlet-excited state and thereafter achieving a triplet-excited state. The excited molecule might further react and then form TPs, or the dissolved oxygen might quench the triplet state, stopping the transformation (Ryan et al., 2010). Self-sensitization can, however, also take place, which leads to the formation of $^1O_2$ and HO• radicals, which, in turn, would therefore lead to a degradation pathway based on ROS (Latch et al., 2003).

Based on the results of our photolysis experiments, it is possible to conclude that once released in the environment, both compounds are expected to remain in the natural aquatic cycle. Whereas CP will not be degraded by sunlight, 5-FU is expected only to be partially eliminated by this natural attenuation mechanism. For this reason, alternatives approaches are needed to eliminate these compounds from the water cycle. Considering specifically CP, UV radiation, as used in our experiments, turned not out to be a good alternative for this purpose. In contrast, the parent compound of 5-FU was completely eliminated with the help of UV radiation after 32 min. However, mineralization degrees lower than 20% after relatively long treatment times (256 min) indicate the formation of stable intermediates.

Taking into account that neither sunlight nor UV radiation are likely to lead to high mineralization rates and will therefore generate stable TPs, the assessment of the treatment's efficiency should be based on the improvement of the biodegradability and/or on the reduction of the toxicity of the photolytic mixture, i.e., whether the transformation products generated during the treatments are more biodegradable and less toxic than the parent compounds. This will be discussed in the following sections.

3.2. Biodegradation

All validity criteria of the OECD guideline for CBT were fulfilled. The results indicated that none of the parent compounds can be classified as readily biodegradable (Fig. S2).

After 28 days, CP showed a biodegradation between 25% and 30% (Fig. S2 A, B). The classification as a non-readily biodegradable compound is in line with the results of previous CBT experiments (Kümmerer et al., 1996). Moreover, even in biodegradation experiments that simulate sewage treatments plants (which contain a much higher density and diversity of bacteria) and at concentrations ranging from low ng/L to a few hundred mg/L, CP was not eliminated (Steger-Hartmann et al., 1997; Kiffmeyer et al., 1997; Buerge et al., 2006). Therefore, the results of these experiments confirm that CP is poorly biodegradable at both low and high concentrations and is expected to remain in the water cycle.

The biodegradation of 5-FU varied between 35 and 44% in the CBT (Fig. S2 C, D). In the literature, there are contradictory results regarding the biodegradability of 5-FU. Kiffmeyer et al. (1997) observed a full elimination of 5-FU (5 mg/L) in an OECD confirmatory test after two days. However, when testing higher initial concentrations (10 and 20 mg/L), the authors found a slower biodegradation rate, which might indicate a possible toxic effect that retards the biodegradation. Kosjek et al. (2013) investigated the degradation of 5-FU in batch biodegradation experiments (0.5 L) using inoculum of activated sludge (AS) with an initial concentration of 5.4 g/L. The results showed that 5-FU was almost completely eliminated at lower concentrations (1 and 10 mg/L) after 40 h, whereas a toxic effect was observed at higher concentrations (20 and 100 mg/L), which retarded or even inhibited biodegradation. Although the results mentioned above suggest a fast elimination of 5-FU, it must be pointed out that all these studies where performed with tests that have a higher bacterial density, which might have a direct influence on the biodegradation rate of a given compound.

CBT is a test that simulates natural surface waters, and it is characterized by low bacterial density (500 CFU/mL), which can explain the differences between the low degrees of biodegradation obtained in our experiments and the higher degrees reported in other studies. Kümmerer and Al-Ahmad (1997) found no biodegradation of 5-FU in the Closed-Bottle-Test (OECD 301 D) and in the Zahn–Wellens-Test (OECD 302 B) for concentrations of 9.02 and 854 mg/L, respectively. Yu et al. (2006) observed a low degree of biodegradation of FU, even at lower concentrations. After 50 days of incubation, the results showed degradations of only 50 and 30% for initial concentrations of 1 and 50 μg/L, respectively.

As can be seen in Figs. 2A and 3A, irradiation with both lamps (Xe and Hg) did not improve the biodegradability of CP after 256 min. These results were expected, since, as already mentioned above, not one of the lamps could eliminate or mineralize CP.

The partially primary elimination of 5-FU by the Xe lamp did also not improve its biodegradability after 28 days (Fig. 2B). However, as can be observed in Fig. 3B, the samples of 5-FU submitted to the UV photolysis showed a great improvement in terms of biodegradability after 28 days. Therefore, considering that the parent compound was completely transformed by the UV lamp and that only 18% mineralization were achieved after 256 min, the improvement of the biodegradability is clearly associated to the formation of more biodegradable PTPs.

3.3. Toxicity assays

As shown in Fig. 4, the samples resulting from treatments with the Xe lamp showed no significant differences in terms of toxicity for the three endpoints (AI, CI and GI). This result could be explained by the partial transformation of the parent compound (only 32% of primary elimination) and the formation of PTPs that exhibit a similar toxicity to 5-FU, since no mineralization was

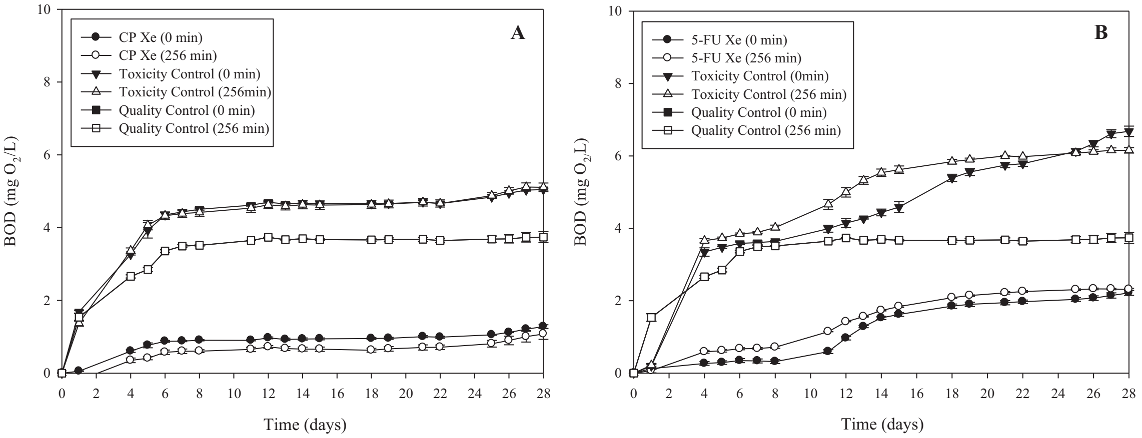


Fig. 2. Degradation in Closed Bottle Test of **A** – cyclophosphamide (4.5 mg/L; irradiation time 0 min) and photodegradation mixture (256 min of irradiation with Xe lamp) **B** – 5-fluorouracil (4.5 mg/L; irradiation time 0 min) and photodegradation mixture (256 min of irradiation with Xe lamp) (n = 2). "Quality control" contained readily biodegradable sodium acetate in a concentration corresponding to 5 mg/L theoretical oxygen demand (ThODNH3, calculated without considering a possible nitrification) as the only organic carbon source besides the inoculum. "Toxicity control" contained in addition to the inoculum, the test compound and sodium acetate in a concentration corresponding to 5 mg/L ThOD, respectively.

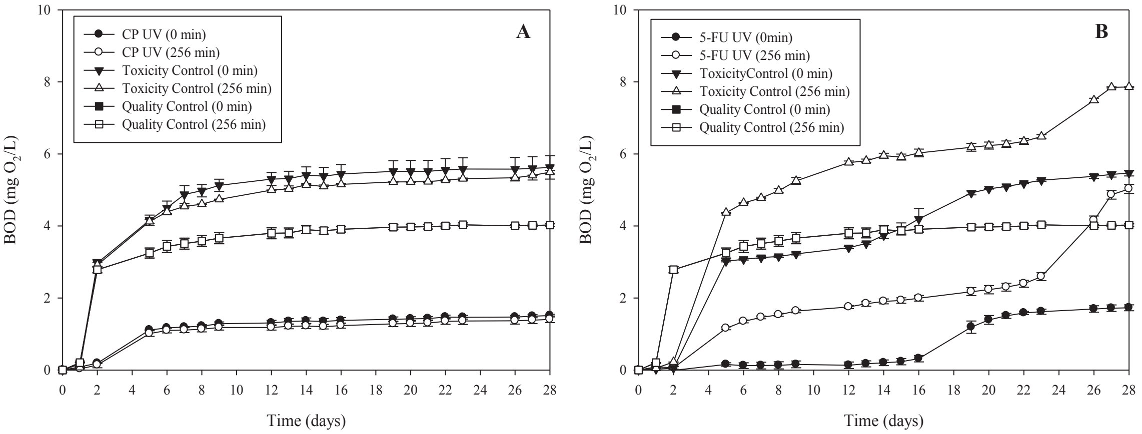


Fig. 3. Degradation in Closed Bottle Test of **A** – cyclophosphamide (4.5 mg/L; irradiation time 0 min) and photodegradation mixture (256 min of irradiation with Hg lamp) **B** – 5-fluorouracil (4.5 mg/L; irradiation time 0 min) and photodegradation mixture (256 min of irradiation with Hg lamp) (n = 2). "Quality control" contained readily biodegradable sodium acetate in a concentration corresponding to 5 mg/L theoretical oxygen demand (ThODNH3, calculated without considering a possible nitrification) as the only organic carbon source besides the inoculum. "Toxicity control" contained in addition to the inoculum, the test compound and sodium acetate in a concentration corresponding to 5 mg/L ThOD, respectively.

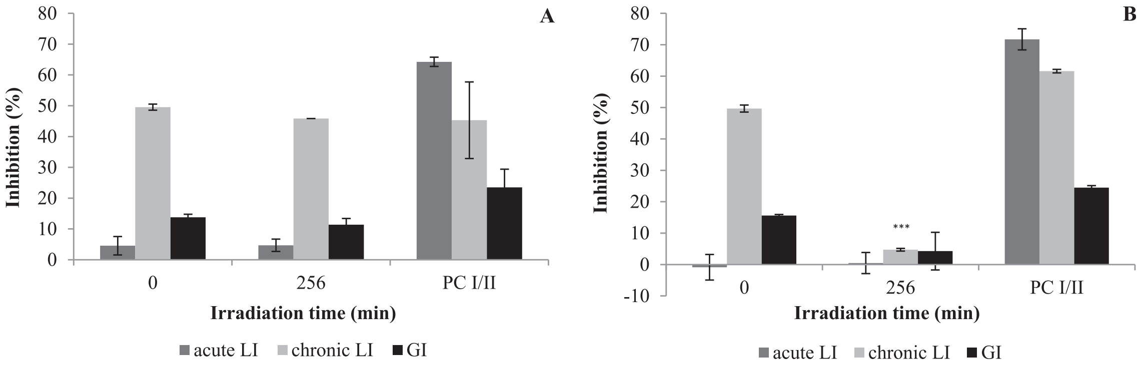


Fig. 4. Toxicity tests with *V. fischeri* considering three endpoints before (0 min) and after (256 min) the treatments with both lamps (n = 2) **A** = Xe Lamp and **B** = Hg. Photolysis was conducted with an initial 5-fluorouracil concentration of 20 mg/L. Considering the EC_{50} for the CI (0.45 mg/L) obtained in previous studies performed in our laboratories, dilutions of 1:2 of the parent compound and photolytic mixtures were done until a dilution of 1/32, which represents a concentration of 0.63 mg L^{-1} (similar to the EC_{50} of 5-fluorouracil). Positive control I (PCI): 4.5 mg/L 3,5-Dichlorophenol (acute LI), Positive control II (PCII): 0.05 mg/L Chloramphenicol (chronic LI, growth Inh.). Statistically significant differences (***) compared to the untreated samples were identified by ANOVA following Tukey's Multiple Comparison Test (P < 0.0001).

Table 3
MS information of the TPs of 5-FU identified by means of LC-HRMS (negative ion mode) and LC-IT-MS (positive ion mode) during direct UV photolysis (Hg medium pressure lamp) and simulated sunlight irradiation (Xe lamp).

TP (LC-HRMS)	Rt (min)[a]	Proposed molecular formula	H-ESI(−) MS $[M-H]^-$ Precursor ions *m/z*[b]	H-ESI(−) MS^2 Product ions *m/z* (Relative abundance, %)
5-FU	4.45	$C_4H_2FN_2O_2$	129.0106	112.0286 (0.06)
TP 105[d,e]	4.23	$C_3H_4FNO_2$	104.01533	84.00930 (0.14)
TP 128[d]	14.23	$C_4H_4N_2O_3$	127.01527	73.04181 (28.03)
TP (LC-IT-MS)	Rt (min)[a]	Proposed molecular formula	ESI(+) MS $[M+H]^+$ Precursor Ions *m/z*[c]	ESI(+) MS^2 Product ions *m/z* (Relative abundance, %)
5-FU	1.4	$C_4H_4FN_2O_2$	130.9	114.0 (33.83), 84.8 (27.96), 59.4 (100.0)
TP 148[d]	1.1	$C_4H_5FN_2O_3$	148.9	130.9 (73.09), 119.0 (0.61), 114.0 (6.02), 106.0 (100.0), 94.9 (2.80), 88.1 (6.40), 63.2 (23.35)

[a] Chromatography retention time.
[b] *m/z* values shown are for deprotonated molecular ions $[M-H]^-$.
[c] *m/z* values shown are for protonated molecular ions $[M+H]^+$.
[d] UV (Hg medium pressure lamp).
[e] Sunlight simulated irradiation (Xe lamp).

verified using this lamp. In contrast, samples submitted to photolysis with the UV lamp showed statistically significant reductions in terms of chronic toxicity (P < 0.0001). The results indicate that the 24 h chronic luminescence inhibition was reduced from 50% to 5% in the assays performed with *V. fischeri*.

Lin and Lin (2014) studied the photo-catalytic degradation of the anticancer drugs 5-FU and CP in ultrapure water. The authors verified an easy elimination of the parent compounds but different toxicity results regarding the assays performed with *V. fischeri*. On the one hand, the toxicity of CP increased during the treatment, and this was attributed to the generation of transformation products that were more toxic than the parent compound, since only 45% of

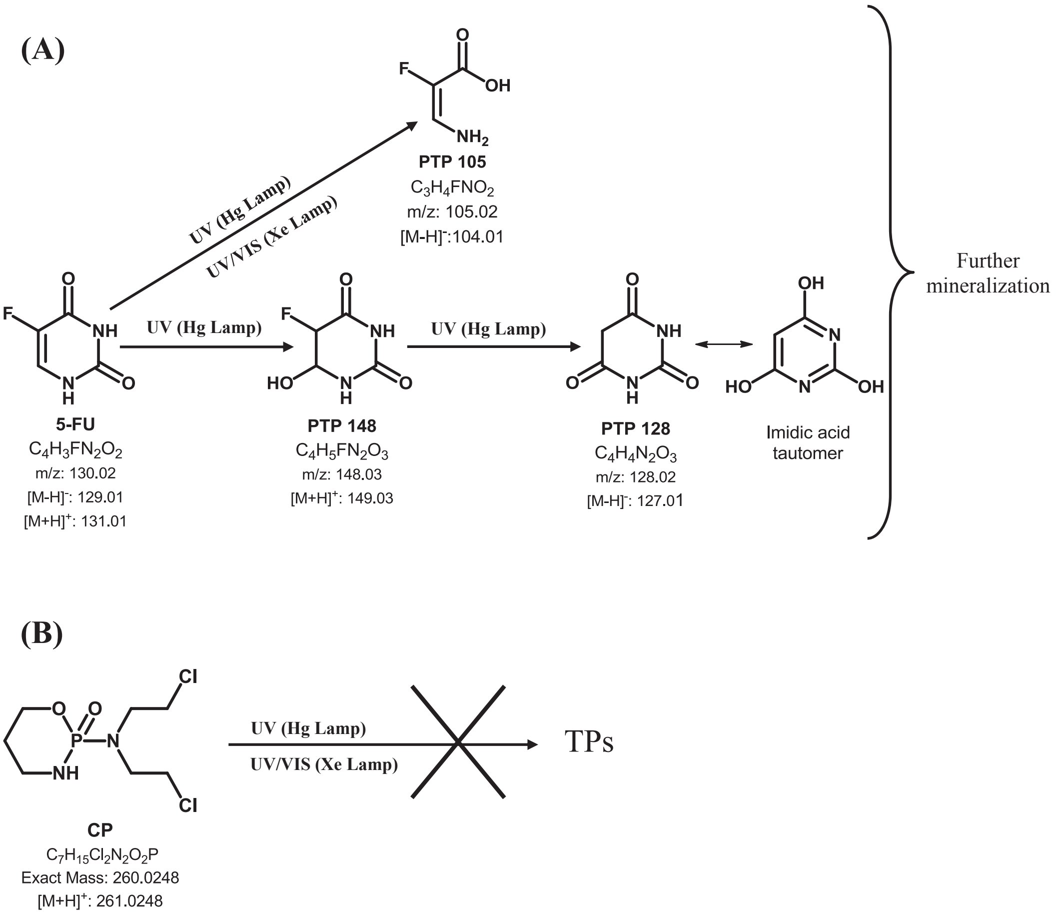


Fig. 5. Degradation pathway of 5-FU (A) and CP (B) by UV (Hg lamp) and Xe photolysis.

mineralization was achieved after 16 h of treatment. On the other hand, the same trend was not observed in the tests performed with 5-FU, indicating that this phenomenon can be compound-dependent. These results reinforce the need of a toxicity assessment of the TPs formed by treatments, which might be even more toxic than the parent compounds. However, because of the special toxicity of anticancer drugs, this point may be of even greater importance.

Furthermore, the results obtained in our assays demonstrate the need for a more comprehensive ecotoxicological evaluation of anticancer drugs (and its TPs) based on long-term assays. Due to the short exposure time of the acute tests, long-term effects present in substances with well-known bactericidal properties, such as antibiotics and anticancer drugs, may not be detected in short-term assays, which, in turn, may then lead to an underestimation of the toxic potential of these compounds (Backhaus and Grimme, 1999). Therefore, the experiments performed in our laboratories, which consider acute, chronic and growth inhibition, represent a more comprehensive and nuanced assessment of the toxic potential of 5-FU and its TPs and of the potential of treatments using Hg and Xe lamps to reduce toxicity.

3.4. Identification of transformation products

The LC-MS/MS analysis allowed for the identification of 3 phototransformation products (PTPs). By using LC-HRMS in negative ion mode, 2 PTPs (PTP 128 and PTP 105) were identified, whereas 1 PTP was identified in positive ion mode (PTP 148) by using LC-IT-MS/MS (Table 3). The MS/MS spectra and the elucidation of the PTPs are available in the supplementary material (Text S3 and S4).

A degradation pathway for 5-FU and CP was proposed in Fig. 5. For 5-FU, a single hydroxylation step formed PTP 148, which might be the result of a previous uracil hydrogenation and further hydroxylation on the C-6 carbon of 5-FU due to its saturation on the C_5-C_6 double bond (Kosjek et al., 2013).

Kosjek et al. (2013) identified PTP 148 as the main phototransformation product of 5-FU. As can be seen in Fig. 6, PTP 148 had a high peak area ratio (A/A_0), where A is the relative peak area of the TP and A_0 is the relative peak area of parent compound, during direct UV photolysis.

PTP 128 is likely to be formed from PTP 148 by means of a defluorination step. PTP 128 was also previously identified by Kosjek et al. (2013) as a transformation product of 5-FU. The A/A_0 profile shows that TP 128 was found at a lower abundance during direct UV photolysis, achieving only 0.27% of the area of the parent compound. TP 128 was also reported as a possible impurity of 5-FU. Fig. 5 shows TP 128 and its imidic acid tautomer.

PTP 105 was the only PTP identified in both direct UV photolysis and simulated sunlight irradiation and, as reported by Lin et al. (2013) the only transformation product formed during direct and indirect photodegradation.

In contrast, CP did not undergo any transformation (Fig. 5B).

3.5. In silico prediction of the mutagenicity and genotoxicity of 5-FU and the generated PTPs

Table 4 presents the results of the *in silico* QSAR predictions for selected endpoints regarding mutagenicity and genotoxicity of 5-FU and the three PTPs identified during the photodegradation tests. As can be seen here, three different endpoints of the software CASE Ultra showed positive alerts confirming the well-known mutagenic and genotoxic effects of 5-FU in the Ames test with different strains of *Salmonella* and *E. coli*. The mutagenic and genotoxic activities of 5-FU were already reported in previous studies (Zounkova et al., 2007; Yasunaga et al., 2006; Lutterbeck et al., 2015b).

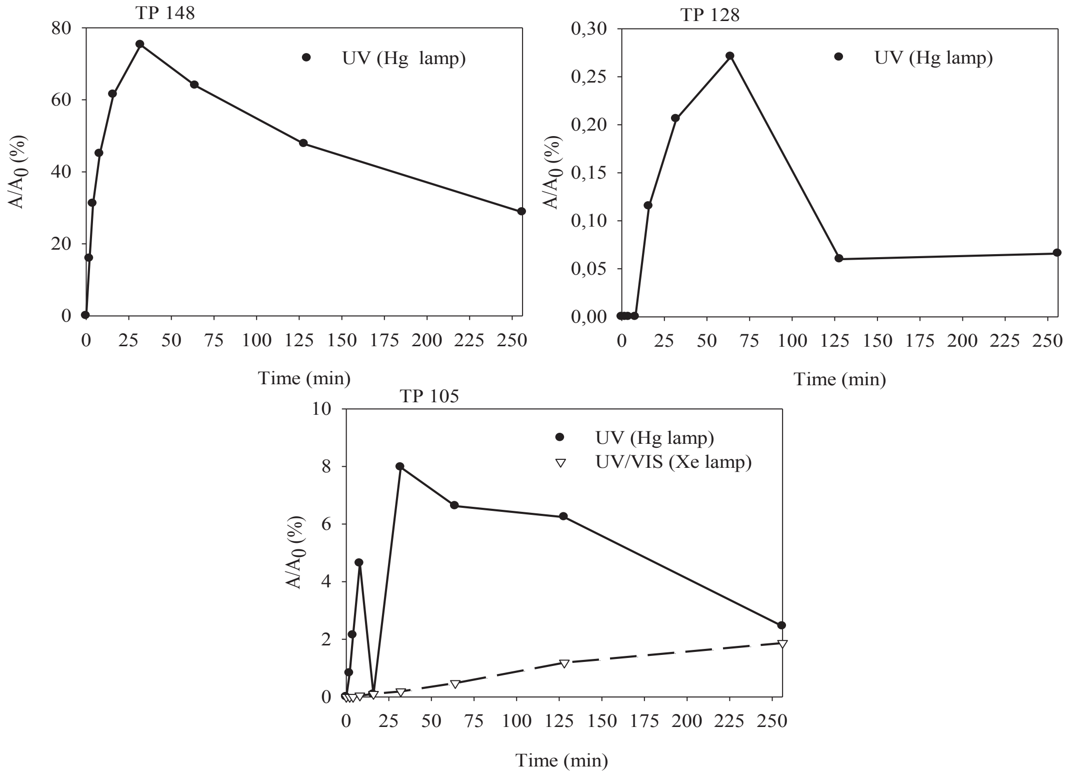
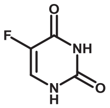
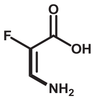
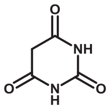
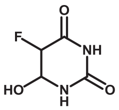


Fig. 6. Profile of the peak area ratio (A/A_0) of the identified PTPs from 5-FU during direct UV photolysis (Hg medium pressure Lamp) and simulated sunlight irradiation (Xe Lamp).

Table 4
In silico toxicity predictions by different models of CASE Ultra for 5-FU and the TPs formed during photodegradation experiments.

Name, MS (m/z), Rt (min)	Structures	Case Ultra QSAR model				
		A	B	C	D	E
5-FU, (130.09); (4.45)		+	IN	+	+	−
TP 105, (104.01); (4.23)		OD	OD	+	OD	OD
TP 128, (127.01); (14.23)		−	−	−	−	−
TP 148, (148.9); (1.1)		−	OD	−	OD	−

(A) Salmonella mutagenicity TA 97,98,100,1535–1538, (B) A–T mutation *E. coli* and TA102, (C) Expert rules for genotoxicity, (D) *E. coli* mutagenicity (all strains) (E) Salmonella mutagenicity (TA97,98,100,1535–1538). Out of Domain [OD] means that the test chemical is not included in the applicability domain of the applied model. 'Inconclusive' means a significant portion of the test chemical is covered by unknown structural fragment. Inconclusive (IN) means both positive and deactivating alerts were found in the same molecule and therefore a clear result cannot be provided.

When considering the TPs generated during the photolysis experiments using both lamps, only PTP 105 showed a positive alert in a rule-based expert system for the genotoxicity endpoint. 5-FU and PTP 105 contain as part of their structures a Michael acceptor which could be a reason for the toxicity. As predicted by the models, the possible genotoxic activity is associated with the presence of haloalkenes structures in the molecule of PTP 105 (Table 4), which may induce DNA adduct formation following cytochrome P450-mediated biotransformation into epoxide metabolites. These epoxides exert their carcinogenic potential by alkylating the DNA (Benigni and Bossa, 2008).

PTP128 and PTP148 formed in the photodegradation experiments with the Hg lamp presented no positive alerts in any of the models, indicating the absence of mutagenic and genotoxic activities and the inactivation of the alerts that were found in the parent compound.

It can be concluded that the photodegradation by UV irradiation (Hg medium pressure lamp) might, besides reducing the toxicity towards *V. fischeri*, also reduce the mutagenic and genotoxic effects of 5-FU. Nevertheless, this observation is likely to require further experimental testing.

4. Conclusions

The present study investigated the photodegradation, the biodegradation and the toxicity of two anticancer drugs and of possible PTPs formed during the photolysis experiments. As indicated by the biodegradation assays, which simulate the conditions of natural surface waters, not one of the parent compounds (5-FU and CP) can be classified as readily biodegradable.

The photodegradation results indicated that the direct photolysis triggered by simulated sunlight will not lead to the elimination and therefore limited presence of CP in aquatic environment. Photodegradation due to UV irradiation (Hg medium pressure Lamp) does not seem to be a suitable treatment for its removal either. For this reason, it might concluded that once released into aquatic environments, CP will not undergo natural attenuation (by sunlight photolysis or biodegradation), and it will not be removed in traditional wastewater treatment plants and is therefore expected to be found in the water cycle. Therefore, different and more efficient alternatives, such as advanced oxidation processes, should be tested in order to remove CP or to improve the biodegradability of substances in wastewater treatment systems.

5-FU was only partially degraded with the Xe lamp, and no mineralization occurred after 256 min. The lack of mineralization indicated the formation of toxic intermediates that were also not biodegradable. It is possible to conclude that sunlight will only play a minor role when it comes to the degradation of 5-FU and, consequently, will not improve its biodegradability and/or reduce the toxicity. In contrast, the experiments performed with UV irradiation completely eliminated the parent compound after 32 min. Three transformation products were formed during the reactions and identified. The photolytic mixture was characterized by a better biodegradability in the CBT than 5-FU, and it was nontoxic to *V. fischeri*. Furthermore, while 5-FU showed positive mutagenic and genotoxic alerts in three models of the *in silico* QSAR predictions, only one PTP still presented a positive alert for genotoxic activity. Hence, the photolysis using UV radiation might have the potential to be applied as a pre-treatment to eliminate 5-FU, an approach that would improve its biodegradability and reduce its toxicity.

Acknowledgments

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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.2015.10.016.

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Supplementary Material

Degradation of Cyclophosphamide and 5-Fluorouracil under UV and Xe irradiations and assessment of the enhancement of the biodegradability and toxicty

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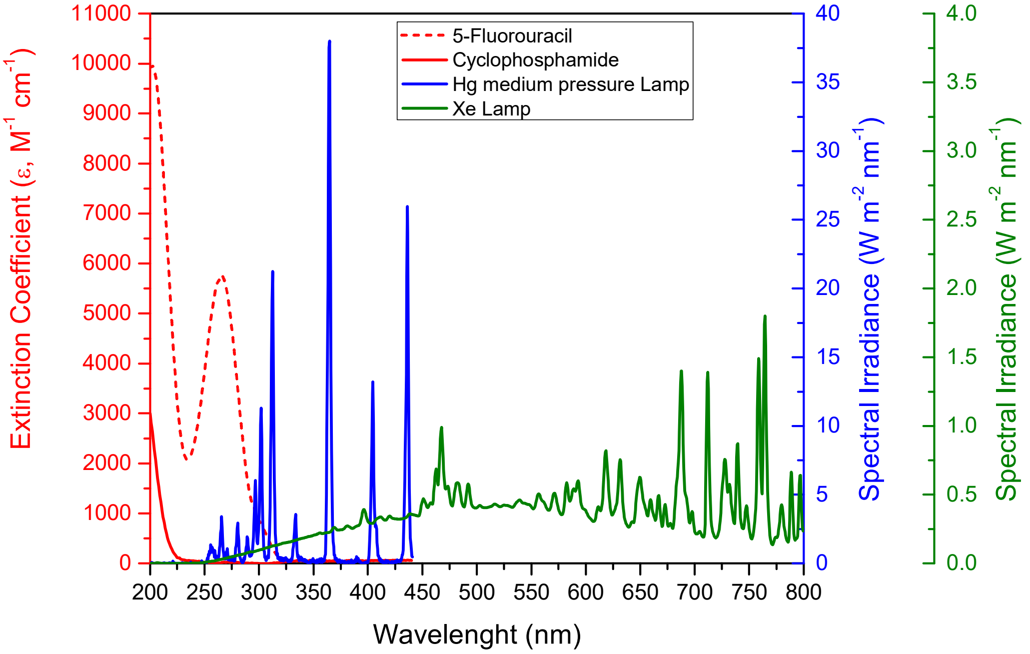


Fig. S1 – Intensity of light emitted by the used Hg and Xe lamps and molar extinction coefficient of 5-FU for the range of 200 nm to 800 nm. (Concentration of 10 mg/L)

Text S1 – Analytical Methods

The chromatographic separation of the CP was performed on a Nucleodur RP-18 endcapped 100-3, 2µm (Macherey-Nagel, Düren, Germany) coupled to a precolumn, Nucleodur C18 ec 4-2, 3µm (Macherey-Nagel, Düren, Germany). For elution, 0.1 % formic acid in deionized water (solution A) and 100% acetonitrile LC-MS grade (solution B) were used by applying the following gradient: isocratic from 0 min until 5 min. 5 % B, linear gradient 20 min 70 % B, isocratic until 25 min 70 % B, 27 min 5 % B, 33 min 5 % B. The sample injection volume was 10 µL and flow rate was 0.3 mL m^{-1}. Standards of CP (0.1, 1, 2.5, 5, 10 and 20 mg/L) were used to establish a linear calibration curve. For LOD we used a signal to noise ratio (S:N) of 3:1 from the extracted ion chromatograms (EICs) peak area and for LOQ a S:N ratio of 10:1 was set. The linearity for CP was r^2 0.997 while the LOD and LOQ were about 100µg/L and 350 µg/L, respectively

The chromatographic separation of 5-FU in the LC-IT-MS was carried out on a Nucleodur RP-18 endcapped 100-3, 2 µm (Macherey-Nagel, Düren, Germany) coupled to a guard column, (Nucleodur C18 ec 4-2, 3 µm; Macherey-Nagel, Düren, Germany). The mobile phase consisted of (A) 0.1 % formic acid in deionized water and (B) 100% acetonitrile. The following gradient was applied: 0-5 min isocratic 5% of B, 5-20 min linear gradient 5-70% of B, 20-25 min isocratic 70% of B, 25-27 min linear gradient 70-5% of B, 27-33 min isocratic 5% of B. Injection volume was 10 µL and the flow rate was settled to 0.3 mL/min.

Elution was performed on a MN Nucleodur® HILIC column (EC 100/3 mm, 3 µm) (Macherey-Nagel, Düren, Germany) by a binary mobile phase consisting of (A) 0.1% of formic acid in ultrapure water and (B) MeOH at a isocratic flow rate of 0.3 mL min^{-1}, oven temperature 30 °C, using a isocratic flow of 30% B during 15 min.

Standards of 5-FU (0.1, 1, 2.5, 5, 10 and 20 mg/L) were used to establish a linear calibration curve. For LOD we used a signal to noise ratio (S:N) of 3:1 from the extracted ion chromatograms (EICs) peak area and for LOQ a S:N ratio of 10:1 was set. The linearity for 5-FU was r^2 0.989 while the LOD and LOQ were about 89 µg/L and 0.29 µg/L, respectively.

Table S1. Operational characteristics of the electrospray ionization Ion Trap-Mass Spectrometer (ESI-IT-MS^n).

Mode		**Tune Source**		**Trap**		**MS/MS Automatic**	
Mass Range Mode	Std/Normal	Trap Drive	46.8	Scan Begin	50 m/z	MS(n) Averages	5 Spectra
Ion Polarity	Positive	Octopole RF Amplitude	266.7 Vpp	Scan End	300 m/z	Depth AutoMS(>2)	3
Ion Source Type	ESI	Lens 2	-58.0 V	Averages	4 Spectra	Auto MS/MS	On
		Capillary Exit	108.3 V	Accumulation Time	200000 µs	Group Length	5 lines
		Dry Temp.	350 °C	(Smart) ICC Target	40000	abs. Threshold Auto MS(2)	10000
		Nebulizer	30.00 psi	ICC	On	rel. Threshold Auto MS(2)	5.0%
		Dry Gas	12.00 L/min			abs. Threshold Auto MS(>2)	1000
		HV Capillary	−4080 V			rel. Threshold Auto MS(>2)	5.0%
		HV End Plate Offset	−500 V				
		Skimmer	33.4 V				
		octopole one	10.71 V				
		octopole two	2.24 V				

Table S2. – Operational characteristics of the heated electrospray ionization Ion Trap-Mass Spectrometer (H-ESI-HRMS/MS).

Tune File Values		Negative polarity		Scan Event Details	
Source Type	HESI	Source Voltage (kV)	3.00	1: FTMS - c norm res=30000	50.0-300.0
Capillary Temp (C)	300.00	Source Current (µA)	100.00	Activation Type	CID
APCI Vaporizer Temp (C)	200.00	Capillary Voltage (V)	-9.50	Min. Signal Required	5.0
Sheath Gas Flow	50.00	Tube Lens (V)	-35.00	Isolation Width	2.00
Aux Gas Flow	5.00	Multipole RF Amplifier (Vp-p)	770.00	Normalized Coll. Energy	20.0
Ion Trap Zoom AGC Target	1000.00	Multipole 00 Offset (V)	4.00	Default Charge State	1
Ion Trap Full AGC Target	10000.00	Lens 0 Voltage (V)	4.20	Activation Q	0.500
Ion Trap SIM AGC Target	5000.00	Multipole 0 Offset (V)	4.50	Activation Time	30.000
Ion Trap MSn AGC Target	5000.00	Lens 1 Voltage (V)	15.00		
FTMS Full AGC Target	200000.00	Gate Lens Offset (V)	35.00		
FTMS SIM AGC Target	10000.00	Multipole 1 Offset (V)	8.00		
FTMS MSn AGC Target	100000.00	Front Lens (V)	5.25		

Text S2. Ready biodegradability by means of Closed Bottle Test (OECD 301D)

The aerobic biodegradability of 5-FU and CP and of possible TP's formed during the photodegradation experiments was investigated in the Closed Bottle Test (CBT) according to the OECD 301D guidelines (OECD, 1992). The CBT consisted of four series running in parallel and in duplicate (i.e., "blank", "quality control", "test", and "toxicity control"). The composition of the CBT series is summarized in Table S1. The concentrations of sodium acetate and test substances, in any of the corresponding test series, were 5 mg/L of theoretical oxygen demand (ThODNH$_3$, calculated without considering a possible nitrification. The inoculum source was the effluent collected from the municipal STP AGL GmbH, Lüneburg, Nord, Germany (73 000 inhabitant equivalents). Two drops of inoculum were added to 1 L of the mineral medium solution.

During the whole test the consumption of oxygen in the bottles was measured daily with an optical oxygen sensor system (Fibox 3 PreSens, Regensburg, Germany) using sensor spots in the bottles. Besides, the temperature and the pH (day 0 and 28) were also controlled. A more detailed description of the test can be found elsewhere (Friedrich et al., 2013; Kümmerer et al., 1996; Mahmoud and Kümmerer, 2012; Trautwein et al., 2008).

According to the OECD guideline, a compound is classified as ‘‘readily biodegradable" if biodegradability, expressed as a percentage of oxygen consumed in the test vessel compared to maximum consumption (ThOD), exceeds 60 % within a period of ten days starting from the day where oxygen consumption reaches 10 % ThOD. A tested compound is considered to be inhibitory for the bacteria if the biodegradation does not reach more than 25% of ThOD within 14 days. Additionally, to determine the toxic effects, the oxygen consumption measured in the toxicity controls was compared with the predicted level calculated from the oxygen consumption in the quality control and in the test vessel, respectively. The ThOD of 5-FU was calculated based on the molecular formula of the compound.

Table S3 – Composition of the aerobic biodegradation test series in the CBT.

Test Series	1 Blank	2 Quality Control	3 Test Compound	4 Toxicity Control
Mineral medium	+	+	+	+
Inoculum	+	+	+	+
Test substance			+	+
Sodium acetate		+		+

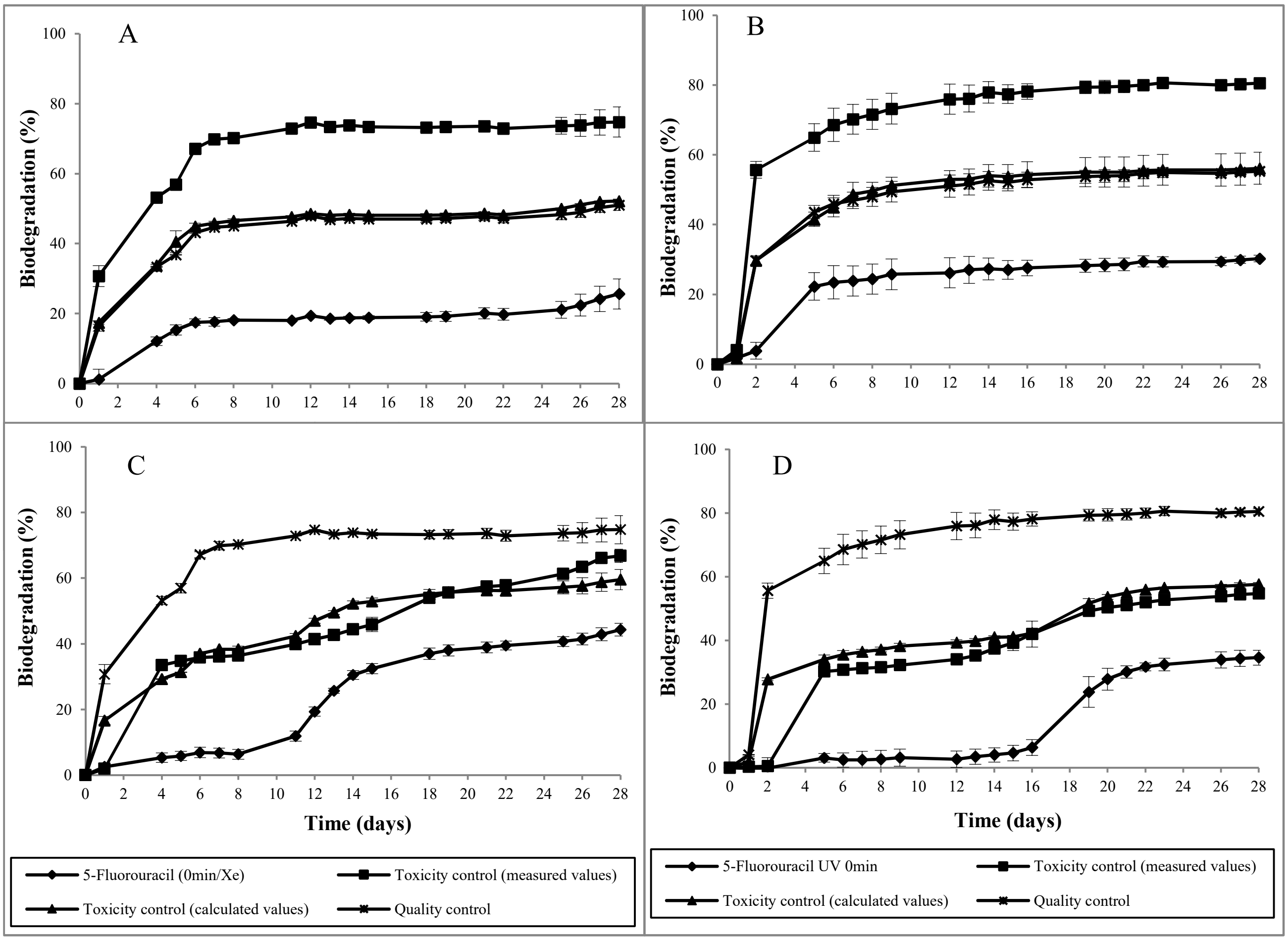
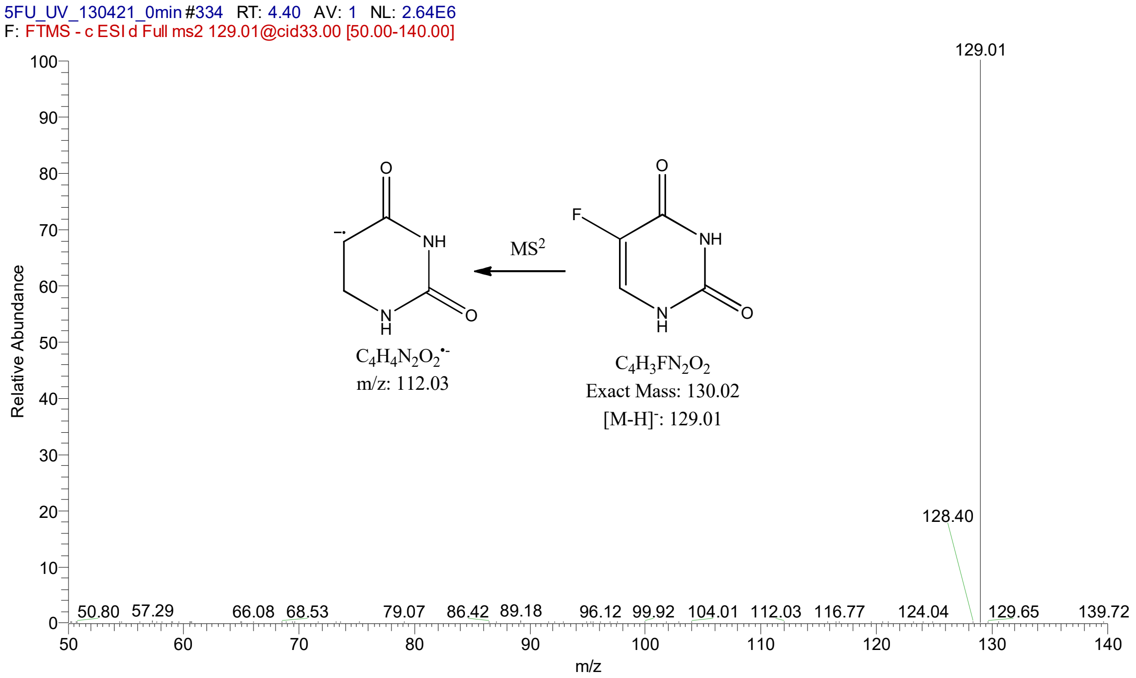


Fig. S2 – Degradation in Closed Bottle Test of **A** – cyclophosphamide (4.5 mg/L; irradiation time 0 min Xe Lamp) and **B** – cyclophosphamide (4.5 mg/L; irradiation time 0 min Hg Lamp) **C –** 5-fluorouracil (4.5 mg/L; irradiation time 0 min Xe lamp) and **D** – 5-fluorouracil (4.5 mg/L; irradiation time 0 min Hg Lamp) (n=2)

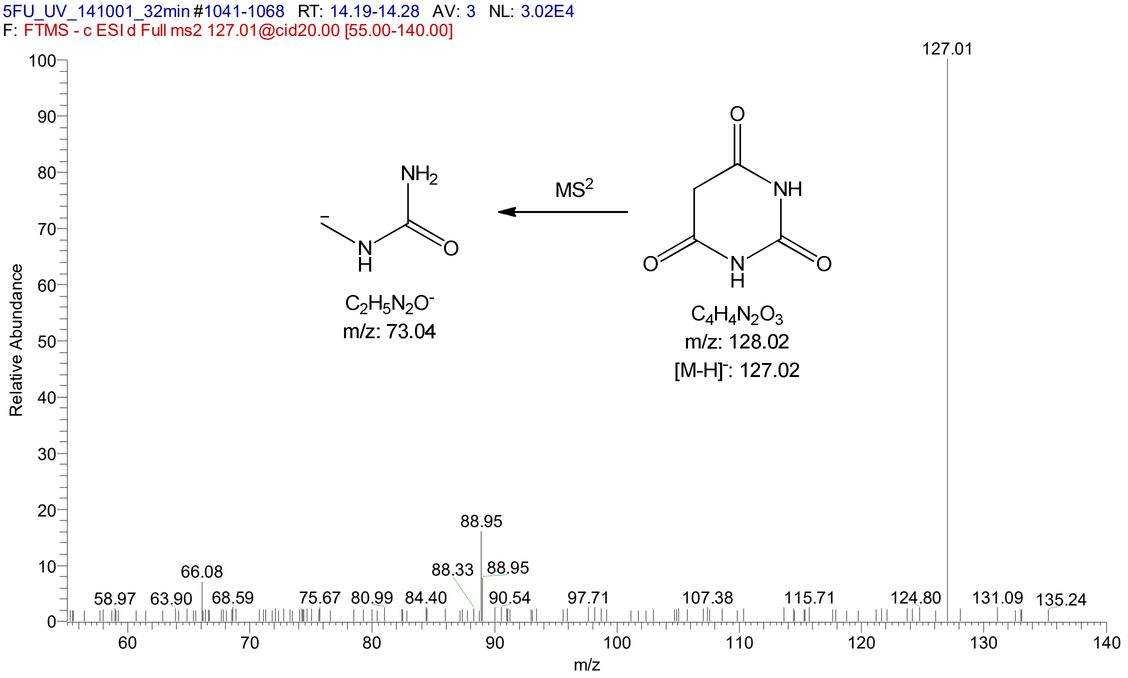
Text S3 – Identification and elucidation of the TPs from 5-Fluorouracil by means of LC-HRMS.

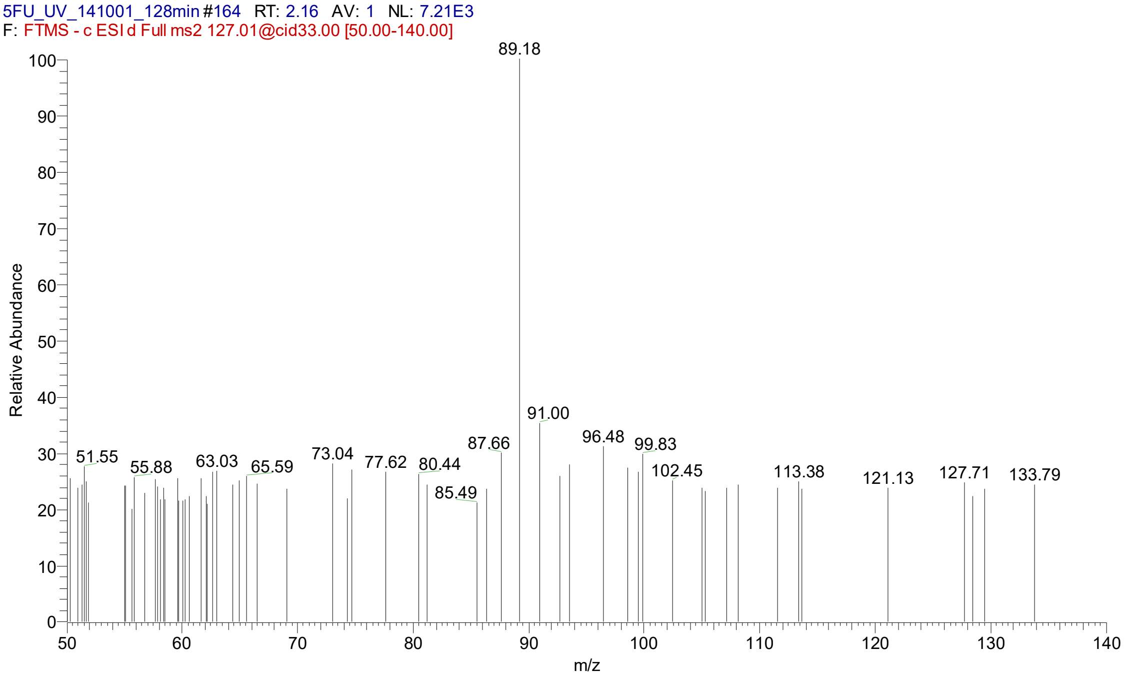
5-FU



m/z $[M-H]^-$	Formula	Intensity	Relative Intensity (%)	RDB	Δmmu	N-rule
129.0106	$C_4H_2O_2N_2F$	2644431.3	100	4.5	0.011	Even
112.02857	$C_4H_4O_2N_2$	1537.7	0.06	4.0	0.744	Odd

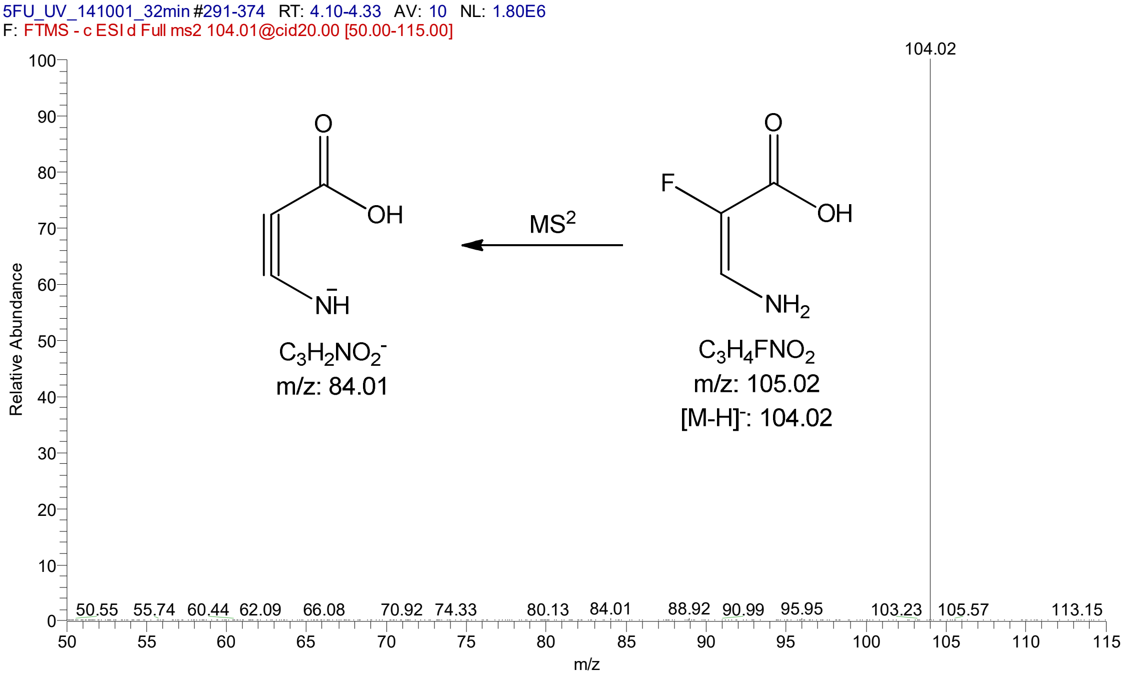
PTP 128



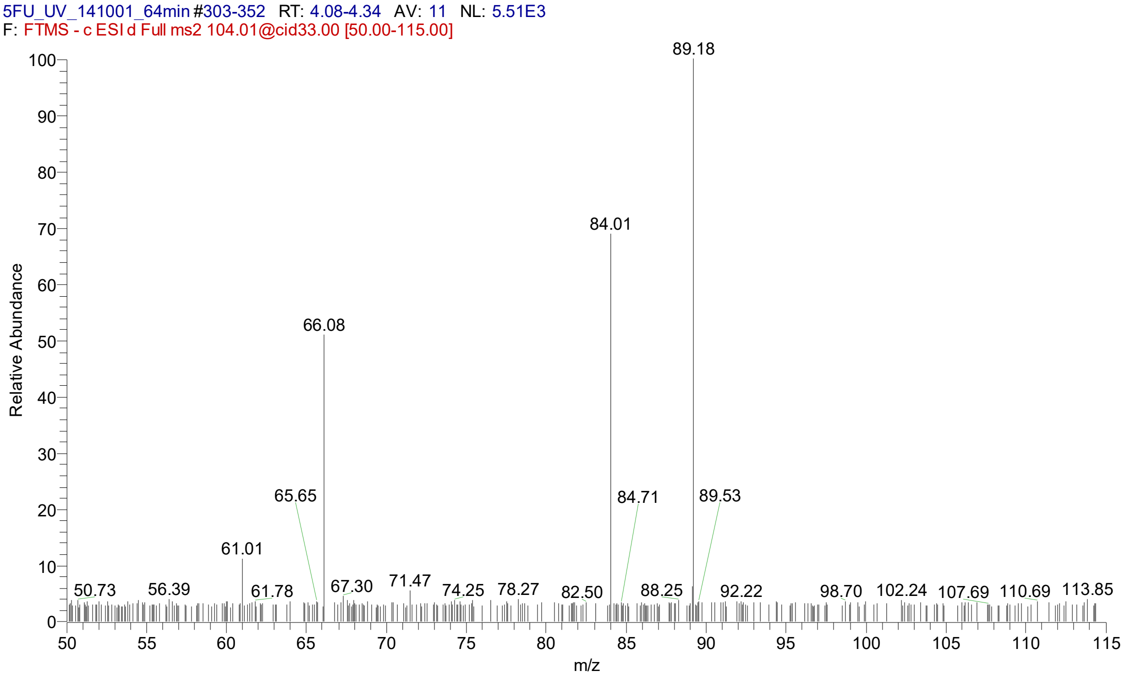


m/z $[M-H]^-$	Formula	Intensity	Relative Intensity (%)	RDB	Δmmu	N rule
127.01527	$C_4H_3O_3N_2$	30205.1	100	4.5	0.355	Even
73.04181	C_2H_5ON2	20209	28.03	1.5	1.074	Even

TP 105

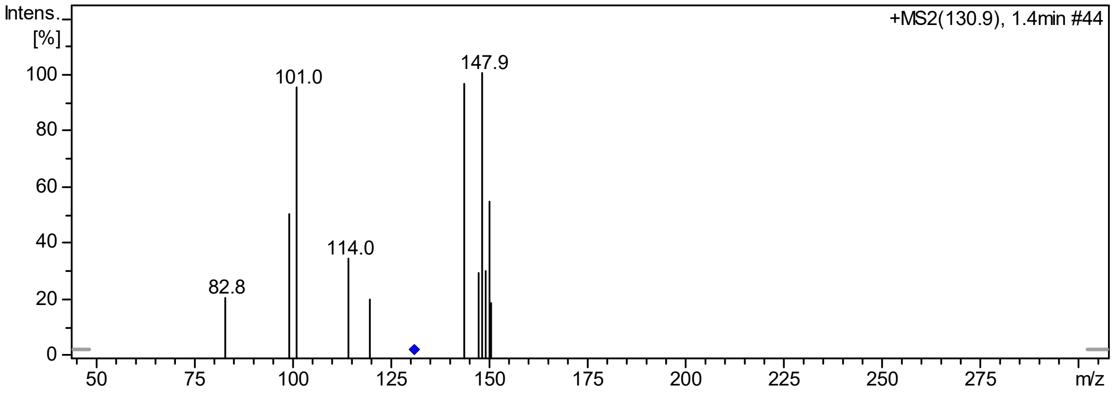


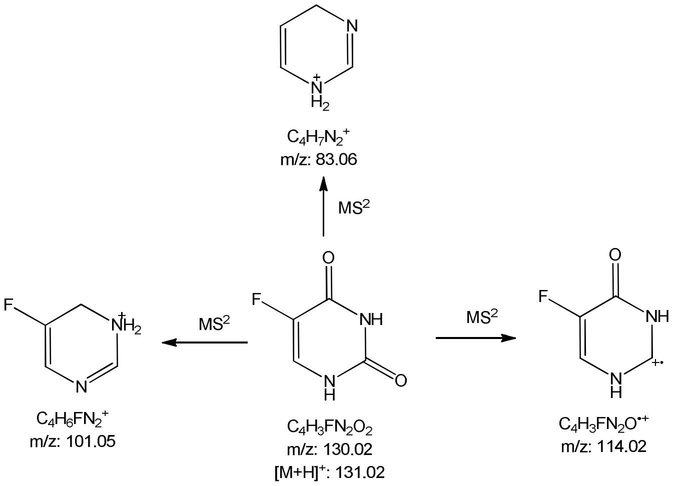
m/z $[M\text{-}H]^-$	Formula	Intensity	Relative Intensity (%)	RDB	Δmmu	N rule
104.01528	$C_3H_3O_2NF$	314564.1	100	2.5	-0.050	Even
84.00905	$C_3H_2O_2N$	447.4	0.14	3.5	-0.052	Even



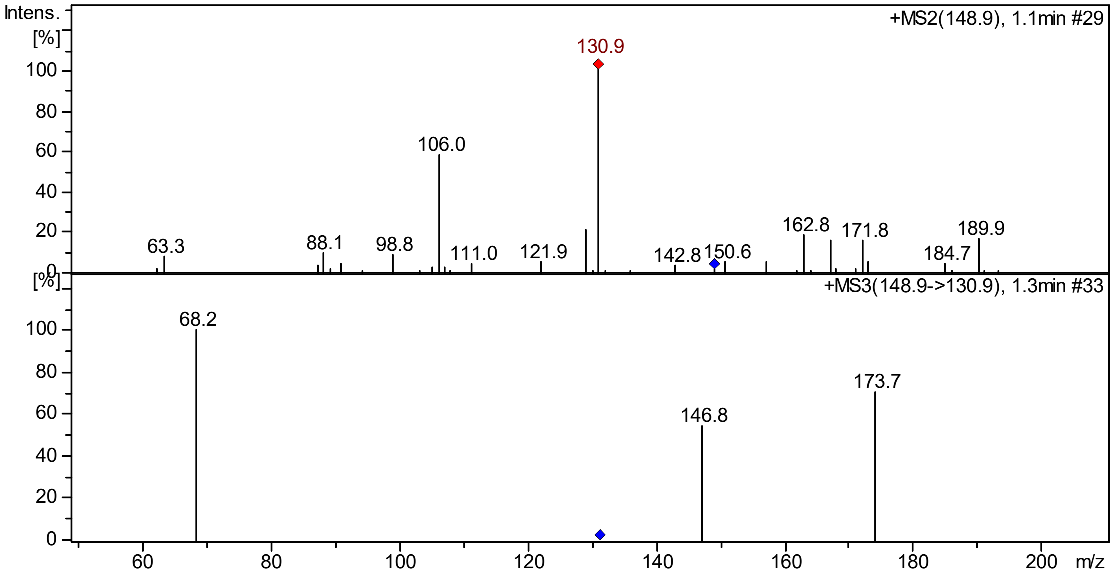
Text S4 – Identification and elucidation of the TPs from 5-Fluorouracil by means of LC-IT-MS/MS.

5-FU





TP 148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98.8
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121.9
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162.8
171.8
184.7
189.9
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+MS3(148.9->130.9), 1.3min #33
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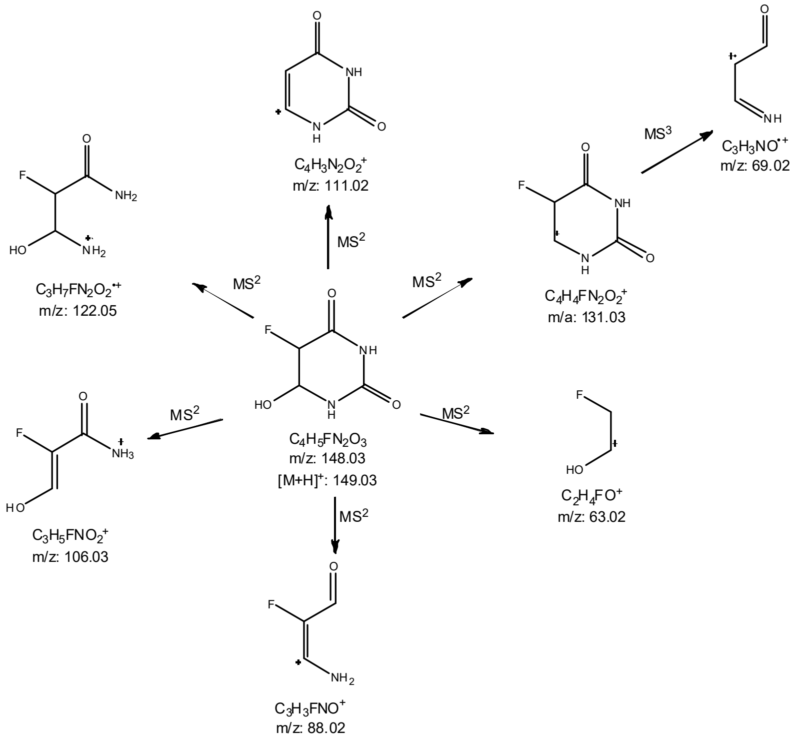
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m/z: 122.05
C4H4FN2O2+
m/a: 131.03
C3H3NO•+
m/z: 69.02
C3H5FNO2+
m/z: 106.03
C2H4FO+
m/z: 63.02
C3H3FNO+
m/z: 88.02

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Trautwein, C., Kümmerer, K., Metzger, J.W., 2008. Aerobic biodegradability of the calcium channel antagonist verapamil and identification of a microbial dead-end transformation product studied by LC–MS/MS. Chemosphere, 72, 442-450.

Article IV

Degradation of 5-FU by means of advanced (photo)oxidation processes: UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2 – Comparison of transformation products, ready biodegradability and toxicity.

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Degradation of 5-FU by means of advanced (photo)oxidation processes: UV/H_2O_2, $UV/Fe^{2+}/H_2O_2$ and UV/TiO_2 — Comparison of transformation products, ready biodegradability and toxicity

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HIGHLIGHTS

- Full primary elimination of 5-FU was achieved in all the treatments.
- None of the processes were able to fully mineralize 5-FU.
- Six transformation products (TPs) were identified during the treatments.
- Photolytic mixture was more biodegradable and non-toxic against *V. fisheri*.
- Several of the formed TPs showed less mutagenic and genotoxic activities.

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ABSTRACT

The present study investigates the degradation of the antimetabolite 5-fluorouracil (5-FU) by three different advanced photo oxidation processes: UV/H_2O_2, $UV/Fe^{2+}/H_2O_2$ and UV/TiO_2. Prescreening experiments varying the H_2O_2 and TiO_2 concentrations were performed in order to set the best catalyst concentrations in the UV/H_2O_2 and UV/TiO_2 experiments, whereas the $UV/Fe^{2+}/H_2O_2$ process was optimized varying the pH, Fe^{2+} and H_2O_2 concentrations by means of the Box–Behnken design (BBD). 5-FU was quickly removed in all the irradiation experiments. The $UV/Fe^{2+}/H_2O_2$ and UV/TiO_2 processes achieved the highest degree of mineralization, whereas the lowest one resulted from the UV/H_2O_2 treatment. Six transformation products were formed during the advanced (photo)oxidation processes and identified using low and high resolution mass spectrometry. Most of them were formed and further eliminated during the reactions. The parent compound of 5-FU was not biodegraded, whereas the photolytic mixture formed in the UV/H_2O_2 treatment after 256 min showed a noticeable improvement of the biodegradability in the closed bottle test (CBT) and was nontoxic towards *Vibrio fischeri*. In silico predictions showed positive alerts for mutagenic and genotoxic effects of 5-FU. In contrast, several of the transformation products (TPs) generated along the processes did not provide indications for mutagenic or genotoxic activity. One exception was TP with m/z 146 with positive alerts in several models of bacterial mutagenicity which could demand further experimental testing. Results demonstrate that advanced treatment can eliminate parent compounds and its toxicity. However, transformation products formed can still be toxic. Therefore toxicity screening after advanced treatment is recommendable.

Abbreviations: 5-FU, 5-fluorouracil; AI, acute inhibition; ANOVA, analysis of variance; AOP, advanced oxidation process; BBD, Box Behnken design; BOD, biochemical oxygen demand; CAP, capecitabine; CBT, closed bottle test; CI, chronic inhibition; COD, chemical oxygen demand; DOC, dissolved organic carbon; DOM, dissolved organic matter; ESI, electrospray ionization; GI, growth inhibition; H_2O_2, hydrogen peroxide; HPLC, high performance liquid chromatography; LC–IT-MS/MS, liquid chromatography tandem mass spectrometry; LBT, luminescent bacteria test; LC, liquid chromatography; mM, milli-molar; MS, mass spectrometry; nm, nanometers; OECD, organization for economic co-operation and development; $HO^•$, hydroxyl radicals; QSAR, quantitative structure activity relationship; ThOD, theoretical oxygen demand; TOC, total organic carbon; UV, ultraviolet; IT, ion trap; HRMS, high resolution mass spectrometry; TiO_2, titanium dioxide; TOC, total organic carbon; TPs, transformation products; UHPLC, ultra high performance liquid chromatography.

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1. Introduction

Anticancer drugs are substances specially designed to act by inhibiting cell growth or directly killing cells (Besse et al., 2012; Mahnik et al., 2004; Kümmerer et al., 2014). Because of the non-selective mode of action, affecting both cancerous and healthy cells, anticancer drugs are reported as potentially carcinogenic, genotoxic, mutagenic and teratogenic compounds (Allwood et al., 2002; Kümmerer et al., 2014). Although, in comparison to other groups of pharmaceuticals, the relatively low consumption rates and thus, relatively low environmental concentrations, it is not possible to establish threshold values for lowest effective concentrations for some of these compounds (Kosjek and Heath, 2011; Kümmerer et al., 2014). Due to their toxicological properties, some authors believe that anticancer drugs might inflict adverse effects on any growing eukaryotic organism, even at very low concentrations (Allwood et al., 2002; Besse et al., 2012; Johnson et al., 2008).

The antimetabolite 5-fluorouracil (5-FU) was introduced into the pharmaceutical market in the late 1950s and, globally, is the most commonly consumed anticancer drug (Kosjek and Heath, 2011; Straub, 2010). 5-FU is also the active substance in the prodrug capecitabine (CAP), which is rapidly metabolized and converted to the active compound (5-FU) in the human body (Deboever et al., 2013). It acts by blocking enzyme activity and disrupting DNA synthesis, and has been widely used in the treatment of solid tumors, such as colorectal and breast cancers. Approximately 15% of the parent compound of 5-FU is excreted unchanged (Zhang et al., 2013). 5-FU may therefore reach different water compartments. Furthermore, it is a very polar compound, which is unlikely to be eliminated by sorption into sewage sludge and is not expected to bioaccumulate in aquatic organisms (Table S1) (Wang and Lin, 2014; Zhang et al., 2013). Contradictory results have been published regarding the biodegradation of 5-FU (Kümmerer and Al-Ahmad, 1997; Straub, 2010). Thus, a general conclusion about its biodegradability cannot be made.

Due to its low absorption at wavelengths above 290 nm, 5-FU (maximum of absorption is 265 nm in ultrapure water) is unlikely to undergo direct solar photolysis. Therefore, it is likely that 5-FU will be found in the water cycle. Accordingly, 5-FU has been detected in the aquatic environment at concentrations ranging from ng/L up to μg/L (Kovalova et al., 2009; Mahnik et al., 2007; Mahnik et al., 2004; Mullot et al., 2009; Weissbrodt et al., 2009).

Since the removal of 5-FU and other pharmaceuticals by conventional wastewater treatment is often incomplete and inefficient (Wang and Lin, 2014; Zhang et al., 2013), other alternatives need to be investigated. The so-called advanced oxidation processes (AOPs) may be suitable methods, which may result in satisfactory elimination. Moreover, they represent an interesting alternative, since they can be employed in association with biological treatments for wastewater remediation, as a pre-treatment, increasing the biodegradability through partial oxidation, or as a post-treatment for the degradation of persistent compounds (De la Cruz et al., 2012). However, a possible incomplete mineralization can generate unwanted, and often unknown, transformation products (TPs) of unknown properties which can be recalcitrant and even more toxic than the parent compound itself (Lutterbeck et al., 2015; Mahmoud et al., 2013; Rastogi et al., 2014). Therefore, the identification of the main TPs generated in the processes is an important issue. Furthermore, the degree of mineralization, the kinetics of formation of TPs and their chemical structure may depend on the type of treatment.

In this way, the present study aims to (i) compare the efficiency of the elimination of 5-FU, as well as its degree of mineralization by means of three different AOPs (UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO_2); (ii) identify and elucidate the structure of TPs formed during degradation processes; (iii) evaluate the biodegradability of 5-FU and the mixture of its post-process TPs by standard OECD tests; (iv) assess the bacterial toxicity of 5-FU and its photo-TPs and (v) assess the mutagenic and genotoxic activities of 5-FU and the TPs formed in the advanced (photo)oxidation processes.

2. Materials and methods

Based on its mode of action 5-FU is a potentially mutagenic and teratogenic agent and thus, should be handled with care by taking the appropriate safety measures (Allwood et al., 2002; Eitel et al., 1999). All the waste generated during the experiments was treated and then disposed as hazardous waste, and the instruments and glassware used were carefully cleaned before and after usage.

2.1. Chemicals

Stock solutions of 5-FU (5-fluoro-1H, 3H-pyrimidine-2,4-dione, CAS Nr: 51-21-8) were kindly provided and handled by the pharmacy of the Hospital Lüneburg, Germany (therapeutic infusions bags were prepared on demand). In order to avoid any scavenging effect of other chemical species, and also to evaluate only the photo degradation of 5-FU, all the solutions were prepared using ultrapure water (Q_1:16.6 MΩ and Q_2:18.2 MΩ Ultra Clear Water, Evoqua Water Technologies GmbH, Barsbüttel, Germany).

All solvents used were of HPLC grade, and all chemicals were of analytical reagent grade or higher. Acetonitrile (HiPerSolv CHROMANORM, LC–MS grade, BDH Prolabo) and formic acid (analytical grade) were acquired from VWR International GmbH (Darmstadt, Germany). Sodium hydroxide (NaOH 1 mol/L), sulfuric acid (H_2SO_4 2 mol/L, 98%) was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Titanium dioxide (TiO_2 P25) was purchased from Evonik Degussa GmbH (Frankfurt, Germany). Hydrogen peroxide (H_2O_2 30% w/w) was obtained from Merck-Group (Darmstadt, Germany). Iron (II) sulfate heptahydrate ($FeSO_4\cdot 7H_2O$), catalase from bovine liver (2000–5000 units/mg protein), ammonium metavanadate (NH_4VO_3), and 1,10-phenantroline ($C_{12}H_8N_2$) were all acquired from Sigma-Aldrich Biochemie GmbH (Hamburg, Germany).

2.2. Setup of the advanced photo degradation experiments

The advanced (photo)oxidation experiments were carried out in an 800 mL reactor, containing 600 mL of a 5-FU solution with an initial concentration of 20 mg/L. The higher initial concentrations were used to allow subsequent biodegradation experiments and enable the identification of transformation products (TPs) that could be formed during the photo treatment, and finally to evaluate the possible effects on *Vibrio fischeri*.

The irradiation source used was a mercury medium pressure lamp (TQ150, UV Consulting Peschl, Mainz) with an ilmasil quartz immersion tube (Fig. S1). An UV-pad Spectral Radiometer (Opsytec Dr. Gröbel GmbH, Ettlingen, Germany) was used to estimate the total photon flow rate of the polychromatic lamp (200–440 nm). The radiometer was positioned at a distance of 4 cm from the emission source in an aluminum box and the integration of the total photon flow rate of the lamp for each wavelength (200–440 nm) was estimated to be 5.71×10^6 mol·photons/$cm^2 \cdot s^1$ (Fig. S2).

The reactor was placed on a magnetic stirrer to maintain the homogeneity of the solution, and the temperature was kept constant at 20 ± 1 °C by using a cooling system (WKL230, LAUDA, Berlin). The experiments were carried out for 256 min and samples (approx. 20 mL) were withdrawn according to a determinate time schedule (2, 4, 8, 16, 32, 64, 128 and 256 min) for further analysis.

The kinetic rates of the three advanced photo treatments were assessed through nonlinear regression models using the software Prism 5 (Graphpad Inc., CA, USA). Exponential decay models with the functions “one phase decay” and “two phase decay” were used to calculate the rate constants and half-lives of the reactions, and to take into account possible first order or second order kinetics. A more detailed description of data analysis is available in the Supplementary material (Text S1).

2.2.1. UV/H_2O_2 photolysis

To set the best initial concentration of hydrogen peroxide, prescreening experiments were performed with three different initial concentrations (9.8 mM, 14.7 mM and 19.6 mM). The evaluation of the UV/H_2O_2 process was based on mineralization degrees. In order to avoid any previous degradation, the lamp was first turned on and H_2O_2 was added. The pH of the samples was monitored during the entirety of all experiments. In order to eliminate the residual H_2O_2 for further analysis, the pH was adjusted to 7 ± 1 with NaOH (0.1 mol/L) after photo treatment, and catalase from bovine liver (activity: 1 unit will decompose 1.0 μmol of H_2O_2 per min at pH 7.0 at 25 °C) was added to remove residual and unreacted H_2O_2 in order to avoid any further interference and the sample was stored at −20 °C until further analysis.

2.2.2. Photo-Fenton

The optimization of the variables of the photo-Fenton process was carried out by means of Box–Behnken Design (BBD). BBD is a model based on a three-level incomplete factorial design, and requires fewer runs. The model prediction covered by a mathematical equation with high degree of confidence allows the estimation of the individual effects of variables and their interactions (Bezerra et al., 2008; Ferreira et al., 2007).

The mathematical model is based on a quadratic polynomial equation as shown below:

$$y = \beta_0 + \sum_{i=0}^{K} \beta_i X_i + \sum_{j=0}^{K} \beta_{ii} X_i^2 + \sum_{i=0}^{K} \sum_{j=0}^{K} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where y is the response of the independent variable, β_0 is the intercept, β_i is the regression coefficient of the experimental y values, and X_i is the coded value of the independent linear or quadratic variables. $X_i \cdot X_j$ and X_i^2 are the interaction terms and the quadratic terms of each independent variable, respectively, and ε is the random error. K is the number of variables (factors) studied.

Table 1
Experimental design based on Box–Behnken Design (BBD) real and coded values of each studied variable and the degradation and mineralization degrees of 5-FU by photo-Fenton treatment.

Code	Levels→ Variables ↓	−1	0	+1
X_1	pH	3	5	7
X_2	[Fe^{2+}] mg L^{-1}	5	10	15
X_3	[H_2O_2] mg L^{-1}	50	100	150

Exp.	pH	[Fe^{2+}] mg L^{-1}	[H_2O_2] mg L^{-1}	NPOC removal (%)	Deg.$_{[5\text{-}FU]}$ (%)
1	3	60	450	71.7	100
2	7	60	450	70.6	100
3	3	120	450	73.2	100
4	7	120	450	73.6	100
5	3	90	300	71.1	100
6	7	90	300	70.0	100
7	3	90	600	73.7	100
8	7	90	600	71.8	100
9	5	60	300	67.3	100
10	5	120	300	73.3	100
11	5	60	600	69.3	100
12	5	120	600	74.0	100
13 (C)	5	90	450	73.7	100
14 (C)	5	90	450	74.7	100
15 (C)	5	90	450	73.9	100
16 (C)	3	60	450	71.5	100

In the present study, three independent variables were chosen for BBD: pH (X_1), and the initial concentrations of hydrogen peroxide (X_2), and ferrous iron (X_3). The pH varied from 3 to 7, the concentration from hydrogen peroxide from 300 to 600 mg/L and the concentration of ferrous iron from 60 to 120 mg/L (Table 1).

The samples were collected and the pH was adjusted to 7 ± 1 with NaOH (0.1 mol/L), and catalase from bovine liver (see above) was added in order to eliminate the residual and unreacted H_2O_2. Thereafter the samples were filtered through 0.2 μm syringe micro filter (CHROMAFIL® Xtra.Typ: PES 20/25, Macherey-Nagel, Germany) and stored at −20° until further analysis.

2.2.3. UV/TiO_2

Three different concentrations of TiO_2 (100, 500 and 1000 mg/L) were used in preliminary tests to verify the impact of their concentration. In order to ensure the adsorption equilibrium of 5-FU on the TiO_2 surface, the solutions containing 5-FU and TiO_2 were constantly stirred, in the dark, for 30 min prior to the beginning of the experiments. The samples collected during the treatments were centrifuged at 4000 rpm for 5 min and then filtered through 0.2 μm membrane filters (CHROMAFIL® Xtra.Typ: PES 20/25, Macherey-Nagel, Germany), and stored at −20 °C until further analysis.

2.3. Analytical methods

The primary elimination of 5-FU and the identification of TPs were monitored by means of liquid chromatography tandem mass spectrometry (LC–IT-MS/MS). The analysis in positive ion mode was performed on an Agilent 1100 module (Agilent Technology, Waldbronn, Germany) LC coupled to a low resolution ion trap mass spectrometer (MS) Bruker Daltonic Esquire 6000^{plus} ion trap (Bruker Daltonik GmbH, Bremen, Germany) in order to conduct a first screening. The ionization was done by electrospray ionization (ESI).

A Dionex Ultimate 3000 UHPLC system (Dionex, Idstein, Germany)–tandem LTQ Orbitrap-XL with H-ESI source (Thermo Scientific, Bremen, Germany) (LC–HRMS) was used for the further analysis of transformation product analysis in negative ion mode. A more detailed description of the chromatographic conditions, as well as limits of detection and quantification can be found in the Supplementary material (Text S2, Tables S2 and S3).

A TOC (total organic carbon) analyzer (TOC VCPN 5050, Shimadzu GmbH, Duisburg, Germany) equipped with an ASI-V auto sampler was used to evaluate the degree of mineralization of the samples. Analyses of the dissolved organic carbon (DOC) were performed in triplicate.

2.4. Biodegradation

The ready biodegradability of 5-FU and of the mixture of TPs formed by the phototreatment was assessed in the Closed Bottle Test (CBT), respectively. This test is recommended as a first test for the assessment of the ready biodegradability of organic compounds (OECD, 1992b; Friedrich et al., 2013). The CBT was performed, following the OECD guidelines, with low nutrient content and under low bacterial density conditions (similar to natural surface water environments), at 20 ± 1 °C, in the dark, for 28 days (OECD,1992b). Each CBT was run in duplicate.

The inoculum was collected from the final effluent of a municipal sewage treatment plant (STP) serving 73,000 inhabitant equivalents (AGL GmbH, Lüneburg Nord, Germany). A more detailed explanation of the principles, procedures and composition of the CBT is described in detail in Text S3.

A threshold of less than 70% mineralization of 5-FU as measured based on DOC removal at the end of each treatment processes (256 min) in the photodegradation experiments was set as a trigger value to perform biodegradation tests after oxidative treatment.

2.5. Toxicity

The toxicity of 5-FU and the mixture of TPs resulting from the photo treatments were evaluated by a modified *V. fischeri* test. The Kinetic Luminescent Bacteria Test (kinetic LBT) combines the conventional short-term luminescence inhibition test according to EN ISO 11348 and the *Photobacterium phosphoreum* growth inhibition test (DIN 38412-37). A more detailed description of the method can be found elsewhere (Menz et al., 2013). The kinetic LBT allows the determination of three endpoints: (i) acute luminescence inhibition (after 30 min), (ii) chronic inhibition (after 24 h), and (iii) growth inhibition (after 14 h). The samples were analyzed before (0 min) and after (256 min) photodegradation experiments. Likewise, as for the ready biodegradation experiments, a threshold of 70% of mineralization was set, i.e., only samples with less than 70% mineralization after treatment were submitted to the toxicity test.

To perform the assays and consequently to evaluate the efficiency of the processes to reduce the toxicity, the results of our previous studies performed indicating an EC_{50} of 0.45 mg/L for the chronic endpoint were taken into account. Thus, the treated and untreated solutions were diluted in a ratio of 1:32 (which results in an initial concentration of 0.625 mg/L, i.e., in the same range of the chronic EC_{50}) in order to evaluate the toxicities of the samples before and after the treatment.

The significance of inhibition between the samples before and after the treatments was calculated by using one way ANOVA, following Tukey's Multiple Comparison Test (overall significance level = 0.05) with aid of the statistical software Prism 5 (Graphpad Inc., CA, USA).

2.6. In silico prediction of the genotoxicity and muteganicity

The mutagenicity and genotoxicity of 5-FU and of the identified TPs generated during the advanced (photo)oxidation treatments was assessed by a set of in silico predictions using the software CASE Ultra V.1.5.2.0. In detail, a combination of statistical and rule-based systems for bacterial mutagenicity according to the recently implemented ICH guideline M7 (International Conference on Harmonization (ICH), 2014) was applied (models: GT1 A7B (*Salmonella* mutagenicity in strains TA 97, 98, 100, 1535–1538)), GT1 AT *Escherichia coli* (A–T mutation *E. coli* and strain TA102), GT Expert (Expert rules for genotoxicity), Pharm *E. coli* (*E. coli* mutagenicity (all strains)), Pharm Salm (*Salmonella* mutagenicity (TA97, 98, 100, 1535-1538)). Structure illustrations were performed by using MarvinSketch 5.8.0. Simplified molecular input line entry specification (SMILES) codes from the molecular TP structures were used for input of molecular structures. CASE Ultra software provides positive (+), negative (−) and out of domain (OD) estimations for the selected models. OD means that the test chemical is not included in the applicability domain of the applied model. Often CASE Ultra software provides alerts for all its selected models like 'Inconclusive' (IN) alert which means that a significant portion of the test chemical is covered by unknown structural fragments or that positive alert was found in the molecule but the calculated probability falls inside the gray zone of the model (below the classification threshold of the model) and therefore a clear result cannot be provided.

3. Results and discussion

Control experiments were performed for the primary elimination of 5-FU in order to verify the contribution of direct UV photolysis and adsorption onto TiO_2, in the dark. The adsorption experiments were carried out for 256 min and showed no removal of 5-FU at the different three catalyst loadings (100, 500 and 1000 mg/L) used. The results of the direct photolysis showed that 5-FU was almost completely eliminated after 256 min, while a DOC removal of only 15% was attained at the end of the experiments (Fig. S3).

3.1. UV/H_2O_2

Similar mineralization degrees of 5-FU were achieved when using 9.8 and 14.7 mmol/L H_2O_2 (Fig. S4). At the highest concentration of H_2O_2 (19.6 mmol/L), the degree of mineralization decreased probably due the preferential formation of perhydroxyl radicals ($HO_2^{\bullet}$) (Litter, 2005), as shown in reaction (2). This radical species (redox potential 1.0 eV), which is much less reactive than the hydroxyl radicals (2.8 V), is formed through an excess of hydrogen in the solution, and can therefore reduce the degradation rate (Augugliaro et al., 2006; De et al., 1999).

$$H_2O_2 + HO^{\bullet} \rightarrow H_2O + HO_2^{\bullet} \tag{2}$$

Thus, in order to avoid an excess of hydrogen peroxide in the photo oxidation, the lowest concentration (9.8 mM) tested was used to carry out subsequent experiments. In the Fig. 1A one can observe that during the UV/H_2O_2 treatment about 38% of the mineralization of 5-FU occurs during the first 128 min. Afterwards the mineralization rate slows down relatively achieving 41.5% until 256 min. So, two different phases can be distinguished in the mineralization of 5-FU: the first where the parent compound will be converted to intermediates which in turn will react on a second phase into the final products. Therefore, this slower mineralization rate might be associated to the formation of newly recalcitrant TPs. The two phased decay kinetic model fitted well to the reaction and showed the two different rate constants: $k_{fast} = 0.35\ min^{-1}$ and $k_{slow} = 0.009\ min^{-1}$ (Table 2). The parent compound of 5-FU was almost completely eliminated after 8 min (Fig. 1B, Table 3). The pH of the solution decreased from 7.2 (0 min) to around 3.9 after the addition of H_2O_2 and remained in this range until the end the reaction (256 min).

Several authors using different operational conditions, studied the degradation of 5-FU by the UV/H_2O_2 treatment. Kosjek et al. (2013) observed pseudo-first order kinetics for the primary elimination of 5-FU in a study performed with a monochromatic low pressure mercury UV lamp (peak emission at 254 nm, with a rate constant and half-life of $0.045\ min^{-1}$ and 15 min irradiation time, respectively). The addition of H_2O_2 increased the reaction rate, and the parent compound was almost completely removed after 10 min. Türk (2006) studied the degradation of 7 anticancer drugs in real wastewater samples spiked with 100 μg/L of each compound. Using a low pressure mercury lamp (15 W) and 1 g/L of H_2O_2, the author observed an almost complete removal of 5-FU after 15 min. By using a medium pressure lamp (800 W) and 90 mg/L H_2O_2, Türk (2006) obtained a complete degradation of 5-FU after 2 min. Anheden et al. (1996) investigated the photocatalytic degradation of a wastewater sample from a 5-FU manufacturing plant. The authors reported a concentration of 900 mg/L of 5-FU and performed UV/H_2O_2 tests over a period of 20 h with different hydrogen concentrations, using a low-pressure mercury lamp (emitting mainly at 350 nm and with a measured UV radiation of 1.5 W), with three different intensities (55, 23 and 15 W/m^2) in a 500 mL reactor. Anheden et al. (1996) verified a great enhancement of the COD reduction, through the addition of 2400 mg/L to the UV/TiO_2/H_2O_2 treatment, which considerably improved the degradation rate.

Based on the above results it has become evident that the elimination of 5-FU is greatly improved by the addition of H_2O_2 to the reactions, and consequently generation of $HO^{\bullet}$ radicals. Furthermore, although the degradation of the parent compound can also occur through direct UV radiation, the reaction rate and the degree of mineralization considerably increased through the action of hydroxyl radicals. However, as shown in our study, the operational conditions of the process need to be optimized in order to set the best concentration and avoid an excess of hydrogen peroxide, which can add toxicity, retard the reaction, reduce the mineralization degree and increase the costs of the process.

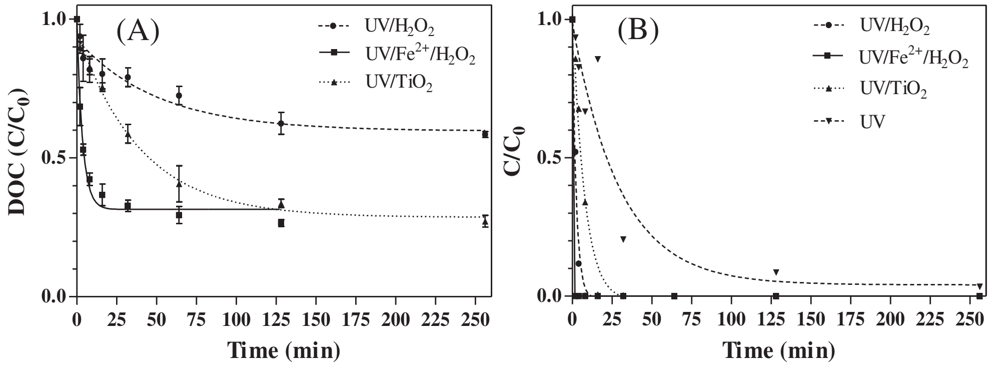


Fig. 1. A — kinetics of the mineralization of 5-FU under the three different treatment reactions. B — kinetics of the primary elimination of 5-FU. (n = 3).

3.2. Photo-Fenton

Table 1 shows the experiments performed during the optimization process reporting the real and coded values of each studied variable on BBD. The efficiency of the process was evaluated based on the DOC removal, i.e., the mineralization in terms of CO_2, H_2O and mineral salts achieved. The results were evaluated using STATISTICA 8 software (StatSoft, Inc. (2007), STATISTICA (*data analysis software system*), version 8.0. www.statsoft.com). As can be seen in Table 1, the higher NPOC removal achieved was 74.7% carried out under an initial pH of 5, $[Fe^{2+}]_0$ 90 mg L^{-1} e $[H_2O_2]_0$ 450 mg L^{-1}. In all the experiments, 5-FU was completely eliminated within 4 min. Therefore, the primary elimination was not considered in the BBD.

3.2.1. BBD statistical analysis

The fitting of the BBD generated model was carried out by means of ANOVA (Table S5). The statistical analysis compared the results of variance of each response and the resulting model. This source of variation was compared to the Fischer's distribution (F-test). The significance of each variable was considered through comparison to the $p < 0.05$ probability (Bezerra et al., 2008). The results are summarized in the Pareto chart of effects (Fig. S5) considering a significance level above 95%. The variables $[Fe^{2+}]_0$ linear (L) and quadratic (Q), $[H_2O_2]_0$ linear (L) and quadratic (Q) and the interaction between pH (Q) and $[Fe^{2+}]$ (L) were found to be statistically significant.

The r^2 of the quadratic model (Eq. (3)) was 0.9896, indicating that only 1.04% of the total variance could not be explained by the model, while the adjusted r^2 was 0.9480. As both values are similar to each other, and near to 1 the result confirms that the quadratic model is adequate to describe the experimental results (Ayodele et al., 2012). The replicate of the central point (Exp. 13–16) shows an RSD of 1.62%, confirming the lower experimental pure error. The quadratic equation considering all variables is shown in Eq. (3):

$$\begin{aligned} NPOC(\%) = 39.03 &- 4.34X_1 + 0.64X_1^2 + 0.32X_2 - 0.003X_2^2 + 0.11X_3 \\ &- 0.00008X_3^2 + 0.07X_1X_2 + 0.0003X_1X_2^2 - 0.01X_1^2X_2 \\ &- 0.008X_1X_3 + 0.0007X_1^2X_3 - 0.00007X_2X_3 \end{aligned} \quad (3)$$

where X_1 is the pH, X_2 is $[Fe^{2+}]_0$, and X_3 is $[H_2O_2]_0$. The originated BBD model does not consider the following effects $X_1^2X_2^2$, $X_1X_3^2$, $X_1^2X_3^2$, $X_2X_3^2$, $X_2^2X_3$ and $X_2^2X_3^2$ once they are considered redundant.

3.2.2. Response surface method and effect of variables for photo Fenton

The response surface of the photo-Fenton process can be observed as a contour plot in Fig. 2.

Fig. 2(A) shows the interaction between pH and $[Fe^{2+}]_0$. As can be seen, the higher degree of 5-FU mineralization was achieved when the initial pH was around 5 and for high concentrations of the ferrous iron. The interaction between pH and $[H_2O_2]_0$ is shown in Fig. 2(B) providing evidence of a high mineralization rate in pH 3–4 and at about 500 mg/L of $[H_2O_2]_0$, which can be related to the stability of hydrogen peroxide in acidic pHs (Lunar et al., 2000).

Fig. 2(C) shows the interaction between $[Fe^{2+}]_0$ and $[H_2O_2]_0$. It can be seen, that the high mineralization rate was achieved by high $[Fe^{2+}]_0$, whereas the concentration of $[H_2O_2]_0$ was around the central

Table 2
Apparent mineralization rate constants of 5-fluorouracil under different AOP's and the corresponding $t_{1/2}$. (95% confidence intervals).

Process	K'_{fast} (min^{-1})	$t_{1/2\ fast}$ (min)	K'_{slow} (min^{-1})	$t_{1/2\ slow}$ (min)	r^2	SS
UV/H_2O_2	0.35 (0.04–0.75)	2.0	0.008 (0.0–0.019)	80.9	0.988	0.001682
$UV/Fe^{2+}/H_2O_2$	0.38 (0.35–0.42)	1.8	0.027 (0.015–0.039)	25.8	0.999	4.585e−005
UV/TiO_2	0.02 (0.02–0.03)	27.4	–	–	0.993	0.004344

Table 3
Apparent primary elimination rate constants of 5-fluorouracil under different AOP's and the corresponding $t_{1/2}$. (95% confidence intervals).

Process	k′ (min^{-1})	$t_{1/2}$ (min)	r^2	SS
UV	0.03 (0.0–0.07)	20.6	0.91	0.1067
UV/H_2O_2	0.41 (0.3–0.52)	1.0	0.99	0.01285
UV/TiO_2	0.13 (0.09–0.18)	5.2	0.98	0.02210

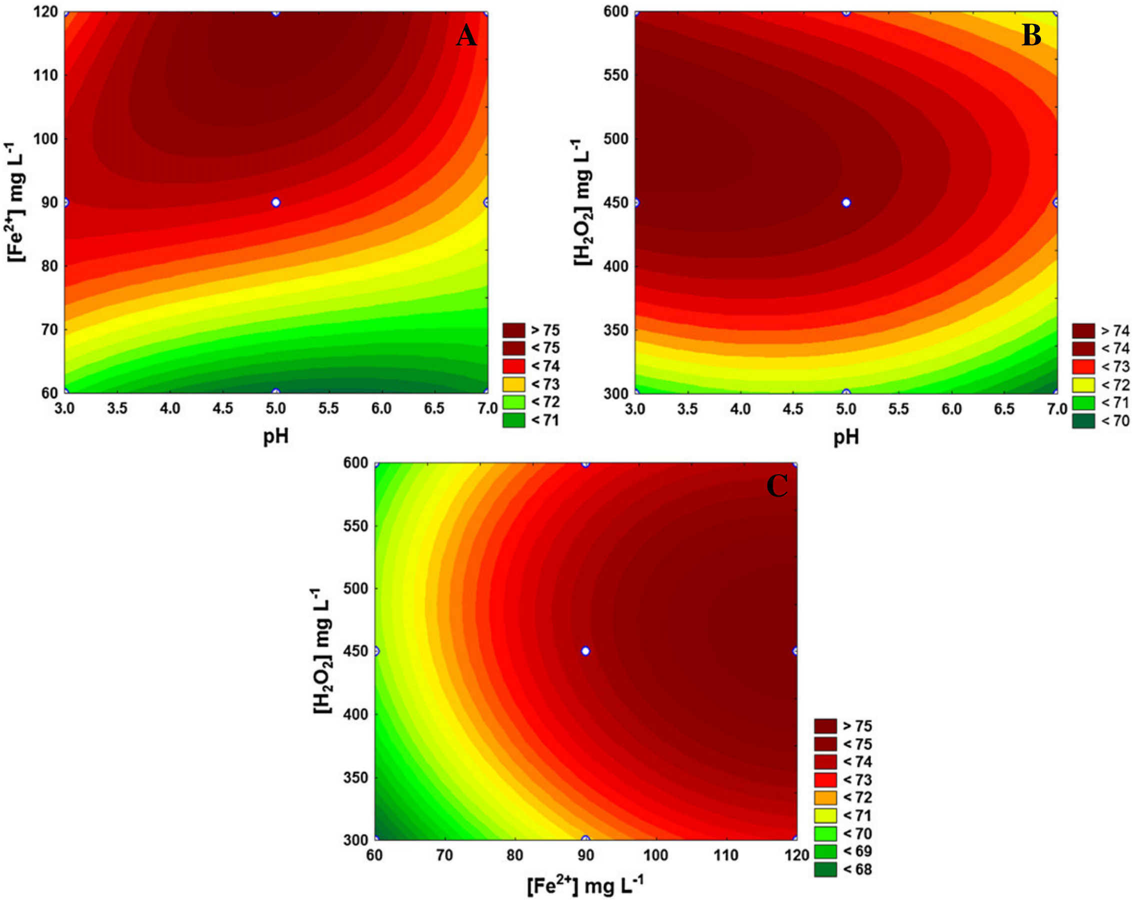


Fig. 2. Contour plot of the response surface of 5-FU mineralization by means of photo-Fenton process. (A) Interaction between pH and $[Fe^{2+}]$; (B) interaction between pH and $[H_2O_2]$ and (C) interaction between $[Fe^{2+}]$ and $[H_2O_2]$.

point of the BBD. The correlations $[Fe^{2+}]_0$:$[H_2O_2]_0$ were between 1:3.5 and 1:4.37 (w/w). They confirm that an excess of hydrogen peroxide is required, once it limits the process after 45 min of photo-Fenton treatment (Gulkaya et al., 2006; Neyens and Baeyens, 2003).

The desirability profile (Fig. S6) is used to aid in the optimization of factorial designs following the equation proposed by Derringer and Suich (Bezerra et al., 2008; Ferreira et al., 2007; Sivakumar et al., 2007). Using a scale of 0 for the undesired response, i.e., for the worst mineralization rate and 1 for the high mineralization rate, it could be concluded that the optimum pH was 6, but no significant difference in relation to pH 5 was found, $[Fe^{2+}]_0$ 120 mg L^{-1} and $[H_2O_2]_0$ 525 mg L^{-1}. Although it is well known that the optimum pH for Fenton and photo-Fenton process is around 2.5–3 (Brillas et al., 2009; Neyens and Baeyens, 2003), the pH 5 was used for further experiments.

The effect of pH on the photo-Fenton process has been widely described (Neyens and Baeyens, 2003; Brillas et al., 2009). However, it must also be pointed out that in the range of the pH values studied (4–7) the predominant micro-species of 5-FU is neutral (MarvinSketch 6.0.3, 2014, ChemAxon (http://www. chemaxon.com)), being available for HO• attack.

At pH 7, besides the neutral form, 5-FU exists approximately at 14% of the negatively charged structure, which is more accessible to an attack by electrophilic $HO^{\cdot}$ (von Sonntag, 2008) because of the nucleophilic character of the positively charged 5-FU.

The kinetics of the reaction of Fe^{2+} and H_2O_2 (reactions (4)–(6)) suggest a rapid generation of hydroxyl radicals, followed by the formation of ferric iron, which in an aqueous medium forms the micro-species $[Fe^{III}(OH)]^{2+}$, $[Fe^{III}(OH)_2]^{+}$. Such species can further catalyze the hydrogen peroxide and regenerate ferrous iron according to reactions (5)–(6), increasing the generation of hydroxyl radicals, and consequently, increasing the mineralization (Neyens and Baeyens, 2003; Brillas et al., 2009).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + HO^- \tag{4}$$

$$HO\bullet + Fe^{2+} \rightarrow HO^- + Fe^{3+} \tag{5}$$

$$Fe^{3+} + H_2O_2 \leftrightharpoons [Fe\text{–}OOH]^{2+} + H^+ \tag{6}$$

$$Fe\text{–}OOH^{2+} \rightarrow HOO\bullet + Fe^{2+} \tag{7}$$

Likewise, under UV–VIS irradiation, the ferric iron aqueous complexes can undergo photochemical reduction, which increases the generation of hydroxyl radicals according to reactions (8)–(9) and results in regeneration of the ferrous iron (Feng and Nansheng, 2000; Zepp et al., 1992). Such behavior was observed accompanying the concentration of ferrous iron during the process (Fig. S7). After the complete consumption of hydrogen peroxide, the concentration of ferrous iron increases, which can also be due the direct photolysis of ferric iron aqueous complexes.

$$Fe^{3+} + H_2O \rightarrow Fe(OH)^{2+} + H^+ \tag{8}$$

$$Fe(OH)^{2+} + h\nu \rightarrow Fe^{2+} + HO\cdot \tag{9}$$

The rapid consumption of hydrogen peroxide (Fig. S8) indicates the fast kinetics within the first minutes of the process, where 5-FU is completely eliminated after 32 min of irradiation. At pH 3 hydrogen peroxide is stable due the formation of the oxonium ion ($H_3O_2^+$) (Nidheesh and Gandhimathi, 2012).

Summarizing, UV/Fenton shows to be an efficient alternative to mineralize pharmaceuticals. In case of 5-FU, a quick elimination of the parent compound and up to 74% of mineralization was achieved during the reactions. Nevertheless, as could be pointed out, it is necessary to optimize the variables of process in order to achieve high mineralization degrees, which should decrease the number of TPs.

3.3. UV/TiO_2

Different TiO_2 initial concentrations were tested in order to find the best catalyst loading. An improvement of about 10% in the mineralization of 5-FU was achieved when the catalyst concentration was increased from 100 mg to 500 mg/L (62.7% to 72.8%) (Fig. S9). A possible explanation is the increased number of catalyst active sites available for photocatalytic reactions at higher TiO_2 loadings. Nevertheless, by increasing the catalyst load up to 1000 mg/L, no significant improvement of the mineralization was attained. It can be attributed to an excess of TiO_2, which above the saturation limit leads to an unfavorable light scattering, and consequently to a reduction of light penetration of the solution; this “loss” of photons usually results in a plateau or even a decrease in the mineralization rate (Hapeshi et al., 2010).

The same behavior has been observed in other studies involving the photo-catalytic degradation of pharmaceuticals. Therefore, all the UV/TiO_2 were conducted with a catalyst loading of 500 mg/L in our study. The results presented in the Fig. 1a show that almost 60% of the DOC removal occurs during the first 64 min, whereas only 14% mineralization was achieved in the remaining 192 min. According to the exponential decay models used in this study, the reactions obeyed the modified pseudo-first order kinetic model well, as was found by regression. This modified first order exponential model considers both the presence of oxidizable and non-oxizidable matter and attributes the remaining DOC of the mixture to the presence of refractory compounds (Martins et al., 2010). A decrease of the pH from 7.4 (0 min) to 4.6 (256 min) was observed in the experiments.

Photocatalytic experiments involving 5-FU have also been performed by Lin and Lin (2014). These authors found an optimal concentration of TiO_2 at 20 mg/L, when studying the degradation of 5-FU. Using a mercury low pressure lamp (8 W) as an irradiation source, the authors observed an almost complete removal of the parent compound (initial concentration of 200 μg/L) after 90 min ($k = 0.0375\ min^{-1}$). Moreover, when increasing the initial concentration of 5-FU to 27.6 mg/L, they verified an optimal catalyst loading at 300 mg/L (similar to the used in our study) and a complete DOC removal only after 24 h.

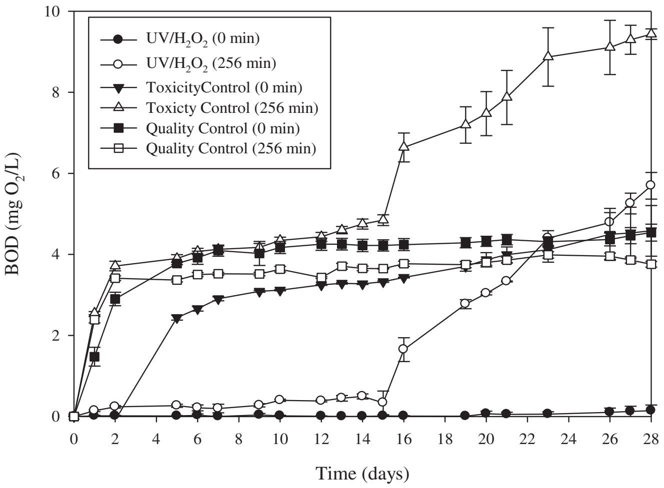


Fig. 3. Aerobic biodegradation (CBT) of 5-FU (0 min) and the photolytic mixture (256 min) formed after the of UV/H_2O_2 process ($n = 2$).

Likewise as the addition of H_2O_2 to the UV reactions, the use of TiO_2 also seems to be an interesting alternative to catalyze the elimination of 5-FU, and to improve the mineralization degree. Nevertheless, the concentration of TiO_2 to be added in the reactions also needs to be optimized in order to avoid a decrease of the efficiency of the process, and also to avoid higher costs for the treatment.

3.4. Biodegradation

Considering the threshold value set for performing biodegradation tests in this study (<70% mineralization), only samples withdrawn from the UV/H_2O_2 experiments were further tested in CBT, as in the other treatments mineralization of 5-FU were always higher than 70%. The CBTs fulfilled all validity criteria established in the OECD guideline 301D.

On the one hand, the obtained results showed a negligible biodegradation of 5-FU after 28 days, and thus classify the compound as not readily biodegradable according to the OECD guidelines. On the other hand, a noticeable increase in the BOD values for the samples from the UV/H_2O_2 treatment was observed at the end of the test (Fig. 3), indicating a better biodegradability of the photolytic mixture. Considering the fact that the parent compound was easily eliminated after 8 min of UV/H_2O_2 photo-degradation, these results can be attributed to the formation of more biodegradable by-products. According to the OECD criteria, neither the parent compound nor the TP mixture after 256 min of UV/H_2O_2 was toxic to the inoculum. Moreover, a clear difference in toxicity control lag phases between samples with the parent compound and the transformation products was observed. In the sample with 5-FU, toxicity control had a 3 day lag phase, whereas samples with transformation products had no lag phase. This is another indication that TPs generated during the UV/H_2O_2 photo-degradation were less toxic than the parent compound itself.

Over the past years, the biodegradability of 5-FU has been studied by several authors, giving rise to different results and contradictory conclusions. Biodegradation values ranging between 38–92% were observed in an OECD confirmatory test (OECD 303 A) after 3 days the best results were obtained at an initial concentration of 10 mg/L (IUTA, 2000). Additionally, in an OECD confirmatory test, Kiffmeyer et al. (1997) verified a full elimination of 5-FU within a few days. According to the authors, the biodegradation rate is dependent on the initial concentration, and the best results were obtained at the lowest 5-FU concentrations (i.e., 5 mg/L). The outcome of research carried out with activated sludge indicated a complete removal of 5-FU from the wastewater of an oncological ward (Mahnik et al., 2007). More recently, Kosjek et al. (2013) investigated the degradation of 5-FU in batch biodegradation experiments at a scale of 0.5 L (inoculum of activated sludge (AS) with initial concentration of 5.4 g/L). The authors found that 5-FU was completely eliminated at lower concentrations (1 and 10 mg/L), whereas a toxic effect occurred at higher concentrations (20 and 100 mg/L), retarding or even inhibiting the elimination.

In contradiction to the above mentioned results, 5-FU was reported as non-biodegradable in the Closed-Bottle-Test (OECD 301D) and in the Zahn–Wellens–Test (ZWT) (OECD 302B) for concentrations of 9.02 and 854 mg/L, respectively (Kümmerer and Al-Ahmad, 1997). Furthermore, no biodegradation of the parent compound of 5-FU (10 and 100 mg/L) was observed in the respirometric ready biodegradability test (OECD 301F) (Gröner, 1983). Yu et al. (2006) observed incomplete removals of 5-FU (<60%), even at lower concentrations (1 and 50 μg/L). After 50 days of incubation, the results showed eliminations of only 50% and 30% for initial concentrations of 1 and 50 μg/L, respectively.

Despite the results of some studies presented above, showing a fast elimination of 5-FU, it is noteworthy that all these tests were performed with higher bacterial densities (2.5 g/L dry mass in the OECD 303A and 5.4 g/L in the study performed by Kosjek et al., 2013), whereas the CBT test is characterized by a low bacterial density (500 CFU/mL), which may explain the different outcomes. Moreover, the results of the ZWT (Kümmerer and Al-Ahmad, 1997), which is also performed with a higher bacterial density (1 g/L dry mass), showed no biodegradation after 28 days. Although performing this test with a high initial concentration of 5-FU (854 mg/L), Kümmerer and Al-Ahmad (1997) observed no toxicity in the toxicity control, which excludes the hypothesis of a toxic effect that could occur at higher concentrations resulting in retarding or inhibiting, as suggested by some authors (Kosjek et al., 2013; Kiffmeyer et al., 1997). Likewise, no toxic effect was observed by the authors in the toxicity control of the CBT.

Besides 5-FU, Kümmerer and Al-Ahmad (1997) also studied the biodegradation of the structurally related anticancer drugs, cytarabine and gemcitabine. The results indicated a biodegradation up to 85% for cytarabine after 40 days in the CBT and over 95% in the ZWT. For gemcitabine, a biodegradation of 42% was observed in the CBT after 40 days and after 45 days in the ZWT. The authors attributed the differences in the biodegradation to the chemical structures of the compounds.

Therefore, considering the above information, the low biodegradability of 5-FU in the CBT is likely more associated with the low bacterial density of the test than to a possible toxic effect, since no toxicity was observed in the toxicity control. The CBT is working with low bacterial densities and mimics in this respect surface water conditions more than that of STPs.

3.5. Toxicity

Based on the set of a 70% mineralization threshold, only samples from the UV/H_2O_2 experiments were analyzed on the kinetic LBT. As can be seen in Fig. 4, the results of the acute toxicity (AI) indicated no difference between the samples at 0 min or at 256 min. Nevertheless, the other two endpoints (CI and GI) showed significant differences: the chronic toxicity was completely eliminated after 256 min (from 51 to −3%), with $p \leq 0.0001$. Moreover, the growth inhibition decreased from 20% to −1% ($p \leq 0.001$).

The application of AOPs to reduce the toxicity of 5-FU and other anticancer drugs has been studied by different authors (Calza et al., 2014; Lin and Lin, 2014). Unfortunately, most of the performed assays are short term tests, which have low environmental relevance due the pseudo-persistent character of this type of micro-contaminants as they miss chronic effects. Consequently, these tests can underestimate the toxic effects of the parent compound and of TPs. Lin and Lin (2014) attributed a gradual increase in the toxicity of cyclophosphamide against *V. fischeri* after 16 h of photo-catalysis to the formation of toxic by-products. The authors did not observe the same trend in the assays involving 5-FU. Calza et al. (2014) studied the photocatalytic degradation of doxorubicin and methotrexate (initial concentrations of 15 mg/L) using a lamp with an intensity of 40 W (maximum emission at 360 nm) and 200 mg/L TiO_2. No significant differences were observed for the doxorubicin samples after 180 min, indicating a similar toxicity of the parent compound and the TPs against *V. fischeri*. On the other hand, the samples of methotrexate submitted to the photocatalytic treatment showed a reduction of the toxicity at around 90% after 2 h.

From these results, generally, it has become evident, that the general conclusion, that the application of AOPs reduces the toxicity of the anticancer drugs, cannot be made, and that there is need for a separate, detailed study for each case.

3.6. Comparison of treatment processes

Similar mineralization degrees were achieved in the UV/Fe^{2+}/H_2O_2 and UV/TiO_2 processes (73.4% and 72.8%, respectively), whereas the UV/H_2O_2 was, by far, the less efficient process. Table 3 presents the mineralization rate constants of 5-FU. Based on the results, it can be assumed that all treatments obeyed pseudo-first order kinetics. The application of the “two phase decay model” equation allowed the identification of two distinct stages in the mineralization of 5-FU by the UV/Fe^{2+}/H_2O_2 and UV/H_2O_2 treatments. The kinetics of the UV/TiO_2 fitted well to the modified pseudo-first order reaction model. The highest k and the smallest half-life time values of the mineralization were reached for the UV/Fe^{2+}/H_2O_2, followed by the UV/H_2O_2 and UV/TiO_2 (Table 2).

When considering only the primary elimination of 5-FU (monitored by HPLC/LC–MS), UV/Fe^{2+}/H_2O_2 was the fastest reaction, followed by UV/H_2O_2 and UV/TiO_2 (Fig. 1b). In the photo-Fenton process, 5-FU was completely eliminated in less than 2 min, whereas in the UV/H_2O_2 and UV/TiO_2 reactions, more than 99% was removed in 8 min and 16 min, respectively. The faster mineralization (DOC) of 5-FU by the UV/Fe^{2+}/H_2O_2 and UV/H_2O_2 can be attributed to the extensive generation of a high

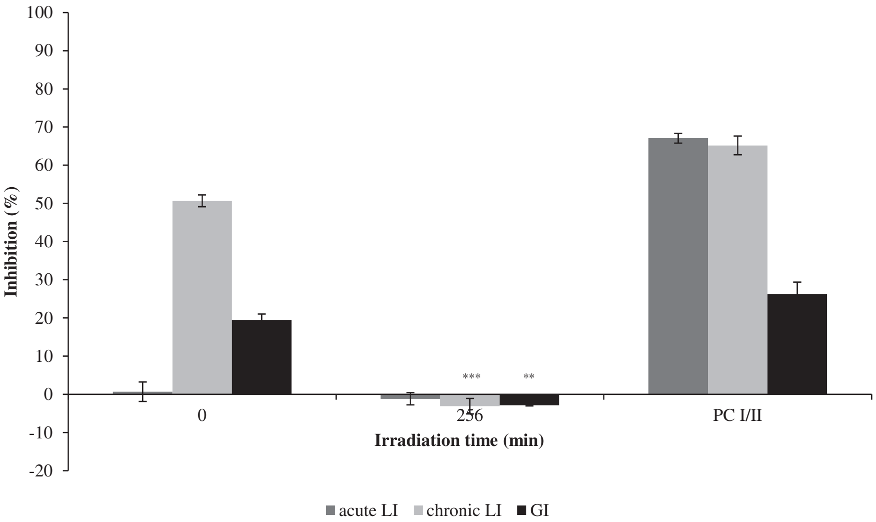


Fig. 4. Ecotoxicological assay tests with *V. fischeri* before (0 min) and after (256 min) the UV/H_2O_2 process (n = 2) considering three endpoints: acute luminescence inhibition after 30 min (acute LI), chronic luminescence inhibition after 24 h (chronic LI) and growth inhibition after 14 h (GI) Positive control I (PCI): 4.5 mg/L 3,5-dichlorophenol (acute LI), Positive control II (PCII): 0.05 mg/L chloramphenicol (chronic LI, Growth Inh.). The untreated and treated samples were submitted to final dilutions of 1:32. Statistically significant differences were compared to the untreated samples and identified by one way ANOVA following Tukey's Multiple Comparison Test (*** = $p < 0.0001$) (** = $p < 0.001$).

Table 4
MS information of the TPs of 5-FU identified by means of LC–HRMS (negative ion mode) and LC–IT-MS (positive ion mode) in different degradation processes UV/H_2O_2, UV/TiO_2 and UV/Fenton.

TP (LC–HRMS)	Rt (min)[a]	Proposed molecular formula	H-ESI(−) MS [M−H]⁻ precursor ions m/z[b]	H-ESI(−) MS² product ions m/z (relative abundance, %)
5-FU	4.45	$C_4H_2FN_2O_2$	129.0106	112.0286 (0.03), 85.05454 (0.031)
TP 105[e]	4.23	$C_3H_4FNO_2$	104.01533	74.01685 (0.35)
TP 117[d,f]	9.02	$C_4H_7NO_3$	116.03437	98.02458 (20.64)
TP (LC–IT-MS)	**Rt (min)[a]**	**Proposed molecular formula**	**ESI(+) MS [M + H]⁺ precursor ions m/z[c]**	**ESI(+) MS² product ions m/z (relative abundance, %)**
5-FU	1.4	$C_4H_4FN_2O_2$	130.9	114.0 (33.83), 84.8 (27.96), 59.4 (100.0)
TP 146[d,e]	2.0	$C_4H_3FN_2O_3$	147.2	130.0 (100.0), 104.0 (44.44), 92.3 (2.53)
TP 151[d,e]	1.6	$C_4H_6FNO_4$	151.9	131.8 (23.30), 107.9 (57.08), 95.1 (37.04), 79.9 (8.83), 57.4 (23.98)
TP 165[f]	1.4	$C_4H_5FN_2O_4$	164.9	143.9 (42.11), 130.1 (56.80), 121.0 (100.0), 107.0 (25.44), 97.9 (19.57), 79.2 (3.95)
TP 167[d]	1.9	$C_4H_6FNO_5$	167.9	150.9 (89.73), 137.7 (27.24), 123.0 (100.0), 109.0 (39.65), 95.0 (41.33), 82.7 (6.28), 71.0 (15.41)

[a] Chromatography retention time.
[b] m/z values shown are for deprotonated molecular ions $[M-H]^-$.
[c] m/z values shown are for protonated molecular ions $[M + H]^+$.
[d] UV/H_2O_2.
[e] UV/TiO_2.
[f] UV/Fenton.

concentration of hydroxyl radicals in Fenton and photo-Fenton reactions. As 5-FU was removed in less than 2 min by UV/Fe^{2+}/H_2O_2, it was not possible to calculate the rate constants of this reaction. For the UV/H_2O_2 and UV/TiO_2 reactions, the rate constants were 0.408 min^{-1} and 0.134 min^{-1} and the half-life times were 1.70 and 5.19 (Table 3).

3.7. Identification and kinetics of transformation products

The applied photocatalytic processes led to the formation of 6 TPs. Positive and negative ionization used, respectively, in order to get best results. Four TPs were identified in positive ion mode $[M + H]^+$ by

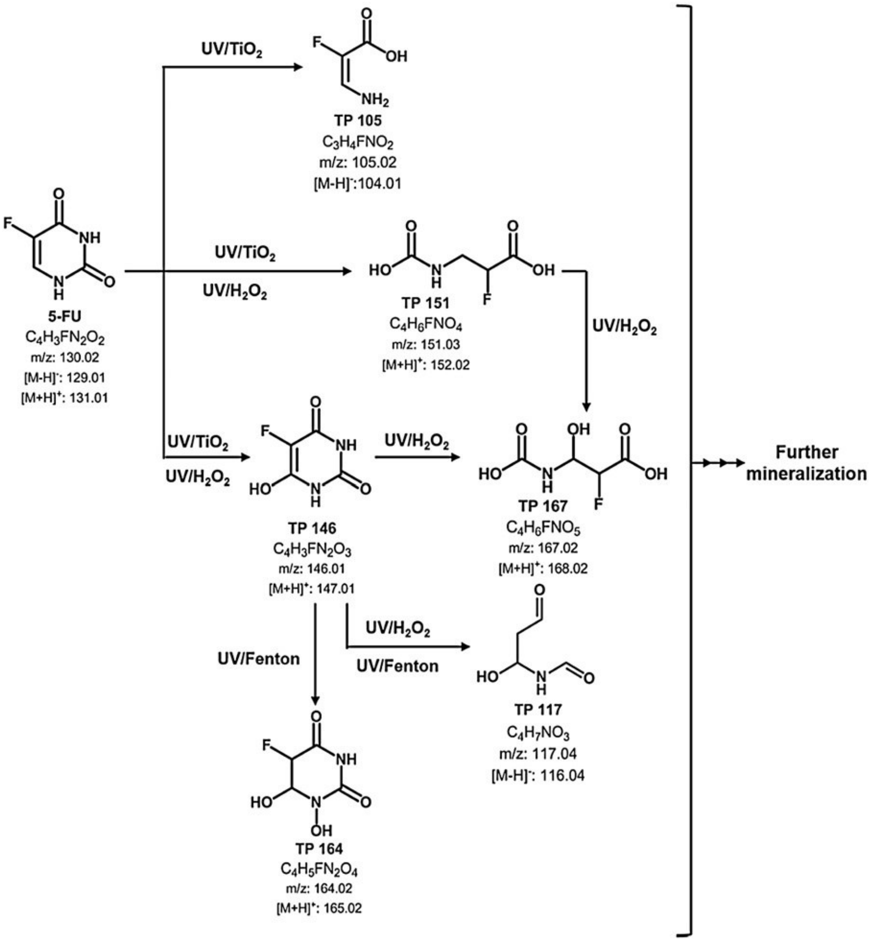


Fig. 5. Proposed degradation pathway of 5-FU by means of the different photocatalytic processes based on HO• generation (UV/TiO_2, UV/H_2O_2 and UV/Fenton).

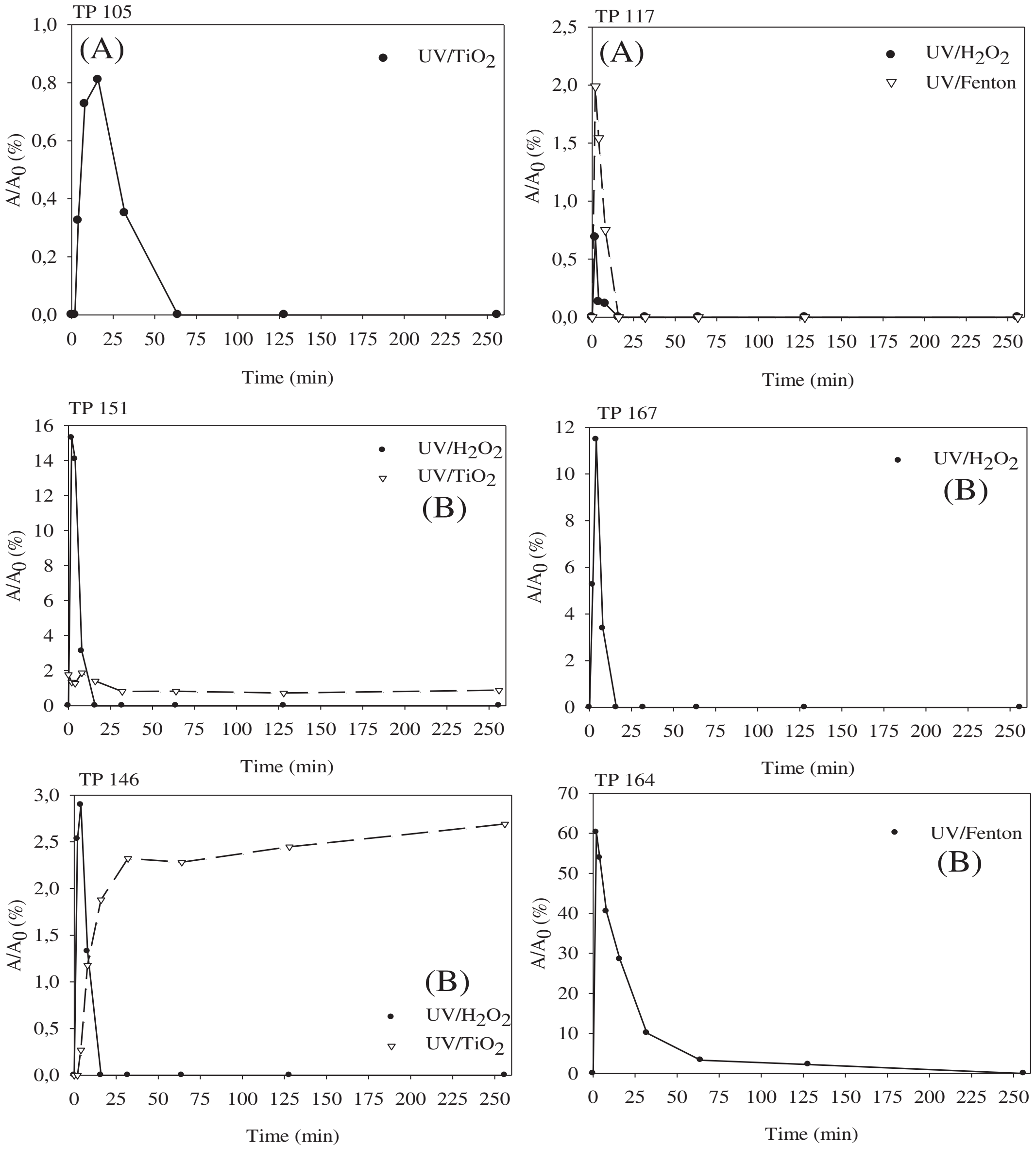


Fig. 6. Profile of the peak area ratio (A/A_0) of the TPs identified in negative ion mode by means of (A) LC–HRMS and positive ion mode by (B) LC–IT-MS during the different photocatalytic process.

using LC–IT-MS/MS (TP 146, TP 151, TP 164 and TP 167; numbers referring to m/z ration found in mass spectrometry), and 2 in negative ion mode $[M - H]^-$ by using LC–HRMS (TP 117 and TP 105), as can be seen in Table 4.

The solvents used in chromatography limit the use of the MS/MS for m/z above 60 Da. As 5-FU consists of small and stable molecule and the processes applied lead to high degrees of mineralization, some TPs were formed in small peak area ratios not allowing its fragmentation up to MS^3. Therefore, the fragmentation in the LC–HRMS was performed just in MS/MS. Different amplitude energies were tested in the collision induced dissociation (CID) −20 V, −25 V, −33 V and −40 V. By using −25 V in CID, 5-FU was rarely fragmented and the product ions were in lower abundance. On the other hand, fragmentation amplitudes of −30 V and −40 V were too high and did not allow distinguishing between product ions and noise.

Taking into account the formed TPs during the processes, lower fragmentation amplitude was applied according to previous reported for Kosjek et al. (2013). Therefore, the fragmentation amplitudes of −25 V and −20 V were tested being the −20 V chosen for further identification and elucidation purpose.

The MS/MS fragmentation pattern and the elucidation of the TPs can be seen in the Supplementary material (Texts S4 and S5). Based on these data the chemical structures were attributed to the specific masses of the identified TPs. In line with the elucidated structures, as all the photocatalytic processes applied are based on the generation of the same oxidant (hydroxyl radical), the degradation pathway as proposed in Fig. 5 covers the different processes applied at one pathway.

A single hydroxylation pathway was proposed to generate TP 146. According to the fragmentation pattern, the hydroxylation is proposed to be on the C-pyrimidine of 5-FU forming the TP 146. The peak area ratio A/A_0 (where A is the TP peak area and A_0 is the peak area of 5-FU at time point 0 min) shows that in the process UV/H_2O_2 TP 146 is formed within the first minutes of the treatment, and then further transformed (Fig. 6). In contrast, in the UV/TiO_2 process the TP 146 was present until the end of the process. TP 146 was not found in the UV/Fenton process.

Table 5
In silico toxicity prediction by different models of CASE Ultra for 5-FU and the TPs formed during the advanced (photo)oxidation treatments.

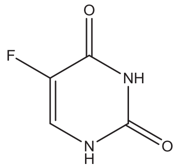
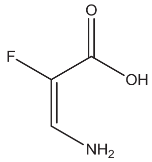
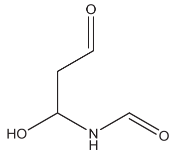
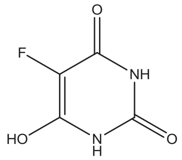
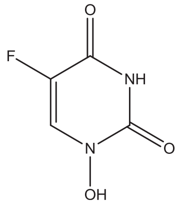
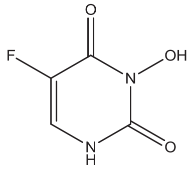
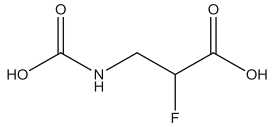
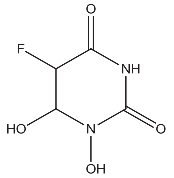
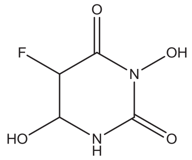
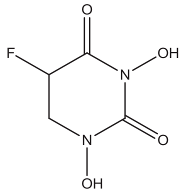
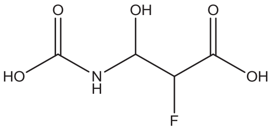
Name, MS (m/z), Rt (min)	Structures	CASE Ultra QSAR model				
		A	B	C	D	E
5-FU, (130.09); (4.45)		+	IN	+	+	−
TP 105, (104.01); (4.23)		OD	OD	+	OD	OD
TP 117, (116.03); (9.02)		−	OD	−	OD	−
TP 146 I, (147.0); (2.0)		OD	OD	+	−	−
TP 146 II, (147.0); (2.0)		IN	+	+	+	IN
TP 146 III, (147.0); (2.0)		IN	IN	+	IN	IN
TP 151, (151.9); (1.6)		OD	OD	−	−	−
TP 164 I, (164.9); (1.4)		IN	IN	−	OD	+
TP 164 II (164.9); (1.4)		IN	OD	−	OD	IN
TP 164 III (164.9); (1.4)		IN	OD	−	−	−
TP 167 (167.9); (1.9)		OD	OD	−	OD	−

Table 5 (*continued*)

Name, MS (m/z), Rt (min)	Structures	CASE Ultra QSAR model				
		A	B	C	D	E
						

(A) *Salmonella* mutagenicity TA 97, 98, 100, 1535–1538 (GT1 A7B), (B) A–T mutation *E. coli* and TA102 (GT1 AT *E. coli*), (C) Expert rules for genotoxicity (GT Expert), (D) *E. coli* mutagenicity (all strains) (Pharm *E. coli*), (E) *Salmonella* mutagenicity (TA97, 98, 100, 1535–1538) (Pharm Salm). Out of domain [OD], Inconclusive (IN) means that a positive alert was found in the molecule which provided a probability below the classification threshold of the model and therefore a clear result cannot be provided, (+) positive prediction for the respective endpoint, (−) negative prediction for the respective endpoint.

According to the peak area profile, TP 151 is formed in the beginning of the UV/TiO_2 and UV/H_2O_2 processes, and further transformed as well. Therefore, TP 151 is proposed to be formed directly from 5-FU through a uracil hydrogenation, followed by lactam hydrolysis, which opens the ring, followed by an amide hydrolysis (Fig. 5).

TP 167 was identified in the UV/H_2O_2 process, and followed the same profile as TP 151 during the process. TP 167 can be formed in several ways, e.g., from TP 151 by a single hydroxylation or from TP 146 through a lactam hydrolysis followed by deamination and then a further hydroxylation step.

Another secondary TP formed is the TP 164, which can be formed by means of N-hydroxylation from TP 148. The profile of the peak area ratio shows that TP 164 is formed in the beginning of the UV/Fenton process due the high mineralization rate of this process.

TP 117 was identified as a secondary TP in UV/Fenton and UV/H_2O_2 by means of LC–HRMS. Like the other TPs, TP 117 is formed within the first minutes of the processes UV/Fenton and UV/H_2O_2 and further eliminated (Fig. 6(A)). TP 117 might be formed through defluorination followed by dihydroxylation from TP 146.

TP 105 was only found in the UV/TiO_2 process. Lin et al. (2013) also identified this TP as the only one during both direct and indirect photodegradation processes despite the constant level of TOC during the photodegradation. In our study, we propose that TP 105 as a primary TP formed through degradation of 5-FU.

Therefore, although the different photocatalytic processes applied generate the same reactant (hydroxyl radical), they formed different TPs, which might be related to efficiency of each process in generating hydroxyl radicals. As pointed out above, the photo-Fenton process tends to be more efficient for generating hydroxyl radicals, whereas UV/H_2O_2 depends mainly on the intensity of the irradiation source at 254 nm to homolyze the hydrogen peroxide. In case of UV/TiO_2, experiments carried out in the dark showed no adsorption of 5-FU onto TiO_2, so the mechanism of degradation is also based on hydroxyl radicals.

The profile of the peak area of the TPs shows that most of them were transient during the treatment once they were formed and further degraded, with exception of TP 146 in the UV/TiO_2 process. As high degrees of mineralization were achieved, it might be proposed that the remaining DOC is due to the low molecular weight of organic acids (Fan et al., 2011).

3.8. In silico prediction of the mutagenicity and genotoxicity of 5-FU and the generated TPs

The in silico assessment of the mutagenicity and genotoxicity of 5-FU and the TPs formed during the processes is presented in Table 5. As one can see in Tables 5, 3 positive alerts from the different models of the ICH M7 guideline conformal set from CASE Ultra indicate possible mutagenic and genotoxic activities of 5-FU in the Ames test with different strains of *Salmonella* and *E. coli*. These results are in line with previous in vivo studies which have already reported the potential mutagenic and genotoxic effects of 5-FU (Zounkova et al. (2007); Yasunaga et al. (2006)).

Taking into account the TPs generated during the reactions, several of them neither showed mutagenic nor genotoxic alerts in the in silico predictions. Moreover, it must be pointed out that almost all the TPs identified were transient species during the application of the processes, i.e., formed and further eliminated during the process (Fig. 6). However, TP 146 was formed during the UV/TiO_2 process and not further eliminated after 256 min of treatment. Here it should be kept in mind that TP 146 provided indications both by the rule-based model (Table 5, C) and the statistical models (e.g., Table 5, E and D) for a retaining or a modulation of the known mutagenicity of the parent compound. These predictions for TP 146 could be a starting point for an experimental assessment of the predicted toxicities.

4. Conclusions

Currently, different treatment alternatives such as ozonation, reversed osmosis, activated carbon, and photolysis are under discussion to remove micro-pollutants like pharmaceuticals from water and wastewater. In the present study, we investigated the application of three different advanced (photo)oxidation processes to eliminate and mineralize 5-FU. The results showed that despite the fast elimination of the parent compound, 5-FU was not fully mineralized in any treatment. Additionally, all the investigated treatments demonstrated that the optimization of the operational conditions is of crucial importance to achieve higher mineralization degrees and consequently reduce the formation of TPs. So, besides achieving the highest mineralization degrees, the photo Fenton reactions also generated only two TPs.

We identified and proposed the degradation pathways and the structures of six TPs formed during the advanced (photo)oxidation treatments. The in silico predictions showed that the parent compound of 5-FU presented positive alerts for mutagenic and genotoxic activities as described in literature, whereas most of the formed TPs did not show any positive alert indicating potentiality of the studied AOPs to reduce the genotoxicity and mutagenicity of 5-FU. One exception was TP 146, an OH-derivative of 5-FU, for which the in silico analysis provided indications for a retaining or modulation of the known mutagenicity of the parent compound.

Finally, despite the high mineralization rates combined with an improved biodegradability and the absence of toxicity against *V. fischeri*, future studies involving the application of the UV/H_2O_2 process to treat hospital wastewaters containing 5-FU should be conducted. Likewise, the combination of UV/H_2O_2 treatment with a biological treatment should be investigated, in order to verify the feasibility of the processes to be used as pre-treatment in hospital wastewaters and ensure the complete mineralization of non-biodegradable and toxic pollutants.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.04.111.

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Supplementary Material

Degradation of 5-FU by means of advanced (photo)oxidation processes: UV/H_2O_2, UV/Fe^{2+}/H_2O_2 and UV/TiO2 – Comparison of transformation products, ready biodegradability and toxicity

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Table S1 – Structure and main physico-chemical properties of 5-fluorouracil

Structure	**Chemical Formula**	**MW**	**WS**	**Log K_{ow}**	**$t_{1/2}$**
	$C_4H_3FN_2O_2$	130.08 g/mol $^{-1}$	11.1 g L^{-1} water (20 °C) (1)	−0.69 (2)	63h (3)

MW: molecular weight; WS: water solubility; log Kow: partition coefficient (octanol/water); $t_{1/2}$: half-life under simulated sunlight

References: (1)(Zhang et al., 2013) (2) (Sanderson and Thomsen, 2009) (3) (Lin et al. 2013)

Fig. S1

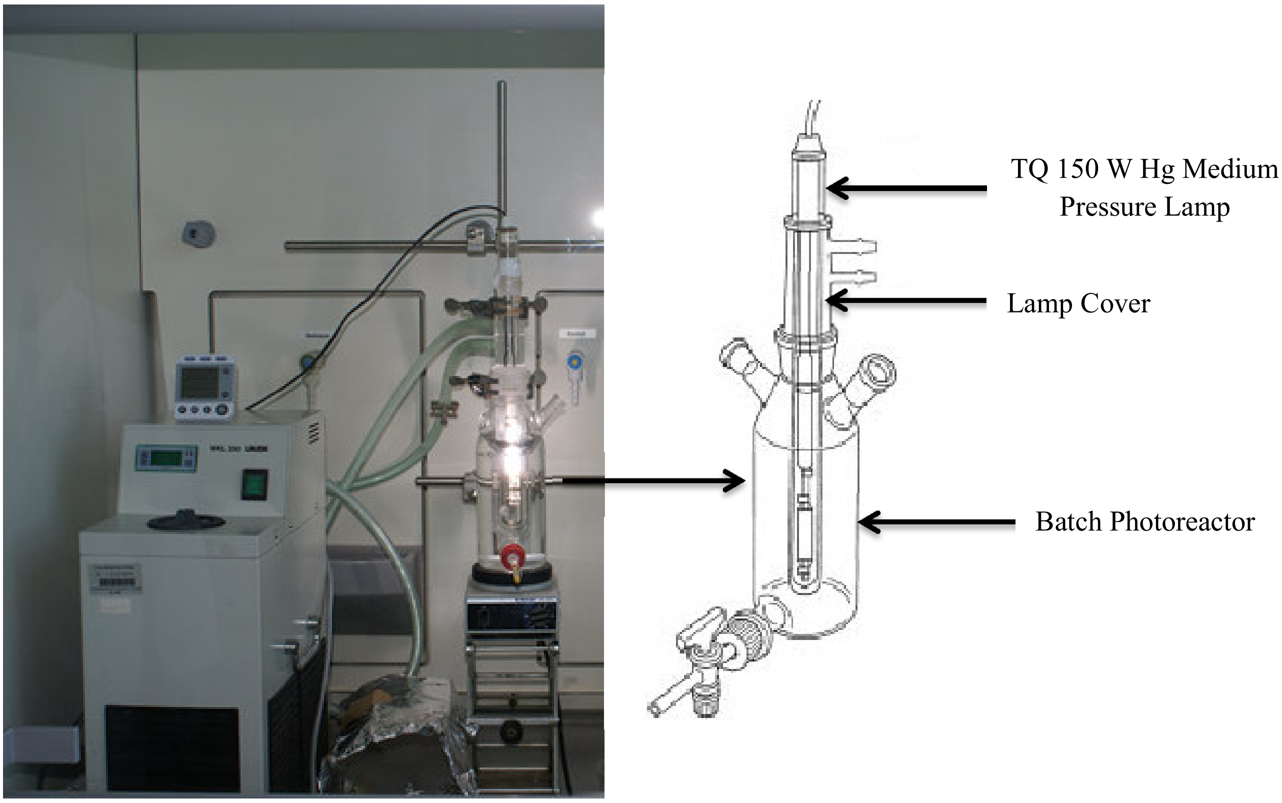


Fig. S1 Schematic of the lab scale plant used to perform the photodegradation experiments.

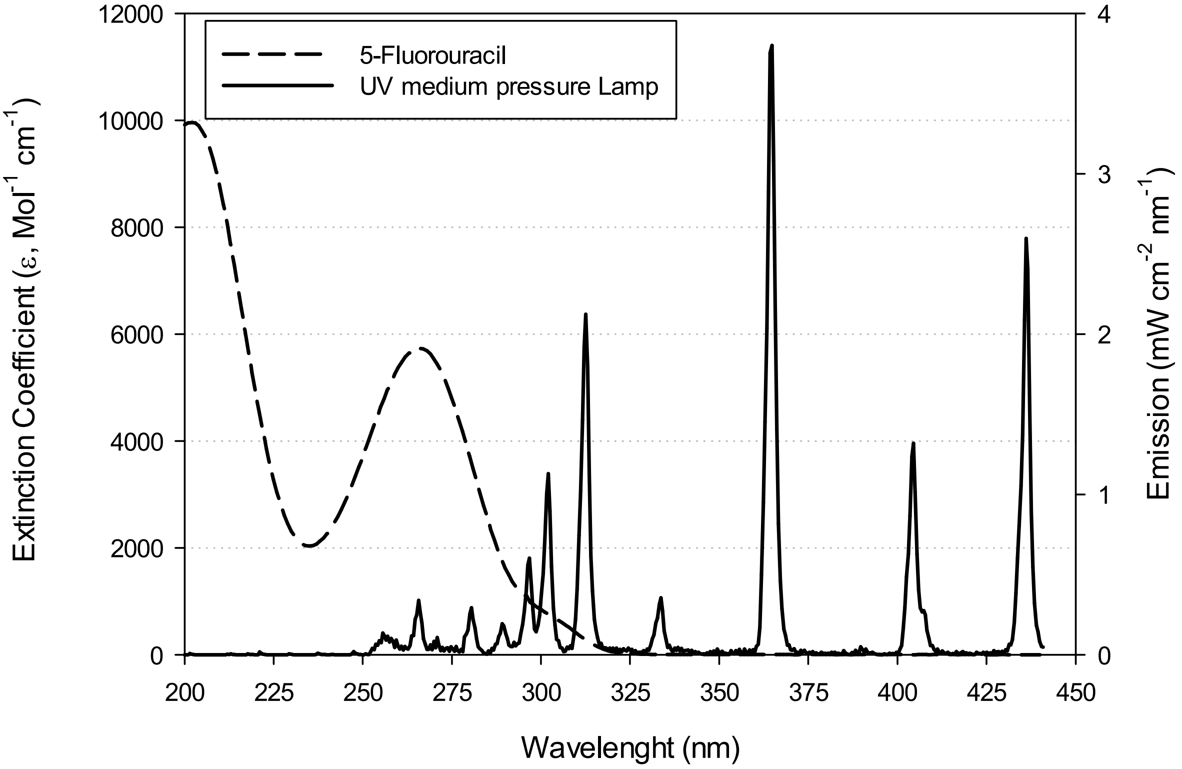


Fig. S2 – UV-VIS absorption spectrum of the 5-FU compounds at pH 7 in the wavelength range of 200 < λ < 440. Concentration 10 mg/L in ultrapure water.

Text S1:

A one phase decay model was applied to calculate the rate constant of the mineralization of 5-FU by the UV/TiO2 process. The following equation was used:

Y = (Y0 - Plateau)*exp (-K*X) + Plateau

Where: y_0 = y value when x (time) is zero and is expressed in the same units as y; plateau= y value at infinite times, expressed in the same units as y; k = the two rate constant expressed in inverse minutes; percent fast is the fraction of the span (from Y_0 to plateau) accounted for by the faster of the two components; half-life (fast) and half-life (slow) are in the time units of the x axis and are computed as ln(2)/K

The two phase decay model was applied to calculate the rate constants of the mineralization of 5-FU by the UV/H_2O_2 and UV/Fe^{2+}/H_2O_2 reactions. The following equation was used:

Spanfast = (y_0 - plateau)*percentfast*.01

spanslow = (y_0 - plateau)*(100-percentfast)*.01

y = plateau + spanfast*exp$^{(-Kfast*X)}$ + spanslow$^{*exp\ (-Kslow*X)}$

Where: y_0 = y value when x (time) is zero and is expressed in the same units as y; plateau= y value at infinite times, expressed in the same units as y; Kfast and Kslow = the two rate constants expressed in inverse minutes; percent fast is the fraction of the span (from Y_0 to plateau) accounted for by the faster of the two components; half-life (fast) and half-life (slow) are in the time units of the x axis and are computed as ln(2)/K

Text S2. LC-MS/MS analysis

In the LC-IT-MS, the main intensities of the MS^1 were used as precursor ions and were further fragmented by using 1.0 V as fragmentation amplitude by means of the option AutoMS(n) in the Esquire 6000^{plus}. The identification of new m/z peaks was performed with aid *Extracted Ion Chromatogram* and *Dissect Compounds* algorithm from the software *Data Analysis* (DataAnalysis 4.0 SP2, Bruker Daltonics GmbH, Bremen, Germany), which permit to identify even overlapped m/z peaks. The main operation conditions of the source can be seen in Table S2.

The chromatographic separation in the LC-IT-MS was carried out on a Nucleodur RP-18 endcapped 100-3, 2 µm (Macherey-Nagel, Düren, Germany) coupled to a guard column, (Nucleodur C18 ec 4-2, 3 µm; Macherey-Nagel, Düren, Germany). The mobile phase consisted of (A) 0.1 % formic acid in deionized water and (B) 100% acetonitrile. The following gradient was applied: 0-5 min isocratic 5% of B, 5-20 min linear gradient 5-70% of B, 20-25 min isocratic 70% of B, 25-27 min linear gradient 70-5% of B, 27-33 min isocratic 5% of B. Injection volume was 10 µL and the flow rate was settled to 0.3 mL/min.

Standards of 5-FU (0.1, 1, 2.5, 5, 10 and 20 mg/L) were used to establish a linear calibration curve. For LOD we used a signal to noise ratio (S:N) of 3:1 from the extracted ion chromatograms (EICs) peak area and for LOQ a S:N ratio of 10:1 was set. The linearity for 5-FU was r^2 0.989 while the LOD and LOQ were about 0.089 mg/L and 0.29 mg/L, respectively.

Elution was performed on a MN Nucleodur® HILIC column (EC 100/3 mm, 3 µm) (Macherey-Nagel, Düren, Germany) by a binary mobile phase consisting of (A) 0.1% of formic acid in ultrapure water and (B) MeOH at a isocratic flow rate of 0.3 mL min^{-1}, oven temperature 30 °C, using a isocratic flow of 30% B during 15 min.

TIC mass spectra for Thermo scientific orbitrap mass spectrometer were obtained in negative ionization mode from 50 to 300 m/z. Fragmentation up to MS^2 was performed by setting collision-induced dissociation (CID) 25 eV for the sample of 0 min and 20 eV for the different sampling times during the treatments. More about the source and tune method can be found in Table S3. The linearity in LC-HRMS for 5-FU was r^2 0.98 whereas the LOD and LOQ were 0.06 mg/L and 0.20 mg/L, respectively.

Table S2 – Operational characteristics of the electrospray ionization Ion Trap-Mass Spectrometer (ESI-IT-MS^n).

Mode		**Tune Source**		**Trap**		**MS/MS Automatic**	
Mass Range Mode	Std/Normal	Trap Drive	46.8	Scan Begin	50 m/z	Precursor Ions AutoMS(2)	4
Ion Polarity	Positive	Octopole RF Amplitude	266.7 Vpp	Scan End	300 m/z	Precursor Ions AutoMS(>2)	1
Ion Source Type	ESI	Lens 2	-58.0 V	Averages	4 Spectra	MS(n) Averages	5 Spectra
		Capillary Exit	108.3 V	Accumulation Time	200000 µs	Depth AutoMS(>2)	3
		Dry Temp.	350 °C	(Smart) ICC Target	40000	Auto MS/MS	On
		Nebulizer	30.00 psi	ICC	On	Group Length	5 lines
		Dry Gas	12.00 L/min			abs. Threshold Auto MS(2)	10000
		HV Capillary	−4080 V			rel. Threshold Auto MS(2)	5.0%
		HV End Plate Offset	−500 V			abs. Threshold Auto MS(>2)	1000
		Skimmer	33.4 V			rel. Threshold Auto MS(>2)	5.0%
		octopole one	10.71 V				
		octopole two	2.24 V				

Table S3 – Operational characteristics of the heated electrospray ionization High resolution Orbitrap Mass Spectrometer (H-ESI-HRMS/MS).

Tune File Values		**Negative polarity**		**Scan Event Details**	
Source Type	HESI	Source Voltage (kV)	3.00	1: FTMS - c norm res=30000	50.0-300.0
Capillary Temp (C)	300.00	Source Current (μA)	100.00	Activation Type	CID
APCI Vaporizer Temp (C)	200.00	Capillary Voltage (V)	-9.50	Min. Signal Required	5.0
Sheath Gas Flow	50.00	Tube Lens (V)	-35.00	Isolation Width	2.00
Aux Gas Flow	5.00	Multipole RF Amplifier (Vp-p)	770.00	Normalized Coll. Energy	20.0
Ion Trap Zoom AGC Target	1000.00	Multipole 00 Offset (V)	4.00	Default Charge State	1
Ion Trap Full AGC Target	10000.00	Lens 0 Voltage (V)	4.20	Activation Q	0.500
Ion Trap SIM AGC Target	5000.00	Multipole 0 Offset (V)	4.50	Activation Time	30.000
Ion Trap MSn AGC Target	5000.00	Lens 1 Voltage (V)	15.00		
FTMS Full AGC Target	200000.00	Gate Lens Offset (V)	35.00		
FTMS SIM AGC Target	10000.00	Multipole 1 Offset (V)	8.00		
FTMS MSn AGC Target	100000.00	Front Lens (V)	5.25		

Text S3. Ready biodegradability by means of Closed Bottle Test (OECD 301D)

The aerobic biodegradability of 5-FU and its TP's was investigated in the Closed Bottle Test (CBT) according to the OECD 301D guidelines (OECD, 1992). The CBT consisted of four series running in parallel and in duplicate (i.e., "blank", "quality control", "test", and "toxicity control"). The composition of the CBT series is summarized in Table S1. The concentrations of sodium acetate and test substances, in any of the corresponding test series, were 5 mg/L of theoretical oxygen demand (ThODNH$_3$, calculated without considering a possible nitrification. The inoculum source was the effluent collected from the municipal STP **AGL GmbH**, Lüneburg, Nord, Germany (73 000 inhabitant equivalents). Two drops of inoculum were added to 1 L of the mineral medium solution. Since some samples after the photodegradation treatment contained the catalase enzyme, which was used to remove the excess of H_2O_2, additional "blank" and "quality control" series were prepared. These series contained in addition 0.21 mg/L of catalase in order to distinguish between the biodegradation of tested compound and possible mineralization of the enzyme which was present in the sample.

During the whole test the consumption of oxygen in the bottles was measured daily with an optical oxygen sensor system (Fibox 3 PreSens, Regensburg, Germany) using sensor spots in the bottles. Besides, the temperature and the pH (day 0 and 28) were also controlled. A more detailed description of the test can be found elsewhere (Friedrich et al., 2013; Kummerer et al., 1996; Mahmoud and Kummerer, 2012; Trautwein et al., 2008).

According to the OECD guideline, a compound is classified as ‘‘readily biodegradable" if biodegradability, expressed as a percentage of oxygen consumed in the test vessel compared to maximum consumption (ThOD), exceeds 60 % within a period of ten days starting from the day where oxygen consumption reaches 10 % ThOD. A tested compound is considered to be inhibitory for the bacteria if the biodegradation does not reach more than 25% of ThOD within 14 days. Additionally, to determine the toxic effects, the oxygen consumption measured in the toxicity controls was compared with the predicted level calculated from the oxygen consumption in the quality control and in the test vessel, respectively. The ThOD of 5-FU was calculated based on the molecular formula of the compound.

Table S4 – Composition of the aerobic biodegradation test series in the CBT.

Test Series	**1** **Blank**	**2** **Quality Control**	**3** **Test Compound**	**4** **Toxicity Control**
Mineral medium	+	+	+	+
Inoculum	+	+	+	+
Test substance			+	+
Sodium acetate		+		+

Fig. S3

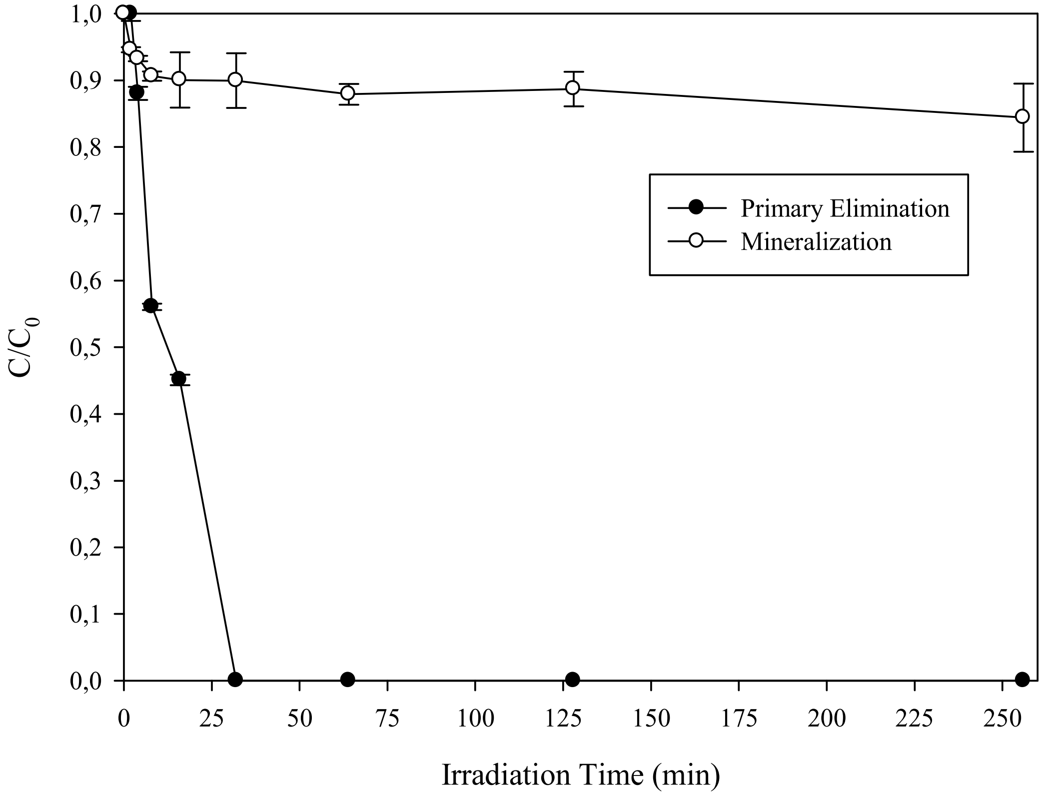


Fig. S3 – Primary Elimination and Mineralization of 5-FU under UV radiation using Hg lamp.

Fig. S4

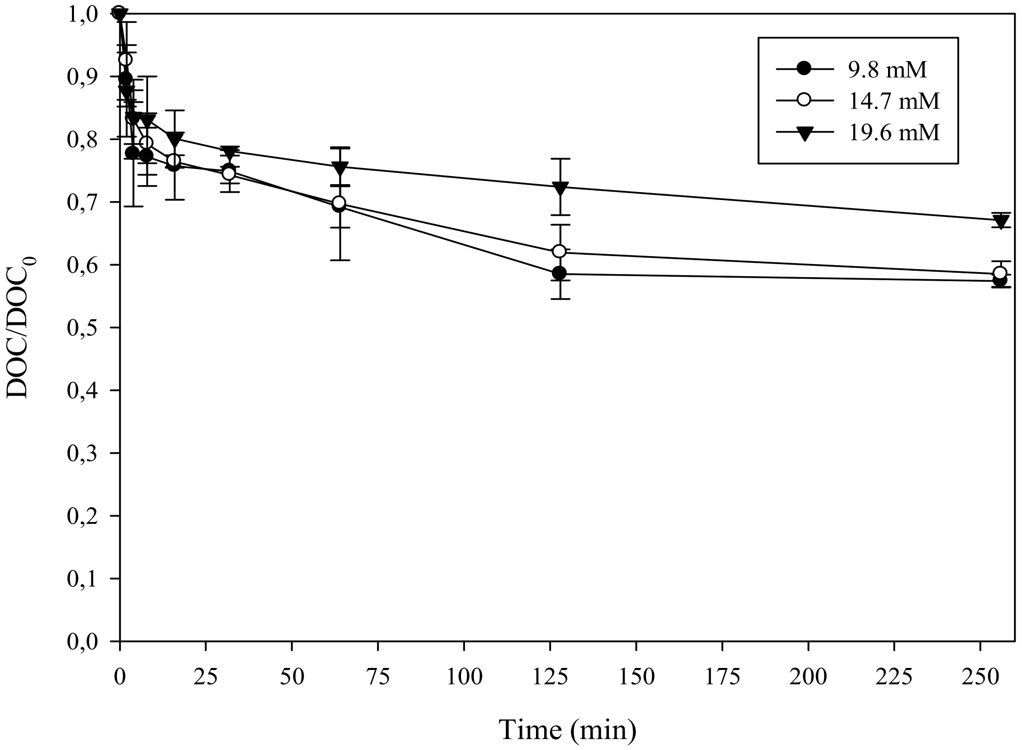


Fig. S4 – Effect of the H_2O_2 concentration on the mineralization rates of 5-Fluorouracil. (Concentration of 5-FU 20 mg/L)

Table S5 – Fitting of the BBD generated model by means of ANOVA comparing the results of variance of each response and the resulting model.

Factor	SS	df	MS	F	p
(1)pH (L)	0.92480	1	0.92480	4.2820	0.130329
pH (Q)	1.30206	1	1.30206	6.0288	0.091243
(2)[Fe^{2+}] mg L^{-1}(L)	22.20001	1	22.20001	102.7910	0.002044
[Fe^{2+}] mg L^{-1}(Q)	6.01409	1	6.01409	27.8466	0.013269
(3)[H_2O_2] mg L^{-1}(L)	6.63552	1	6.63552	30.7240	0.011576
[H_2O_2] mg L^{-1}(Q)	12.92743	1	12.92743	59.8569	0.004490
1L by 2L	0.54018	1	0.54018	2.5011	0.211906
1L by 2Q	0.70227	1	0.70227	3.2517	0.169131
1Q by 2L	4.69656	1	4.69656	21.7461	0.018612
1L by 3L	0.16403	1	0.16403	0.7595	0.447626
1Q by 3L	0.39161	1	0.39161	1.8133	0.270817
2L by 3L	0.44890	1	0.44890	2.0785	0.245053
Error	0.64792	3	0.21597		
Total SS	62.23079	15			

Fig. S5

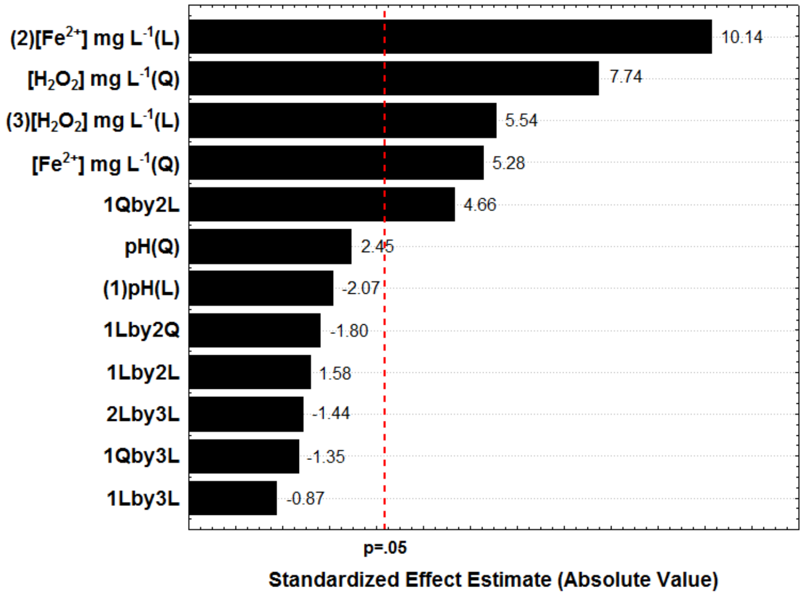


Fig. S5 – Pareto chart of effects of BBD applied for photo-Fenton treatment with a significance above 95% (p = 0.05).

Fig. S6

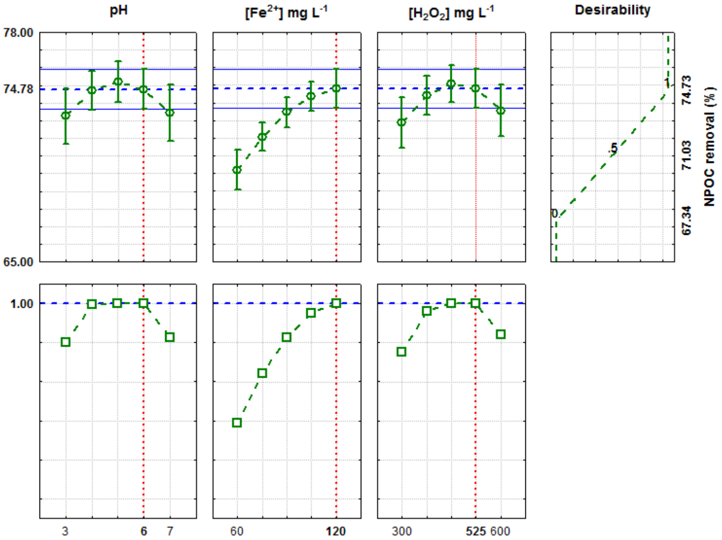


Fig. S6 – Desirability profile of the variables pH, [Fe^{2+}] and [H2O$_2$] of photo-Fenton process considering the optimized results of NPOC removal by means of BBD.

Fig. S7

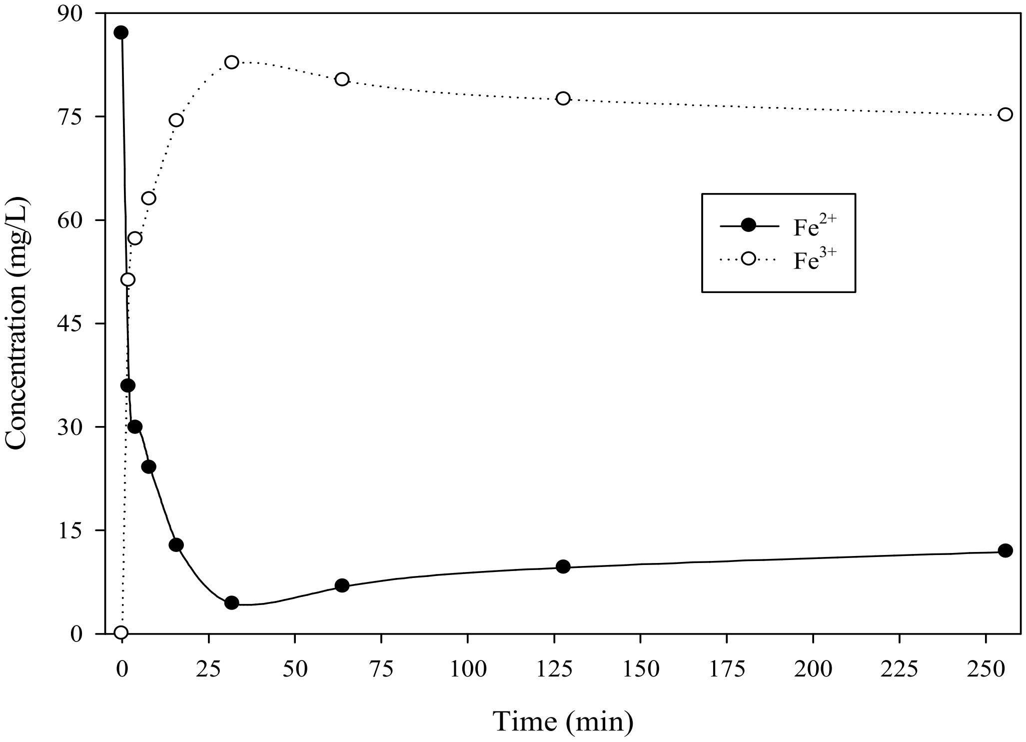


Fig. S7 – Consumption of ferric and ferrous ions during the UV/Fe^{2+}/H_2O_2.

Fig. S8

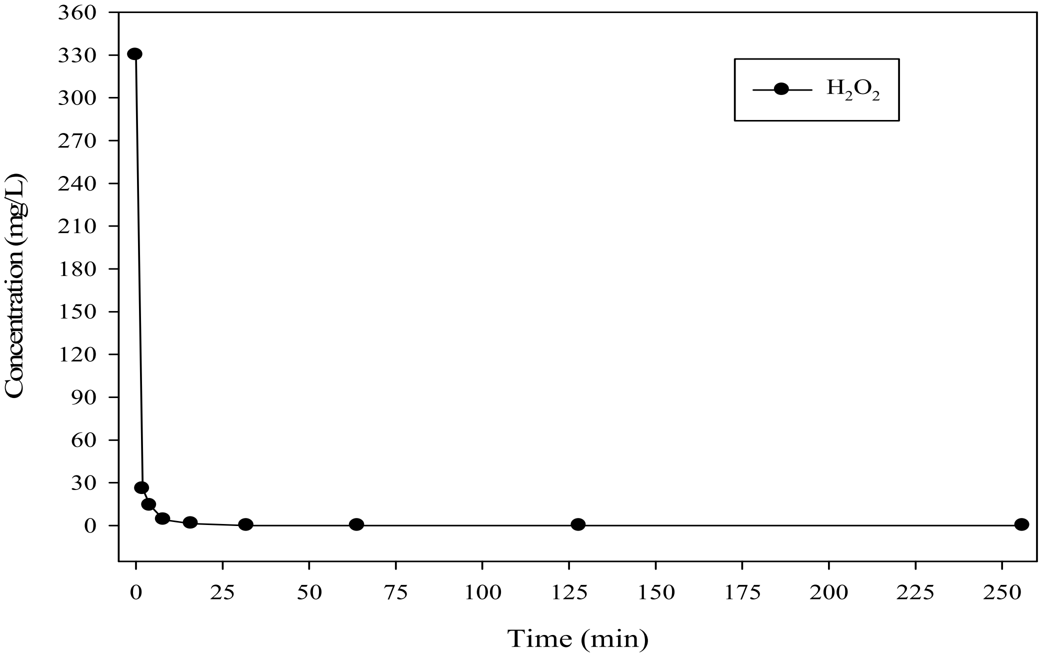


Fig. S8 – Consumption of H_2O_2 during the photo-Fenton treatment

Fig. S9

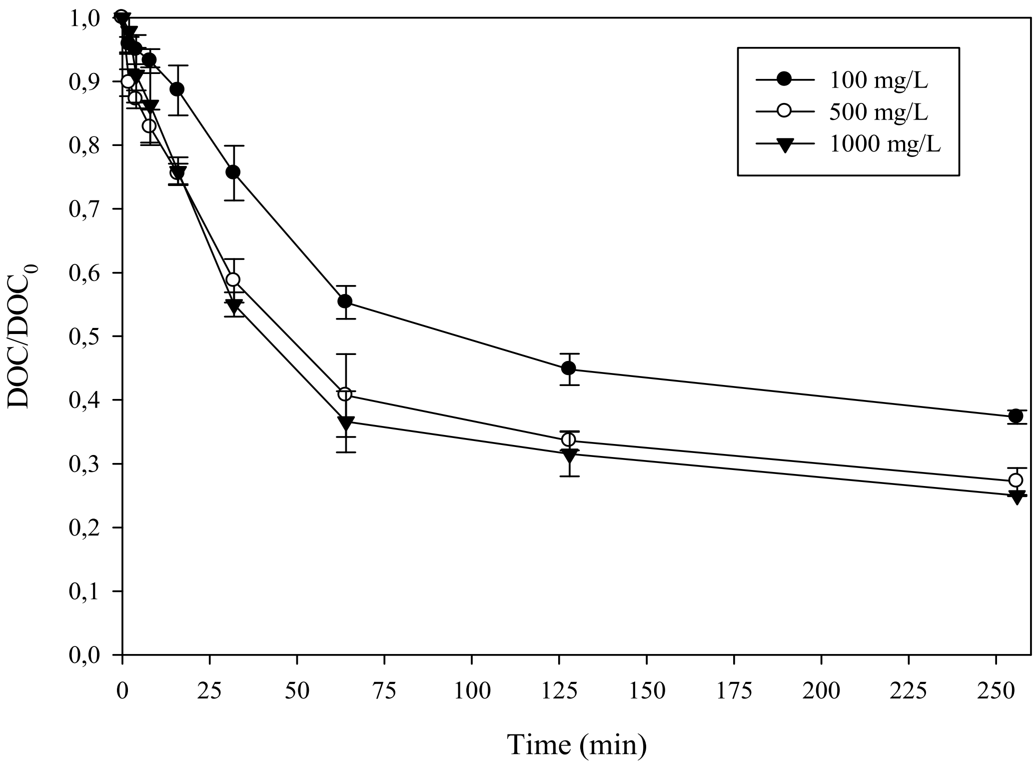
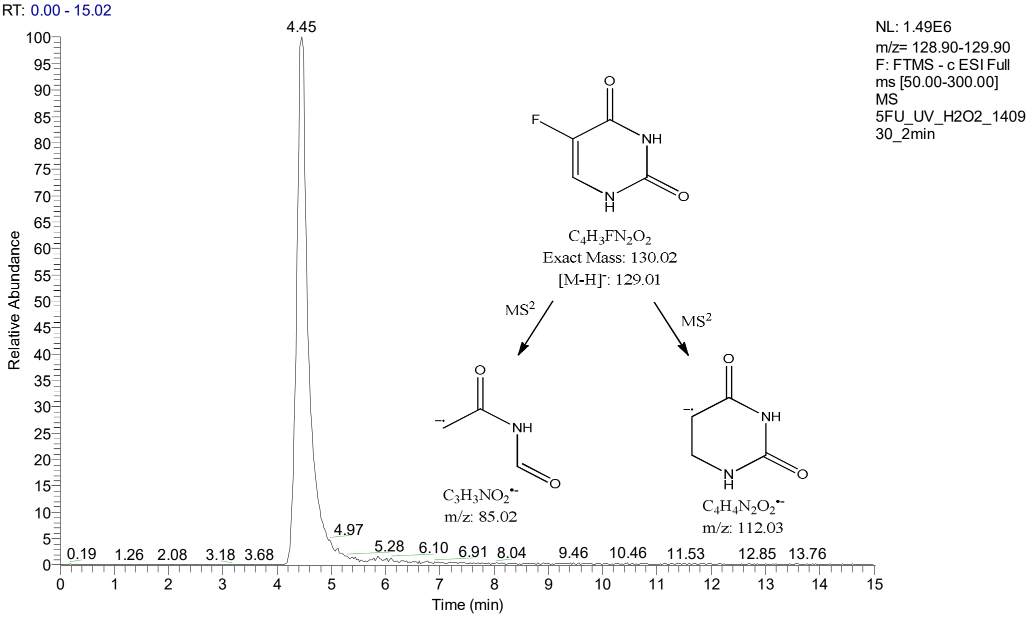
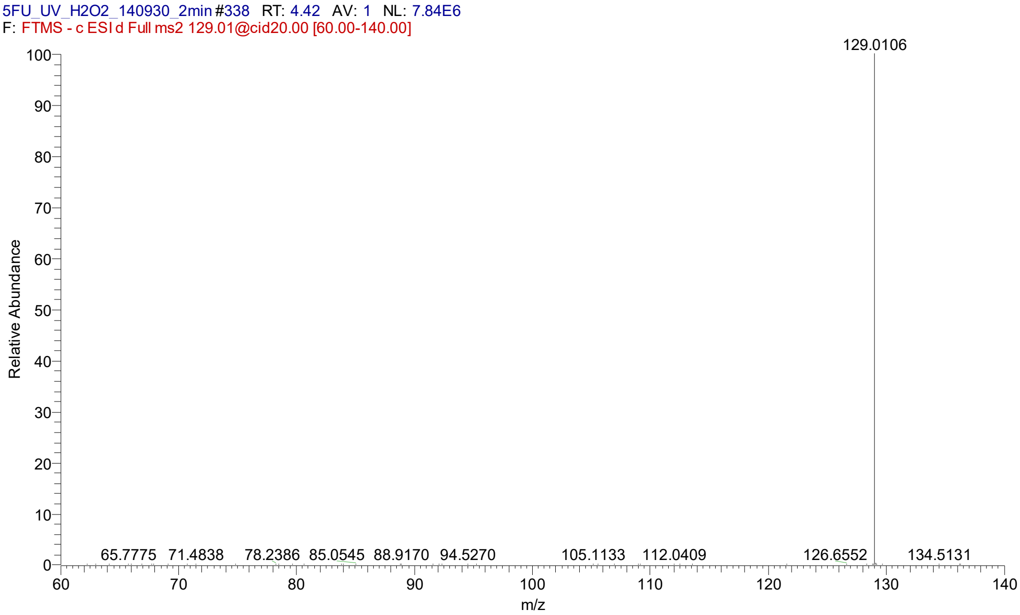


Fig. S9 – Effect of catalyst loading on the mineralization rates of 5-Fluorouracil. (Concentration of 5-FU 20 mg/L), n = 3

Text S4 – Identification and elucidation of the TPs from 5-Fluorouracil by means of LC-HRMS.

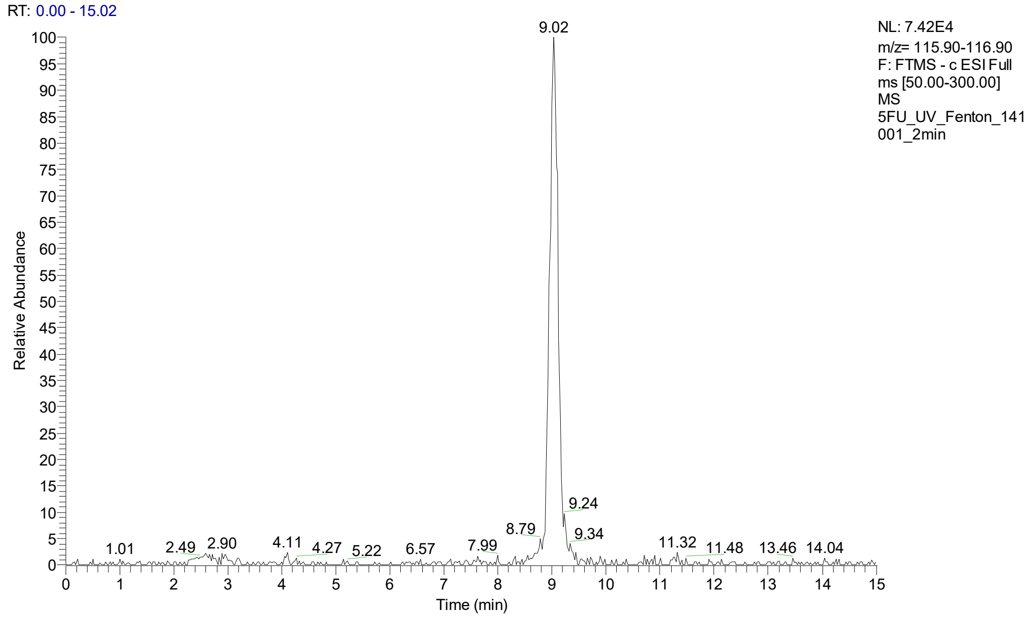
5-FU: Fragmentation pathway

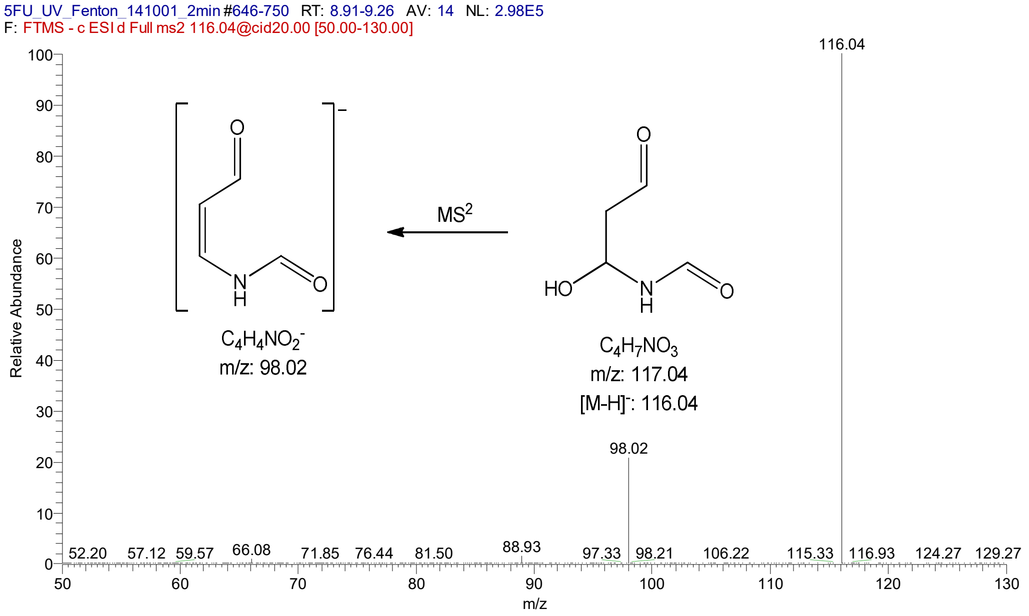




m/z [M-H]-	**Formula**	**Intensity**	**Relative Intensity (%)**	**RDB**	**Δmmu**	**N-rule**
129.0106	$C_4H_2O_2N_2F$	7842945	100	4.5	0.011	Even
112.0286	$C_4H_4O_2N_2$	2007	0.03	4.0	0.744	Odd
85.05454	C_4H_7ON	2492	0.031	2.0	1.228	Odd

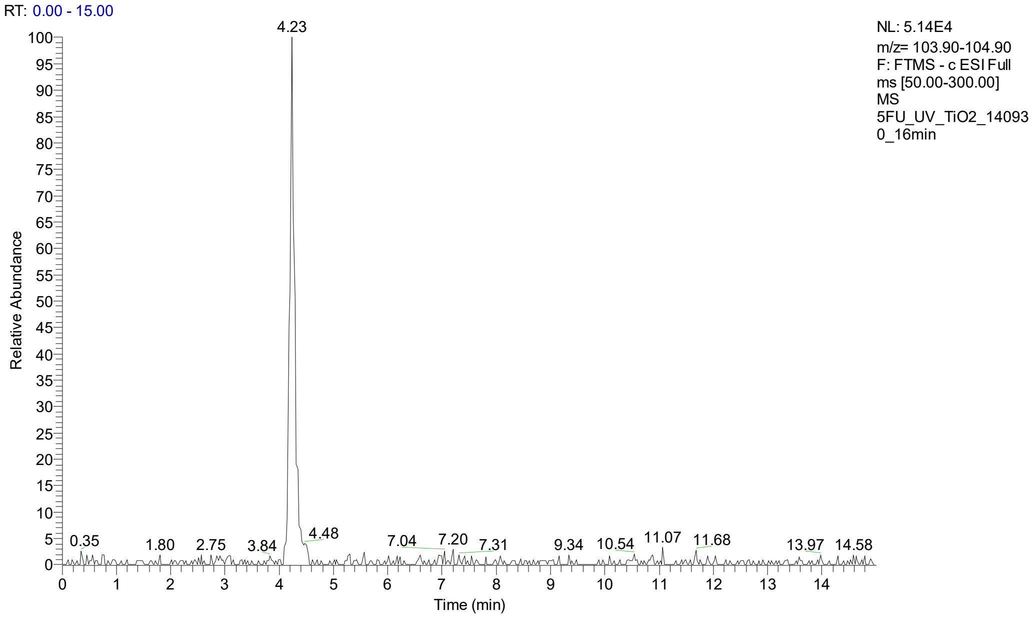
TP 117: Fragmentation pathway

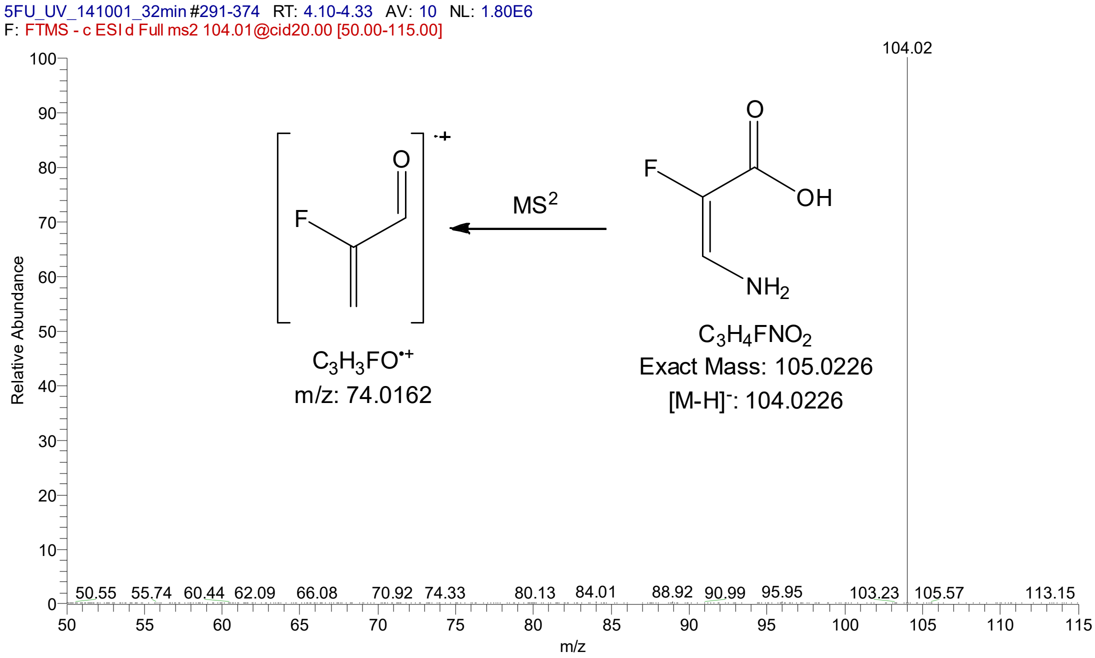




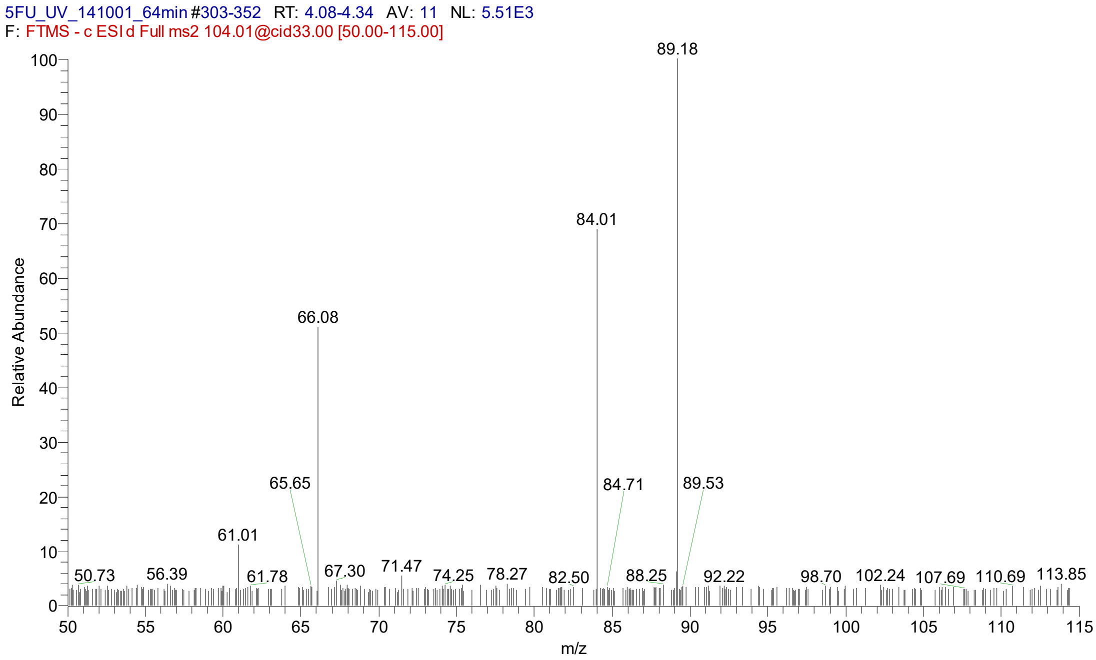
m/z [M-H]$^-$	Formula	Intensity	Relative Intensity (%)	RDB	Δmmu	N rule
116.03437	$C_4H_6O_3N$	298101.5	100	2.5	-0.946	Even
98.02458	$C_4H_4O_2N$	61540.4	20.64	3.5	-1.315	Even

TP 105: Fragmentation pathway



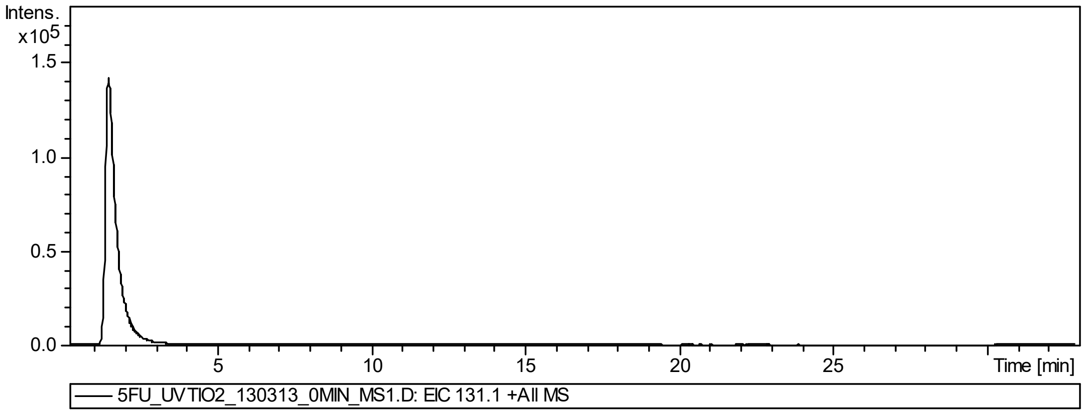


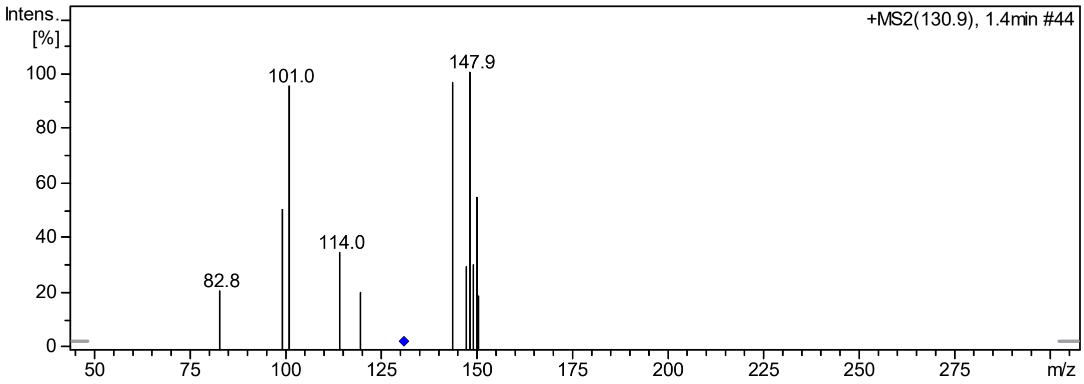
m/z $[M\text{-}H]^-$	Formula	Intensity	Relative Intensity (%)	RDB	Δmmu	N-rule
104.01528	$C_3H_3O_2NF$	568393.5	100	2.5	-0.050	Even
74.01685	C_3H_3OF	1982	0.35	2.0	-0.491	Odd

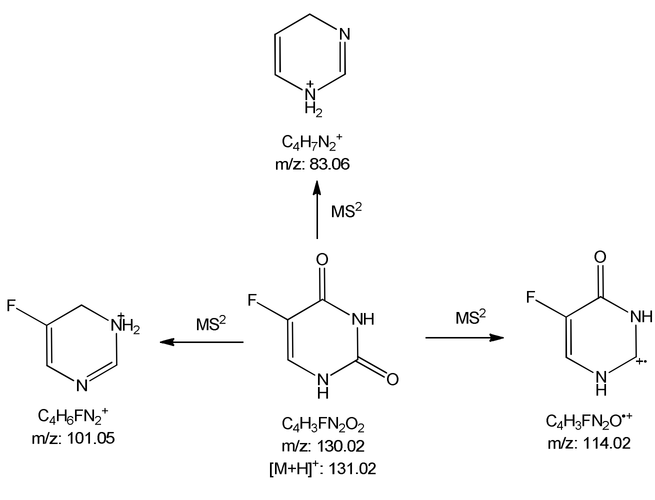

5FU_UV_141001_64min #303-352 RT: 4.08-4.34 AV: 11 NL: 5.51E3
F: FTMS - c ESI d Full ms2 104.01@cid33.00 [50.00-115.00]
Relative Abundance
m/z
0
10
20
30
40
50
60
70
80
90
100
50
55
60
65
70
75
80
85
90
95
100
105
110
115
89.18
84.01
66.08
65.65
84.71
89.53
61.01
50.73
56.39
61.78
67.30
71.47
74.25
78.27
82.50
88.25
92.22
98.70
102.24
107.69
110.69
113.85

Text S5 – Identification and elucidation of the TPs from 5-Fluorouracil by means of LC-IT-MS/MS.

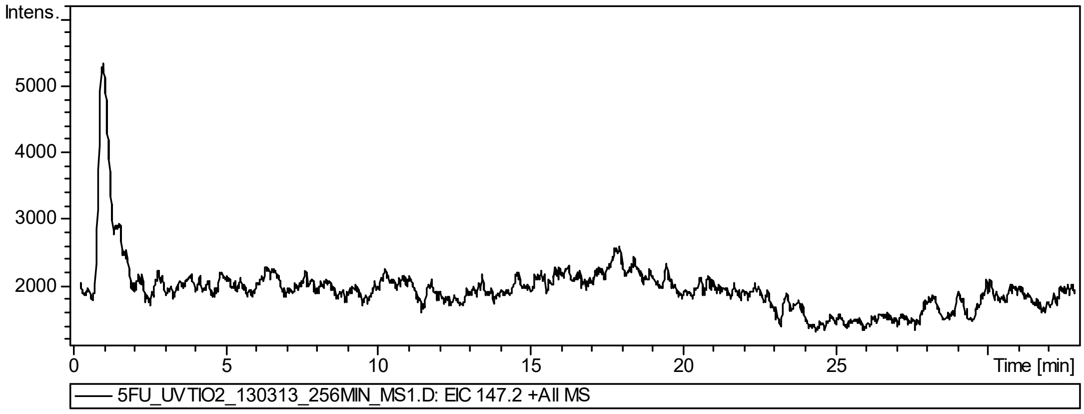
5-FU

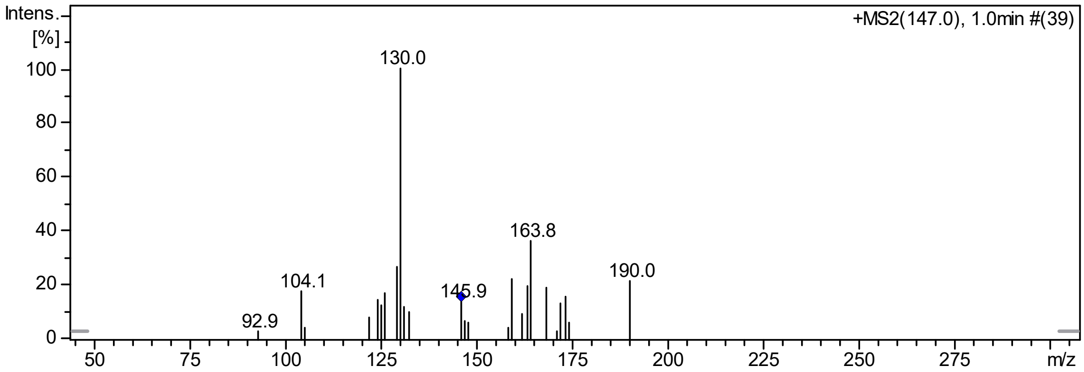


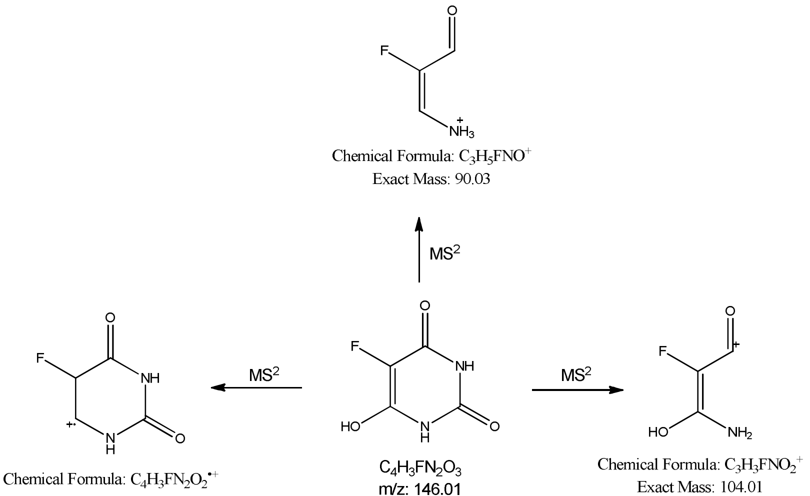




TP 146: Fragmentation pathway







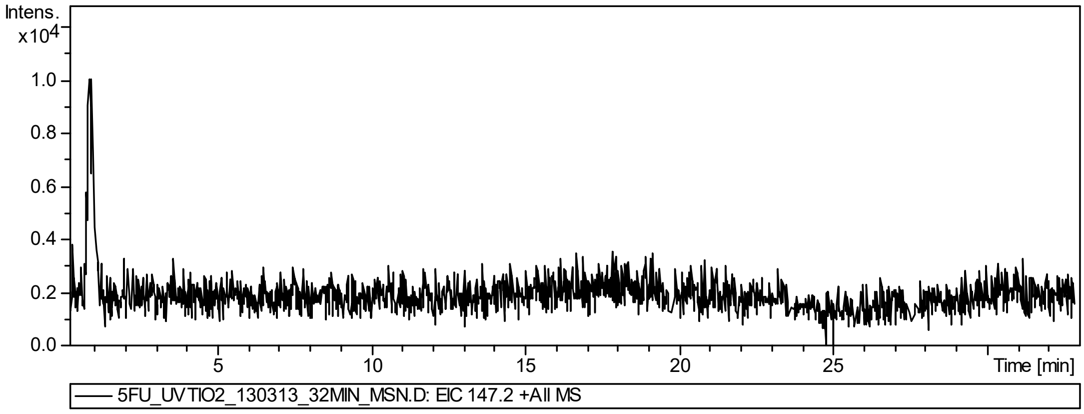
Chemical Formula: $C_3H_5FNO^+$
Exact Mass: 90.03

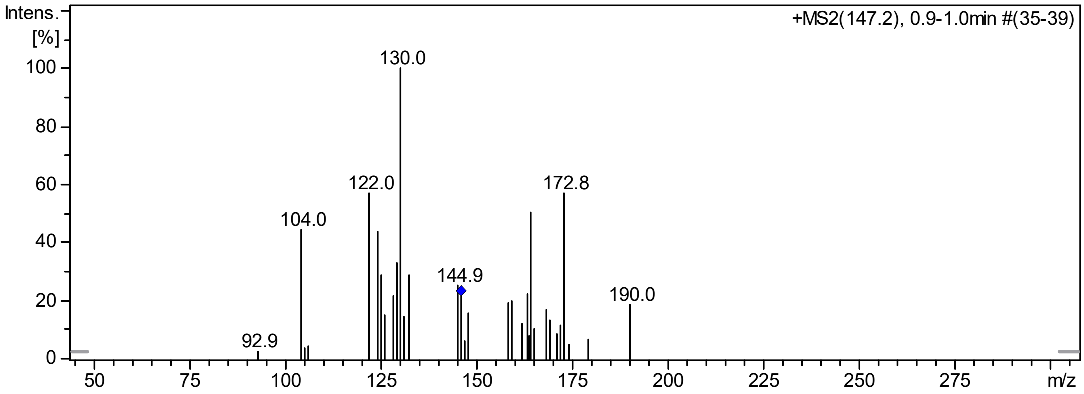
Chemical Formula: $C_4H_3FN_2O_2^{\bullet+}$
Exact Mass: 130.02

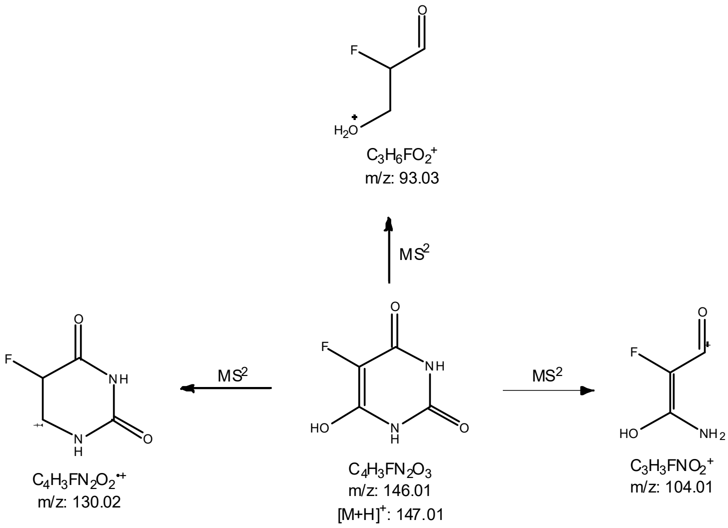
$C_4H_3FN_2O_3$
m/z: 146.01
$[M+H]^+$: 147.01

Chemical Formula: $C_3H_3FNO_2^+$
Exact Mass: 104.01

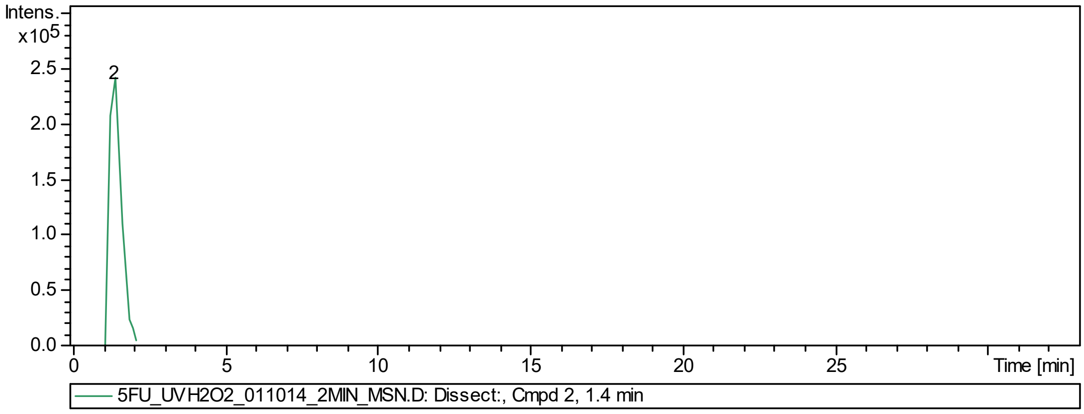
TP 148: Fragmentation pathway

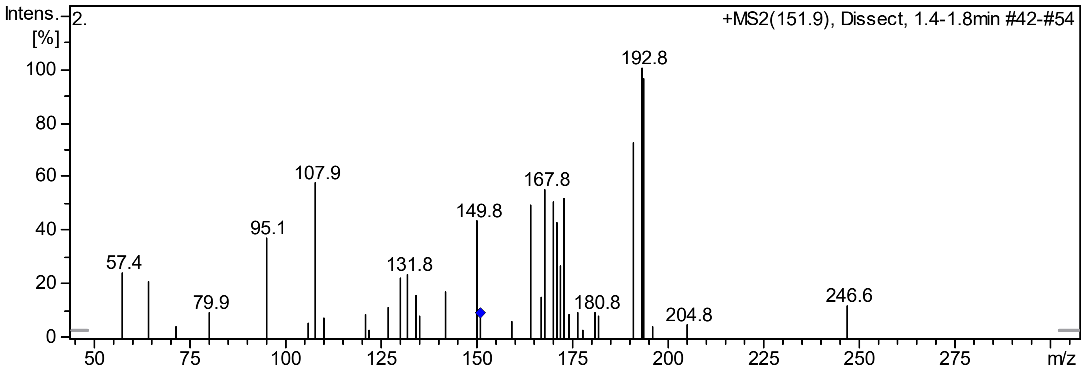


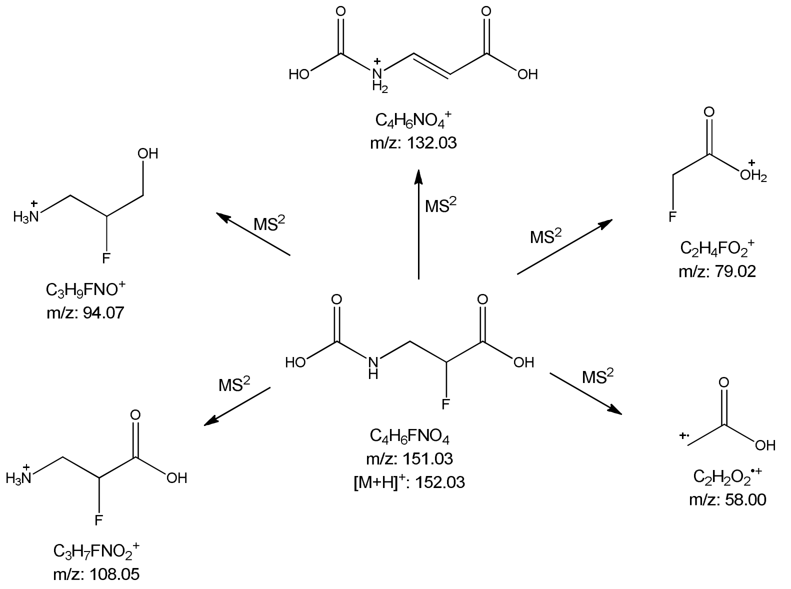




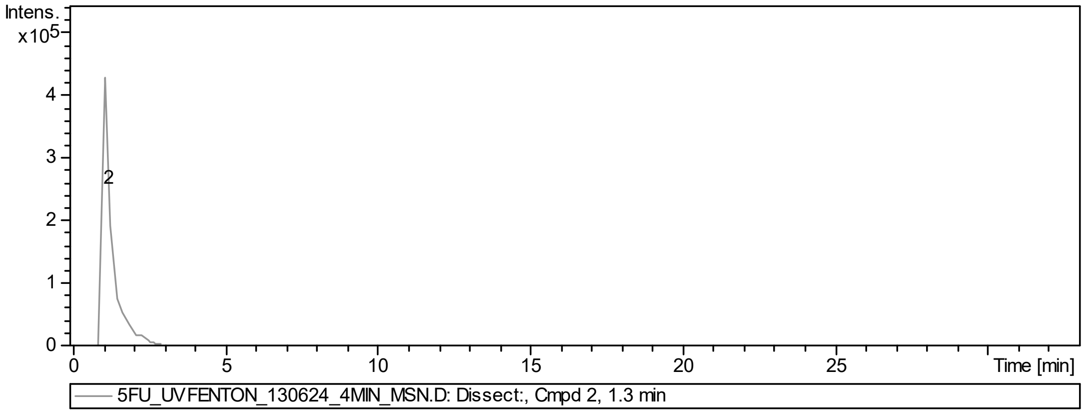
TP 151: Fragmentation pathway

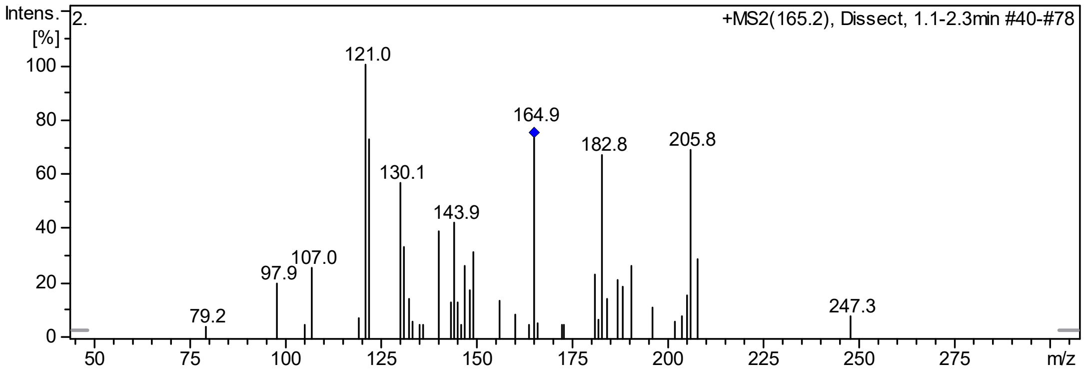


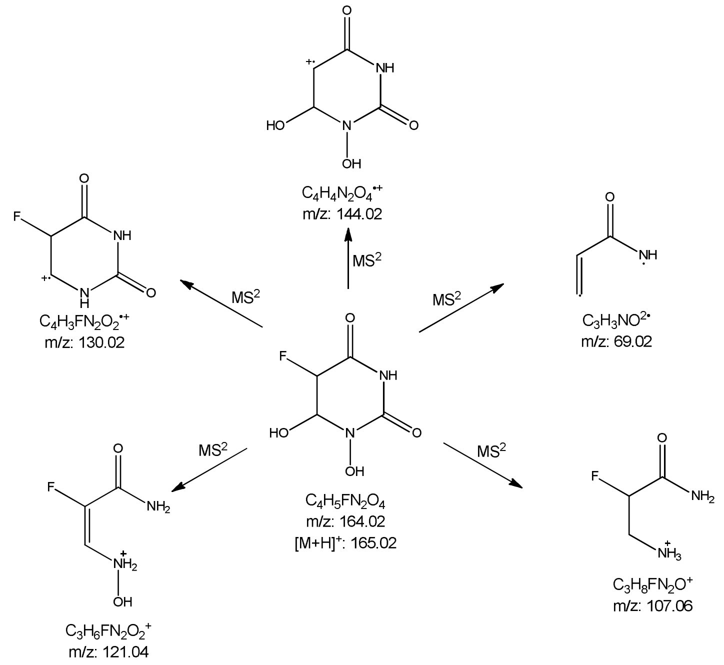




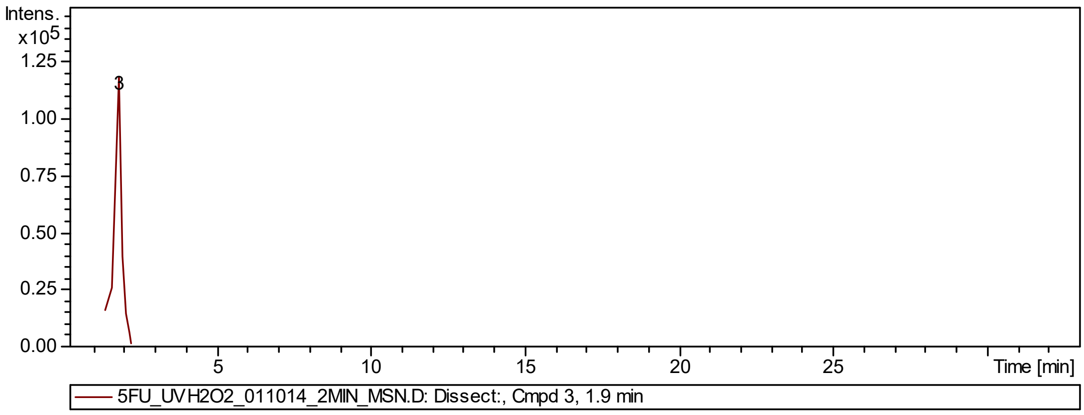
TP 164: Fragmentation pathway

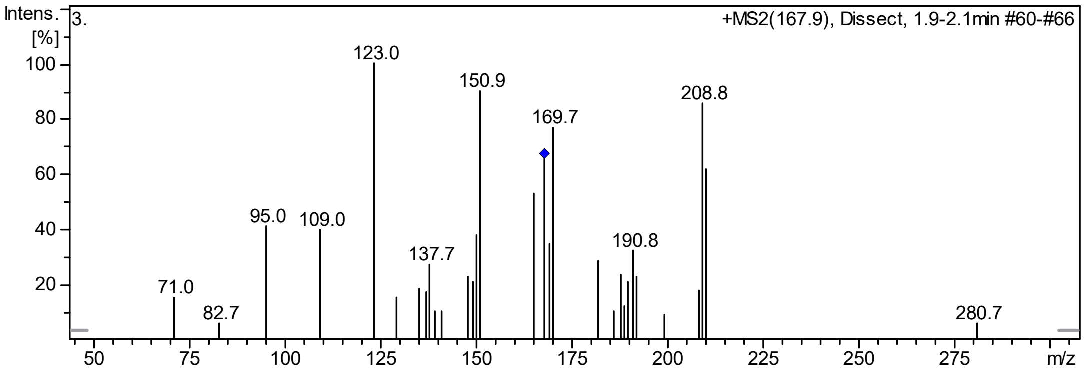


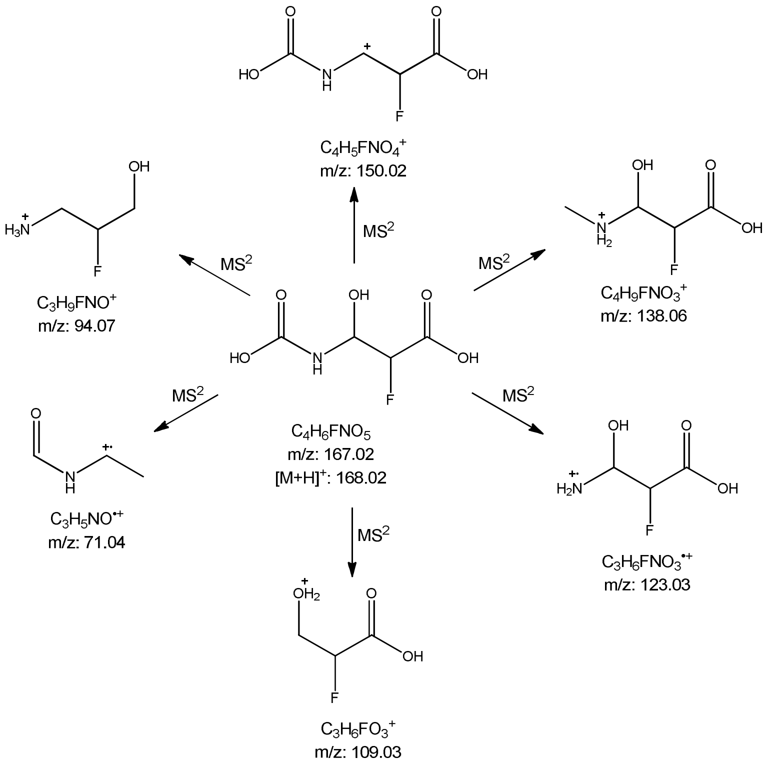




TP 167: Fragmentation pathway







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Article V

Biodegradation screening of chemicals in an artificial matrix simulating the water–sediment interface.

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Biodegradation screening of chemicals in an artificial matrix simulating the water–sediment interface

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HIGHLIGHTS

- New approach for screening biodegradation testing of chemicals was developed.
- Water–sediment interface was incorporated into screening test conditions.
- Artificial medium was applied to increase reproducibility of the data.
- Acetaminophen was eliminated by biodegradation.
- Ciprofloxacin was removed from water phase by sorption onto sediment.

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ABSTRACT

Biodegradation is the most important attenuation process for most of organic chemicals in the environment. This process decides whether the organic substance itself or its degradation products rests in the environment and should be considered for a further risk assessment. This work presents the development of a water sediment screening test, based on OECD guideline 308, with a high significance to environmental conditions and with a good reproducibility and consistency of results. The increased reproducibility was achieved by creating an artificial and standardized medium, based on the existing OECD guidelines OECD 302C, 301D and 218. Each test consisted of five different series: blank, quality control, test, toxicity control and abiotic control. Biodegradation was assessed by measurement of pressure difference in closed vessels using the OxiTop® system. Aniline, diethylene glycol and sodium acetate were used to optimize and validate test conditions. Additionally, two pharmaceuticals: Acetaminophen and ciprofloxacin (CIP) were tested as an example of possible test application. Acetaminophen was mainly removed from the system by biodegradation whereas CIP was removed from water phase by sorption onto sediment. Water sediment test proved to be a promising tool for the biodegradation investigation of chemicals in the water–sediment interface.

1. Introduction

Biodegradation is one of the main natural attenuation processes for most of organic chemicals in the aquatic environment. Chemicals that are characterized as being biodegradable are broken down into smaller compounds by bacteria or fungi during their growth; in the best case they are fully mineralized (i.e., transformed to inorganic compounds and/or used for biomass production). The biodegradability and rate of biodegradation of chemicals are important for the estimation of parameters such as exposure to aquatic organisms, persistency, toxicity and bioaccumulation (Blok and Booy, 1984; Raymond et al., 2001; OECD, 2006). Biodegradation is one of the most desirable processes in the environment, especially since products released during this bio-reactions, can be once again assimilated into the natural element cycles (Cowan et al., 1996).

Among different biodegradation tests developed, OECD guidelines for ready biodegradability testing represent the most prominent group of internationally used screening biodegradation tests (301A-F) (Strotmann et al., 1995; OECD, 2006; Guhl and Steber, 2006). All these ready biodegradability screening tests are very simple in their designs and use indirect measurement of elimination e.g., oxygen decrease, carbon dioxide evolution or pressure

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measurement, which allows for fast and cheap screening of biodegradability (OECD, 2006). However, because of their stringency, the test conditions are not close to simulating environmental conditions and may lead to unrealistic results and therefore the results of these tests should be considered as guiding only. They allow only for the classification of the compound as ready/not ready biodegradable, but not for the prediction of its full behaviour in natural environment (Alexander, 1985; Cowan et al., 1996).

The OECD simulation tests are designed to overcome these limitations since they allow the investigation of the behaviour of chemicals in specified environmentally relevant compartments e.g., soil (OECD 307), sediment (OECD 308), aerobic sewage system (OECD 303A). Further, the concentrations used in these tests should be in the same range as found in the environment. Despite the fact that simulation tests give more insight into fate of chemicals in the environment, they are not applied frequently as they are often tedious, time consuming and expensive. One of the reasons for that might be that it involves working with radiolabeled substances, which requires specific apparatus, knowledge and safety measures as well as the safe disposal of radioactive waste which is causing additional costs. The OECD 308 guideline (OECD, 2002) describes a simulation test designed to investigate aerobic and anaerobic transformation in aquatic sediment systems. The main notion represented in the guideline for simulation water sediment test is the matrix for the test, which should be taken directly from the environment. This may not give repetitive results over biodegradation rate and other parameters since the characteristic of the test matrix (i.e., chemical and physical composition of sediment, water, and bacterial community structure) may differ by each sampling time and place. Moreover, the same compound tested in one laboratory but in different seasons (therefore with different characteristics of the matrix) might not give the same results (Kalsch et al., 1999).

The aim of the presented research was to establish an aerobic screening water sediment test (WST) that will be characterized by good data repeatability. The increased reproducibility was achieved by creating an artificial and standardized medium for the test based on the existing OECD guidelines and the work of Junker et al. (2010). Junker et al. (2010), to authors best knowledge, was the first to propose the incorporation of the artificial matrix to the OECD 308 test. In this article we further develop and improve this artificial matrix and testing conditions. What is more, we introduce an additional test series (toxicity control and sterile control) to improve interpretation of the data. Moreover, in this test no radiolabeled test compounds were used and the degradation monitoring was focused on an indirect measurement method (pressure change in the closed vessels) which is cheap and simple.

2. Materials and methods

2.1. Materials

Aniline (CAS 62-53-3) was purchased from VWR International; diethylene glycol (DEG; CAS 111-46-6), calcium carbonate (CAS 471-34-1), quartz (CAS 14808-60-7) and clay (CAS 1318-74-4) were purchased from Carl Roth, Germany. Sodium acetate (CAS 127-09-3) was acquired from Bernd Kraft GmbH, Germany; sodium azide (CAS 26628-22-8), ciprofloxacin (CIP; CAS 5721-33-1) and acetaminophen (CAS 103-90-2) were purchased from Sigma–Aldrich, Germany. Peat (from sphagnum moss) was obtained from Aurich-Wiesmoor-Torfvertriebs GmbH, Germany.

2.2. Methods

2.2.1. Screening water sediment test (WST)

The biodegradation water sediment test consisted of five different series: 'blank', 'quality control', 'test', 'toxicity control' and 'sterile control' (Supplementary material Table S1); each run in three parallels. All of the series contained artificial sediment (230 g; wet weight), water with mineral medium (500 mL) and, with the exception of 'sterile control', inoculum (2 mL per one litre of water). 'Blank' series was used to subtract background readings (e.g., resulting in bacterial respiration not connected to the substance degradation) for the evaluation of the substance degradation. The 'test' series contained additionally the tested compound. The 'Quality control' series contained readily biodegradable aniline (appropriate handling and disposal of aniline as mutagenic and toxic substance was applied). 'Toxicity control' in addition to tested compound contained aniline; this series monitors co-metabolism and possible inhibitory effects of the test substance against bacteria and therefore allowed for the recognition of possible false negative results. The 'sterile control' contained additionally to the test compound sodium azide in a concentration of 400 mg L^{-1} in water phase and 800 mg kg^{-1} in sediment. 'The sterile control' allows monitoring the removal of the test compound by non-biotic processes such as sorption or hydrolysis.

2.2.2. Test design

Each of the series were placed in glass bottles (1 L) equipped with two septum sealed bottle nozzles (Fig. 1). Aniline and test compound concentration in all series was 40 mg L^{-1} expressed as theoretical oxygen demand (ThOD). All assays were incubated in the dark at 20 °C in closed vessels. Test duration was 28 d. The water phase in the bottles was stirred to improve water exchange between water and sediment phase but not vigorously to avoid disturbance of the sediment. Water to sediment ratio was 1:5. During the experiment pressure change inside the vessels are measured by pressure sensors using OxiTop® system (WTW Weilheim, Germany).

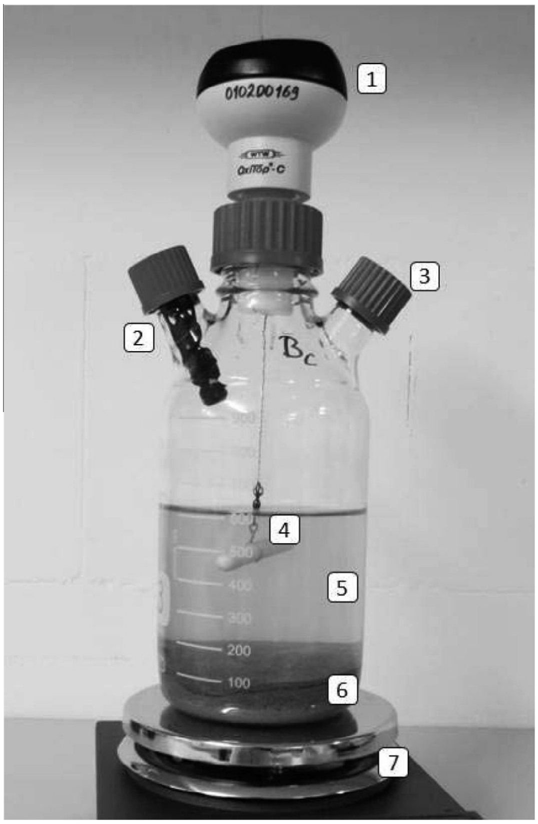

Fig. 1. Vessel used for the water sediment test. 1 – OxiTop® measuring head, 2 – a septum sealed nozzle with CO_2 trap with KOH, 3 – a septum sealed nozzle used as sampling port, 4 – stirring bar on the stainless-steel wire, 5 – water, 6 – sediment and 7 – magnetic stirrer.

2.2.2.1. Composition of the artificial matrix.

All components of artificial medium were standardized and based on OECD guidelines for testing of chemicals. Inoculum (OECD guideline 302C (OECD, 1981)) prepared from mixed sources of surface water and sediment as well as effluent from sewage treatment plant. Since the low bacterial density was suspected in WST the mineral medium composition, which contained of mineral salts, trace elements, and growth factors, was based on the OECD guideline 301D (OECD, 1992)) where low bacterial density is used. Artificial sediment (OECD guideline 218 (OECD, 2004)) was composed of kaolin clay (5% dry weight), quartz sand (grain size 0.8–0.2 mm; 92% dry weight), sphagnum moss peat (2% dry weight), and calcium carbonate (<0.1% dry weight). Details of the preparation of the above matrix are included in Supplementary material.

2.2.3. Endpoints

The assessment of biodegradation is based on the measurement of a pressure difference with the OxiTop® system (WTW, 2006). During the biodegradation, the oxygen is used by the microorganisms, and carbon dioxide is released. CO_2 is then captured by the KOH pellets, placed in the rubber hood in the bottle above the water phase. Eq. (1) had been used for calculations of the biological oxygen demand (BOD) values.

$$\mathrm{BOD} = \frac{\mathrm{M(O_2)}}{RT_{\mathrm{m}}}\left(\frac{V_{\mathrm{t}} - V_{\mathrm{l}}}{V_{\mathrm{l}}} + \alpha\frac{T_{\mathrm{m}}}{T_{\mathrm{o}}}\right)\Delta p(\mathrm{O_2}) \tag{1}$$

where BOD – biological oxygen demand (mg L^{-1}); $M(O_2)$ – molecular weight (32,000 mg mol^{-1}); R – gas constant (83.144 L mbar mol^{-1} K^{-1}); T_0 – reference temperature (273.15 K); T_m – measuring temperature (K); V_t – bottle volume (mL); V_l – sample volume (mL); α – Bunsen adsorption coefficient (0.03103); $\Delta p(O_2)$ – difference of the oxygen partial pressure (mbar).

Additionally, to monitor the bacterial toxicity of inoculum the oxygen consumption was measured in the toxicity control and compared with the predicted level computed from the oxygen consumption in the 'quality control' and in the 'test'. Substance is considered to be toxic if measured toxicity control is lower than 25% which corresponds to less than 50% degradation of aniline. If the measured 'toxicity control' is lower than calculated one substance is assumed to have negative influence on inoculum

2.2.4. Optimization and validation

Two pre-tests were conducted to optimize the test based on the design. Because the indirect measurement method of compound mineralization (i.e., change in pressure of the head space) used in the WST, the first pre-test was conducted to determine the optimum ThOD with minimum background influence, for the given test conditions. The concentrations of test compounds where optimized to the best fitting in terms of ThOD. In this pre-test, where aniline was tested at four different concentrations: 4, 10, 20, and 40 mg L^{-1} which in terms of ThOD correspond to 9.6, 24.1, 48.2, and 96.4 mg L^{-1} of ThOD, respectively. Secondly, for the choice of the optimum quality control, biodegradation of three compounds were investigated, namely: Aniline (99.96 mg L^{-1}); diethylene glycol (DEG; 80.28 mg L^{-1}), and sodium acetate (20.16 mg L^{-1}).

Additionally, to determine the change in bacterial density during the inoculum cultivation, colony forming units (CFUs) were determined according to ISO EN 6222:1999 standard. Three time points were considered for CFUs: (1) directly after sample collection mixing and filtration, (2) at the third day of cultivation, when inoculum was used for sediment conditioning and (3) at the tenth day when it was used for the test. Also, for the comparison, the CFUs of effluent from sewage treatment plant (STP) were determined. Agar plates were inoculated with tenfold dilution of inoculum samples in 1% peptone water solution. All dilutions were inoculated in triplicates and incubated at 20 °C for 68 ± 4 h. After that time CFUs were counted.

2.2.5. Biodegradation of acetaminophen and ciprofloxacin

Biodegradation of two pharmaceuticals, namely acetaminophen and ciprofloxacin, in the WST was investigated. The concentrations used in the test were 22.4 mg L^{-1} for acetaminophen and 24.0 mg L^{-1} for ciprofloxacin which corresponds each to a ThOD concentration of 40 mg L^{-1}. Ciprofloxacin was expected to be influenced by sorption, therefore removal from water phase was investigated. For that, water samples were collected with a syringe through sample port, at days 0, 1 and 28. The samples were stored at −20 °C until they were measured by the HPLC-fluorescence detection (FDA) (Shimadzu Prominence HPLC system; Duisburg, Germany) at excitation wavelength as set at 278 nm and the emission was monitored at 445 nm. The analytical method is described elsewhere in detail (Haddad and Kümmerer, 2014). Briefly, the chromatographic separations was carried out on 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. Prior to analysis, samples were filtered through 0.2 μm PES filters (Macherey–Nagel GmbH & Co. KG).

3. Results and discussion

3.1. WST design

3.1.1. Substance concentration

Following the OECD guideline 308 (OECD, 2006), test substance concentration in simulation tests should reflect the expected or measured environmental concentrations. On the other hand, higher concentrations are used in screening tests, which are constructed to investigate only biodegradation potential through the use of indirect measurement methods that are less sensitive than analytical methods. In the WST, the concentration of tested substance was depended on obtaining an indirect measurement that would be able to distinguish the mineralization of the compound, from the high background degradation of the organic fraction (peat) in the artificial sediment. Aniline in all four concentrations achieved similar rates and levels of biodegradation; 64 ± 17%; 80 ± 4%; 62 ± 10%; 68 ± 84% for 40, 20, 10, and 4 mg L^{-1} respectively (Supplementary material Fig. S1). With increasing concentration, the measurement was more stable and clear biodegradation curves were observed. With the exception of the highest concentration, the lower the concentration the higher the standard deviation was observed. Best values in regards to standard deviation were obtained at a test concentration of 20 mg L^{-1} aniline which corresponds to a ThOD of 48.2 mg L^{-1}. Optimum test substance concentration was therefore decided to be 40 mg L^{-1} in terms of ThOD.

3.1.2. Quality control

In the OECD guideline 308 (OECD, 2002), compounds that could be used as reference for inoculum quality, are not specified. However, this guideline recommends reference compounds that are radiolabeled for the identification and quantification of removal and transformation products by different analytical methods. Consequently, the results strongly depend on the part of the molecule where the radio-label is present. In the WST presented here, investigation of the biodegradation level is conducted by the indirect measurement of pressure development inside the test system;

therefore there is a need for a reference substance to give an indication of inoculum activity. In many screening biodegradation tests the level and the rate of biodegradation of the ready biodegradable substance is used as validity criteria (quality control). Reference substances proposed for WST should also meet ready biodegradability criteria and have good biodegradation kinetics. Therefore aniline and sodium acetate, proposed by OECD guideline as reference compounds for ready biodegradability testing (OECD, 1992) were also considered for quality control. In addition, diethylene glycol (DEG) was taken under consideration for quality control. This compound is known to be of intermediate biodegradability (Zgajnar Gotvajn and Zagorc-Koncan, 2003). During the conducted water sediment pre-test, all three substances achieved similar levels of biodegradation (>70%) at day 28 of the experiment (Fig. 2). In case of sodium acetate the degradation was rapid and no lag phase could be observed. In contrast, the degradation curve of aniline showed good separation into a three day lag phase, an exponential degradation phase of six days, and the stationary plateau phase. In case of DEG, biodegradation rate was much lower than these of the two other compounds and plateau phase was achieved only at the day 22. The ready biodegradability of aniline along with the clearly separated and structured biodegradation curve makes this compound the optimum choice for a reference compound in the WST.

3.1.3. Toxicity and sterile controls

As it was mentioned before, in WST the 'toxicity' and 'sterile' controls series were introduced to understand the behaviour of tested substances. The application of this series are presented and discussed together with examples in Chapter 3.3.

3.2. Artificial matrix

3.2.1. Artificial sediment

In OECD guideline 308, it is preferable to choose two different types of sediments, with different textures and organic carbon content, which are directly sampled from chosen sites. For this purpose, the additional analysis of collected sediments is required. In OECD guideline 218, adopted by Junker et al. (2010), and applied also in this WST, there is a defined composition of sediment components. Therefore no additional analysis is required. Additionally, in this WST some changes have been introduced. First of all, artificial sediment content had been adjusted to improve the oxygen penetration into sediment. For that, content of clay, which is the component influencing oxygen penetration (Booij et al., 1991, 1994), was reduced from 20% to 5%. This improved the water exchange between water and sediment phases and therefore exchange of oxygen. Moreover, to reduce the BOD of the sediment, and therefore high background readings, the organic fraction was decreased to 1% (2% of peat containing of 50% of carbon).

3.2.2. Inoculum

Another element of artificial matrix is the inoculum. In OECD guideline 308, inoculum is a part of sediment and water collected from the environment. However, taking the inoculum directly from surface waters may result in high deviation of bacterial composition, depending on geographical location, climate, characteristics of water and catchment area and season. Junker et al. (2010) adapted the OECD guideline 302, where they used bacteria from mixed sources that are cultivated for a period of one month before used in the test. However, this has also a negative drawback on the quality of the inoculum (decreasing the quality and increasing the quantity) and consequently may influence the test readings (Nyholm, 1991; Forney et al., 2001). Therefore in this WST, the cultivation time was decreased from one month to 10 d. The shorter cultivation period should limit the decrease in bacterial diversity, while still increasing the bacterial density and its adaptation to WST conditions. The higher bacterial density allows the volume of the inoculum suspension added to be low enough to avoid introduction of any additional carbon source or contaminates, which could affect the performance of the test. CFUs at the day of collection were $1.2 * 10^4$ CFU mL^{-1}, after three days it reached $1.6 * 10^7$ CFU mL^{-1} and at the 10th day they were $1.1 * 10^6$ CFU mL^{-1}. In comparison, CFUs of secondary effluent from STP was $1.3 * 10^4$ CFU mL^{-1}. These results indicate that the inoculum reached the highest measured bacterial density possible for the applied conditions (feed type and amount) already after 3 d of incubation. This '3 d inoculum' was used for the sediment conditioning (Supplementary material).

To investigate possible discrepancy of inoculum performance, two WSTs with aniline, sodium acetate and DEG, were conducted one and half years apart and in two different seasons (summer

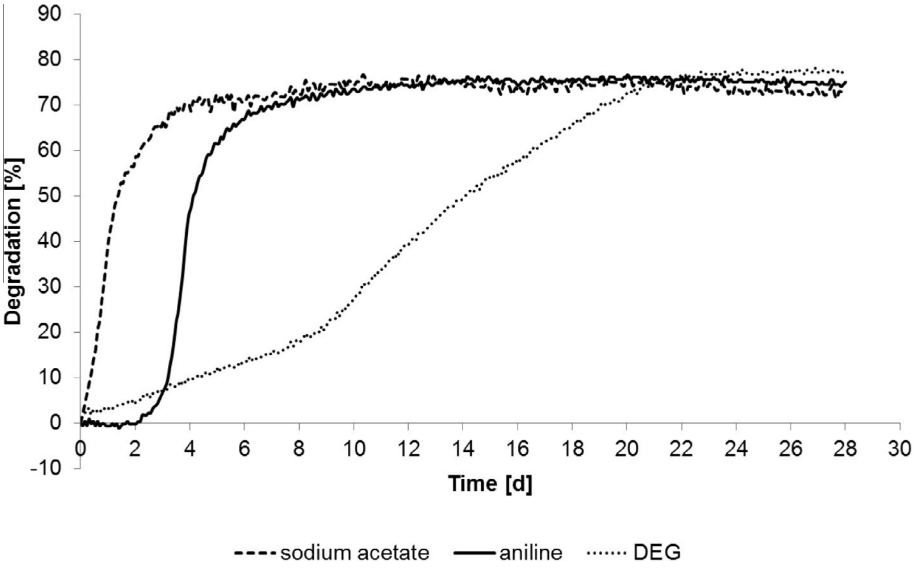

Fig. 2. Biodegradation of sodium acetate 80.64 mg L^{-1}; aniline 39.98 mg L^{-1} and diethylene glycol (DEG) 64.22 mg L^{-1} in screening water sediment test conducted in December.

Table 1
Overview of biodegradation of diethylene glycol (DEG), aniline and sodium acetate in water sediment test (WST; I – December; II – July).

Compound	Test	Concentration (mg L^{-1})	Lag phase (d)	Degradation (%)
DEG	WST I	64.22	4	77 ± 11
	WST II	32.00	5	78 ± 1
Aniline	WST I	39.98	3	75 ± 10
	WST II	20.00	3.5	80 ± 14
Sodium acetate	WST I	80.64	0	73 ± 19
	WST II	62.00	0	81 ± 6

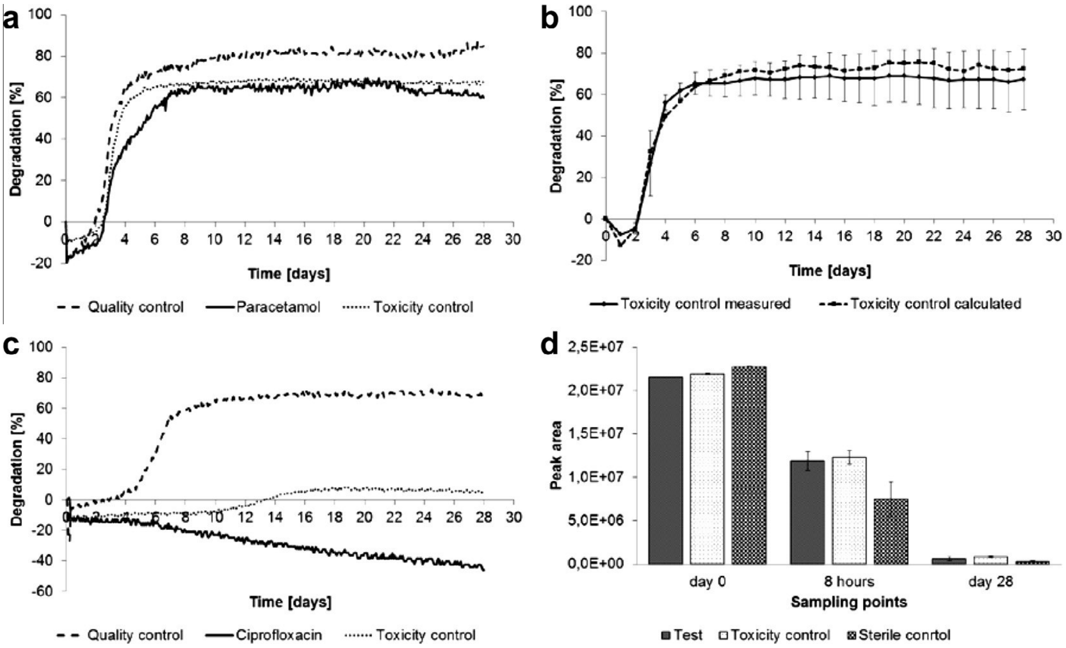

Fig. 3. a – Biodegradation of acetaminophen (22.4 mg L^{-1}) in screening water sediment test; b – Comparison between measured and calculated toxicity control, c – Biodegradation of ciprofloxacin (24.0 mg L^{-1}) in screening water sediment test, and d – ciprofloxacin removal, measured by HPLC, from water phase of three test series: Test, toxicity control and sterile control, at different time points in screening water sediment test.

and winter). As expected, there was no significant difference of biodegradation rate of sodium acetate since it is ready biodegradable compound. DEG and aniline also reached similar levels of biodegradation in both tests, with only slight difference in the adaptation time of inoculum expressed as lag phase (Table 1, and Supplementary material Fig. S2). Since DEG and aniline biodegradation kinetics is usually a result of inoculum potency (Nyholm, 1991; van Ginkel et al., 1995; Ahtiainen et al., 2003), the result of these two tests gives a proof of a good reproducibility of conditions (especially inoculum) in WST regardless the season when experiments are performed.

3.3. WST application to acetaminophen and ciprofloxacin

3.3.1. Acetaminophen

The inoculum in the WST was in an optimum condition since ‘quality control’ was biodegraded in 85 ± 14%. Acetaminophen was degraded to 60 ± 28% (Fig. 3a). Acetaminophen in tested concentration was not toxic to inoculum with biodegradation level in ‘toxicity control’ of 67 ± 15%. Since the ‘Toxicity control calculated’ (sum of oxygen demand in the test and quality assay) and ‘toxicity control’ measured corresponded well to each other, concluding that degradation of both compounds occurred in parallel and was not disturbed (Fig. 3b). Therefore, the ‘Toxicity control’ proved to be a good tool to investigate bacterial inhibition on the tested substances. The potential of acetaminophen to be biodegraded is in agreement with literature data. Lin et al. (2010) tested biodegradation and sorption of acetaminophen in natural environment simulating systems in a concentration of 50 μg L^{-1}. In their study, biodegradation was the main attenuation mechanism and complete biodegradation was achieved on 13th day of the experiment. Furthermore, no sorption and good biological removal of acetaminophen was found in a nitrifying membrane bioreactor by Gusseme et al. (2011). Many other authors have also reported a high rate of biodegradation in simulation tests of different environmental compartments (Wu et al., 2012b). Since the biodegradation achieved in the WST was similar to those in simulation test systems it can be suggest that the WST is a good indicator system for screening under simulating environment biodegradation conditions.

3.3.2. Ciprofloxacin (CIP)

Similarly in this test series, the inoculum was in optimum condition since ‘quality control’ reached 66 ± 18% of degradation. CIP showed no biodegradability with a negative biodegradation curve that reached -46 ± 12% at day 28 (Fig. 3c). The negative development of the biodegradation curve for CIP is a result of normalization of the test results by the ‘blank’ series, therefore this result should be considered as 0% degradation as less than zero biodegradation is not possible. Furthermore, low biodegradability in ‘toxicity control’ 5 ± 11% and correlation of toxicity controls between calculated and tested indicated that the test substance has inhibited the bacterial activity during the WST (Supplementary material, Fig. S3). These results are in agreement with literature data. CIP was not biodegraded in screening biodegradation closed bottle test (Al-Ahmad et al., 1999) and no toxic effect against bacteria was observed. However, the concentration of CIP was lower than that applied in WST. Inhibition of bacterial activity was observed by Girardi et al. (2011) in soil and aqueous systems with only minor mineralization in soil. It was also found that sorption of CIP does not completely eliminate toxic effects of the compound; however it can potentially reduce bioavailability and its antibiotic potency (Córdova-Kreylos and Scow, 2007). The CIPs' EC_{50} for activated sludge bacteria was estimated as 0.31–1.22 mg L^{-1} (Halling-Sorensen, 2000) and the MIC_{50} (minimum inhibition concentration) for pathogenic bacteria were reported to be 2–8000 μg L^{-1} (Al-Ahmad et al., 1999). CIP concentration in WST was from 20 to 77 times higher than EC_{50}, which could explain bacterial inhibition observed in this test.

The comparison, of substance removal from water phase, between test and sterile controls, performed by analytical screening of compound concentration in water phase, gave more information on the influence of the abiotic processes e.g., sorption or hydrolysis to CIP. CIP concentration in water phase during the test, demonstrated that CIP was partially removed from water phase in all three test series after 8 h and almost completely removed from water phase at the end of the test (Fig. 3d), which indicates that sorption will be the main removal process of CIP from aquatic environment. The more rapid CIP elimination was obtained in the sterile control which could be explained by the zwitterionic nature of CIP. Decreasing the pH value by sodium azide promote the share of cationic form of CIP and increase sorption effects. Numerous studies showed CIP having a strong potential to be adsorbed on clay (Li et al., 2011; Wu et al., 2012a; Wang et al., 2011) and peat (humic substances) (Aristilde and Sposito, 2010).

4. Summary and outlook

The WST provides testing conditions (presence of water–sediment interface) that are more complex than those in standardized screening tests, where only biodegradation performed by bacteria suspended in water phase is taken into account. The additional sediment compartment is especially of interest for testing those chemicals which are known to be persistent in the environment and the ones that may sorb to suspended solids and sediments. These compounds are more likely to have contact with sediments and therefore eliminated by sorption or by biodegradation through sediment bacteria, which are different to those present in the water phase. An indirect method for the evaluation of compound mineralization, adopted from biodegradation screening tests, makes WST less laborious than standardized simulation OECD tests where analytical methods validated for each compound and radiolabelling are required. The limitation for application of the WST test is the toxicity against inoculum of the tested compound. However, the inclusion of a 'toxicity control' allows to exclude false negative results. The applied indirect measurement system (OxiTop®) makes the WST not suited for volatile compounds. Furthermore, compounds, which have low water solubility, are also not suited for this method.

Further advantages of WST can be achieved by integration of a 'sterile control' into the test system. This, together with screening the water phase for the test substances e.g., by HPLC, gives a good indication of possible sorption and other non-biotic processes of the tested compounds. Consequently, WST can be a good starting point for further investigations of sorption and desorption in different sediment compartments. Summarizing, the WST is more efficient than other screening tests, since it gives a better understanding of the environmental fate of organic compounds, which is essential to estimate its effects and risks for an environmental risk assessment, and at the same time affordable workload level.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.09.103.

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Supplementary material

Biodegradation screening of chemicals in artificial matrix simulating water-sediment interface

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1. Supporting methods

Screening water sediment test (WST)

Table S1

Screening water sediment test vessels content accordingly to test series.

Test series	Blank	Quality control	Test	Toxicity control	Sterile control
Sediment	■	■	■	■	■
Mineral medium	■	■	■	■	■
Inoculum	■	■	■	■	
Aniline		■		■	
Test substance			■	■	■
Sodium azide					■

Inoculum

One of the main characteristics of the presented WST is the artificial medium which is composed of defined proportions of components of known sources. All components of artificial medium i.e. inoculum, water with mineral medium and sediment were standardized based on existing OECD guidelines for testing of chemicals.

Inoculum sampling, preparation and culture methods were conducted in accordance to OECD guideline 302 C (OECD, 1981). The three sampling sites for the inoculum were chosen: (1) effluent from a municipal WTP (Lüneburg, 73,000 population equivalents; N: 53° 16' 0" E: 10° 25' 19"), (2) water and sediment from river Ilmenau in Lüneburg (upstream from WTP; N: 53° 12' 31" E: 10° 24' 45") (3) and water and sediment from Lake Loppau in Ammelinghausen (N: 53° 7' 57" E: 10° 13' 41"). At each sampling site 1 L of water and 1 L of sediment were taken. The upper layer of sediment, not deeper than 10 cm, was collected to be sure that only bacteria from the aerobic zone were present. After sample collection and transportation to the laboratory all sediment and water samples were mixed together in one container by shaking vigorously for around 5 min. Then the container was left alone for

30 min for the settlement of the sediment. After that the supernatant was filtrated through filter paper (5-8 µm; Rotilabo® typ113P; CarlRoth). The filtrate (3 L) was transferred into a culture tank and adjusted to pH 7.0 ± 1.0 with sodium hydroxide or phosphoric acid and aerated overnight. From the next day on, the inoculum was daily fed by replacing 1/3 of the reactor volume with an equal volume of 0.1% synthetic sewage (OECD 302 C: aquatic solution of glucose, peptone and KH_2PO_4). The culturing was carried out at 25 ± 2°C in dark for 10 days before the inoculum was used in the test. The amount of inoculum used for the test was 2 mL per 1L of water.

Artificial sediment

Artificial sediment was prepared according to OECD guideline 218 (OECD, 2004). In brief, first the peat suspension was conditioned in demineralised water for two days in dark at 20 ± 1°C (peat to water ratio 1:5.8 (w/v)). Then the pH of this suspension was adjusted to 6.0 ± 0.5 with calcium carbonate ($CaCO_3$). Next the peat suspension was mixed with the other constituents and deionised water to obtain homogenous sediment with a water content of 10-20%. The complete sediment mixture was moist but not wet. Then the pH of the final artificial sediment was controlled and adjusted to 7.0 ± 0.5 with $CaCO_3$. The final artificial sediment composition contained 93% quartz sand (grain size 0.8-0.2 mm), 5% clay, peat 2% and $CaCO_3$<0.01% (dry weight). Directly after preparation of the artificial sediment and prior to the test a sediment conditioning was performed. For that purpose, the sediment was placed in the test vessels with a water layer above containing mineral medium and inoculum and acclimated for 7 days under the test conditions. Conditioning allows stabilization of important parameters e.g. pH, redox potential, and adaptation of bacteria and their growth on the sediment. During the conditioning the pressure development and BOD were measured to monitor the processes inside the sediment. Additionally, the bulk density of the artificial sediment and its dry weight were determined according to DIN EN 12880:2000. Carbon content of both peat and sediment was measured by a C/N analyser.

2. Supporting results and discussion

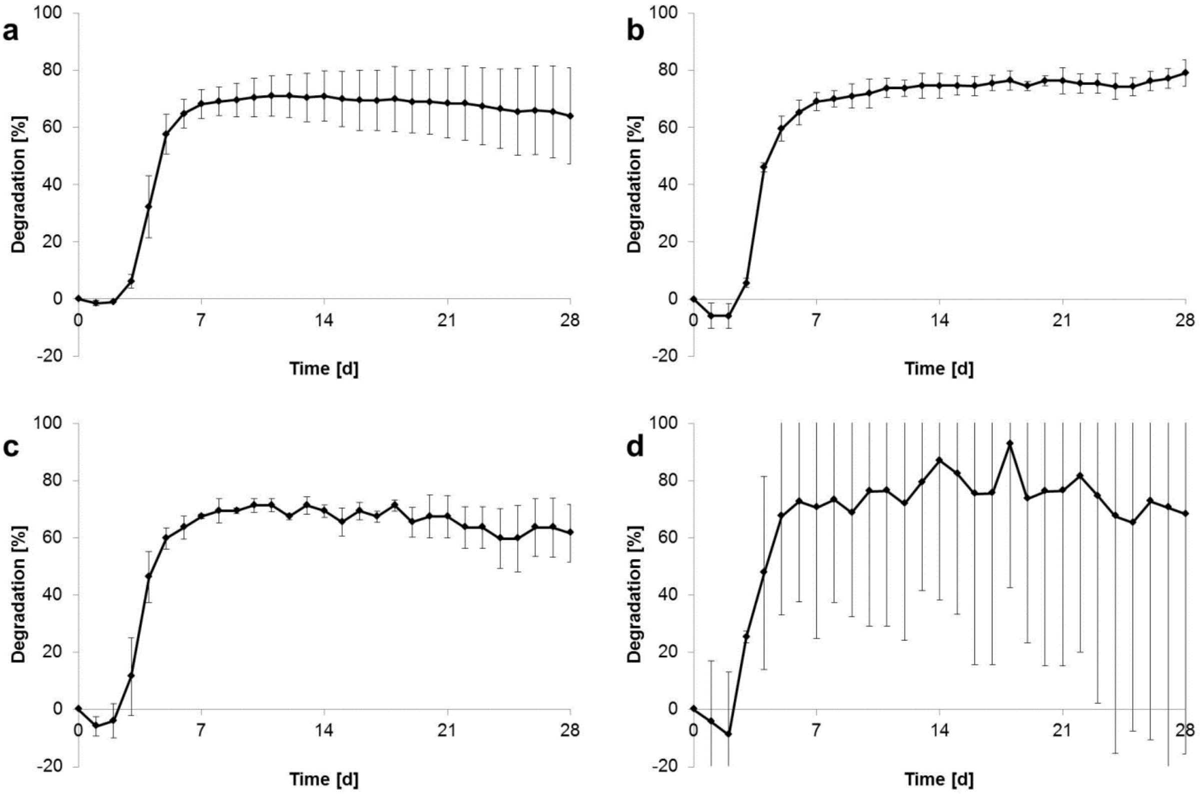

Fig. S1. Comparison between aniline biodegradation in four different concentrations 40 mg L^{-1} (a), 20 mg L^{-1} (b), 10 mg L^{-1} (c), and 4 mg L^{-1} (d), during the screening water sediment test.

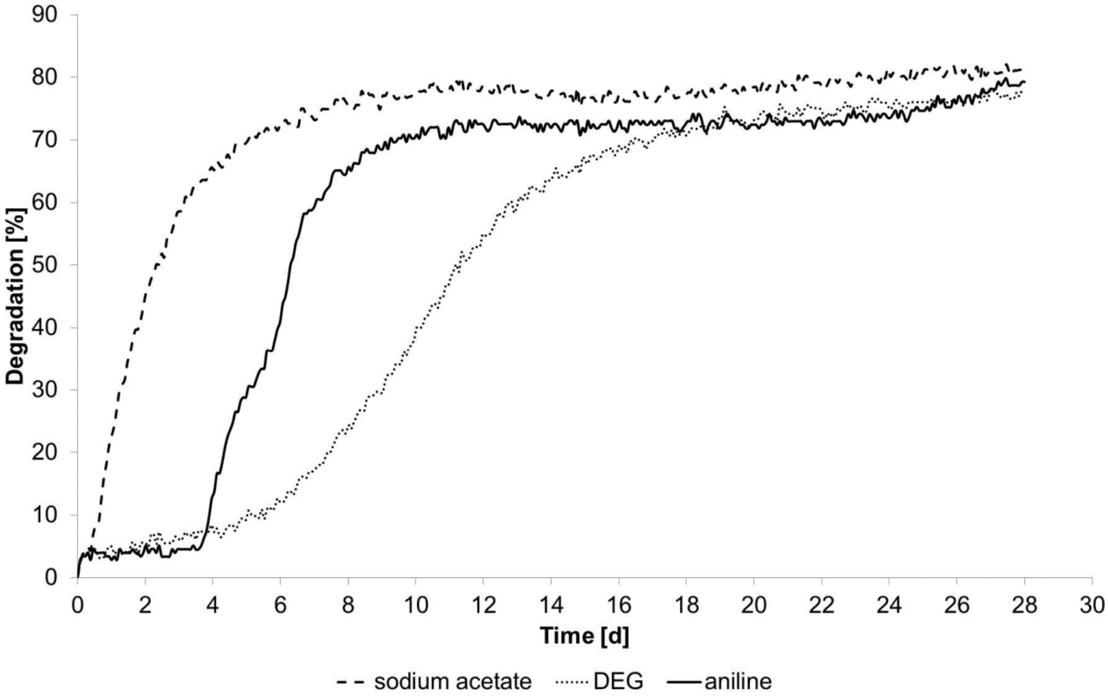

Fig. S2. Biodegradation of sodium acetate (62 mg L^{-1}), diethylene glycol (DEG) (32 mg L^{-1}) and aniline (20 mg L^{-1}) in screening water sediment test (conducted in July).

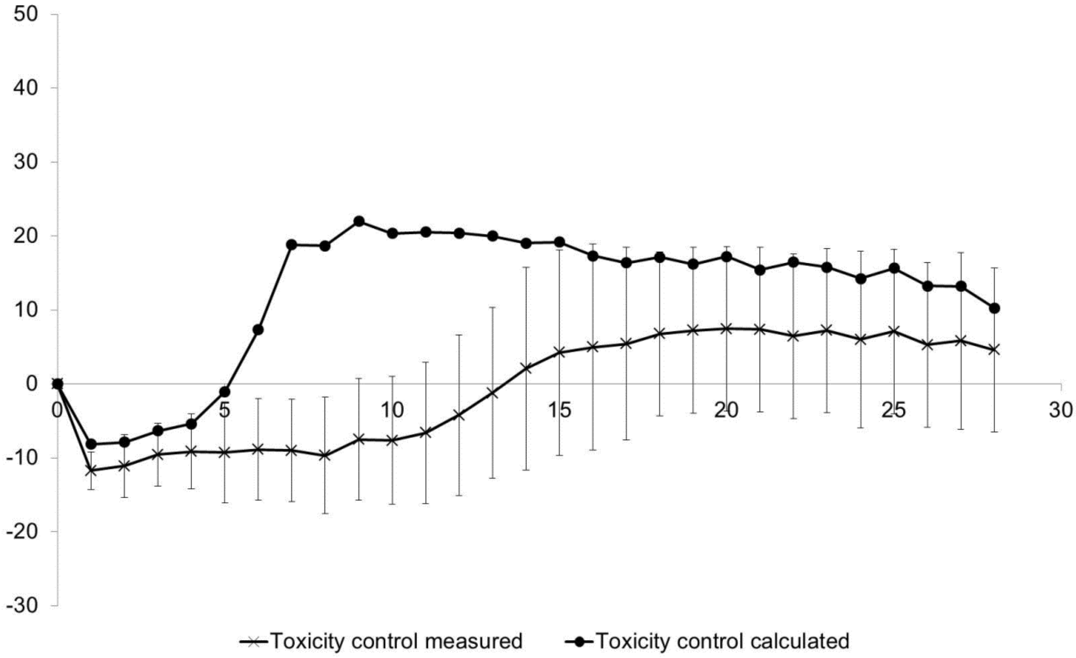

Fig. S3. Comparison between measured and calculated toxicity controls during the ciprofloxacin biodegradation (24 mg L^{-1}) in screening water sediment.

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Article VI

Assessing the environmental fate of S-metolachlor, its commercial product Mercantor Gold® and their photoproducts using a water–sediment test and *in silico* methods.

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Assessing the environmental fate of S-metolachlor, its commercial product Mercantor Gold® and their photoproducts using a water–sediment test and *in silico* methods

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HIGHLIGHTS

- SM and MG were not biodegraded in the water–sediment system.
- New bio-TPs were generated by aquatic micro-organisms from SM and MG photo-TPs.
- Adjuvants in MG had no significant influence on biodegradation but on sorption of SM.
- 50% of SM was irreversibly adsorbed onto sediment.
- *In silico* assessment of the new bio-TPs indicates toxicity to water organisms.

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ABSTRACT

Pesticides enter surface and groundwater by several routes in which partition to sediment contributes to their fate by abiotic (e.g. photolysis, hydrolysis) and biotic processes. Yet, little is known about S-metolachlor (SM) transformation in water–sediment systems. Therefore, a newly developed screening water–sediment test (WST) was applied to compare biodegradation and sorption processes between pure SM and Mercantor Gold® (MG), a commercial formulation of SM. Photolysis in water was performed by Xe lamp irradiation. Subsequently, the biodegradability of SM and MG photolysis mixtures was examined in WST. The primary elimination of SM from water phase was monitored and structures of its TPs resulting from biotransformation (bio-TPs) were elucidated by LC-MS/MS. SM was extracted from sediment in order to estimate the role of sorption in WST for its elimination. A set of *in silico* prediction software tools was applied for toxicity assessment of SM and its bio-TPs. Obtained results suggest that the MG adjuvants do not significantly affect biodegradation, but do influence diffusion of SM into sediment. 50% of SM could not be re-extracted from sediment with 0.01 M $CaCl_2$ aqueous solution recommended in OECD test guideline for adsorption. Neither the parent compound nor the photo-TPs were biodegraded. However, new bio-TPs have been generated from SM and MG photo-TPs due to bacterial activity in the water–sediment interphase. Moreover, according to *in silico* assessment of the bio-TPs the biotransformation might lead to an increased toxicity to the water organisms compared with the SM. This might raise concerns of bio-TPs presence in the environment.

1. Introduction

The estimated annual worldwide usage of pesticides is about 2.5 million tons. Herbicides account for the largest portion of total use, oscillating about 1 million tons (EPA, 2011). S-metolachlor (2-chloro-N-(2-ethyl-6-methylphenyl)-N-[(1S)-2-methoxy-1-methylethyl] acetamide) is a selective chloroacetanilide herbicide intensively applied for annual grassy weeds, corn, soybeans, peanuts, and other crops. From the chloroacetamides class, S-metolachlor (SM) is in the top three most used worldwide corresponding to 4.2% of the global pesticide use (Fenner et al., 2013; Martins et al., 2007). Mercantor Gold® (MG) as commercial product consists of SM (86.5% w/w) mixed with different formulation additives (adjuvants).

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Research on the transport of pesticides from their source (i.e. agricultural application) to surface bodies of water and groundwater by processes such as spray drift, wash-off from plants, surface runoff, infiltration, lateral subsurface flow, leaching or via industrial waste water discharge has been conducted by numerous authors and the dominating processes are largely known (Gassmann et al., 2013; Reichenberger et al., 2007; Remucal, 2014; Shibata et al., 2011). Approximately 1 to 5% of field applied herbicides are removed by surface runoff and reach the aquatic compartment (Scott et al., 1999; Wauchope, 1978). The application of pesticides introduces to the environment not only the active compound itself, but also other chemicals that make up the commercial product that is applied. In commercial products such as Mercantor Gold®, adjuvants have been developed not only to maximize pesticide efficacy but also to prevent any unfavorable environmental contamination from the active compound and its transformation products (TPs) (Katagi, 2008). Surfactants are some of the most important components among many other adjuvants such as stabilizers, thickeners or disperse and antifreeze agents (Katagi, 2008). Surfactants modify spray droplet size thus improving biological activity together with the retention, spreading on the leaf, or even the enhancing of pesticide uptake by crops (Knowles, 2001). Adjuvants can affect the solubility, biodegradation, and sorption properties (Katagi, 2006, 2008) and may act as a photosensitizer (Malouki et al., 2009).

Once in the water environment, pesticides in commercial products or as pure compounds are subject to biotic and abiotic transformation. Resulting TPs can be more toxic and present at higher concentrations than their parent compounds (Mañas et al., 2009; Olsson et al., 2013). Gómez et al. (2012) stated that the majority of the TPs have most likely not even been identified yet and much less is known about their environmental relevance.

To assess the ecological impact of pesticides and their TPs on water organisms and distribution between water and sediment phase, the investigation of pesticide behavior in laboratory water–sediment system would deliver valuable information (Katagi, 2008). Some authors have addressed chloroacetanilide herbicides' degradation and dissipation in simulated water–sediment systems (Mersie et al., 2004; Rice et al., 2004). Few studies focused on pesticides and their commercial formulations' biodegradation in the water–sediment systems (García-Ortega et al., 2006; Krieger et al., 1989). Only Gutowski et al. (2015) studied the environmental fate of SM and MG in a combined consecutive photolysis – biodegradation test. However, no sediment phase was included in this study. It should be noted that information and data regarding the SM, MG, and their photolysis products' behavior in the water–sediment environment is still plainly lacking in the international scientific literature. Knowledge regarding a possible transformation of photo-TPs and the products' fate and effects in the water–sediment system is especially limited. Furthermore, the influence and effects of the adjuvants on the pesticide and its TP's behavior in the water–sediment system is largely unknown. TPs are usually formed in low concentrations, within complex matrices so that isolation and purification is very challenging. Also many of these TPs are not available commercially, which makes the individual experimental analysis of their toxicity impossible. *In silico* predictions are versatile tools used to fill this gap. Hence, the generation of data based on quantitative structure activity relationships (QSARs) are gaining importance especially for analysis and assessment of environmental TPs (Mahmoud et al., 2014; Rastogi et al., 2014a, 2014b). This paper will address this lacuna by an in depth analysis of the fate of parent compound and photo-TPs in water–sediment test and the potential eco-toxicity against aquatic organisms of bio-TPs found in the study presented here. This publication addresses specifically:

(i) The indirect impact of MG's adjuvants on the active substance fate in the simulated water–sediment system (WST).
(ii) The biodegradability of photo-TPs in the water sediment system using WST to evaluate the elimination of the parent compound, its photo-TPs and their fate.
(iii) The structure elucidation of the generated bio-TPs, using LC-MS/MS. (iv) The initial eco-toxicity assessment of the found bio-TPs supported by *in silico* data generation using QSAR for the endpoints microtoxicity in environmental bacteria, bioconcentration factor and toxicity in rainbow trouts.

2. Materials and methods

2.1. Chemicals

The analytical standard of S-metolachlor (98.4% chemical purity, CAS Nr. 87392-12-9) was obtained from Fluka (Sigma–Aldrich, Steinheim, Germany) and the commercial product Mercantor Gold® from Syngenta Crop Protection, France. This product consists of S-metolachlor (86.5% w/w), a mixture of not further specified aromatic hydrocarbons (2–12% w/w), dodecylbenzenesulfonic acid calcium salt (a surfactant), 1–5% w/w), poly(oxy-1,2-ethanediyl),alpha-2,4,6-tris(1-phenylethyl)phenyl-omega-hydroxy (1–5% w/w, CAS Nr. 70559-25-0), and 2-methyl-1-propanol (1–2% w/w). Sodium azide (CAS Nr. 26628-22-8) was purchased from Sigma–Aldrich, Germany. HPLC grade acetonitrile (ACN, CAS Nr. 75-05-8) was purchased from VWR (VWR International, GmbH, Darmstadt, Germany). Aniline (CAS Nr. 62-53-3) was purchased from the same supplier; calcium carbonate (CAS Nr. 471-34-1), quartz (CAS Nr. 14808-60-7) and clay (CAS Nr. 1318-74-4) were purchased from Carl Roth, Germany. All aqueous solutions were prepared using ultrapure water 18.2 MΩ cm (Ultra Clear UV TM, Barsbüttel, Germany).

A flow chart of the experimental procedures applied in this study can be found in Supplementary Information (SI), Fig. S1.

2.2. Simulated solar photolysis experiments in aqueous solution

Photolysis in water was performed in an ilmasil quartz immersion tube using a xenon lamp (TXE 150, UV consulting Peschl, Mainz, Germany) as the source of radiation. The lamp emits spectra similar to natural sun light (200–800 nm) with the highest intensity in the visible range (200–280 nm: $1.61\,e^{-2}$ W/m^2, 280–315 nm: $1.16\,e^{-2}$ W/m^2, 315–380 nm: $3.75\,e^{-2}$ W/m^2, 380–780 nm: $5.58\,e^{-1}$ W/m^2) (data provided by the manufacturer). Photo-transformation products were generated by irradiation of the pesticides solution (SM and SM in MG, respectively) for 8 h time in ultrapure water. Samples before (0.0 h) and after (8.0) h of photolysis were collected and subsequently submitted to the WST. The final concentration of SM and MG was adjusted by measuring NPOC (non-purgeable organic carbon) of the tested substance (i.e. before photolysis) and photolysis treated samples, to provide required carbon content, and to reach adequate theoretical oxygen demand (ThOD), (described further in Section 2.3) Details on the experimental methods and the structural elucidation of the photo-TPs can be found in Gutowski et al. (2015).

2.3. Water sediment test (WST)

Because of the test design, processes such as biodegradation, sorption, elimination from water phase, and abiotic degradation could be investigated in this test simultaneously. All components of the artificial medium (sediment, inoculum, mineral medium) were standardized and based on OECD guidelines for testing of chemicals (218, 301 D and 302 C) (OECD, 1981, 1992, 2004).

Briefly, the WST consisted of five different series (blank, quality control, test, toxicity control and sterile control each was run in three parallels, details can be found in SI, Table S1. Glass bottles (1 L) were used as test vessels, each equipped with two septum sealed bottle nozzles. With water phase (500 mL) and artificial sediment (230 g) volumetric ratio was 1:5. Individual constituents of the artificial sediment are shown in Table S2 (SI). The aniline (used as quality control) and test substance concentrations were prepared so that they would correspond to 40 mg L^{-1} of theoretical oxygen demand (ThOD). The nominal concentrations were 17.2; 18.6; 16.9 mg L^{-1} for aniline, SM pure and SM in MG, respectively. A sterile control was used to account for abiotic elimination of test compounds. Therefore, sodium azide was added to one set of vessels in a concentration of 400 mg L^{-1} in water phase and 800 mg kg^{-1} in sediment. All assays were incubated in the dark at 20 °C in closed vessels. Test duration was 28 days as in related OECD tests (OECD, 2002, 2006). The water phase in the vessels was gently stirred to improve water exchange between water and sediment phase without disturbing the sediment. During the experiment pressure change as a proxy for oxygen consumption inside the vessels was monitored by pressure sensors (OxiTop®, WTW Weilheim, Germany).

Additionally, in order to avoid false negative results, the bacterial toxicity of test compounds against the inoculum was monitored. Therefore, oxygen consumption was measured in the toxicity control and compared to the predicted level, computed from the oxygen consumption in the quality control and in the test series. The dissipation of the SM from water phase was investigated in each of the test series (test, toxicity control, and sterile control). Samples were collected at days 0, 1, and 28 through sample port and analyzed by HPLC and LC-MS/MS analysis (for method see Chapter 2.4). The full method and preparation steps are described in detail by Baginska et al. (2015).

2.4. Extraction of S-metolachlor from WST sediment

Sediment samples were collected from WST vessels at the end of the test and air dried. An extraction solution (4 mL) containing a mixture of acetonitrile and water (9:1) was added to glass centrifuge tubes containing 1 g (dry weight) of sediment. Each sample was then vortexed for 30 s and placed into an ultrasonic bath for 30 min. The samples were then centrifuged (15 min, 4600 rpm, 20 °C). Then, the liquid phase was transferred to another centrifuge tube and extraction procedure was repeated an additional 2 times with the addition of 3 mL of extraction solution, successively. The combined extracts were filtered through 0.2 μm PES filter (Macherey–Nagel, Germany). The filtrate was collected and stored at −20 °C until analysis. In parallel, the extraction from sediment with 0.01 M $CaCl_2$ aqueous solution was performed to evaluate the strength of sorption of SM onto sediment (OECD, 2000).

2.5. Analysis of S-metolachlor and TPs by HPLC and LC-MS/MS

The primary elimination of the parent compound in WST and during photolysis was monitored by means of HPLC-UV (Prominence series Shimadzu, Duisburg, Germany). The chromatographic separation was achieved on RP-18 column. The identification and structure elucidation of the bio-TPs were performed with a LC-MS/MS Bruker Daltonic Esquire 6000+ ion-trap mass spectrometer (IT-MS) with electrospray ionization (ESI) equipped with the Bruker data analysis system (Bruker Daltonic GmbH, Bremen, Germany). The mass spectrometer was connected to an Agilent LC 1100 series (Agilent Technologies, Böblingen, Germany, HPLC 1100 series). The complete analytical method, operating parameters of the source, and ion-trap are described in detail by Gutowski et al. (2015) and were summarized in Text S2 (SI).

2.6. In silico QSAR models for ecotoxicity

In silico ecotoxicity predictions of SM and its bio-TPs were done using Case Ultra V.1.5.0.1 (MultiCASE Inc.) (Saiakhov et al., 2013). For the ecotoxicity assessment, the models "Microtoxicity in environmental bacteria (*V. fischeri*), AUA" and "Rainbow trout toxicity, AUE" as well as bioconcentration factor (BCF) in *Cyprinus caprio* (CITI) were applied. Structure illustrations were performed by using MarvinSketch 5.8.0. Simplified molecular input line entry specification (SMILES) codes were derived from the molecular TP structures and used as input for the software.

CASE Ultra software provides positive, negative and out of domain (OD) estimations for the selected models. Out of domain means that the test chemical is not included in the applicability domain of the model used and therefore predictions are not valid in this case. The above mentioned models and software are described in detail elsewhere (Mahmoud et al., 2014; Rastogi et al., 2014a, 2014b).

3. Results and discussion

3.1. Biodegradation of pure S-metolachlor, Mercantor Gold®, and their photo-TPs in WST

The inoculum was of sufficient activity since the biodegradation of aniline in the quality control reached 75 ± 9% (results from two independent tests). The results shown in Fig. 1A demonstrate for SM no biodegradability (6 ± 14%) likewise for the photolysis

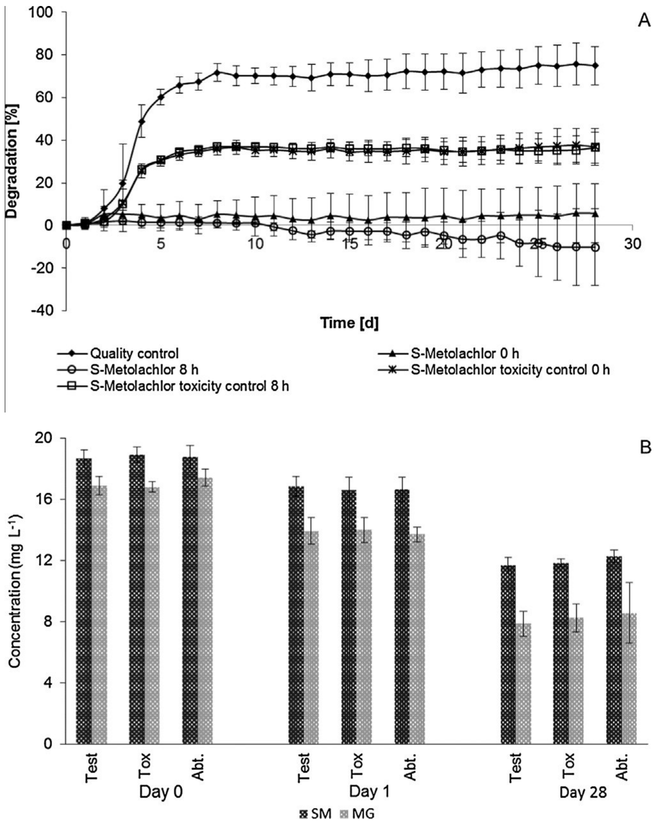


Fig. 1. Biodegradation and dissipation from water phase in water–sediment test. A – Degradation of SM and photodegradation mixture of SM (Xe-lamp irradiation time 8.0 h), in screening water sediment test ($n = 2$, each bottle measured three times). B – Primary elimination of SM and SM in MG in WST water phase monitored with HPLC. Start concentration varies due to different determined theoretical oxygen demand (ThOD) of the MG compared to the pure SM. All values represent the means ± SD ($n = 3$).

mixture of SM after 8 h of irradiation (-10 ± 18%). The negative development of the SM biodegradation was a result of normalization of the test blank series and should therefore be considered as 0%. No toxic effects on bacteria (biodegradation in toxicity control >25%, Fig. 1A) were observed by any tested substance in the toxicity control (biodegradation level in 'toxicity control' of 37 ± 9% for SM and 37 ± 7% for the photolysis mixture). Moreover, in both cases, calculated toxicity controls (sum of theoretical oxygen demand in the test and quality assay) and toxicity controls measured corresponded well to each other, concluding that degradation of aniline was not inhibited (SI, Fig. S2).

Mercantor Gold® was resistant to biodegradation in WST. The inoculum was of sufficient activity (quality control was biodegraded in 79 ± 9%, $n = 2$). No difference was observed between biodegradation of MG and its photolysis mixture (SI, Fig. S3). The biodegradability of MG reached 11 ± 16% and 11 ± 6% of photolysis mixture. Both, MG and its photo-TPs were not toxic to the test bacteria as biodegradation in toxicity control reached 44 ± 7% and 48 ± 10%, respectively. Additionally, the obtained toxicity control corresponded well to calculated toxicity values, concluding that degradation of both compounds (aniline and MG) occurred in parallel and was not inhibited (biodegradation in toxicity control >25%, SI, Fig. S2). The slightly higher degradation rates of MG compared with pure grade SM and their photolysis mixtures could be within measurement error and therefore were not significant, especially when biodegradation was evaluated based on indirect measurements such as monitoring pressure. No difference was found in biodegradability between the commercial formulation MG and the pesticide SM alone in other biodegradability tests (Gutowski et al., 2015). The main difference between these tests and the current study was the presence of a sediment layer and the diversity of inoculum used. The WST inoculum was a mixture of microbial communities from natural water bodies and secondary effluent from sewage treatment plant whereas for the other tests only inoculum from secondary effluent of sewage treatment plant was used.

The photo-TPs were also resistant to biodegradation in the WST. Although both substances and their photolysis mixtures were not mineralized, it is worthy to mention that there was always a small but not significant difference in biodegradation in favor of the commercial product. This finding might be interpreted as the influence of the adjuvants on the biodegradation process. The commercial formulation consist of a mixture of hydrocarbons and the surfactant dodecylbenzenesulfonic acid calcium salt. These adjuvants could have contributed to biodegradation in a low extent (Khleifat, 2006; Scott and Jones, 2000), dodecylbenzenesulfonic acid is known to be biodegradable in the environment by anaerobic and aerobic bacteria (Denger and Cook, 1999; Manousaki et al., 2004). This is in agreement with the results of Katagi (2006, 2008). In another study by Mersie et al. (2004), metolachlor solution was found to be partially degraded in a water–sediment test. As a result the formation of oxanilic acid and ethane sulfonic acid transformation products was reported in the first 30 days of the experiment. For their 112 day study period Mersie et al. (2004) used Bojac sandy loam to prepare the sediment (22.5% clay and 77.5% silt), which was mixed with river water. Moreover, those authors reported a decline in concentration of the detected TPs after 56 days of incubation, suggesting further transformation to new products. However, these metabolites were not found in the WST. The main reason might be the use of artificial sediment (SI, Table S2) or another test design (e.g. no addition of river water) applied in the present study.

3.2. Dissipation of the SM from water phase

HPLC measurements revealed that SM was similarly removed from the water phase in all three test series of the WST (test, toxicity and sterile controls). The compound was gradually dissipating, starting from the 1st day to reach 33.6% (±2.8%) removal on 28th day (Fig. 1B). Likewise the pure compound, the SM in MG was continuously dissipating to reach higher removal of 52.8% (±5.1%) on the 28th in all three test series (Fig. 1B). This result could be explained by the presence of the MG adjuvants (dodecylbenzenesulfonic acid calcium salt), which is an anionic surfactant that would directly interact with the sediment. Katagi (2008) indicated that due to their structures, surfactants can determine the distribution properties of pesticides in soil and sediment systems (i.e. mobility, leaching, sorption etc.). Generally, this can be confirmed by a study of Gonzalez et al., 2010, who reported increased soil mobility of organochlorine pesticides due to the presence of anionic surfactants (sodium dodecyl sulfate). Therefore SM in MG could easier be distributed to sediment than pure SM.

The WST allowed tracking the elimination of the parent substance from the water phase which probably occurred due to its sorption onto sediment particles. The extraction of SM was conducted in order to investigate whether SM could be re-extracted form sediment taken from WST. This information is especially important since desorption processes play also an important role in the fate and distribution of chemicals in the environment. For this purpose two different solvents were selected. One polar "organic" consisting of ACN/water (9:1) and as a second one a solution of 0.01 M $CaCl_2$ as suggested by OECD Test Guideline 106 (OECD, 2000). Recovery rate for SM and MG with $CaCl_2$ was 55.4% and 50.0%, respectively (summarized in Table S4). Extraction with ACN/water turned out to be more effective with recovery rates of 96.9% and 93.1% (MG). Log P of SM is 3.05 (PPDB, 2014). This might suggest, that part of SM amount in both formulations can be immobilized by the sediment under natural conditions. The nature of SM sorption onto artificial sediment might differ depending on the individual fraction that it consists of: mineral, clay and organic. Thus, it might be assumed that SM was sorbed to one of the three sediment components under natural conditions. Therefore, only extraction with highly polar ACN, allowed obtaining high recovery compared with $CaCl_2$ solution. One can say that in natural aquatic environment SM might display similar properties and could partially be immobilized in bottom sediments.

3.3. Determination of transformation products in WST

To investigate the bio-transformation of photo-TPs in WST, an LC-MS/MS analysis of samples after 0 and 28 days was performed. The results are shown in Fig. 2A for the WST samples after 0 days, and in Fig. 2B after 28 days.

Compared to Fig. 2A, the results from WST after 28 days (Fig. 2B) show that the photolysis product with the specific mass m/z 266.2 was eliminated, and it revealed that two products with the m/z 264.2 (Rt 9.7 and 12.0 min) were formed (indicated with arrows). The abiotic control of the photolysis mixture, after 28 days (Fig. 2C) showed no changes compared to Fig. 2A. This demonstrates, that these new peaks resulted from microbial activity, and not from non-biotic elimination. Moreover, the peak areas of the photo-TPs were compared for the start and end of WST, as shown in Fig. 3 ($TP1_{a–g}$ are isomers). Both photolysis mixtures, showed identical behavior in WST. It is worthy to mention, that the peak area does not precisely indicate the concentrations of photo-TPs as their molar extinction coefficient and ionization rate, respectively, are not known and have to be assumed to be different from the parent compounds. However, the relative change in concentration can be measured anyway and their elimination due to biodegradation was calculated. The LC-MS/MS results, shown in Fig. 3 indicate for the photolysis mixture that the photo-$TP1_a$ was eliminated, whereas intensity of the photo-$TP1_b$ increased, by co-elution of the second bio-TP. In other words new bio-TPs

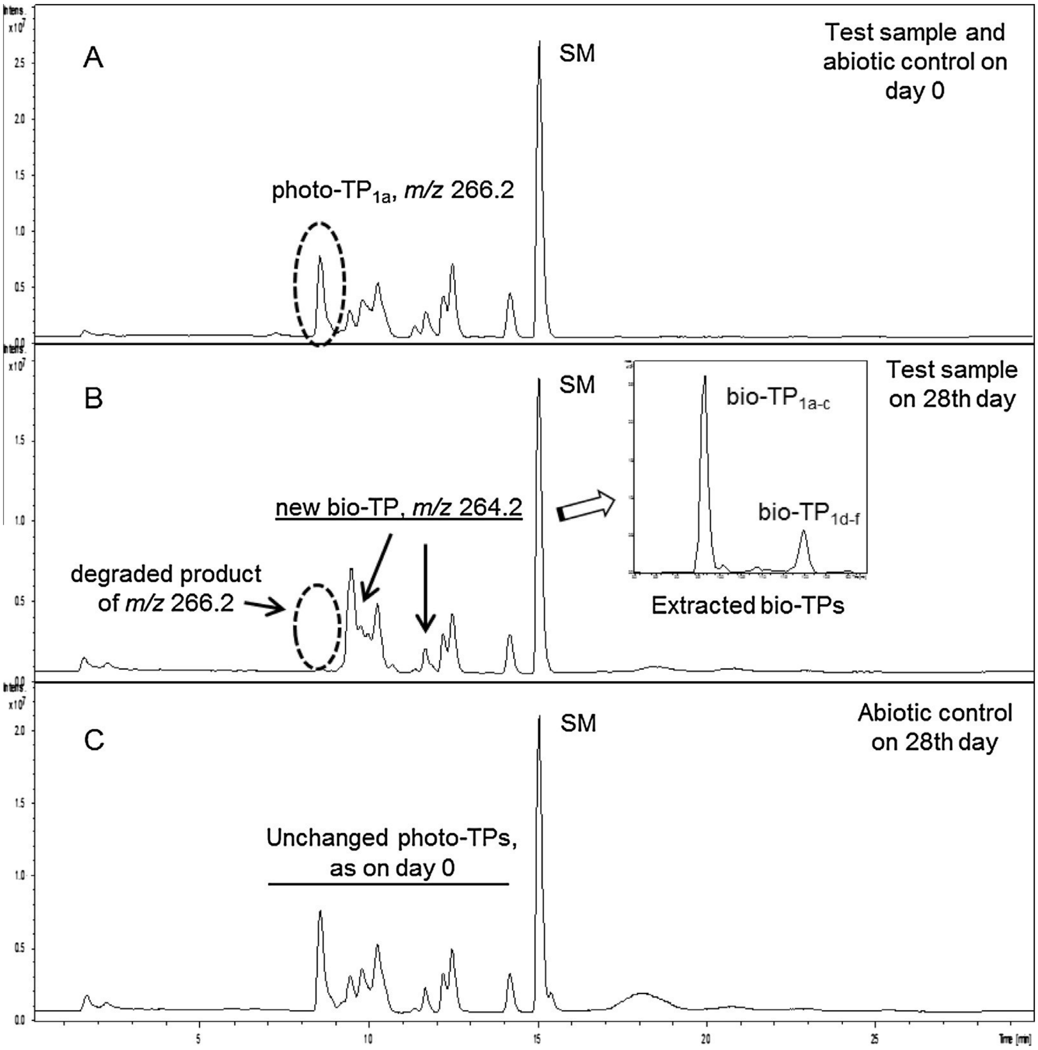


Fig. 2. Total on chromatogram of the photolysis mixture of SM obtained from the WST at day 0 (A) and 28th (B), and the abiotic control at day 28th (C). Dashed ellipse indicates the biodegraded TP (TP1a), arrows indicate newly formed bio-TPs (Bio-TP1a and Bio-TP1b, which co-elutes with present photo-TPs).

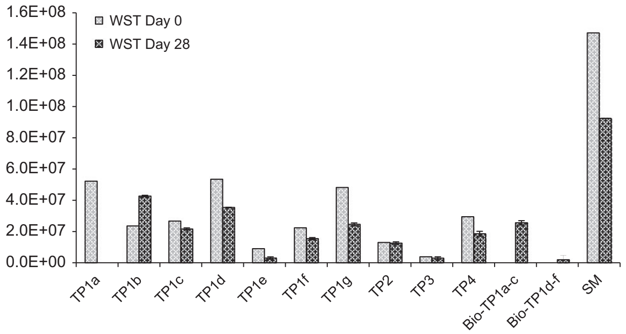


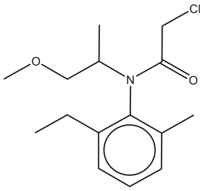
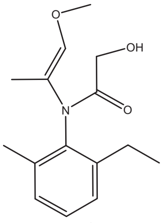
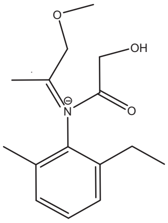
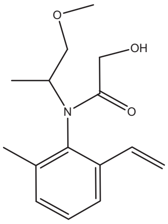
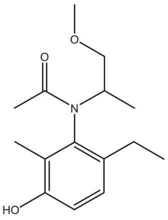
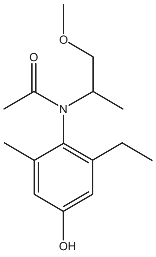
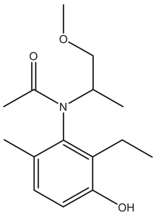
Fig. 3. Relative peak area of the photo-TPs in photolysis mixture (8 h irradiation time) at the start and end of biodegradation test assays. WST of SM in MG formulation. Day 0 ($n = 1$), day 28 ($n = 2$), respectively (lower case letters indicate isomers of the respective TPs, see also Fig. 4).

(a–c and d–f are isomers, respectively, see below) were formed as found in samples of the 28th day for pure SM, as for the MG formulation. SM and SM in MG showed similar behavior. Aforementioned photo-TPs and bio-TPs tend to be of higher polarity than the parent compound itself, as they do elute before the parent on the non-polar chromatographic column. Retention times and TPs in the photo mixture, resulting from commercial formulation were identical to these found in the treatment of the pure SM.

The formation of bio-TPs from photolysis mixtures of SM in water was also demonstrated by Gutowski et al. (2015) using a Manometric Respiratory test, OECD 301F. Since this study shows an identical degradation of photo-TPs with the m/z 266.2 and the formation of two new products with the m/z 264.2 (R_t 9.7 and 12.0 min), it confirms that WST delivers similar conditions for biotic transformation as a Manometric Respiratory test (Gutowski et al., 2015). What is more, this demonstrates that the bio-TPs are likely to be formed in the natural water environment as they were found in water phase in two different biodegradation tests.

The observed bio-TPs were found only in microbiologically active test, and toxicity control series, but were not detected in the sterile control. Based on analysis of mass spectra, it can be assumed that the bio-TPs' formation occurred through a formation of a double bond within the structures of dechlorinated, and monohydroxylated m/z 266.2 photo-TPs of SM, as reported in literature (Coffinet et al., 2012; Gutowski et al., 2015). Moreover, due to possible isomerization and position of the double bond within the structure, a total of 6 possible bio-TPs are presented in Table 1 and Fig. 4. Neither a specific mass of m/z 264.2 was found in the abiotic control nor were the 6 photo-TPs of m/z 266.2 degraded in the sterile control (Fig. 2C). The latter ones were still detected

Table 1
In silico prediction by different models of Case Ultra for S-metolachlor and the observed newly formed bio-TPs in WST. For ecotoxicity assessment the models "Microtoxicity in environmental bacteria (*V fischeri*), AUA" and "Rainbow trout toxicity, AUE" as well as bioconcentration factor (BCF) in *Cyprinus caprio* (CITI) were applied.

Name, MS (m/z), R_t (min)	Structure	Model (Case Ultra)		
		BCF (CITI)	Microtox (V fischeri) (AUA)	Rainbow trout (AUE)
S-metolachlor 284.4; 15.2		POSITIVE	NEGATIVE	NEGATIVE
bio-TP1$_a$, 264.2; 9.6		POSITIVE	OUT OF DOMAIN	OUT OF DOMAIN
bio-TP1$_b$, 264.2; 9.6		POSITIVE	OUT OF DOMAIN	OUT OF DOMAIN
bio-TP1$_c$, 264.2; 9.6		POSITIVE	OUT OF DOMAIN	OUT OF DOMAIN
bio-TP1$_d$, 264.2; 12.0		POSITIVE	NEGATIVE	POSITIVE
bio-TP1$_e$, 264.2; 12.0		POSITIVE	POSITIVE	OUT OF DOMAIN
bio-TP1$_f$, 264.2; 12.0		POSITIVE	NEGATIVE	POSITIVE

POSITIVE: positive alert for the respective endpoint, NEGATIVE: no positive alert for the respective endpoint; OUT OF DOMAIN: Structure of the tested compound not included into the applicability domain of the model.

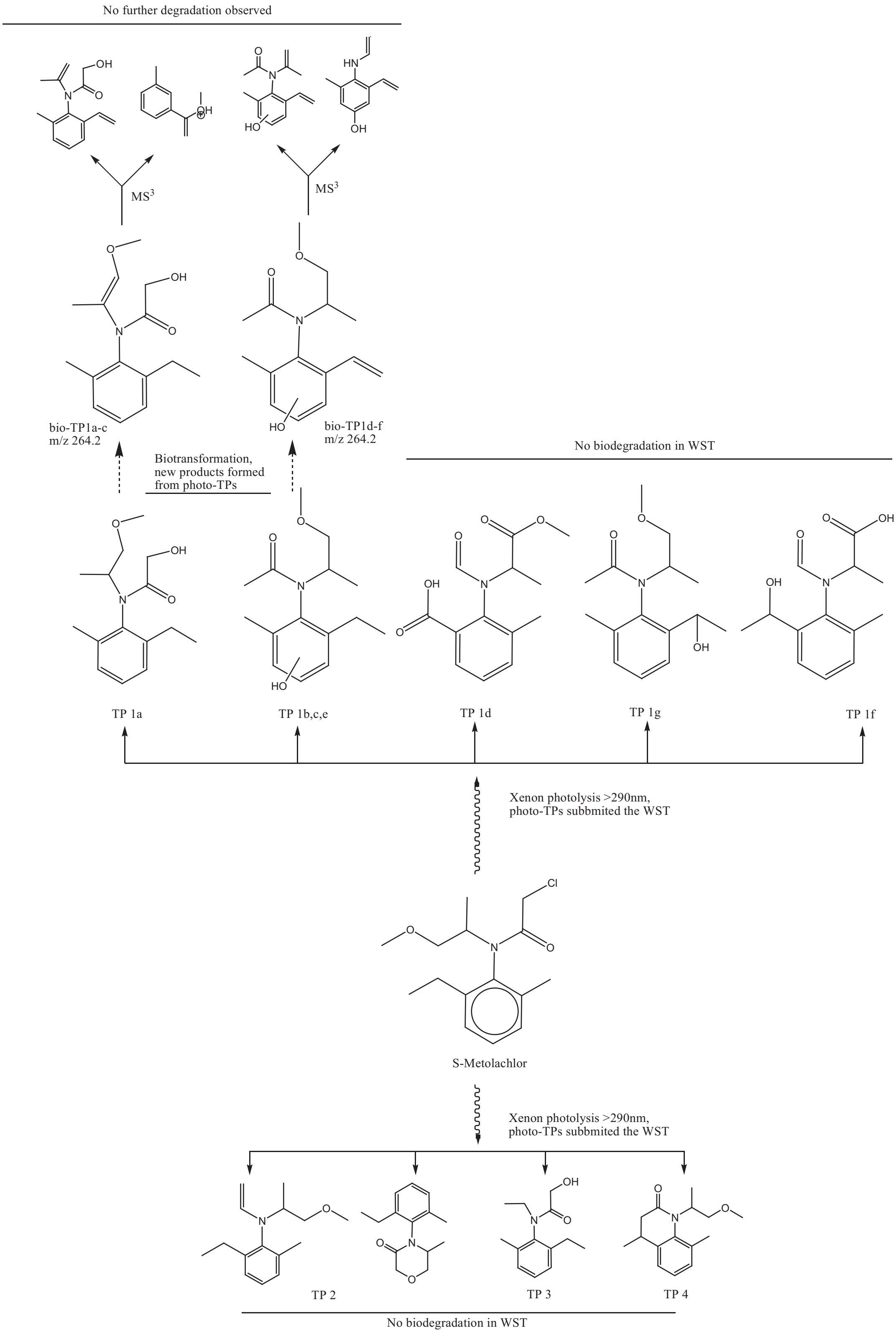


Fig. 4. Proposed abiotic (photo-) and biotic (WST) pathway for most intensive photo-TPs (*m*/*z* 266.2) of SM and SM in MG. Bio-TPs were further fragmented up to MS^3 spectra. All photoproducts were submitted to the WST. Only some the most intensive TPs of *m*/*z* 266.2 were further transformed into two new bio-TPs of *m*/*z* 264.2.

by LC-MS/MS at the end of the test, at the very same intensity. Therefore, it can be concluded, that found bio-TPs of *m*/*z* 264.2 are resulting from microbial transformation of photo-TPs *m*/*z* 266.2. The results for product ions and percentage of relative abundance of the biotransformation products are given in the SI (Table S5). Therefore, abiotic processes like hydrolysis or other abiotic chemical transformations can be excluded, to play a significant role, in the parent compound and its photo-TPs fate in water.

Suggested abiotic and biotic degradation pathway for SM and SM in MG is shown in Fig. 4. Liu et al. (1995) had reported the formation of numerous biotransformation products of metolachlor, by a rot fungus (*P. chrysosporum*) and its inability to fully mineralize the parent compound. Thus, it appears that found bio-TPs can result in the environment from direct transformation of the parent compound, or by further transformation of the photo-TPs, not leading to the direct mineralization as yet.

3.4. In silico eco-toxicity assessment for S-metolachlor and its bio-TPs

The BCF is predicted to be positive for both parent compound and transformation products (Table 1), but an additional refined analysis with several available models revealed indications, that the BCF might be mitigated after biotransformation (SI, Table S7). Moreover, *in silico* prediction provided initial indications that some of the bio-TPs might have an increased ecotoxicity compared with the parent compound SM. Of note are results of the bio-TPs (Table 1), which showed alerts for toxicity towards environmental bacteria (bio-TP1_e) and towards rainbow trouts (bio-TP1_d and bio-TP1_f), respectively. Thus, the biotransformation may have increased the ecotoxicological potential of SM and its photo-TPs, which would deserve further experimental attention. Besides, for some transformation products, such as bio-TP1a or bio-TP1b, the selected QSAR models cannot provide a prediction, since the molecules are out of the applicability domain. Therefore, it would be worth to study these compounds in the corresponding *in vivo* or *in vitro* assays, once they are available in suitable amounts in order to corroborate the prediction and/or to fill the data gaps.

4. Conclusions

This study shows that WST proved to be an appropriate tool for the first screening of a substance's behavior in water–sediment interface and a good starting point providing the information that allows to plan direction of further research especially in combination with *in silico* tools.

Experiments carried out in the laboratory showed that pure SM and in Mercantor Gold® formulation were resistant to biodegradation in the WST. However, some of their most abundant photolysis products were transformed into new bio-TPs due to bacterial activity, as found in our previous research. Adjuvants did not significantly influence the biodegradation process, but due to the present surfactants in MG's formula they might increase the mobility of the active substance compared to the pure grade of SM. In fact, this could result in increased diffusion to the sediment layers. This study demonstrates that SM, MG, and especially their photo-TPs should be considered as persistent. Moreover, some of the photo-TPs could undergo biotransformations and result in new bio-products in the aquatic environment. Applied QSAR models provided preliminary information that the observed bio-TPs might be of higher eco-toxicity than the parent compound. Therefore it is highly recommended that their ecological impact should be further investigated and should be taken into account for a detailed risk assessment of the chemical. This calls for a more detailed investigation and inclusion of follow up transformations into risk assessment in general.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.08.013.

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Supplementary material for article:

“Assessing the impact of adjuvants in commercial formulations on the fate of the pesticide S-metolachlor and its photoproducts using a water-sediment test and *in silico* methods”

1. Methods

1.1. Description of the different experimental methodology applied in this study

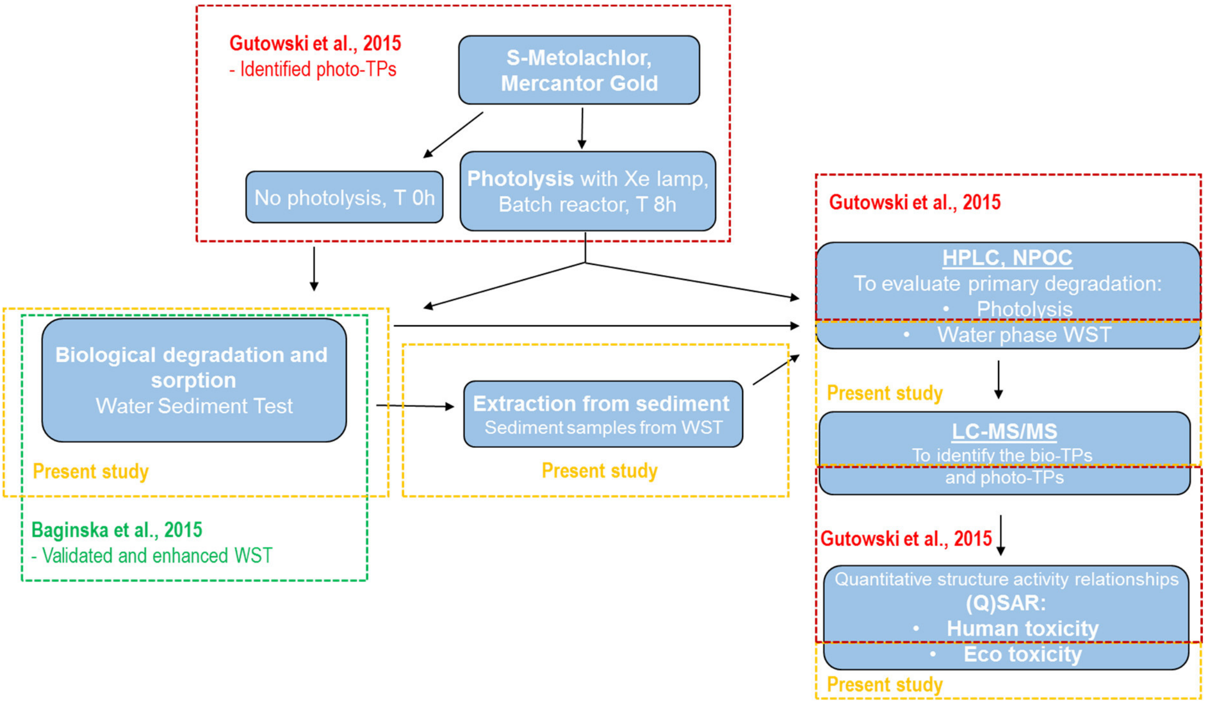


Fig. S1. Flow chart description of the experimental procedures, referred to the different published articles.

1.2. Water-sediment test conditions

<u>Different series of the water-sediment test.</u>

Table S1. Screening water sediment test vessels content accordingly to test series

Test series	Blank	Quality control	Test	Toxicity control	Sterile control
Sediment	■	■	■	■	■
Mineral medium	■	■	■	■	■
Inoculum	■	■	■	■	
Aniline(reference substance)		■		■	
Test substance			■	■	■
Sodium azide					■

Constitutes of the artificial sediment.

Table S2. Composition of the artificial sediment used in screening water sediment test

Constituent	Characteristics	Content [% dry weight]
Peat	from sphagnum moss	2
Clay	kaolin type	5
Quartz sand	grain size 0.8 – 0.2 mm	93
Calcium carbonate	powder	0.01

1.3. Sediment conditioning (Text S1)

The sediment was placed in the test vessels (230 g wet weight) with a water layer above containing mineral medium and inoculum. Separately, in vessels conditioned for ‘sterile control’ contained no inoculum and addition of sodium azide in sediment and water. Prepared like this vessels were acclimated for 7 days under the test conditions. Conditioning allows stabilization of important parameters e.g. pH, redox potential, and adaptation of bacteria and their growth on the sediment. During the conditioning the pressure development and BOD were measured to monitor the processes inside the sediment.

Table S3. Localization of inoculum sampling sites for the WST

Sample type	Details	GPS location
Effluent from a municipal WTP	Lüneburg, 73,000 population equivalents	N: 53° 16' 0" E: 10° 25' 19"
water and sediment from river	Ilmenau in Lüneburg; upstream from WTP	N: 53° 12' 31" E: 10° 24' 45"
water and sediment from lake	Lake Loppau in Ammelinghausen	N: 53° 7' 57" E: 10° 13' 41"

1.1.HPLC method and mass spectrometer settings (Text S2)

The primary elimination was monitored by means of HPLC-UV (Prominence series Shimadzu, Duisburg, Germany). The chromatographic separation was achieved with RP-18 column (EC 125/4 NUCLEODUR 100-5 C18 ec, Macherey and Nagel, Düren, Germany) protected by an EC 4/3 NUCLEODUR 100-5 C18 ec guard column. The mobile phase consisted of ultrapure water (solution A) and 100% acetonitrile (solution B). For elution, the following gradient was used: 0.01 min 20% B, 3.0 min 20% B, 13.0 min 80% B, 20 min 80% B, 24 min 20% B. Sample injection volume was 20 μL and the oven temperature was set at 40 °C, flow rate was set at 0.7 mg L^{-1}. Total run time was 30 min and the wavelength was set at 220 nm. SM and MG standards (1.25, 2.5, 5, 10, 20, 40 and 80 mg L^{-1}) were used to obtain calibration curves. Retention times for SM and SM in MG were 14.20 min. Linear concentration-signal relationships were obtained. Regression coefficients for SM and MG were r^2 = 0.999 and r^2 = 0.999; n=2, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) for SM were 0.02 mg L^{-1} and 0.06 mg L^{-1}, respectively, and for SM in MG 0.07 mg L^{-1} and 0.2 mg L^{-1}, respectively. The mass spectrometer was operated in positive polarity. Flow rate was 0.7 mg L^{-1} in LC part, before MS a T cap was applied reducing the flow to the half (0.35 mg L^{-1}). Injection volume was 20 µL and oven temperature was set to 40°C. The retention time for SM was 14.25 min and molecule ion was found at 284.5 m/z. Analysis of total ion chromatogram and corresponding mass spectrum was used for structural identification of the bio-TPs. The structural identification of the biotransformation products was first based on the analysis of the total ion chromatogram (TIC) and the corresponding mass spectrum. Furthermore, to obtain structural elucidation the bio-TPs were isolated, used as precursor ions and further fragmented up to MS^3 using the Auto MS^n mode. The operating conditions of the source were: -500 V end plate, - 4833 V capillary voltage, 30 psi nebulizer pressure, and 12 L min^{-1} dry gas flow at a dry temperature of 350 °C. The selected lens and block voltages were: + 95.8 V capillary exit, 245.8 Vpp octopole reference amplitude and -61.0 V lens two. The scan range was determined from *m/z* 100 to 900 and the scan time was 200 ms.

2. Results:

2.1. Calculated and measured toxicity controls in WST

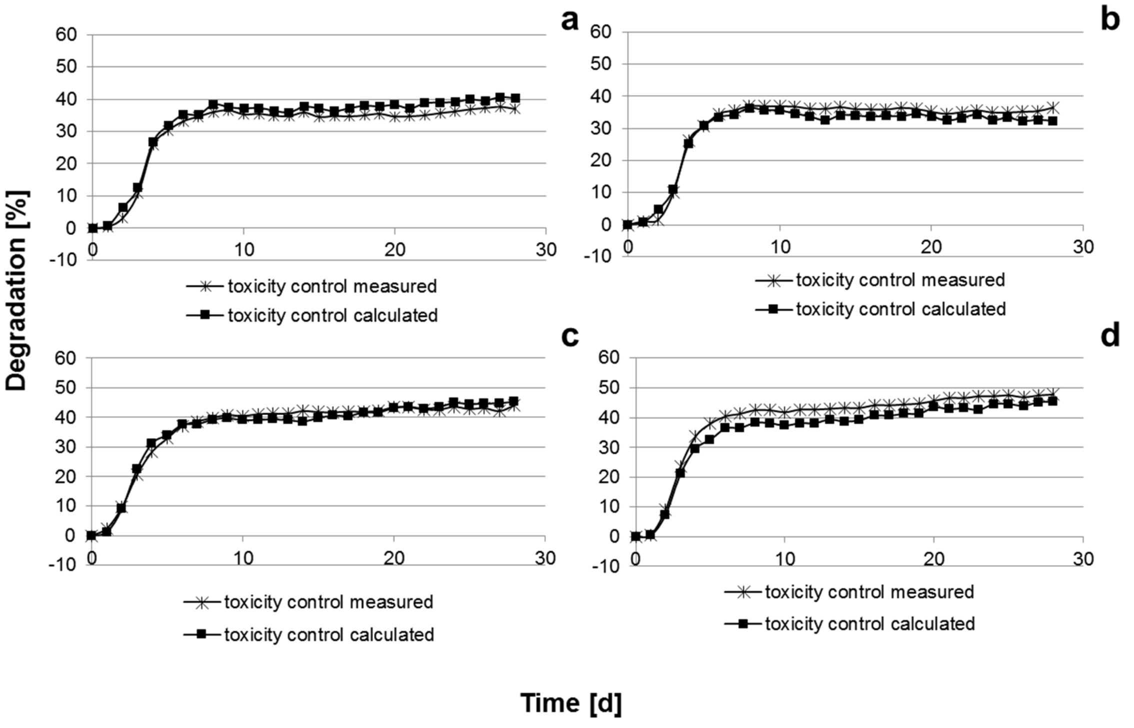


Fig. S2. Correlation between toxicity control series measured and calculated during WSTs, between samples of S-Metolachlor (SM) (a) sample from photodegradation experiment time point 0 h, and (b) sample from photodegradation experiment time point 8 h; Mercantor Gold (MG) samples from photodegradation experiments time points 0 (c) and 8 hours (d) (Xe-Lamp).

A substance was considered to be toxic if measured toxicity control was lower than 25% which corresponds to less than 50% degradation of aniline. If the measured toxicity control was lower than calculated a substance is assumed to have inhibitive or toxic impact on the inoculum.

2.2.Biodegradation of Mercantor Gold in WST:

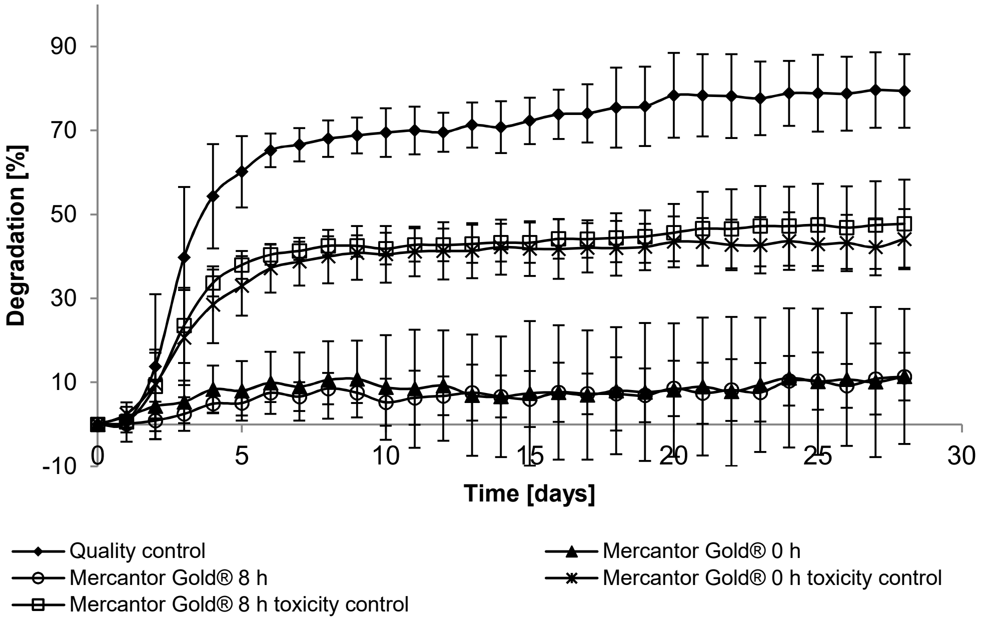


Fig.S3. Degradation of MG and its photodegradation mixture (Xe lamp irradiation and time 8.0 h), in screening water sediment test (n = 2, each bottle measured three times).

2.3. Extraction from sediment

Table.S4. Effect of extraction solvent on the recovery rates of SM and MG from the water phase.

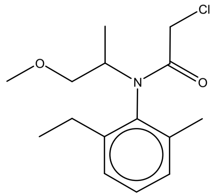
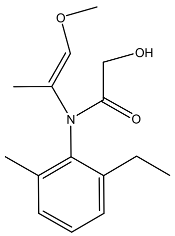
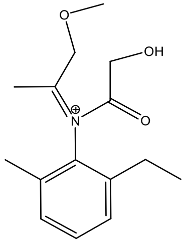
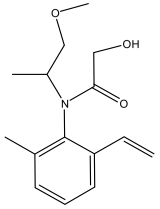
Solvent composition	**pH**	**Extraction cycle**	**Recovery (%)**		**dt dev**	
			SM	**MG**	**SM**	**MG**
0.01 M water solution of $CaCl_2$	7.0	3	55.4	50.0	1.5	1.1
ACN and water (9:1)	7.2	3	96.9	93.1	0.0	1.9

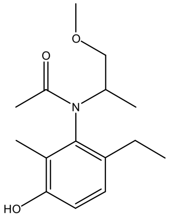
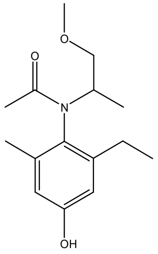
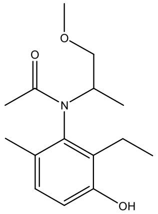
2.4.LC-MS/MS parameters of the bio-TPs

Table S5. Product ions and percentage of relative abundance, of the biotransformation products.

Compound	Rt (min)	Main precursor ion (*m/z*)	Product ions (*m/z*), % of relative abundance in brackets
WST product day 28, bio-TP1$_{a-c}$	9.6	264.2	232.2 (100), 149.2 (17.72), 175.0 (18.54), 204.0 (100)
WST product day 28, bio-TP1$_{d-f}$	12.0	264.2	232.2 (100), 175.0 (100), 204.0 (18.97)

Table S7. Additional BCF predictions by different models of EpiSuite, Oasis Catalogic and Vega Cesar, KNN and Mylan for SM and its biotransformation products.

	EpiSuite		Oasis Catalogic BCF 02.05			Vega BCF Caesar		Vega BCF KNN	Vega BCF Mylan		
Chem. Name	**log BCF (EpiSuite 4.1.1, BCFBAF model)**	**log P (EpiSUITE)**	**log Kow**	**logBCF corrected**	**± logBCF corrected**	**Predicted BCF [log(L/kg)]**	**Predicted BCF [L/kg]**	**Predicted BCF [log(L/kg)]**	**Predicted BCF [log(L/kg)]**	**Predicted BCF [L/kg]**	**Predicted LogP (Meylan/Kowwin)**
SM 	1.58	2,9 (database)	3.236	0.8724	0.154	1.17	15	0.92	1.66	46	3.02
bioTP1a 	0.735	2.01	2.006	0.5326	0.124	0.58	4	0.92	0.5	3	0.7
BioTP1b 	0.5	-4.3	-4.296	0.5464	0.083	0.6	4	0.92	0.5	3	1.4
BioTP1c 	0.701	1.95	1.954	0.4757	0.084	0.56	4	1.22	0.5	3	0.65

BioTP1d 	0.871	1.82	1.824	0.4974	0.081	0.77	6	1.24	0.6	4	1.42
BioTP1e 	0.871	1.82	1.824	0.4972	0.081	0.84	7	1.24	0.37	2	1.07
BioTP1f 	0.871	1.82	1.824	0.4974	0.081	0.77	6	1.24	0.6	4	1.42